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Natural Disease Resistance in Strawberry Fruit and Geraldton Waxflower Flowers

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ABSTRACT

Antifungal activity against *Botrytis cinerea* and *Cladosporium cladosporioides* declined with increasing fruit maturity as shown by thin layer chromatography (TLC) bioassay. Preformed antifungal activity was also demonstrated in flower tissue. Decline in fruit antifungal compounds was correlated with a decline in natural disease resistance (NDR) against *B. cinerea*. Crude extracts of green stage I fruit contained at least two previously unreported preformed antifungal compounds ($R_f = 0.44$ and 0.37) that were not present in white and red stage fruit. These compounds were confirmed by TLC reagent sprays not to be phenolics or alkaloids. Positive reactions to Ehrlich's reagent suggested that $R_f = 0.37$ was a terpene. The majority of antifungal activity was found in the achenes of green stage I fruit. However, antifungal activity was found in all tissue types (viz. pith, cortex, epidermis) of green stage I fruit. TLC bioassays showed that all fruit stages showed antifungal activity at the origin ($R_f =$ 0.00). The approximate area of fungal inhibition at origin in green stage 1 fruit extracts was 90 and 70% greater than in white and red stages. TLC reagent sprays confirmed that antifungal compounds at origin contain phenolics. This is consistent with previously reported phenolic compounds in strawberry fruit that are inhibitory to *B. cinerea*.

An investigation into the potential of enhancing NDR using different chemical (acibenzolar), biological (*Aureobasidium pullulans*) and physical (UV-C) elicitors was conducted with a view to developing an integrated pest management (IPM) strategy. The most promising results were achieved with pre-harvest treatments of the chemical plant activator acibenzolar. Seven glasshouse trials were conducted over a three year period. Preharvest application of acibenzolar (0.25 – 2.0 mg AI ml^{-1}) were effective in suppressing grey mould on strawberry fruit harvested from winter grown plants. Conversely, acibenzolar was ineffective at suppressing grey mould on fruit harvested from summer grown plants. However, where acibenzolar was effective, disease development was delayed by as much as 2 days. This delay was equivalent to a 15-20% increase in shelf-life. If systemic acquired resistance and/or other inducible mechanisms are to be implemented as part of an IPM strategy for controlling *B. cinerea* more research is required on how environment and management factors affect the efficacy of elicitors such as acibenzolar.

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Geraldton waxflower is the most economically important native Australian cut flower export. Infection of Geraldton waxflower by *B. cinerea* can lead to unacceptable levels of flower abscission after harvest. Thus, an investigation was conducted into the nature and identities of constitutive antifungal compounds in imported Geraldton waxflower flower and leaf tissues. Antifungal activity against *B. cinerea* and *C. cladosporioides* was observed in both Geraldton waxflower leaf and flower tissue. Leaf tissue contained considerably less antifungal activity than flower tissue. Some antifungal compounds were common to the three different waxflower cultivars studied. Through TLC reagent sprays and NMR GC-MS spectra, these antifungal compounds were identified as the sesquiterpenes, globulol and grandinol. There were also at least two unidentified phenolics. Notwithstanding similarities in antifungal profiles, it was also evident from TLC bioassays that significant variations exist between different waxflower cultivars.

Further work is required to fully characterise the preformed antifungals compounds found in strawberry and Gerladton waxflower tissues and to elucidate pathways involved in their biosynthesis. In addition, work is also required to look at the full spectrum of antifungal activity of these antifungal compounds. Such information will allow precise definition of the roles that these compounds may play in suppression of in strawberry and waxflower NDR against *B. cinerea*. In turn, this knowledge should enable the introduction of improved and/or novel IPM strategies that enhance levels of these compounds.

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LIST OF ABBREVIATIONS

ACC	aminocyclopropane-1-carboxylic acid
AI	active ingredient
ANOVA	analysis of variance
BABA	DL-3-aminobutyric acid
BTH	benzothiadiazole
ca	approximately
CBSC	carbon based secondary compounds
cfu	colony forming units
CHS	chalcone synthase
cm	centimetre
CNB	carbon nitrogen balance
CRD	completely randomised design
cv.	cultivar
cvs	cultivars
°C	degrees Celsius
d	day
1-D	one dimension
2-D	two dimension
Da	Dalton
DAA	days after anthesis
DACP	diazocyclopentadiene
DAP	days after planting
DW	dry weight
EC	electrical conductivity
ECe	electrical conductivity of soil saturation extract
e.g.	for example
et al.	and others
exp	exponential
FB	full bloom
frc	fraction
FW	fresh weight

g	gram
GDB	growth differentiation balance
GRP	glycine-rich proteins
h	hour
ha	hectare
H°	hue angle
HPLC	high performance liquid chromatography
HR	hypersensitive response
HRGP	hydroxyproline-rich glycoproteins
IAA	indole-3-acetic acid
i.e.	that is
INA	2,6-dichloroisonicotinic acid
IPM	integrated pest management
IR	induced resistance
JA	jasmonic acid
kg	kilogram
kJ	kilojoules
kPa	kilo Pascal
l	litre
LAR	local acquired resistance
L	leaf
LHS	left hand side
LOX	lipoxygenase
LSD	least significant difference
m	metre
M	molar (moles/L)
1-MCP	methylcyclopropene
MeSA	methyl salicylate
MHz	mega hertz
MJ	methyl jasmonate
µg	microgram (10^{-6} kg)
µl	microlitre (10^{-6} L)
µM	micromole (10^{-6} mol)

mg	milligram (10^{-3} g)
min	minutes
MJ	methyl jasmonate
ml	millilitre (10^{-3} l)
mm	millimetre (10^{-3} m)
mM	millimolar (10^{-3} M)
mol	mole
mS	milli-siemens
n	number of observations comprising a value
nl	nanolitre (10^{-9} L)
N	Newton
N/ N-N	nectiferous / non-nectiferous
NBD	norbornadiene
NDR	natural disease resistance
N.I.	not identified
nm	nanometre
NMR	nuclear magnetic resonance
$\leq$	less than
ns	not significant at $P \leq 0.05$
P	probability
PA	proanthocyanidins
P-A	post-anthesis
PAL	phenylalanine ammonia lyase
PDA	potato dextrose agar
pers. comm.	personal communication
PG	polygalacturonase
PGIP	polygalacturonase inhibiting protein
pH	relative proton concentration in a solution
PMA	phosphomolybdic acid
PR	pistils and receptacle tissue
PRP	pathogenesis-related protein
pv.	pathovar
%	percent

±	plus or minus
®	registered trademark
R	resistance
RCBD	randomised complete block design
RH	relative humidity
R_f	retardation factor
RP	reverse phase
RP-HPLC	reverse phase - high performance liquid chromatography
Rpm	revolutions per minute
s	second
SA	salicylic acid
SAH	salicylate hydroxylase
SAR	systemic acquired resistance
s.e.	standard error
SOD	superoxide dismutase
sp. /spp.	species
STS	silver thiosulfate
t	tonnes
TL	thaumatine-like
TLC	thin layer chromatography
™	trademark
TMV	tobacco mosaic virus
TSS	total soluble solids
UV-C	short-wave ultraviolet
UDPGFT	uridine diphosphate glucose:flavonol O^3-D-glucotransferase
v	volume
var.	variant
viz.	that is
w	weight
WF	whole flower

CHAPTER 1 INTRODUCTION

During the course of co-evolution, plants and fungal pathogens have developed intricate relationships (Benhamou and Nicole, 1999). Pathogens have an array of attack strategies in order to parasitise plants, including fruits, vegetables and ornamentals. Horticultural crops and other plants have evolved constitutive and/or inducible defense mechanisms. These natural defence systems that contribute to preventing or retarding post-infectional disease development for varying periods of time. During the development of harvestable plant organs and after harvest the natural disease resistance (NDR) of horticultural produce declines leading to inevitable infection. Often, the decline in NDR during storage can lead to activation of quiescent infections. Limited research has been conducted into characterising preformed antifungal compounds that occur in horticultural produce. In contrast, inducible biochemical defences are better understood. The association between the patterns of change in preformed antifungal compounds requires more thorough study.

The following literature review (Chapter 2) discusses the physiology and biochemistry of natural disease resistance in horticultural crops. Emphasis is upon constitutive and inducible defence mechanisms that are non-proteinaceous in nature. Preformed antifungal compounds and phytoalexins discovered in horticultural crops are reviewed in detail. Strategies for control of postharvest pathogens, such as SAR, elicitation of NDR and influence of environment are discussed. Also, in conclusion, the potential for exploitation of NDR and associated caveats are considered.

The overall aim of this study was to develop improved understanding of the host-pathogen interaction between two different horticultural products and *Botrytis cinerea* Pers. More specifically, the aim was to characterise preformed antifungal compounds in strawberry cv. Elsanta fruit and flowers at different stages of development (Chapter 3) and in different tissues of harvested Geraldton waxflower (Chapter 5). This work was undertaken with a view to contributing to the development of strategies for improved control of grey mould.

Grey mould disease is caused by the fungal pathogen *B. cinerea*. This disease is one of the most important affecting horticultural produce after harvest. Although grey mould can be partially controlled by certain pre-harvest cultural methods (e.g. growing strawberry fruit undercover) and postharvest storage techniques (e.g. low temperature), industry is still heavily reliant upon chemical fungicides (e.g. iprodione) applied during flowering. However, there are strengthening concerns over increasing loss of efficacy of conventional fungicides. This change is due to pathogen resistance. Additionally, there less public acceptability of fungicide usage in terms of both health and environmental issues. These issues favour the increased adoption of integrated pest management (IPM) programmes by growers.

Exploitation of systemic acquired resistance (SAR), a sub-set of NDR processes, is a potential strategy for achieving IPM. That is, elicitation of SAR involves enhancing natural defence mechanism in crops. Induced resistance through enhancement of plant defence metabolism has been shown to suppress development of a variety of crop diseases (Adikaram, 1990; Wilson *et al.*, 1994; Sticher *et al.*, 1997). However, most data on activation of SAR concern control of pre-harvest diseases of arable and horticultural crops (Sticher *et al.*, 1997). In contrast, relatively little attention has been paid to the potential for controlling postharvest fruit diseases through enhancing plant NDR (Joyce and Johnson, 1999).

Various biological, physical or chemical elicitors can be used to activate and/or boost NDR in non-infected plant tissue (Adikaram, 1990; Wilson *et al.*, 1994; Prusky, 1998). Amongst the natural and synthetic chemicals that can activate SAR, acibenzolar (S-methyl benzo [1,2,3] thiadiazole-7-carbothioate; BTH; used as the 50% WG formulation Bion®; Actigard™) is perhaps the most potent elicitor (Friedrich *et al.*, 1996). The potential for postharvest and preharvest use of acibenzolar to suppress grey mould in harvested strawberry cv. Elsanta fruit and the effect of acibenzolar on growth of *B. cinerea in-vitro* were investigated in this work (Chapter 4, Part A). In addition, preliminary studies were conducted into the efficacy of *Aureobasidium pullulans* (de Bary) Arnaud, a biocontrol elicitor, and UV-C treatment, a physical elicitor, for control of grey mould on strawberry cv. Elsanta fruit (Chapter 4, Part B).

CHAPTER 2 LITERATURE REVIEW

NATURAL DISEASE RESISTANCE IN HARVESTED HORTICULTURAL CROPS

2.1 Introduction

A number of constitutive and inducible defence mechanisms have evolved in plants as a result of pressures imposed by virulent pathogens. Thus, pathogens that infect plants are confronted by an array of defence mechanisms of structural and/or biochemical nature. Many of these mechanisms have been implicated in natural disease resistance (NDR) of certain fruit, vegetables (Prusky, 1996; 1998; Elad, 1997; Mercier, 1997; Barkai-Golan, 2001) and ornamentals (Meir *et al.*, 1998).

NDR is defined here as the degree to which a plant inherently is able to resist pathogen attack and subsequent infection. The level of NDR within a crop is a function of genotype and physiological age, which can be modified by environment and management factors. During development and after harvest, the NDR of horticultural produce generally declines leading to the increased likelihood of infection, principally by fungi (Prusky, 1996). This decline in NDR can lead to quiescent infections in horticultural produce continuing their infection process. The rate of decline in NDR is principally governed by duration of storage and storage regime employed as these will effect respiration rate of pathogen and host. This chapter reviews past and current research on the physiology and biochemistry of NDR in harvested horticultural produce and discusses how NDR may be manipulated to reduce postharvest losses and maintain produce quality.

2.2 Physiology of NDR

In general, plants defend themselves against pathogens by a combination of structural characteristics and biochemical reactions. The nature of these defences can be specific to different host-pathogen interactions and can vary according to the kind of

plant organ and tissue attacked. In horticultural produce, postharvest diseases caused by fungi usually begin as either latent infections established in the field or wound infections during subsequent harvesting and handling. The mechanisms affecting the decline of NDR of produce after harvest were summarised into four hypotheses (Prusky, 1996): 1) the lack of nutritional requirements for the pathogen; 2) the presence of preformed antifungal compounds that decrease in ripening and senescing horticultural produce; 3) the presence of inducible antifungal compounds (phytoalexins) that decrease after harvest; and 4) activation of fungal pathogenicity factors occurring as horticultural produce ripen or senescence.

The time at which NDR mechanisms are overcome by a pathogen will depend on the type of infection, physiological age of the product and handling and storage conditions employed. None-the-less, regardless of how infection is derived, fruit, vegetables and ornamentals will generally exhibit a period before or just after harvest when NDR is relatively high. Thus, pathogen invasion or development is often restricted until the fruit, vegetable or ornamental matures (Prusky, 1996; Labavitch, 1998). On approaching maturation, ripening or senescence, NDR declines leading to active infection and subsequent fungal colonisation (Prusky, 1996). This pattern in NDR suggests that horticultural crops have evolved constitutive and/or inducible mechanisms that limit or retard disease development for a given physiological period. These mechanisms allow the fruit, vegetable or ornamental to protect the developing organ. However, with fruit and ornamentals a balance between maintaining NDR through costly metabolic processes and attracting seed and pollen dispersers is thought to be important (Joyce *et al.*, 1998; Labavitch, 1998).

2.2.1 Constitutive Defence

2.2.1.1 *Pre-existing defence structures*

Structural defences contribute to NDR of horticultural produce by preventing or restricting pathogen infection and/or growth. Fruit and vegetables are protected by the pre-existing structures of the peel or skin. Thus, infection after harvest can only occur if this defence barrier is breached through direct penetration, wounding or natural openings such as stomata and lenticels and/or growth cracks.

The amount and nature of the wax layer and cuticle tissue that covers epidermal cells and the structure of epidermal cells can influence NDR (Elad, 1997). A thicker cuticle may enhance NDR by providing increased mechanical resistance and being more resistant to cracking and thus penetration by wound invading pathogens (Elad and Everson, 1995). Greater cuticle and/or cell wall thickness have been positively correlated with increased NDR in tomato (*Lycopersicon esculentum*) (Rijkenberg *et al.*, 1980), sweet cherry (*Prunus avium*) (Adaskaveg *et al.*, 1989; 1991), nectarines (*Prunus persica* var. *nucipersica*) (Michaillidis and Johnson, 1992), peaches (*Prunus persica* var. *persica*) (Adaskaveg *et al.*, 1989; 1991), roses (*Rosa hybrida*) (Hammer and Evensen, 1994) and grapes (*Vitis vinifera*) (Commenil *et al.*, 1996). Epicuticular wax may prevent spore germination on surfaces of produce as this hydrophobic plane prevents free water from collecting. Specialised structures, like trichomes on peaches, may exert a similar water repellent effect. A compound found in epicuticular waxes on young and resistant grape berries cv. Pinot Noir was also shown to have direct antifungal activity against *Botrytis cinerea* (Commenil *et al.*, 1996). However, contrasting work on gourd (*Cucurbita maxima*) suggests that there is no relationship between amount of surface wax and incidence of rots during storage (Hawthorne, 1998). Moreover, Kerssies and Frinking (1996) found no relationship between mass and thickness of epicuticular wax on petals of gerbera (*Gerbera jamesonii*) and roses and NDR toward *B. cinerea*. In fact, plant-surface lipids may induce spore germination and appressorium formation (Podilia *et al.*, 1993). This stimulation indicates that specific waxes and other constitutive factors can play an important role in inhibiting or triggering appressorium germination (Kolattukudy *et al.*, 1995).

Many pathogens, such as the necrotroph *B. cinerea*, are able to avoid many structural barriers by infecting senescing anther tissue and pistils at anthesis. They then remain quiescent until NDR in the non-senescent host tissues fall beneath a threshold allowing the infection process to continue (Prusky, 1996; Mercier, 1997). Certain strawberry cvs., have relatively limited infection through 'phenological escape'. Disease incidence in strawberry fruit (*Fragaria ananassa*) derived from pistillate inflorescences sprayed with a condial suspension of *B. cinerea* was less than for hermaphrodite genotypes (Simpson, 1989; 1991). Anthers on hermaphrodite flowers provide a major route for *B. cinerea* to enter the developing receptacle. Thus, flower structure differences between the two strawberry genotypes may be loosely

considered a constitutive structural resistance factor. Similarly, ornamentals are afforded protection by sepals, which when receded fail to contribute to NDR. On the other hand, remnants of sepals and bractioles, and the wounds they leave following abscission, can be prime infection sites in both fruit and ornamentals (Prusky, 1998).

2.2.1.2 *Pre-inflectional chemical defence*

Horticultural products contain hundreds of secondary metabolites, many of which possess antifungal properties (Grayer and Harborne, 1994; Mercier, 1997). Many of these compounds are constitutive and are present in their biological active form (Osborne, 1996; 1999). Ingham (1973) refers to these as 'pro-inhibitins', as they are pre-infectional plant metabolites which are normally present in concentrations high enough to limit most fungi. Other antifungal compounds occur as inactive precursors that are activated by mild physical stress or pathogenic attack. These latter compounds are still considered as being constitutive as they are immediately derived from pre-existing constituents (Mansfield, 1983; Grayer and Harborne, 1994). VanEtten *et al.* (1994) proposed that these inactive compounds should be referred to as 'phytoanticipins' or 'post-inhibitins' (Ingham, 1973) rather than phytoalexins. Phytoalexins are synthesised from non-immediate precursors in response to pathogenic attack (Hammerschmidt, 1999a).

Relatively little research has been conducted on identifying preformed antifungal compounds in horticultural produce compared to inducible biochemical defence (Table 2.1) (Mercier, 1997). Difficulties arise in determining *in-vivo* concentrations, location and antifungal activity of preformed antifungals (Morrissey and Osbourn, 1999). Where preformed antifungal compounds have not been fully characterised in certain products antifungal activity can still often be demonstrated. Wilson *et al.* (1997b) showed that crude extracts of 3 species of pepper, *Capsicum annum*, *C. chinense*, and *C. frutescens* all strongly inhibited conidial germination of *B. cinerea*.

Levels of preformed antifungal compounds in certain fruit and vegetables tissues decline after harvest (Kim *et al.*, 1991; Ben-Yehoshua *et al.*, 1992; Prusky and Keen 1993; Mercier, 1997; Caccioni *et al.*, 1998). The decline in natural defence capability is often associated with ripening and/or senescence (Verhoeff, 1974; Brown, 1989;

Prusky, 1996). This decline has been correlated with the activation of both latent (Prusky, *et al.*, 1991a) and wound pathogens (Ben-Yehoshua *et al.*, 1992; 1998), such as *Colletotrichum gleosporioides* and *Penicillium digitatum*, respectively. However, the concentration of existing preformed antifungal compounds in fruit tissue can be induced using biotic or abiotic elicitors (Prusky *et al.*, 1991a; b; Prusky and Keen, 1993).

The distribution of preformed antifungal compounds is often not uniform within horticultural produce. Most preformed antifungal compounds tend to be found in vacuoles and oil glands in the outer layers of plant organs (Prusky, 1998; Table 2.1). This distribution suggests that these compounds may only be 'released' if the cell is disrupted either through physical damage or direct penetration by the pathogen. However, release of compartmentalised compound(s) may be induced through recognition of pathogen-derived signals (Prusky, 1998).

2.2.2 Inducible Defence

Attempted infection by an avirulent pathogen leads to the activation of an array of biochemical defences which have evolved to halt the infection process (Hammerschmidt, 1999b). Upon infection, an oxygen burst occurs. The rapid generation and accumulation of activated oxygen species is thought to provide resistance to the host plant against the pathogen (Lamb and Dixon, 1997). As a result of decompartmentalisation, super oxide dimutase (SOD) and hydrogen peroxide are released, and influence the release of signal molecules within and around the cell. This leads to the hypersensitive response (HR). HR is an ubiquitous defence mechanism that is aspecific to all plants.

Table 2.1: Examples of constitutive antifungal compounds associated with postharvest disease resistance of horticultural produce

Species	Tissue	Compound(s)	Chemical class	Inhibited Pathogen	Reference
Allium cepa (onion)	scales	protocatechuic acid; catechol	phenolics	*Colletotrichum circinans*	Walker *et al.*, 1925; Angell *et al.*, 1930
Allium sativum (garlic)	bulb	ajoene	unsaturated sulfoxide disulfide	*Aspergilus niger*	Yoshida *et al.*, 1987
Apium graveolens (celery)	stalk	marmesin; columbianetin	furanocoumarins (psoralen) precursors	*Botrytis cinerea*	Afek *et al.*, 1995
Capsicum frutescens (cayenne pepper)	whole	capsidiol	terpene	*B. cinerea*	Stoessl *et al.*, 1972; Wilson *et al.*, 1997b
Carica papaya (papaya)	latex, fruit pulp and seed	benzyl isothiocyanate	isothiocyanate	*Colletotrichum gleosporioides*	Tang, 1973 Patil *et al.*, 1973; Giordani *et al.*, 1996; Adikaram *et al.*, 1998
Cichorium intybus (chicory)	whole	lactucin	lactone	Not in abstract	Monde *et al.*, 1990
Citrus aurantifolia (lime)	flavedo	citral (3,7-dimethyl-2,6-octadienal); limettin (5,7-dimethyoxycoumarin); 5-geranoxy-7-methxycoumarin; isopimpinellin (5,8-dimethoxypsoralen)	monoterpene aldehyde; coumarin and furanocoumarins	*Penicillium digitatum; Cladosporium cladosporioides*	Ben-Yehoshua *et al.*, 1992
Citrus limon (lemon)	flavedo	citral limettin; 5-geranoxy-7-methxycoumarin; isopimpinellin; psoralen derivative (unidentified)	monoterpene aldehyde coumarins and furanocoumarins	*P. digitatum; C. cladosporioides*	Ben-Yehoshua *et al.*, 1992; Rodov *et al.*, 1995; Caccioni *et al.*, 1998

Species	Tissue	Compound(s)	Chemical class	Inhibited Pathogen	Reference
Citrus grandis (pomelo)	flavedo	Osthol (7-methoxy-8-prenylcoumarin); auratene (2,3-epoxy-7-methoxy-8-7-8-prenylcoumarin); 7-[(6,7-epoxy-3,7-dimethyl-2-octyl)oxy] coumarin; 7-geranoxycoumarin	coumarins	*C. cladosporioides P. digitatum*	Ben-Yehoshua *et al.*, 1988; 1992; Kumpoun *et al.*, 1998
Citrus paradisi (grapefruit)	flavedo	7-geranoxycoumarin	coumarin	*P. digitatum*; *Penicillium italicum*	Angioni *et al.*, 1998
Citrus reticulata (mandarin)	calyx	hesperidin (hesperitin-7-rutinoside)	flavanoid	*Phomopsis (Diaporthe) citri*	Homma *et al.*, 1989
Citrus sinensis (orange)	flavedo	similar antifungal profile to lemon	monoterpene aldehyde coumarins and furanocoumarins	*P. digitatum*	Ben-Yehoshua *et al.*, 1992; Caccioni *et al.*, 1998
		dehydroalbietic acid; linoleil monoglyceride	flavone	*Geotrichum candidum*	Vargas *et al.*, 1999
Citrus unshiu (satsuma)	fruit tem end	hesperidin; derivatives of cinnamic acid	flavanoid; phenolic acid	*Phomopsis (Diaporthe) citri*	Homma *et al.*, 1998
Daucus carota (carrot)	slice	falcarindiol (cis-pentadeca-6-ene-1,3-dyn-5,15-diol)	polyacetylene	*Mycocentrospora acerina*	Garrod *et al.*, 1978; Davies and Lewis, 1981
Dianthus caryophyllus (carnation)	leafy stems and roots	kaempferide triglycoside	glycoside	*Fusarium oxysporum* f. sp. *dianthi*	Curir *et al.*, 2001;
Dioscorea batatas (Chinese yam)	tuber	3-hydroxy-5-methyoxybibenzyl; 3,2'-dihydroxyl-5-methoxybibenzyl (batatasin IV) 6-hydroxy-2,4,7-trimethoxyphenanthrene (batatasin I); 6,7-dihydroxy-2,4-dimethoxyphenanthrene; 2,7-dihydroxy-4,6-dimethoxyphenanthrene	oxygenated bibenzyl phenanthrenes	Examined against 24 fungi	Takasugi *et al.*, 1987

Species	Tissue	Compound(s)	Chemical class	Inhibited Pathogen	Reference
Ecballium elaterium (squirting cucumber)	whole fruit	cucurbitacin I	triterpene	*B. cinerea*	Bar-Nunn and Meyer, 1990
Fragaria ananassa (strawberry)	green fruit	proanthocyanidins including - catechin; epicatechin; gallic acid	proanthocyanidins (hydrolyzable and condensed tannins; phenolic acids)	*B. cinerea*	Jersch *et al.*, 1989; Di Veneri *et al.*, 1998; Herbert *et al.*, 2001
Ipomoea batatas (sweetpotato)	periderm and outer cortex	3,5-dicaffeoylquinic acid (isochlorogenic acid)	phenolic	*Rhizopus stolonifer*	Stange *et al.*, 2001
Lupinus albus (lupin)	leaf surface	luteone	isopentenyl isoflavone	Not in abstract	Harborne *et al.*, 1976
Lycopersicon esculentum (tomato)	green fruit	α-tomatine	steroidal alkaloid	*Fusarium solani* *B. cinerea*	Roddick, 1974; 1976; Défago and Kern 1988; Sandrock and VanEtten 1998;
	seeds	not identified	phenolic	*C. gleosporioides; Cladosporium cucmerinum*	Nidiry, 1999
Malus domestica (apple)	peel of unripe fruit	p-coumaryl-quinic acids chlorogenic acid	phenolic acids	*Alternaria sp.; B. cinerea; Penicillium expansum*	Ndubizi, 1976
Mangifera indica (mango)	peel unripe fruit	5,12-cis heptadecenyl resorcinol; 5-pentadecyl resorcinol;	alkylated phenol	*Alternaria alternata; C. gleosporioides; C. cladosporioides*	Cojocaru *et al.*, 1986; Droby *et al.*, 1986; Kobiler *et al.*, 1998; Zanuri *et al.*, 2001;
		di-2-ethylhexyl phthalate+ alkyl phthalates	phthalates derivatives	*C. cladosporioides*	Supyen *et al.*, 1998

Species	Tissue	Compound(s)	Chemical class	Inhibited Pathogen	Reference
Musa (banana)	peel of unripe fruit	dopamine (oxidation products)	amine	*Colletotrichum musae*	Muirhead and Deverall, 1984
Persea americana (avocado)	pericarp and mesocarp	diene/ Persin (cis,cis 1-Acetoxy-2-hydroxy-4-oxo-heneicosa-12,15-diene) 1-acetoxy-2,4-dihydroxy-n-heptadec-16-ene 1,2,4-Trihydroxyheptadec-16-yne; 1,2,4-trihydroxyheptadec-16-ene; 1-acetoxy-2,4-dihydroxy-heptadec-16-yne	long-chain alcohol	*C. gleosporioides; C. cladosporioides*	Prusky *et al.*, 1982; Prusky *et al.*, 1991ac; Kobiler *et al.*, 1994; Sivanathan and Adikaram *et al.*, 1989; Adikaram *et al.*, 1992; 1993
	idioblasts	1-acetoxy-2-hydroxy-4-oxo-heneicosa-5,12,15-triene			Domergue *et al.*, 2000
Prunus persica (peach)	fruit skin	chlorogenic and caffeic acids	phenolic acids	*Monilinia fructicola*	Bostock *et al.*, 1999
Solanum tuberosum (potato)	tuber	α-solanine; α-chaconine	steroidal alkaloids	*Erwinia carotovara*	Allen and Kuć, 1968
Vitis viniferea (grape)	whole immature fruit	tannic acid, chlorogenic acid; caffeic acid; gallic acid	proanthocyanidins (hydrolyzable and condensed tannins)	*B. cinerea*	Nyerges *et al.*, 1975; Hills *et al.*, 1981
Zingiber officinale (ginger)	rhizome	gingerenones A, B and C; isogingerenone	diaryheptenone	*Pyricularia oryae*	Endo *et al.*, 1990

HR is an early feature of the infection process following the perception of pathogen avirulence signals. Thus, HR is a specific response that will only take place if a virulent pathogen infects a susceptible host. For an incompatible reaction to take place and for infection to be unsuccessful some recognition between the host and the pathogen must take place. According to the 'gene for gene' hypothesis a resistance gene (R) is triggered by an elicitor molecule produced by an avirulence gene in the pathogen (Flor, 1971).

HR results in a localised induced cell death in the host plant at the site of infection, and an oxidative burst. Although not always visible, HR is characterised by localised tissue becoming necrotic. This leads to local acquired resistance (LAR) and the subsequent release of host and pathogen derived elicitors. This is accompanied by a cascade of biochemical reactions in attacked and surrounding plant cells (Dixon *et al.*, 1994; Kombrink and Somssich, 1995). Upon infection, the activity of phenylalanine ammonium-lyase (PAL) and chalcone synthase (CHS), the regulating enzymes for phenylpropanoid biosynthesis respectively, increase. The phenylpropanoid pathway plays a significant role in LAR as many potentially protective compounds are synthesised including structural lignins and many phenylpropanoid derived phytoalexins (Lamb *et al.*, 1989).

2.1.1.1 *Induced structural defence*

Upon infection or mild abiotic stress, plant cells can respond by modifying their cell walls to restrict the pathogen. Mechanisms include increasing the mechanical strength of cell walls through lignification, suberization and deposition of hydroxyproline-rich glycoproteins (HRGPs), glycine-rich proteins (GRP) and increase in phenolic compounds (Nicholson and Hammerschmidt, 1992; Agrios, 1997) and/or increasing the resistance of the cell wall to pathogen derived hydrolytic enzymes. As many postharvest pathogens need to penetrate pre-existing differentiated peel or skin, these responses are often critical in limiting disease incidence. Charles *et al.* (1999) reported that UV-C treatment or inoculation of tomato fruit with *B. cinerea* lead to the biochemical reinforcement of the cell walls through lignification and suberization. These induced responses led to increased NDR. Similarly, Ray and Hammerschmidt (1998) found that inoculation of potato tubers (*Solanum tuberosum*) with *Fusarium*

sambucinum resulted in an increase in phenolic acids. These phenolic acids were converted to lignin or cross linked with cell walls. However, microscopy indicated that this induced physical barrier was repeatedly circumvented by *F. sambucinum*. This suggests that either lignin deposition is not an effective defence against dry rot or that its induction is too slow to restrict pathogen growth (Ray and Hammerschmidt, 1998).

2.1.1.2 *Induced chemical defence*

Following the early work by Müller and Börger (1940) and Cruickshank and Perrin (1960), more than 300 chemicals with phytoalexin-like properties have been isolated from 30 plants families (Harborne, 1999). Phytoalexins are chemically diverse and include phenylpropanoid derivatives, alkaloids and terpenes. Certain classes of phytoalexin-like compounds are known to be common in certain genera (Harborne, 1999). For example, terpenoid phytoalexins predominate in the Solanaceae, isoflavonoids in the Leguminosae and sulfur containing phytoalexins in the Cruciferea (Bailey and Mansfield, 1982; Pedras *et al.*, 2000; Grayer and Kokubun, 2001). However, some relationships between family and phytoalexin class are not always as clear-cut (Hammerschmidt, 1999a; Harborne, 1999).

Phytoalexins are antifungal non-protein compounds of low molecular weight (< 1000 Da) that accumulate locally in plants as a result of elicitation through infection or stress (Kuć, 1995; Grayer and Kokubun, 2001). Phytoalexin production is an integral part of the HR. Resistance only occurs when one or more phytoalexins reach a threshold concentration at a sufficiently rapid rate to restrict pathogen development. In most cases, phytoalexin accumulation suppresses disease rather than prevents it.

Because most phytoalexin research (Mansfield 1983; Harborne, 1999) has not been carried out on stored products it has little relevance to postharvest pathology (Mercier 1997). However, an increasing body of work has demonstrated that phytoalexin accumulation is correlated with NDR of horticultural produce (Table 2.2) (Grayer and Harborne, 1994; Mercier, 1997). However. little work has examined how phytoalexins change during storage and/or according to developmental age (Mercier, 1997). Most of this limited work has been conducted on fruit, vegetables and bulbs.

In contrast, no work has specifically looked at the role of phytoalexins in NDR of cut flowers.

Induced NDR is determined by the speed, magnitude and timing of defence response, as influenced by the environment (Kuć, 1991). During ripening and storage, the rate and potential of *de-novo* phytoalexin accumulation declines (Mercier, 1997). The rate of decline will depend on the storage regime employed, product maturity and cultivar. For example, the level of production of hydroxyanigorufone, a phytoalexin, in ripe banana fruit (*Musa* sp.) treated by wounding and/or inoculation with conidia of *Colletotrichum musae* was much lower than in unripe fruit (Kamo *et al.*, 2000; 2001). Thus, the accumulation of hydroxyanigorufone decreased, after its transient maximum, on ripening by exposure of the wounded fruit to ethylene (Kamo *et al.*, 2000). A drop in the capability of carrot (*Daucus carota*) and apple (*Malus domestica*) to produce phytoalexins after inoculation was also correlated with a decline in NDR (Goodliffe and Heale, 1978; Noble and Drysdale, 1983). The same pattern of reduction in phytoalexin potential was seen in stored carrot treated with the physical elicitor UV-C light, with concomitant decrease in NDR to *B. cinerea* (Mercier *et al.*, 1993a; b). Akazawa and Wada (1961) and Minamikawa *et al.* (1963) demonstrated that resistant cultivars of sweet potato (*Ipomoea batatas*) accumulated higher levels of phytoalexins after inoculation with *Ceratocystic fimbriata* compared to susceptible cultivars. This suggests that a reduction in phytoalexin accumulation can determine whether a product is susceptible or resistant.

Phytoalexin accumulation has also been reported to decline with fruit development before storage (Creasy and Coffee, 1988; Bais *et al.*, 2000; Adikaram *et al.*, 2002). In grapes, skins of berries after set, but before maturation, had a high concentration of the phytoalexin stilbene following elicitor treatment (Creasy and Coffee, 1988). In contrast, buds, flowers and mature fruit accumulated lower stilbene concentrations. The observed reductions in phytoalexin production potential, that is, the amount of phytoalexin that can be accumulated in tissue following elicitor treatment during storage, further supports the importance of phytoalexins in specific plant-pathogen interactions in stored products (Mercier, 1997).

Table 2.2: Examples of phytoalexins associated with postharvest disease resistance of horticultural produce

Species	Tissue	Compound(s)	Chemical class	Inhibited pathogen	Reference
Allium cepa (onion)	bulb	5-octyl-cyclopenta5-1,3-dione ; hexylcyclopenta-1,3-dione	cyclic dione	*Botrytis cinerea*	Dmitriev *et al.*, 1990; Tverskoy *et al.*, 1991
Apium graveolens (celery)	stalk	psoralen; angelicin; bergapten (5-methoxypsoralen); xanthotoxin (8-methoxypsoralen); isopimpinellin	furanocoumarins	*Sclerotinia sclerotiorum; Erwinia carotovara pv. carotovara*	Chaudhary *et al.*, 1985; Surico *et al.*, 1987; Afek *et al.*, 1992; 1995; Harborne, 1999
Brassica oleracea (cabbage)	head	4-methoxybrassimin	sulphur containing indole	Not in abstract	Monde *et al.*, 1990
Capsicum annum (bell pepper)	immature fruit sections	capsicannol	complex of sesquiterpenes	*Glomerella cingulata* *Cladosporium cladosporioides* *B. cinerea*	Stoessl *et al.*, 1972; Adikaram *et al.*, 1982; 1988
Carica papaya (papaya)	fruit slices without peel	danielone (3,5-dimethoxy-4-hydroxy-(2-hydroxy)acetophenone)	hydroxyacetophenone	*Colletotrichum gleosporioides*	Echeverri *et al.*, 1997
Cichorium intybus (chicory)	whole	cichoralexin	sesquiterpene lactone (guaianolide)	Not in abstract	Monde *et al.*, 1990
Citrus aurantiifolia (lime)	flavedo	scoparone (6,7-methoxycoumarin)	coumarin	*Penicillium digitatum*	Ben-Yehoshua *et al.*, 1992
Citrus limon (lemon)	flavedo	3-[4-hydroxy-3-(methyl-2-butenyl) phenyl]-2-(E)-propenal; scoparone; aesculetin 6,7-dimethyl ether;	coumarins	*P. digitatum*	Ben-Yehoshua *et al.*, 1992; Kim *et al.*, 1991; Stange *et al.*, 1993;

Species	Tissue	Compound(s)	Chemical class	Inhibited pathogen	Reference
Citrus limon (lemon) continued	flavedo	xanthyletin;	furanocoumarin	*P. digitatum*	Khan *et al.*, 1985
		4-(3 methyl-2-butenoxy) isonitroactofenone	hydroxyacetophenone		Dubery *et al.*, 1988; 1999
		xanthoxylin (phloracetophenone 4,6-dimethyl ether)			Afek and Sztejmberg, 1988
Citrus paradisi (grapefruit)	flavedo	scoparone;	coumarins	*P. digitatum;*	Ben-Yehoshua *et al.*, 1992;
	albedo of immature fruit	umbelliferone (7-hydroxycoumarin)		*B. cinerea; Alternaria alternata*	Afek *et al.*, 1999
Citrus sinensis (orange)	flavedo	scoparone	coumarin	*P. digitatum; P. italicum; C. gleosporioides;*	Tatum and Berry, 1977; Ben-Yehoshua *et al.*, 1992; Stange *et al.*, 1993
				Phytophthora gummosis	Afek *et al.*, 1986
Cucurbita maxima (squash)	mature whole fruit	trans-p-coumaryl aldehyde	coumarin	*Cladosporium cucumerinum*	Stange *et al.*, 1999
Daucus carota (carrot)	mature whole and sliced	8-methoxypsoralen; 5- methoxypsoralen 6-methoxymellein, p-hydroxybenzoic acid; 6 hydroxymellein; euginin; scopoletin;	furanocoumarins; isocoumarin;	*B. cinerea; Mycocentrospora acerina; S. sclerotiorum*	Ceska *et al.*, 1985 Coxon *et al.*, 1973; Goodliffe and Heale, 1978; Davis and Lewis, 1981; Harding and Heale, 1980;
		falcarinol	polyacetylene		
Dianthus caryophyllus (carnation)	leafy stems and roots	anthranilic acid derivatives; dianthalexins; dianthramides; 3-hydro-xyacetophenone;	nitrogen containing phytoalexins; flavanol	*Fusarium oxysporum* f. sp. *dianthi*	Niemann *et al.*, 1992; Niemann, 1993; Curir *et al.*, 1996

Species	Tissue	Compound(s)	Chemical class	Inhibited pathogen	Reference
Fortunella margarita (kumquat)	flavedo	scoparone	coumarin	*P. digitatum*	Ben-Yehoshua *et al.*, 1992; Kim *et al.*, 1991
Fragaria ananassa (strawberry)	green fruit	euscaphic acid; tormentic acid; myrianthic acid	triterpenes	*Colletotrichum musae*	Hirai *et al.* (2000)
Helianthus annuus (sunflower)	stems	ayapin; scopoletin	coumarins	*Alternaria helianthi*	Tal and Robeson, 1985
Ipomoea batatas (sweet potato)	periderm	ipomeamarone;	furanoterpene;	*Ceratocystis fimbriata*	Suzuki *et al.*, 1952; Oguni and Uritani 1974; Schneider *et al.*, 1984;
		umbelliferone; scopoletin;	coumarins;		Akazawa and Wada, 1961 Minamikawa *et al.*, 1963
		ipomeamaronol	sesquiterpene		Kato *et al.*, 1971
Lactuca sativa (lettuce)	leaves	costunolide;	sesquiterpene lactone	*B. cinerea*	Takasugi *et al.*, 1985;
		lettucenin A	unsaturated lactone		Bennett *et al.*, 1994
Lilium maximowiczii (edible lily)	bulb scales	yurinelide (3-benzodioxin-1,4-benzodioxin-2(3H)-one derivative);	phenolic;	*Fusarium oxysporum* f.sp. *lilii*	Monde *et al.*, 1992
		2,4,6-trichloro-3-hydroxy-5-methoxytoluene; 2,6-dichloro-3,5,dimethoxytoluene; 2,4-dichloro-6-hydroxy-3,5-dimethoxytoluene; 2,4-dichloro-5,6-dihydroxy-3-methoxytoluene; 4-chloro-2,5-dihydroxy-3-methoxytoluene; 4-chloro-3,5-dihydroxytoluene; 2-chloro-3,5-dihydroxytoluene; orcinol	organochlorine orcinol derivatives		Monde *et al.*, 1998

Species	Tissue	Compound(s)	Chemical class	Inhibited pathogen	Reference
Lycopersicon esculentum (tomato)	green fruit	rishitin falcarindol; cis-tetradeca-6-ene-1,3-diyne-5,8-diol	sesquiterpene; polyacetylenes	*Fulva fulva* *C. cucmerinum*	De Witt and Flack, 1979; De Witt and Kodde, 1981
Malus domestica (apple)	peel	benzoic acid	phenolic	*Sclerotinia fructigena; Nectria galligena; Pezicula malicorticus*	Fawcett and Spencer, 1966; 1967; Brown and Swinburne, 1971; Bykova et al., 1975; Nobel and Dyrsdale, 1983
	cell suspension	biphenyl	phenolic		Borejsza-Wysocki et al., 1999
Mangifera indica (mango)	peel of unripe fruit	5,12-cis heptadecenyl resorcinol; 5-pentadecyl resorcinol; 5(7,12-heptadecadienyl) resorcinol	alkylated phenol	*Alternaria alternata*	Droby *et al.*, 1987; Kobiler *et al.*, 1998
Musa (banana)	peel of unripe fruit	2-(4'-hydroxyphenyl)-naphthalic anhydride; methyl 2-benzimidazole carbamate;	phenalenone –type	*C. musae*	Brown and Swinburne, 1980; Hirai *et al.*, 1994 Luis *et al.*, 1993;
		irenolone; emenolone; musanolones C-F		*Mycosphaerella fijiensis; Phyllosticta musarum*	Abayasekara *et al.*, 1998;
		other phenylphenalenones (e.g. hydroxyanigorufone)		*C. musae*	Kamo *et al.*, 2000; 2001
Narcissus pseudonarcissus (daffodil)	bulb	7-hydroxyflavan; 7,4'-dihydroxyflvan; 7,4'-dihydroxy-8-methylflavan	flavans	*B. cinerea*	Coxon *et al.*, 1980

Species	Tissue	Compound(s)	Chemical class	Inhibited pathogen	Reference
Pastinaca sativa (parsnip)	whole	xanthotoxin; psoralen; bergapten (5-methoxypsoralen); xanthotoxin (8-methoxypsoralen); isopimpinellin	furanocoumarins	*Phoma complanata*	Cerkauskas and Chiba, 1990
Phaseolus vulgaris (bean)	pod tissue	phaseollin	pterocarpan	*Colletotrichum lindemuthianum; Monilinia fructicola*	Cruickshank and Smith, 1988
Prunus persica (nectarine)	peel of unripe fruits	1β,2α,3α,24-tetrahydroxyurs-12-en-28-oic acid; 1β,2α,3α,24-tetrahydroxyolean-12-en-28-oic acid; 2α,3α,24-trihydroxyolean-12-en-28-oic acid; 2α,3α,24-trihydroxyurs-12-en-28-oic acid; 2α,3β,24-trihydroxyolean-12-en-28-oic acid; 2α,3α,-dihydroxyolean-12en-28-oic acid; 2α,3α,-dihydroxyurs-12-en-28-oic acid	triterpenes	*C. musae*	El Lahlou *et al.*, 1999
Solanum melongena (aubergine)	whole fruit	lubumin; aubergenone; 9-oxonerolidol; 9-hydroxynerolidol, 11-hydroxy-9,10-dehydronerolidol;	sesquiterpenes	*B. cinerea; Fusarium oxysporum*	Ward *et al.*, 1975
		falcarindiol (cis-pentadeca-6-ene-1,3-dyn-5,15-diol)	polyacetylenes		Harborne, 1999
Solanum tuberosum (potato)	periderm	rishitin; lubimin; solavetivone; phytuberin; phytuberol; anhydro-β-rotunol; isofraxidin + 14 other compounds	sesquiterpenes coumarins	*E. carotovora; Phytophthora infestans; Phoma exigua*	Lyon and Bayliss, (1975); Sato *et al.*, 1971; Varns *et al.*, 1971; Malmberg and Theander, 1980; Ghanekar *et al.*, 1984; Engstrom *et al.*, 1999; Ampomah and Friend, 1988
	tuber disc	chlorogenic; p-coumaric; ferulic acids	insoluble phenolics		
Vitis vinifera (grape)	grape skin	resveratol (3,5,4'-trihydroxystilbene)	stilbene	*B. cinerea*	Langcake 1981; Creasy and Coffee, 1988; Jeandet *et al.*, 1991

Although phytoalexin levels have been correlated with NDR *in-vitro,* few studies have been able to demonstrate conclusive evidence of their importance *in-planta.* This is especially true in horticultural produce (Prusky, 1998). Reasons for this lack of direct evidence include the difficulties in accurately assessing the quantities of antifungal compound that may be in direct contact with the pathogen, determining the *in-planta* activity of the compound and, relating temporal changes in phytoalexin concentration to NDR. Questions also arise over whether phytoalexins accumulate before cell death or as a consequence of cell death (Nicholson and Hammerschmidt, 1992; Hammerschmidt, 1999a).

2.2.3 Systemic acquired resistance

Systemic acquired resistance (SAR), like the HR and LAR, is an inducible plant defence response (Ross, 1961a; b). SAR is loosely analogous to immunisation in animals (Chester, 1933), where a prior pathogen infection or elicitor treatment activates long lasting non-specific resistance in uninfected tissue (Kuć, 1982; Ryals *et al.*, 1996; Schneider *et al.*, 1996; Sticher *et al.*, 1997). This broad spectrum resistance occurs throughout the whole plant and is independent of resistance genes (Lawton *et al.*, 1995). SAR is characterised by a reduction in subsequent infection by an avirulent pathogen and so acts to prime host cells for a more rapid deployment of plant defences (Kessman *et al.*, 1994; Katz *et al.*, 1998). At the molecular level, SAR is characterised by the expression and accumulation of pathogenesis-related proteins (PRPs).

The accumulation of salicylic acid (SA) is thought to play a major role in both the initiation and establishment of SAR (Dempsey *et al.*, 1999). SA levels increase on treatment with elicitors of SAR. In tobacco (*Nicotiana tabacum*) and cucumber (*Cucumis sativa*), SA levels increased several hundred fold after treatment with an inducing pathogen (Malamy *et al.* 1990; Métraux *et al.*, 1990; Rasmussen *et al.*, 1991). This increase was correlated with SAR against Tobacco Mosaic Virus (TMV) in tobacco (Rasmussen *et al.*, 1991). Gaffney *et al.* (1993) argued that if an endogenous increase in SA is required for SAR establishment then a barrier to SA accumulation should block the development of SAR. Gaffney *et al.* (1993) transformed tobacco with the nahG gene in *Pseudomonas putida* which encodes salicylate hydroxylase (SAH). The SAH enzyme catalyses the conversion of SA to

catechol. After subsequent TMV inoculation the nahG mutants accumulated little or no SA and were defective in their ability to induce SAR against TMV. This example using SAR-compromised mutants provides compelling evidence that SA is required for SAR. Work using *in-vivo* SA ^{14}C labelling suggests that SA produced in the TMV-infected leaves of tobacco is translocated throughout the plant (Yalpani *et al.*, 1991). This transport has also been observed in cucumber (Métraux *et al.*, 1990). Thus, SA may be the signal molecule that translocates from an infection site to activate SAR elsewhere in the plant. However, while it seems likely that SA is essential for SAR signal transduction (Ryals *et al.* 1996), it is probably not the primary translocated signal that triggers SAR in distal plant organs (Willits and Ryals, 1998). Grafting experiments using wild-type rootstock challenged with TMV provide evidence that the induction of SAR is SA dependent (Vernooji *et al.*, 1994). SAR was found to be induced in wild-type scions but not in nahG scions. This indicates that SA is required downstream of a currently unknown long distance signal (Ryals *et al.*, 1996).

SAR is a potentially desirable strategy in achieving integrated pest management (IPM) (Lucas, 1999; Lawton *et al.*, 2001). However, there has been little research into controlling postharvest diseases through eliciting SAR (Joyce and Johnson, 1999). Biotically-induced SAR has been reported in stored carrot roots pre-inoculated with *B. cinerea* (Mercier *et al.*, 2000) and avocado (*Persea americana*) pre-inoculated with a non-pathogenic *Colletotrichum magna* strain (Prusky *et al.*, 1994). Huang *et al.* (2000) demonstrated that a pre-harvest foliar spray of the SAR activator acibenzolar substantially decrease disease incidence of stored rock and hami melons (*Cucumis melo*).

2.3 Biochemical Components of NDR in Horticultural Crops

As described above, NDR is manifest as physical and biochemical barriers that may be preformed or inducible upon infection. Thus, NDR is determined in part by the presence of antifungal carbon-based secondary antifungal metabolites and the level and toxicity of those metabolites present. Key metabolic enzymes control the biochemical pathways that produce these compounds. The *de-novo* synthesis of these enzymes is a function of ontogenesis and the environment.

2.3.1 Metabolic pathways

Secondary antifungal metabolites can be divided into three general groups according to their structure and biosynthesis; these are phenolics, terpenes and nitrogen containing compounds (Grayer and Harborne, 1994). Three secondary metabolic pathways are involved in the production of these non-protein structural and antifungal compounds. In order of importance, these are the shikimate, mevalonic and malonic pathways. Antifungal metabolites are derived from either the allocation of carbon to the pentose phosphate pathway or acetyl CoA (Fig. 2.1). Nitrogen-containing compounds, such as alkaloids and toxic proteins, are primarily synthesised from amino acids (Fig. 2.1).

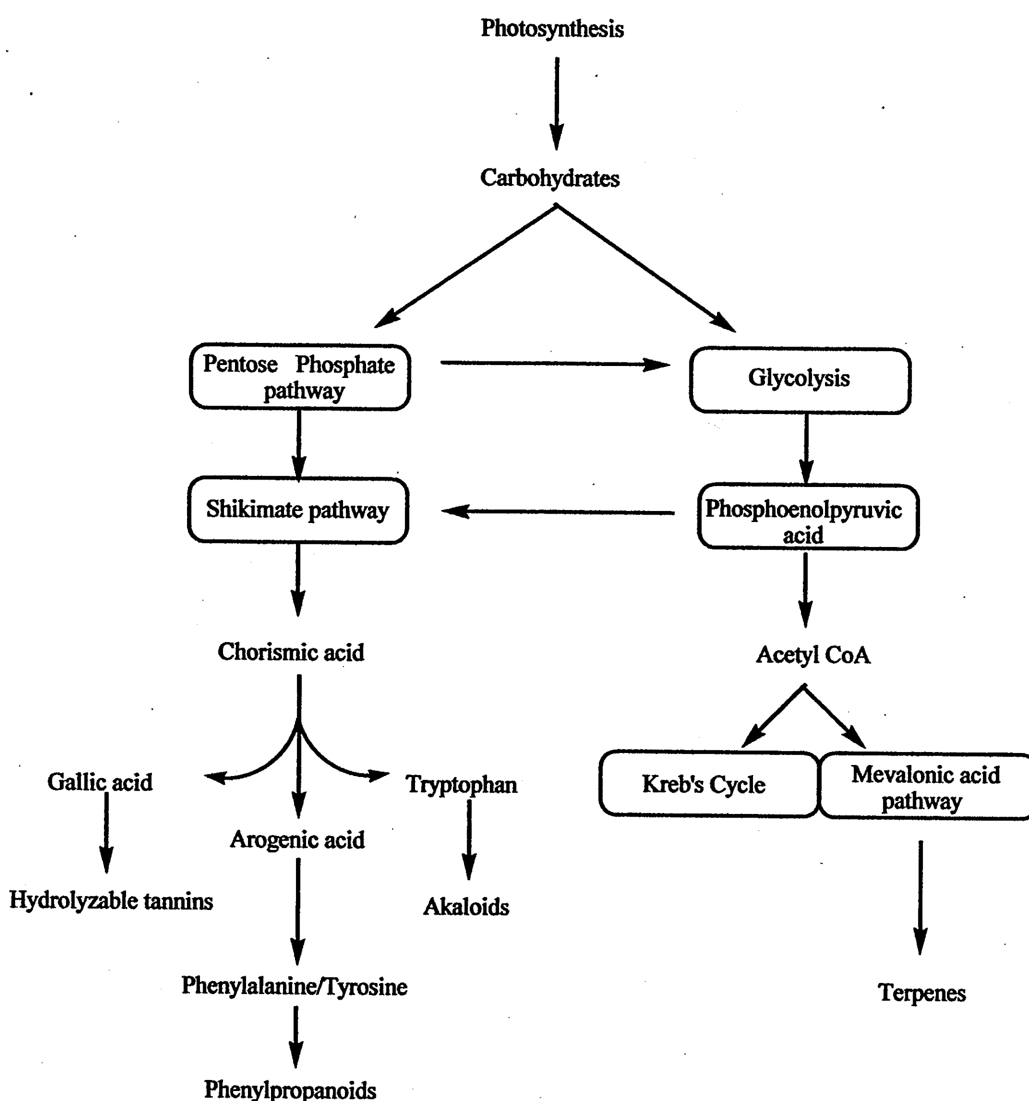


Figure 2.1: General pathways of carbon based secondary compound synthesis. The arrows indicate pathways and not necessarily single reactions

The phenylpropanoid pathway, derived from the shikimate pathway, leads to the majority of defence–related compounds in plants, including the aromatic phenolics, and condensed tannins. Phenylalanine ammonia-lyase (PAL), the branch-point enzyme between primary and secondary metabolism is the key regulatory enzyme of the phenylpropanoid pathway. PAL catalyses the conversion of phenylalanine into cinnamic acid from which all phenylpropanoids are derived downstream (Fig. 2.2). Many of these phenylpropanoids are important in pathogen growth restriction (Benhamou and Nicole, 1999).

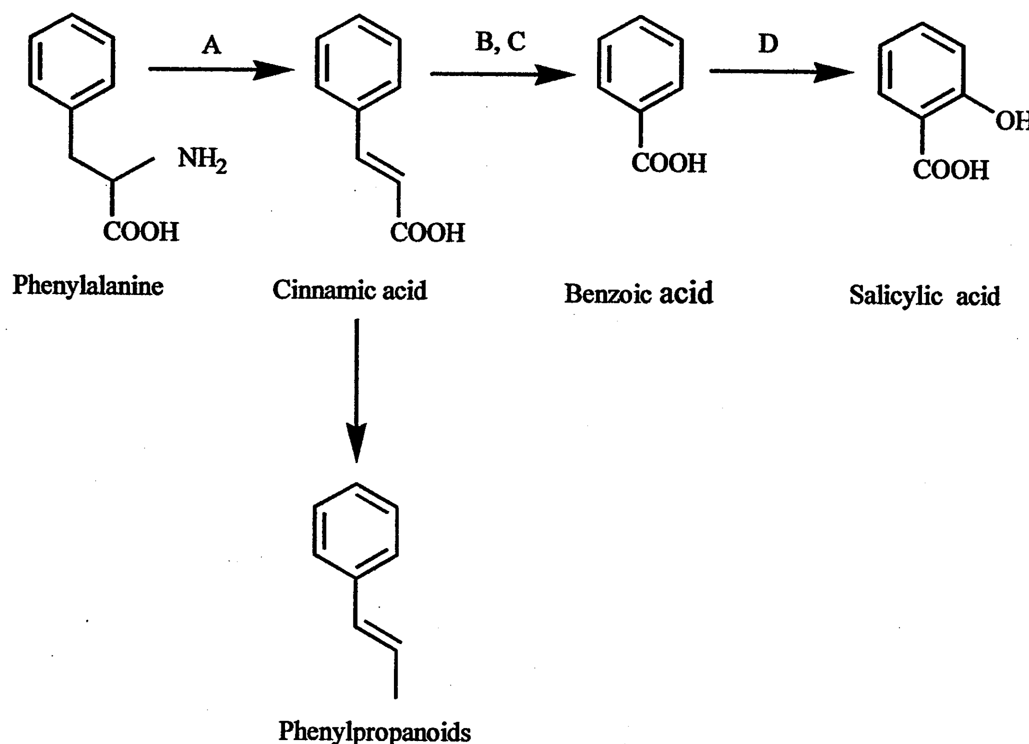


Figure 2.2: A simplified view of the proposed biosynthesis of salicylic acid in plants from phenylalanine (adapted from Dixon and Paiva (1995)). A = phenylalanine ammonia-lyase, B = cinnamyl CoA ligase, C = β oxidation of cinnamyl CoA to benzoic acid, D = benzoic acid-2-hydroxylase.

2.3.2: Non-proteinaceous defence compounds

2.3.2.1 *Phenolics and other aromatic compounds*

Phenolics are a heterogeneous group of secondary metabolites that contain a phenol group; that is, a hydroxyl function on an aromatic ring (Fig. 2.3). In keeping with their chemical and functional diversity phenolics are derived either from the shikimate

and/or malonic acid pathways (Van Sumere, 1989). The majority of defence related phenolics are phenylpropanoids (Paiva, 2000).

Antifungal phenolics have been found in all plants examined to date (Nicholson and Hammerschmidt, 1992). Plant phenolics are active against pathogens (Nemec, 1976; Jersch *et al.*, 1989; Wang *et al.*, 1989). For example, phenolics are involved in inhibition of activities of extracellular fungal enzymes (e.g. cellulase, pectase, laccase, xylanase), substrate nutrient deprivation (e.g. metal complexation, protein insolubilization), antioxidant activity, and direct action on fungal membranes (e.g. inhibition of oxidative phosphorylation) (MacRae and Towers, 1984; Wang *et al.*, 1989; Scalbert, 1991; Elad, 1992).

Specific preformed and inducible phenolics are integral components in NDR of horticultural produce (Tables 2.1 and 2.2). Lattanzio *et al.* (1994) reported that many common phenolics found in fruit and vegetables such as benzoic acid derivatives (Fig. 2.3), cinnamic acid derivatives and flavanoids, demonstrate *in-vitro* antifungal activity against common storage pathogens, such as *B. cinerea*, *P. digitatum* and *Alternaria* spp. Nyerges *et al.* (1975) showed that some polyphenols commonly found in grape berries such as tannic acid, chlorogenic acid and cinnamic acid, inhibit *in-vitro* radial growth of *Botrytis* spp. Boonyakiat *et al.* (1986) reported that that both chlorogenic acid (0.5 mg ml^{-1}) and arbutin (0.5 mg ml^{-1}) reduced *in-vitro* spore germination of *Penicillium expansum* and mycelial growth of *Mucor piriformis*. Both *P. expansum* and *M. piriformis* are two common storage pathogens of pear (*Pyrus communis*). Both chlorogenic acid and arbutin are major phenolics found in both the peel and flesh of pear cv. d'Anjou fruit (Ranadive and Haard, 1971; Boonyakiat *et al.*, 1986). However, in contrast to Nyerges *et al.* (1975), chlorogenic acid increased both germination and *in-vitro* mycelial growth of *B. cinerea* (Boonyakiat *et al.*, 1986). Ndubizu (1976) on the other hand, reported that chlorogenic acid was inhibitory to germination *B. cinerea* and mycelial growth of *B. cinerea*, *P. expansum* and *Alternaria* sp. The concentrations of both chlorogenic acid and another antifungal phenolic acid, p-coumaryl-quinic acid, found in apple cv. Red Delicious fruit decreased with fruit development (Ndubizu, 1976). This decrease coincided with decline in NDR to *B. cinerea.*

Early research demonstrated that catechin and protocatechuic acid are preformed antifungal compounds which diffuse onto the surfaces of coloured dried scales of coloured onion bulbs (*Allium cepa*). These water soluble phenolics are inhibitory to *Colletotrichum circinans*, the causal agent of onion smudge (Walker, 1925; Angell *et al.*, 1930; Walker and Stahmann, 1955). These phenolic compounds were absent or in low concentration in susceptible white skinned cultivars. More recent work has shown that the phenolic acids, catechin, epicatechin and gallic acid (Fig. 2.3), are preformed antifungal compounds in strawberry fruit (Herbert *et al.*, 2001) that decline in concentration during fruit development. This decline is associated with increased susceptibility to *B. cinerea* in mature fruit. Similarly, the decline in concentration of chlorogenic acid and caffeic acid in peach fruit epidermis during ripening has been correlated with lower NDR against brown rot (*Monilinia fructicola*) (Bostock *et al.*, 1999). However, Bostock *et al.* (1999) suggested that the correlation between NDR and high phenolic concentrations in both immature fruit and highly resistant cultivars was not a result of direct toxicity to the pathogen but rather interference with pathogen-derived cuticular degradation (Bostock *et al.*, 1996; 1999).

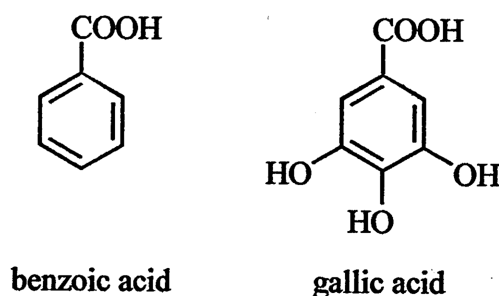


Figure 2.3: Two phenolic acids, benzoic and gallic acid, implicated in the natural disease resistance of apples and strawberries, respectively (Noble and Drysdale, 1983; Herbert *et al.*, 2001)

Commonality in host defence also exists between pathogens and insects (Coley, 1986) as concentration of foliar catechol-based phenolic compounds was found to be negatively correlated with development of two spotted spider mite in strawberry cv. Totem plants (Luczynski *et al.*, 1990).

In addition to phenolic acids, phenylpropanoid acids have been identified as preformed antifungal compounds in a variety of plant species. However, few

examples have been identified as important in postharvest host-pathogen interactions. One exception is isochlorogenic acid, which is believed to confer resistance against *Rhizopus stolonifer* in sweet potato periderm tissue (Stange *et al.*, 2001). Little information exists on precisely how phenylpropanoid acids inhibit pathogens, but it is believed that the aromatic rather than the carboxyl group is essential for antifungal activity as these acids are still antifungal in their esterified form (Grayer and Harborne, 1994). In support of this assertion, aromatics without carboxyl groups have been reported as antifungal components in horticultural produce, including ginger and mango (Endo *et al.*, 1990; Koblier *et al.*, 1998).

The levels of resorcinols (alkylated phenols; Fig. 2.4) found in unripe mango (*Mangifera indica*) peel (154 - 232 µg ml^{-1} FW) decline after harvest and during ripening (Cojocaru *et al.*, 1986; Droby *et al.*, 1986). *In-vitro* tests showed that the concentration of the 5-substituted resorcinols mixture becomes non-toxic to common mango storage pathogens at the time of initiation of the black spot disease (*Alternaria alternata*) development (Kobiler *et al.*, 1998). Mango cv. Tommy Atkins fruit are more susceptible to decay than cv. Hayden and show a faster decrease in concentration of antifungal compounds during ripening (Droby *et al.*, 1986). However, as with most host-pathogen systems, evidence on the specific role of these antifungal compounds in NDR is only through correlation. Given the astringency of unripe mango fruit it might be expected that peel tissue may contain considerable amounts of proanthocyanins, which may either inhibit pathogen derived extracellular hydrolases or affect the pathogen directly (Schlösser, 1994).

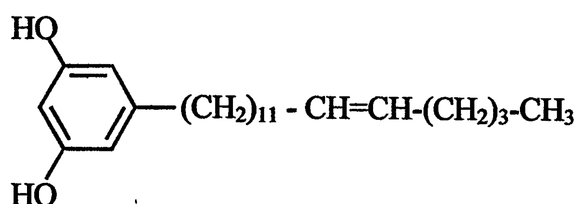


Figure 2.4: Structure of dominant resorcinol from mango peel: 5-(12-heptadecenyl) resorcinol implicated in natural disease resistance of mango (after Kobiler *et al.*, 1998)

Other antifungal compounds derived from the phenylpropanoid pathway include the coumarins and furanocoumarins, which are defined by possessing a phenylpropanoid

nucleus (Fig. 2.5). Coumarins are structurally regarded as lactone derivatives of *o*-hydroxy-cinnamic acid (Ibrahim and Barron, 1989). Many coumarins have been reported to play a role in plant protection against pathogens, both as preformed antifungal compounds and phytoalexins. In this sense they differ from phenolic and phenylpropanoid acids in that many of coumarins are inducible. Many preformed and inducible antifungal coumarins and furanocoumarins have been reported in carrot, parsnip, potato, squash tissue and flavedo extracts of citrus spp. (Tables 2.1 and 2.2). For example, levels of phytoalexins, scoparone (6,7-methoxycoumarin) (Fig 2.5) and umbelliferone (7-hydroxycoumarin) induced by abiotic elicitors have been shown to increase NDR after harvest and during prolonged storage (Ben Yehoshua *et al.*, 1992; Afek *et al.*, 1999). Related phytoalexin compounds, like stilbenes and dihydrostilbenes, that are derived from *p*-coumaric acid (hydroxycinnamic acid) and malonyl-CoA sub units, are also important in pathogen restriction in grapes and Chinese yam (Table 2.2). Levels of resveratol (α-viniferin) (Fig. 2.6) found in grape skin have been closely correlated to grey mould disease incidence caused by *B. cinerea* (Creasy and Coffee, 1988). Hain *et al.* (1993) confirmed the importance of resveratol in providing resistance against *B. cinerea*. Stilbene synthase genes from grapevine were transformed into tobacco which thereby conferred greater resistance to infection by *B. cinerea* in regenerated tobacco plants.

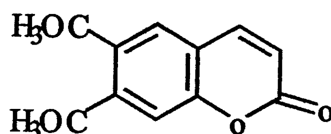


Figure 2.5: Structure of coumarin phytoalexin, scoparone (6,7-dimethoxycoumarin), implicated in the natural disease resistance of citrus spp. (Ben-Yehoshua *et al.*, 1998)

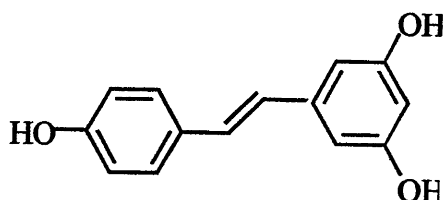


Figure 2.6: Structure of stilbene phytoalexin, trans-resveratrol implicated in the natural disease resistance of grapes (Creasy and Coffee, 1988; Douillet-Breuil, *et al.*, 1999)

2.3.2.2 *Condensed tannins*

Tannins are water soluble polyphenolic compounds derived from the phenylpropanoid pathway that either inactivate pathogenic enzymes produced by some pathogens (Jersch *et al.*, 1989; Schlösser, 1994) or affect pathogens directly (Scalbert, 1991; Herbert *et al.*, 2001). In addition, tannins can inhibit pathogen growth through iron deprivation (Mila and Scalbert, 1994). Tannins also play an important role in insect resistance (Coley, 1986).

Tannins differ from other natural phenolic compounds in their ability to precipitate proteins. Polyphenols, like proanthocyanidins (PA), are found in many fruits, including mango, guava (*Psidium guajara*), persimmon (*Diospyros kaki*), grapes and strawberries (Schlösser, 1994). PAs inhibit cellulases, pectin methylesterases and polygalacturonases of many plant pathogenic fungi (Huth, 1980). For example, PA concentrations in immature grapes and strawberry fruit are sufficiently high to inhibit the extracellular hydrolases of *B. cinerea* (Hills *et al.*, 1981; Jersch *et al.*, 1989). However, with berry development, PAs present become biologically inactive, probably through polymerisation (Schlösser, 1994) and lead to reduced NDR (Jersch *et al.*, 1989). Although stilbene phytoalexins in grapes are active against many fungi, *B. cinerea* can breakdown phytoalexins into non-toxic products by producing hydroxystilbene-degrading enzymes (Pezet *et al.*, 1991; Pezet *et al.*, 1998). However, recent evidence suggests that the concentration of condensed tannins and phenolic acids may inhibit the activity of stilbene oxidase (Goetz *et al.*, 1999). Thus, immature grapes may be relatively more resistant to grey mould due to higher levels of PA which inhibit *B. cinerea* from breaking down stilbene phytoalexins.

2.3.2.3 *Aliphatic compounds*

Aliphatic molecules include all acyclic, cyclic, saturated or unsaturated carbon compounds, excluding the aromatic compounds. As yet, only the polyacetylene falcarinol has been identified in carrot and tomato as an aliphatic phytoalexin in horticultural produce (Table 2.2). A similar polyacetylene falcarindiol has been identified as a phytoalexin in aubergine and a preformed compound in carrot.

The most comprehensively studied case where preformed aliphatic compounds have been shown to influence postharvest NDR is in avocado. The major antifungal compound responsible for this temporal change in NDR is the diene, cis, cis 1-acetoxy-2-hydroxy-4-oxo-heneicosa-12,15-diene. (Prusky *et al.*, 1982). Four other preformed antifungal compounds with weaker activity have been extracted from avocado peel (pericarp) (Sivanathan and Adikaram, 1989; Prusky *et al.*, 1991ac; Adikaram *et al.*, 1992; Adikaram *et al.*, 1993). These long-chain alcohols, some of which are acetylenic, have synergistic antifungal activity with one another (Prusky *et al.*, 1991a; c).

Colletotrichum gleosporioides is the causal agent of anthracnose disease. The pathogen infects unripe avocado fruits preharvest but remains quiescent until after harvest (Prusky and Keen, 1993). C. *gleosporioides* is activated as avocado fruit ripen and NDR declines. This leads to anthracnose disease expression. The concentration of diene in pericarp of immature avocado cv. Fuerte fruit was 1600 $\mu g\ g^{-1}$ fresh weight (FW) (Prusky and Keen, 1993; Prusky *et al.*, 1998). The concentration decreased to 120 $\mu g\ g^{-1}$ FW in ripe avocado fruit. A 10-fold reduction was concomitant to an appreciable fall in NDR. Cultivars that retain higher levels of dienes during ripening have relatively delayed disease expression. Peel extracts of freshly harvested avocado fruits inhibited *in-vitro* growth of *C. gleosporioides* by 78% compared with 7% for peel extract from ripe fruits showing anthracnose disease symptoms (Prusky and Keen, 1993).

Treatment of avocado fruit with α-tocopherol, which inhibits diene oxidation by lipoxygenases (LOX), delayed the appearance of disease symptoms (Prusky *et al.*, 1993). Similarly, preharvest treatment of avocado cv. Fuerte fruits with compounds exhibiting cytokinin-like activity (thidiazuron and benzylaminopurine) reduced anthracnose development after harvest (Beno-Moualem *et al.*, 2001). Treatment of avocado callus tissue originating from the avocado pericarp with thidiazuron enhanced levels of epicatechin and stimulated the phenylpropanoid pathway (Beno-Moualem *et al.*, 2001). Epicatechin is a natural antioxidant present in the avocado pericarp (Prusky and Keen, 1993). Epicatechin is therefore a non-specific inhibitor of LOX and is thought to mediate diene degradation (Prusky *et al.*, 1988; Ardi *et al.*,

1998). During ripening, epicatechin levels fall from around ca. 500 to 8 μg g^{-1} FW (Prusky and Keen, 1993). The decline in epicatechin during ripening is correlated with a decline in NDR (Prusky and Keen, 1993). Treatments such as thidiazuron (Beno-Moualem *et al.*, 2001), carbon dioxide (Prusky *et al.*, 1993b) or ethylene (Leikin-Frenkel and Prusky, 1998) that maintain NDR through delaying senescence and/or induce epicatechin lead to higher concentrations of diene and thus higher NDR. Although, the case for diene influencing NDR in avocado is compelling, concentration of diene in peel tissue during storage have been shown to fluctuate widely with no obvious affect on disease incidence (Prusky *et al.*, 1991b).

Another aliphatic compound that has been implicated in NDR of horticultural produce is ajoene. This preformed unsaturated sulfoxide disulfide was demonstrated to have antifungal activity toward *Aspergillus niger* in garlic (*Allium sativum*) (Yoshida *et al.*, 1987). Similarly, crude extracts of related *Allium* spp. demonstrated *in-vitro* antifungal activity against of *B. cinerea* (Wilson *et al.*, 1997). Early research showed that extracts of garlic could be used as a prophylactic postharvest treatment (Ark and Thompson, 1959).

2.3.2.4 *Terpenes*

Terpenes and the related plant secondary metabolites, such as steroids, are derived from the melavonic pathway (Fig. 2.1). These compounds are important NDR factors in many horticultural crops (Table 2.2). Terpenoids are ultimately derived from either isopentenyl pyrophosphate or its isomer dimethylallyl pyrophosphate (Paiva, 2000). When these C_5 compounds combine, the derivative terpene is a monoterpene (C_{10}). Derivations on the basic 5-carbon structure lead to sesquiterpenes (C_{15}), triterpenes (C_{30}) and polyterpenes $(C_5)n$ (Fig. 2.6).

Terpenes are important in reduction of insect herbivory in plants (Brooks and Watson, 1991). Terpenes also act as phytoalexins in NDR in aubergine (*Solanum melongena*), bell pepper, nectarine, potato, strawberry and tomato (Table 2.2). Sesquiterpene lactones have been reported as phytoalexins in chicory (*Cichorium intybus*) and lettuce (*Lactuca sativa*). A role for terpenes in NDR has been demonstrated in citrus

spp. by Ben-Yehoshua *et al.* (1992). The monterpene aldehyde citral is the major preformed antifungal compounds in citrus spp. active against green mould disease caused by *P. digitatum* (Table 2.1). For example, during prolonged storage of lemon fruit (*Citrus limon*), the decline in citral concentration is in parallel to a reduction in antifungal activity and an associated increase in green mould incidence (Rodov *et al.*, 1995). Rodov *et al.* (1995) found that of the two citral isomers (Fig. 2.7), geranial declined more rapidly during storage than neral (Fig. 2.7). The inherent antifungal activity of flavedo extracts of yellow fruit was almost half that of green fruit viz. 25-38 and 48-57% citral, respectively (Kim *et al.*, 1991; Ben Yehoshua *et al.*, 1998).

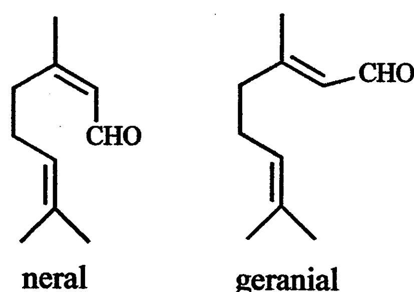


Figure 2.7: Two isomers of the preformed monoterpene aldehyde, citral (3,7-dimethyl-2,6-octadienal), implicated in natural disease resistance of citrus spp. (Ben-Yehoshua *et al.*, 1998)

Synthetic terpenes have also been used as constituents in an antifungal spray treatment for fruit and vegetables (Sardo, 1997). A triterpene-based preformed antifungal compound called cucurbitacin I that was identified in squirting cucumber was found to prevent fungal infection by *B. cinerea* of cucumber fruit and cabbage leaves (*Brassica oleracea*) (Bar-Nun and Meyer, 1990). Suppression of *B. cinerea,* through inhibition of laccase production by the pathogen, was achieved when cucurbitacin I was applied prior to inoculation (Bar-Nun and Meyer, 1990).

2.3.3 Nitrogen containing defence compounds

2.3.3.1 *Alkaloids*

Alkaloids are organic nitrogen-containing compounds derived from one of a few common amino acids, in particular, aspartic acid, tryosine and trytophan. However, much of the carbon skeleton is derived from the melavonic pathway (Fig. 2.1). The

nitrogen atom in alkaloids is usually part of a heterocyclic ring (Paiva, 2000). Only a few reports have implicated specific alkaloids in NDR for horticultural crops (Tables 2.1 and 2.2). However, steroidal glycoalkaloids with antifungal activity are present in the solanaceaous crops tomato and potato. For example, α-tomatine ($C_{50}H_{83}NO_{21}$; lycopersicin) is found in the vacuoles of immature green tomato fruit (Roddick 1974; 1976). It is thought to restrict the development of *B. cinerea* in immature tomato fruit (Defago and Kern, 1988). It is normally not detectable in ripe fruit (Eltayeb and Roddick, 1984). The toxicity of α-tomatine is in part due to its ability to bind to 3β-hydroxy sterols in fungal membranes (Sandrock and VanEtten, 1998). Fungal pathogens of tomato are generally more tolerant of α-tomatine than non-pathogens of tomato (Sandrock and VanEtten, 1998). Tolerance of α-tomatine is thought to arise either from the ability of the pathogen to detoxify this compound or from membrane characteristics (Arneson and Durbin, 1968). However, like many preformed antifungal compounds, the evidence of the importance of α-tomatine as a general barrier to microbial infection has been circumstantial (VanEtten *et al.*, 1994). Despite considerable variation in α-tomatine levels between *Lycopersicon* spp., relationships between preformed antifungal content and NDR are not well documented (Osbourn, 1996). Similar findings have been observed in potato where the concentration of glycoalkaloids in the peel of potato tubers correlated poorly with resistance to wound inoculation with *Fusarium solani* var. *coeruleum* and *Phoma exiguea* var. *foveata* (Olsson, 1987).

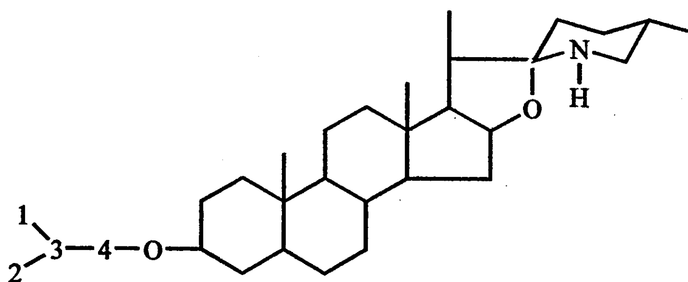


Figure 2.8: Structure of preformed alkaloid, α-tomatine: 1 = *O*-β-D-xylopyranosyl-(1,3 glu), 2 = *O*-β-D-glucopyranosyl-(1,2 glu), 3 = *O*-β-D-glucopyranosyl-(1,4 gal), 4 = β-D-galactopyranosyl, implicated in natural disease resistance of green tomato fruit (after Sandrock and VanEtten, 1998)

2.3.3.2 *Polygalacturonase-inhibiting proteins*

Polygalacturonase-inhibiting proteins (PGIPs) are relatively heat stable extracellular glycoproteins that inhibit fungal polygalacturonase (PG) *in-vitro* by both competitive and non-competitive mechanisms (De Lorenzo *et al.*, 2001). Because PG is thought to be an important component in of a fungal pathogen's infection process, PGIPs are important components of NDR (Labavitch *et al.*, 1998; De Lorenzo *et al.*, 2001). PGIPs are found in horticultural produce including pear (Abu-Goukh *et al.*, 1983a; Stotz *et al.*, 1993), pepper (Brown and Adikaram, 1983), onion (Favaron *et al.*, 1993) raspberry (*Rubus idaeus*) (Johnson *et al.*, 1993), tomato (Stotz *et al.*, 1994) and apple (Yao *et al.*, 1995). According to Labavitch *et al.* (1998), PGIPs may contribute to NDR by either inhibiting PG directly or altering the signals that influence the host response to the pathogen. Thus, if the pathogen relies on PG to digest pectin polymers in the cell wall, the PGIP would be a biochemical barrier to infection.

Falling PGIP levels have been correlated to decreased in NDR in pear (Abu-Goukh *et al.*, 1983b; Elgi, 1987) and raspberry (Johnston *et al.*, 1993; 1994). Pear PGIP shows differential activity against PGs of several fungi *in-vitro* (Abu-Goukh *et al.*, 1983b), being especially effective against *B. cinerea*. Labavitch *et al.* (1998) demonstrated that glasshouse-grown tomato transformed with pear PGIPs were more resistant to *B. cinerea*.

2.3.3.3 *Pathogenesis-related proteins*

Many plants synthesise a group of inhibitory proteins called pathogenesis-related proteins (PRPs) in response to pathogenic attack, mild stress or elicitor treatment (White, 1979; Van Loon, 1997). PRPs are a structurally diverse group of plant proteins that are inhibitory to many invading fungi, bacteria and viruses. Currently, there are 10 recognised groups of PRPs (Van Loon, 1997). These groups are classified according to their function and structure. For example, PR-2, 3 and 5 have been identified as β-1,3-glucanases, chitinases and thaumatine-like (TL) proteins, respectively. Generally, PRPs, like chitinase and β-1,3-glucanases, play a role in limiting the spread of infection by catalysing the hydrolysis of the main components

of fungal cell walls. PRPs are predominately localised in the intercellular spaces and appear to be resistant against proteolytic enzymes (Van Loon, 1997).

Most research on PRPs has concentrated on their role in preharvest disease resistance. However, chitinases and β-1,3-glucanase have been identified in roots, leaves and flavedo tissue of grapefruit cv. Marsh (*Citrus paradisi*) and in the leaves and blossoms of navel oranges (*Citrus sinesis*) (Niedz *et al.*, 1994). Niedz *et al.* (1994) demonstrated that citrus PRPs exert a direct *in-vitro* antifungal activity against *P. digitatum* and *Phoma tracheiphila*. Recently a hot water treatment at 62°C for 20 seconds that induced resistance against green mould in red grapefruit cv. Star Ruby was correlated to the accumulation of PRPs in flavedo tissue (Pavoncello *et al.*, 2001). Rodov *et al.* (1996) also demonstrated that PRPs accumulated in citrus albedo tissue following pathogen inoculation and heat treatment. In both studies, these PRPs were identified as chitinase and β-1,3-glucanase. Similarly, in non-inoculated and non-heat treated Valencia oranges, two polypeptides were found (Ben-Yehoshua *et al.*, 1998). These constitutive PRPs were tentatively identified as chitinases as they reacted positively to antibodies specific to PRP chitinase from citrus callus cultures. During storage these polypeptides tended to decline. However, in contrast to Pavoncello *et al.* (2001) and Rodov *et al.* (1996) heat treatment accelerated this decline. Moreover, inoculation with *P. digitatum* caused the rapid disappearance of chitinase polypeptides from albedo tissue within one day of inoculation. However, accumulation of one of these polypeptides (27 kDa) was restored by heat treatment (36°C for 72 h) of inoculated fruit and caused pathogen resistant areas on the albedo (Ben-Yehoshua *et al.*, 1998). Subsequent storage at 20°C of inoculated plus heat treated fruit resulted in far greater chitinase accumulation than in non-elicited fruit after 4 and 11 days. In addition, injection with chitinase fragments into albedo of Valencia oranges lead to a linear increase in the phytoalexin, scoparone (Ben-Yehoshua *et al.*, 1998). Ben-Yehoshua *et al.* (1998) argue that elicitation of scoparone by exogenous chitinase treatment may point towards a relationship between chitinase and phytoalexin accumulation in terms of enhancing NDR.

2.4 Exploitation of NDR

Information concerning preformed and inducible defences may be important in enhancing or boosting NDR in horticultural crops (Adikaram, 1990; Wilson *et al.*, 1994; Joyce *et al.*, 1998; Joyce and Johnson, 1999). Given the growing public concern over the continued use of traditional chemical fungicides, the introduction of integrated pest management (IPM) for plant protection is widely favoured. Enhanced protection through induced/acquired resistance of the host during periods of susceptibility is considered as being one of the preferred strategies in achieving IPM (Kuć, 1987; Tuzun and Kuć, 1991; Benhamou, 1996; Lucas, 1999).

2.4.1 Elicitation of NDR

Elicitors may be biological, chemical or physical and may induce local acquired resistance (LAR) or systemic acquired resistance (SAR). Luckey (1980) defined the concept of inducing natural disease resistance as 'plant hormesis'. Hormesis involves a stimulation of a beneficial plant response by low or sub-lethal doses of an agent, such as a chemical inducer or a physical stress (Luckey, 1980). The importance of induced or acquired resistance in plants has long been documented (Chester, 1933). However, only recently has the potential of utilising such plant responses within plant protection been recognised (Adikaram, 1990; Wilson *et al.*, 1994; Lucas, 1999). Several elicitors applied pre- and/or postharvest have been shown to enhance NDR in a number of horticultural crops. Enhancement of constitutive and inducible compounds through the use of elicitors has been demonstrated in peel of avocado and mango fruit (Table 2.3).

2.4.2 Elicitors of SAR and induced resistance

2.4.2.1 *Chemically induced resistance*

Various natural and synthetic substances can activate SAR in horticultural produce. Most research on chemical activators has concentrated on preharvest diseases (Table 2.3). However, few compounds demonstrated true resistance inducing activity when

applied as a prophylactic treatment (Métraux *et al.*, 1990). A true chemical activator should modify the plant-pathogen interaction so that it phenotypically resembles an incompatible interaction, which includes defence-related mechanisms induced prior to or after challenge (Sticher *et al.*, 1997). Chemical elicitors can be divided into three categories depending on their chemical nature (Sticher *et al.*, 1997). These include natural organic, inorganic and synthetic elicitors. Many natural and synthetic elicitors not only have structural similarity to each other but also to the natural stress metabolites nicotinamide and trigonelline (Schneider *et al.*, 1996) (Fig 2.9).

Natural organic elicitors

- Salicylates

Salicylic acid (SA), is a well known natural inducer of disease resistance in plants (Fig. 2.9). White (1979) was the first to observe that treatment of tobacco plants with SA increased PRPs and reduced disease symptoms caused by tobacco mosaic virus (TMV). Van Loon and Antoniw (1982) confirmed these findings and, also demonstrated that exogenous application of SA to tobacco leaves mimics the pathogen-induced SAR response by inducing the same set of SAR genes (Van Loon, 1983). Similarly, PRPs were found to accumulate in grapevines after SA treatment and/or *B. cinerea* infection (Renault *et al.*, 1996). The signalling mechanism by which SA induces SAR is as yet unknown (Ryals *et al.*, 1996).

Preharvest and/or postharvest applications of SA (2.0 mg ml^{-1}) tended to suppress *C. gleosporioides* in mango cv. Kensington Pride fruit (Zanuri *et al.*, 2001) and *Alternaria sp. and Epicoccum sp.* in Geraldton waxflower cv. CWA Pink (*Chamelaucium uncinatum*) flowers (Beasley *et al.*, 1999), respectively. However, in both cases it was not clear whether SAR was induced. Zanuri *et al.* (2001) attributed the effects of SA to inhibition of mango skin ripening possibility through an anti-ethylene affect, as seen in banana (Srivastava and Dwivedi, 2000). An ethylene-suppression role may extend the shelf life of the fruit, and thereby delay development of disease symptoms that typically develop as fruit ripen (Zanuri *et al.*, 2001). Preharvest treatment of kiwifruit cv. Hayward (*Actinidia deliciosa*) vines with SA (0.28 mg ml^{-1}) induced a 10-fold rise in PAL activity after 2 days in leaf tissue.

Concomitant to induced PAL activity, treatment with SA (0.14 mg ml^{-1}) alongside a chlorinated SA analogue, 4-chlorosalicylic acid, reduced leaf disease severity caused by post-treatment inoculation with *Sclerotinia sclerotiorum* (Reglinski *et al.*, 1997). Similarly, NDR against postharvest *B. cinerea* was enhanced in kiwifruit fruit dipped in SA (0.14 mg ml^{-1}) before storage (Poole and McLeod, 1994; Poole *et al.*, 1998). SA treatment resulted in significant increases in PAL and peroxidase activity relative to untreated control fruit (Poole *et al.*, 1998). However, SA (0.14 mg ml^{-1}) can cause phytotoxicity problems at leaf margins where runoff accumulates (Reglinski *et al.*, 1997).

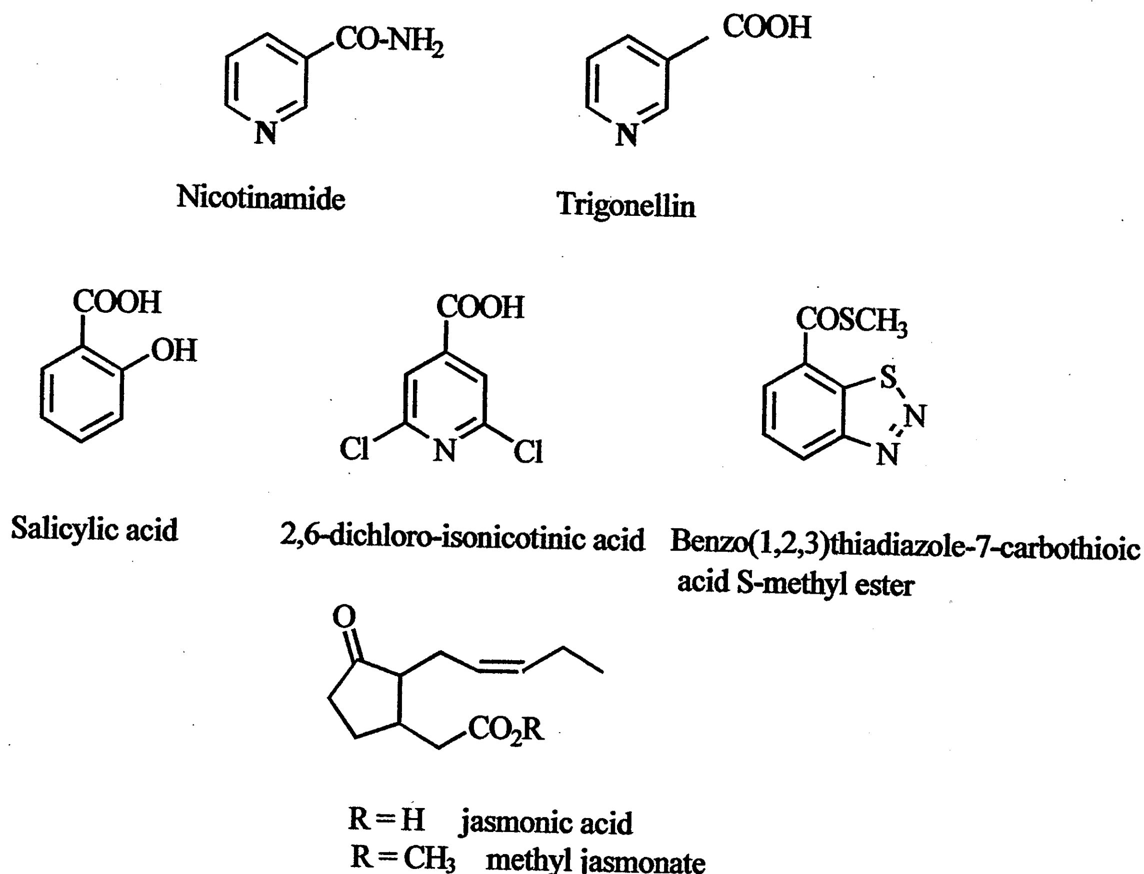


Figure 2.9. Structural formulae of chemical activators of SAR and chemically related natural stress metabolites nicotinamide and trigonellin (after Schneider *et al.*, 1996).

Methyl salicylate (MeSA) is a volatile metabolite of SA. It is produced by pathogen-inoculated plant parts. MeSA is possibly an airborne signal which can activate disease resistance in neighbouring plants and in remote healthy tissue of an infected plant (Shulaev *et al.*, 1997). It is thought that MeSA is converted to SA in targeted

tissues, thereby inducing SAR (Shulaev *et al.*, 1997). As yet, MeSA has not been used as a postharvest treatment.

- Jasmonates

Jasmonic acid (JA) and methyl jasmonate (MJ) (Fig. 2.9) are known as the jasmonates. Jasmonates are naturally occurring plant growth regulators involved in various aspects of plant development and responses to biotic and abiotic stresses (Ellard-Ivey and Douglas, 1996). Their ubiquity in higher plants suggests a role for these molecules in plant metabolism. Ryan (1992) proposed that JA and MJ act as secondary messengers in SAR systems. There are reports of exogenously applied jasmonates protecting plants against disease development and insect herbivory (Creelman and Mullet, 1997). Jasmonates have been shown to elicit SAR in the field (Jackson, 1998) and after harvest (Droby *et al.*, 1999).

Treatment with jasmonate have been shown to reduce storage diseases on cut rose flowers (Meir *et al.*, 1998), grapefruit (Droby *et al.*, 1999) and apple (Saftner *et al.*, 1999). However, MJ was not effective in controlling *Monilinia fructicola* on sweet cherry (Tsao and Zhou, 2000). Treatment with MJ (200 μM) enhanced the accumulation of responsive LOX in the peel of freshly harvested avocado cv. Fuerte fruit. The treatment resulted in a fall in the antifungal diene and enhancement of anthracnose disease development caused by *C. gloeosporioides* (Prusky *et al.*, 1998). Although jasmonates have been previously reported to have a direct antifungal activity against *B. cinerea* (Meir *et al.*, 1998), they were not inhibitory to *P. digitatum* (Droby *et al.*, 1999). Suppression of *P. digitatum* by MJ or JA on grapefruit was indirect, and operated through the enhancement of NDR through phytoalexin accumulation (Droby *et al.*, 1999).

- Chitosan

Chitosan is a high molecular weight cationic polysaccharide, β-1,4-glucosamine, that is produced by the deacetylation of chitin. Chitosan is usually extracted from crustacean shell wastes (Hadwiger *et al.*, 1988). It has been used as a surface coating

on fruit and vegetables and as a seed dressing (Benhamou *et al.*, 1994). Chitosan has been reported to delay ripening, prolong shelf-life, and limit fungal decay of several commodities, including bell pepper (El Ghaouth *et al.*, 1991b; 1997), cucumber (El Ghaouth *et al.*, 1991a) and strawberry fruit (El Ghaouth *et al.*, 1992; Reddy *et al.*, 2000). These effects has been attributed to its direct antifungal activity (Allan and Hadwiger, 1979; Leuba and Stossel, 1986), modified atmosphere effects and/or its ability to induce postharvest resistance responses in plant tissues. Chitosan has been shown to elicit accumulation of chitinases (Mauch *et al.*, 1988), proteinase inhibitors (Walker-Simmons *et al.*, 1983), specific phytoalexins (Walker-Simmons *et al.*, 1983) and lignification (Pearce and Ride, 1982). However, chitosan treatment of strawberry cv. Chandler fruit, however, showed only direct fungistatic properties against *B. cinerea* and *Rhizopus stolonifer* (El Ghaouth *et al.*, 1992). That is, chitosan did not stimulate accumulation of host-derived chitinase, chitosanase or β-1,3-glucanase as tested by polyacrylamide gel assays.

- *Inorganic elicitors*

Phosphonates have been shown to induce resistance in cucumber, bean and maize plants (Sticher *et al.*, 1997). Phosphonates are usually applied as a foliar spray. They are thought to generate an endogenous SAR signal through calcium sequestration at the site of application (Sticher *et al.*, 1997). Pre-harvest truck injections and foliar sprays of avocado and pineapple (*Ananas comosus*), respectively, have been shown to control preharvest *Phytophthora* diseases (Pegg *et al.*, 1990). However, pre-harvest and/or postharvest applications of potassium phosphonate (1.0 – 0.1 mg ml^{-1}) failed to significantly induce resistance to *C. gleosporioides* in harvested mango fruit (Zanuri *et al.*, 2001). In contrast, postharvest potassium phosphonate (10 mg ml^{-1}) treatment after wounding reduced decay caused by *P. expansum* in apple cv. Red Delicious fruit (Wild *et al.*, 1998). Phosphonate (1 mg ml^{-1}) when applied to wound-inoculated apple cvs. Granny Smith and Golden Delicious fruit at the time of inoculation was also effective in reducing disease incidence caused by *P. expansum, B. cinerea* and *Mucor piriformis* (Holmes *et al.*, 1998). Phosphonate showed no *in-vitro* antifungal activity (Wild *et al.*, 1998) against P. expansum but was incompatible with $CaCl_2$,

diphenylamine (DPA) and biocontrol yeast antagonists (Holmes *et al.*, 1998). Thus, phosphonate may not be an appropriate treatment in a IPM strategy.

potassium phosphonate induced resistance in oranges to *P. digitatum* through inducing the rapid production of scoparone (Forbes-Smith *et al.*, 1998). The concentration of the phytoalexin, scoparone, in crude methanol extracts of flavedo tissue in control fruit and oranges treated with potassium phosphonate was 13.2 and 335 $\mu g\ g^{-1}$ FW after 6 days storage, respectfully. The phytoalexins scopoletin and umbelliferone were not induced (Forbes-Smith *et al.*, 1998).

Silicon and powdered silicon oxide have been shown to induce SAR in cucumber and tobacco as shown by enhanced activities of pathogenic host-derived enzymes (Cherif *et al.*, 1993; Schneider and Ullrich, 1994). However, the use of silicon has again been limited to pre-harvest disease control.

- *Synthetic elicitors*

The potential benefits of SAR in agronomic systems is becoming more widely recognised (Lucas, 1999). Research has focussed upon developing novel synthetic chemical activators with increased efficacy (Gorlach *et al.*, 1996; Tally *et al.*, 2000). Many of the modern synthetic compounds confer broad spectrum efficacy against pathogens on a number of crops (e.g. banana, cucumber, melon, tobacco) (Huang *et al.*, 2000; Tally *et al.*, 2000). However, a number are more species specific. 2,2-Dichloro-3,3-dimethylcyclopropane carboxylic acid (WL 28325) is a plant protectant that is mostly limited to rice (*Oryza sativa*) (Cartwright *et al.*, 1980). Rice produces a specific phytoalexin momilactone, which WL 28325 effectively elicits.

2,6-dichloroisonicotinic acid (INA; CGA 41396) and its methyl ester were the first synthetic chemicals shown to activate SAR (Métraux *et al.*, 1990) (Fig. 2.9). INA is weakly fungistatic *in-vitro*, but effectively elicits expression of SAR genes in tobacco prior to TMV challenge inoculation (Ward *et al.*, 1991). It is thought to act independently of SA, as it is still an effective elicitor in an SA-insensitive tobacco mutant. This persistence of effect indicates that INA operates downstream of SA.

Although broad spectrum protection against pre-harvest diseases has been achieved in many plants (e.g. pear and pepper) (Métraux *et al.*, 1991), practical use of INA and indeed SA as plant protection compounds is not favoured due to phytotoxicity problems (Sticher *et al.*, 1997).

DL-3-aminobutyric acid (BABA) is an amino acid which has no direct antifungal activity (Cohen, 1994). BABA induces resistance to pre-harvest diseases such as *Phytophthora infestans*, *Peronospora tabacina* and *Plasmopara viticola* in tomato, tobacco and grapes, respectively (Cohen and Gisi, 1994; Cohen, 1994; Cohen *et al.*, 1998). However, it has not been used as a treatment against postharvest diseases. The mode of action of BABA is not known (Cohen *et al.*, 1998). It does not induce all the characteristics of SAR. When applied as a spray to tomato plants, BABA enhanced PRP accumulation. However in tobacco, where BABA strongly protected against infection the accumulation of PRPs was not observed. No evidence of phytoalexin accumulation was recorded in either crop (Cohen, 1994). Cohen and Gisi (1994) suggest that BABA may afford protection by strengthening cell wall structure so making the host tissue more resistant to infection.

Acibenzolar (benzo-(1,2,3)-thiadiazole-7-carbothioic acid S-methyl ester; BTH; CGA 245704; Bion™; Actigard™) is the most potent synthetic SAR activator discovered to date (Kessman *et al.*, 1994; Friedrich *et al.*, 1996; Gorlach *et al.*, 1996; Lawton *et al.*, 1996) (Fig. 2.9). Acibenzolar is a functional analogue of SA (Gullino *et al.*, 2000; Tally *et al.*, 2000). Like INA, acibenzolar has practically no direct *in-vitro* antifungal effect, it acts downstream of SA (Friedrich *et al.*, 1996), and leads to the accumulation of the same SAR genes and PRPs as SA. Unlike INA, acibenzolar is not phytotoxic and has proven to be an effective SAR elicitor of both monocotyledons, such as wheat, and dicotyledons, including tobacco, *Arabidopsis thaliana* (Gorlach *et al.*, 1996) and cucumber (Tally *et al.*, 2000) when applied as a foliar spray. Acibenzolar has been reported to have a dual role in direct elicitation and/or potentiation of plants defences (Katz *et al.*, 1998). Acibenzolar was reported to augment the sensitivity for low doses elicitation of coumarin phytoalexin in cultured parsley cells (Katz *et al.*, 1998). Thus, acibenzolar renders plant cells more responsive to very low elicitor concentrations, analogous to the priming seen in biologically activated SAR. Katz *et*

al. (1998) observed that enhanced coumarin secretion was associated with potentiated activation of genes encoding for PAL. The augmentation of PAL gene induction was proportional to the length of pre-treatment of acibenzolar, thus, indicating time-dependent priming.

As previously mentioned SAR research on synthetic elicitors has concentrated on controlling preharvest diseases. Only three published studies have shown that acibenzolar used as a pre-harvest spray can suppress postharvest disease (Bokshi *et al.*, 2000; Huang *et al.*, 2000; Terry and Joyce, 2000) (Table 2.3). This limited research demonstrates that acibenzolar could prove valuable in the commercial management of various postharvest diseases. Continued research in this area is needed.

Most published literature has shown positive effects of elicitors in inducing SAR. However, marginally effective results may obscure the fact that in some plant-pathogen systems or environments SAR elicitors are relatively ineffectual in inducing an appreciable defence response. For instance, the efficacy of acibenzolar (0.588 mg AI ml^{-1}) has been observed to be weak in eliciting the defence response in cucumber against powdery mildew (*Sphaerotheca fuliginea*) regardless of whether it was applied before after artificial inoculation (Wurms *et al.*, 1999). Similarly, acibenzolar was not effective in reducing the incidence of camellia flower blightcaused by *Ciborinia camelliae*, when applied as a foliar spray to Camellia bushes (*Camellia japonica*) (Van Toor *et al.*, 2001). Thus, in some plant-pathogen systems elicitors of SAR may not be a commercially viable. In addition, trade-offs in plant defence have been shown to occur between pathogens and herbivores using acibenzolar or jasmonic acid (Thaler *et al.*, 1999). Herbivore resistance was compromised in acibenzolar treated tomato plants that SAR against bacterial speck disease caused by *Pseudomonas syringae* pv. tomato. Equally, treatment of plant with jasmonic acid induced resistance (IR) against insects but reduced PRP expression and resistance to bacterial speck disease. Thus, effective utilisation of chemical plant activators of SAR or IR for reducing postharvest disease requires more understanding on possible trade-offs that might be encountered.

Table 2.3: Examples of chemical elicitors of host plant resistance in horticultural produce

Species	Elicitor	Target Pathogen	Experiment type	Reference
Actinidia deliciosa (kiwifruit)	Salicylic acid	*Botrytis cinerea*	field/postharvest	Poole and McLeod, 1994
			as above	Poole *et al*., 1998
		Sclerotinia sclerotiorum	as above	Reglinski *et al*., 1997
Antirrhinum sp.	Methyl jasmonate	*Puccinia antirrhini*	glasshouse	Jackson, 1998
Apium graveolens (celery)	Gibberellic acid	*B. cinerea*	postharvest	Afek *et al*., 1994
Capsicum fructescens (pepper)	DL-β-amino-n-butyric acid	*Phytophthora capsici*	glasshouse	Sunwoo *et al*., 1996
		Colletotrichum coccodes	as above	Hong *et al*., 1999
Chamelaucium uncinatum (Geraldton waxflower)	Salicylic acid	*Alternaria sp.; Epicoccum sp.*	field/postharvest	Beasley *et al*., 1999*

* reduced *Alternaria sp.; Epicoccum sp.* but increased *B. cinerea*

Species	Elicitor	Target Pathogen	Experiment type	Reference
Citrus paradisi (grapefruit)	Jasmonic acid	*Penicillium digitatum*	postharvest	Droby *et al.*, 1999
	Methyl Jasmonate	as above	as above	Droby *et al.*, 1999
Cucumis melo (rock and hami melon)	Acibenzolar	*Alternaria sp.; Fusarium sp.*	field/ postharvest	Huang *et al.*, 2000
Cucumis sativus (cucumber)	2,6-dichloro-isonicotinic acid	*Sphaerotheca fuliginea*	glasshouse	Hijwegen and Verhaar, 1994
	Milsana™	as above	as above	Daarf *et al.*, 1997
	Acibenzolar	*Cladosporium cucumerinum*	as above	Narusaka *et al.*, 1999
Diospyros kaki (persimmon)	Gibberellic acid	*Alternaria alternata*	field/postharvest	Perez *et al.*, 1995; Eshel *et al.*, 2000
Fragaria ananassa (strawberry)	Acibenzolar	*B. cinerea*	glasshouse/ postharvest	Terry and Joyce, 2000

Species	Elicitor	Target Pathogen	Experiment type	Reference
Helianthus annuus (sunflower)	DL-β-amino-n-butyric acid	*Plasmopara helianthi*	glasshouse	Tosi *et al.*, 1998
	Acibenzolar	*Plasmopara helianthi*	as above	Tosi *et al.*, 1999
Mangifera indica (mango)	Carbon dioxide	*Colletotrichum gleosporioides*	postharvest	Kumpoon *et al.*, 1998
	Salicylic acid	as above	postharvest	Willingham *et al.*, 2000
			field/ postharvest	Zanuri *et al.*, 2001
Persea americana (avocado)	Carbon dioxide	*C. gleosporioides*	postharvest	Prusky *et al.*, 1991b; 1993b
	Cytokinins	as above	field/postharvest	Beno-Moualem *et al.*, 2001
Phaseolus vulgaris (green bean)	2,6-dichloro-isonicotinic acid	*Uromyces appendiculatus*	field	Dann and Deverall, 1996
Pisum sativum (pea)	Salicylic acid	*Erysiphe pisi*	glasshouse	Frey and Carver, 1998
	Acibenzolar	*Mycosphaerella pinodes*	as above	Dann and Deverall, 1999

Species	Elicitor	Target Pathogen	Experiment type	Reference
Pyrus communis (pear)	Calcium	*Phialophora malorum*	field/ postharvest	Sugar *et al.*, 1992
Pyrus pyrifolia (nashi/ Japanese pear)	Acibenzolar	*Gymnosporangium asiaticum*	field	Ishii *et al.*, 1999
Rosa hybrida (rose)	Gibberellic acid	*B. cinerea*	postharvest	Shaul *et al.*, 1995
	2,6-dichloro-isonicotinic acid	*Sphaerotheca pannosa*	glasshouse	Hijwegen *et al.*, 1996
	Methyl jasmonate	*B. cinerea*	postharvest	Meir *et al.*, 1998
	Acibenzolar	*Diplocarpon rosae*	*in-vitro*	Suo and Leung, 2001
Solanum tuberosum				
(potato disks)	Acibenzolar	*Fusarium semitectum*	postharvest	Bokshi *et al.*, 2000
(tubers)	Acibenzolar	as above	field/postharvest	Bokshi *et al.*, 2000

2.4.2.2 *Physically induced resistance*

Induction of NDR of horticultural crops using physical elicitors has received increasing attention over recent years (Wilson *et al.*, 1994). The primary mode of action of most physical treatments is to disinfect the commodity from fungal spores and from latent infections that occur in the outer cell layers of the fruit or vegetable surface. However, some physical treatments impart a mild stress on the commodity. This stress, in some species, can lead to induced resistance against future infection (Barkai and Phillips, 1991). Examples of physical methods of enhancing NDR include low temperature storage, wounding (Ismail and Brown, 1979), CO_2 treatment (Prusky *et al.*, 1993b), heat treatment (Schirra *et al.*, 2000), ionising irradiation (McDonald *et al.*, 2000), UV-C irradiation (Wilson *et al.*, 1994) and ozone (Eckey-Kaltenberg *et al.*, 1994; Kangasjarvi *et al.*, 1994).

- Heat treatment

Pre-storage heat treatment appears to be a promising postharvest method for reducing disease incidence and severity (Lurie, 1998; Schirra *et al.*, 2000). Heat treatments may be applied to fresh produce after harvest by hot water dips (Garcia *et al.*, 1995) hot dry air (Lurie *et al.*, 1997a) vapour heat (Lydakis, 1999) or very short water rinse and brushing (Fallik *et al.*, 1999; Pavoncella *et al.*, 2001). Postharvest heat treatments increase the resistance of cucumbers against *Cladosporium cucumerinum* (Stermer and Hammerschmidt, 1984), tomatoes to *B. cinerea* (Fallik *et al.*, 1993; Lurie *et al.*, 1997a) and reduce development of *P. expansum* in apple fruit (Fallik *et al.*, 1995). Heat treatments have also been used to control *B. cinerea* and *Alternaria alternata* on red bell pepper (Fallik *et al.*, 1996), *Monilinia fructicola* in nectarines (Anthony *et al.*, 1989) and *B. cinerea* on table grapes in storage (Lydakis, 1999). A thermal dip treatment for flower heads was found effective against grey mould in five rose cultivars and one carnation cultivar (*Dianthus caryophyllus*), naturally infected with *B. cinerea* (Elad and Volpin, 1991). Heat treatment of citrus have been reported to induce resistance against green mould caused by *P. digitatum* (Schirra *et al.*, 2000). This induced response has been associated with increases in lignin (Nafussi *et al.*, 2000), heat shock proteins, PRPs (Pavoncella *et al.*, 2001), phytoalexins, like scoparone and scopoletin (D'hallewin *et al.*, 1997), and inhibition of decline in preformed antifungals such as citral (Ben-Yehoshua *et al.*, 1998). On the other hand,

hot water treatment at 55°C for 10 min increased susceptibility of avocado to *C. gleosporioides* (Plumbley *et al.*, 1993). This was correlated to a decline in diene concentration in pericarp tissue. In contrast, dry heat treatment delayed anthracnose disease development in avocado (Lurie *et al.*, 1997b).

- UV-C treatment

Non-ionising radiation offers great potential amongst alternative methods for controlling postharvest diseases (Wilson *et al.*, 1994). Low doses of short-wave ultraviolet light (UV-C, 190-280 nm wavelength) have been used to control many storage rots in fruit and vegetables (Table 2.4). UV-C irradiation at low doses (0.25 – 8.0 kJ m^{-2}) targets the DNA of micro-organisms. For this reason it has been used as a germicidal or mutagenic agent (Wilson *et al.*, 1997a). In addition to this direct germicidal activity (Marquenie *et al.*, 2001a), UV-C radiation can affect physiological processes in plants (Luckey, 1980). Given the appropriate wavelength and dose, UV-C irradiation can cause accumulation of stress-induced phenylpropanoids (Dixon and Paiva, 1995). Many of these phenylpropanoids have been associated with induced disease resistance (Hadwinger and Schwochau, 1971; Fritzenheimer and Kindl, 1981). However, visible damage caused by high doses UV-C (254 nm) has been reported in banana (Wade *et al.*, 1993), citrus spp. (Ben-Yehoshua *et al.*, 1992), bell peppers (Mercier *et al.*, 2001) and strawberries (Marquenie *et al.*, 2002).

Ben-Yehoshua *et al.* (1992) showed that low UV-C illumination reduced lemon fruit susceptibility to *Penicillium digitatum*. UV-C dose was directly related to the concentration of scoparone induced in flavedo tissue. Similar phytoalexin-mediated responses and associated increases in NDR have been observed in other citrus fruit (Droby *et al.*, 1993; Rodov *et al.*, 1992), in carrot (Mercier *et al.*, 1993a; 2000) and tomato (Charles *et al.*, 2001). The responsiveness of harvested horticultural produce to UV-C treatment declines with increase fruit ripeness (Liu *et al.*, 1993) and is influenced by harvesting date (D'hallewin *et al.*, 1999). Where phytoalexins have not been measured in other fruit, such as strawberry, correlative evidence demonstrates that UV-C treatment can increase PAL activity (Nigro *et al.*, 1998b; 2000). However, UV-C (0.5 – 15 kJ m^{-2}) was ineffective in reducing *Monilinia fructigena* in sweet cherry (Marquenie *et al.*, 2002).

Table 2.4: Examples of postharvest UV-C (254 nm) treatment reducing storage diseases in horticultural produce

Species and Cultivar	Optimum UV-C dose (kJ m^{-2})	Targeted Pathogen	Reference
Actinidia deliciosa (kiwifruit)			
cv. Hayward	0.5	*Botrytis cinerea*	Nigro *et al*., 1998b
Allium cepa (onion)			
cv. Walla Walla	3.58-7.33	Not specified	Lu *et al*., 1987
Capsicum annuum (bell pepper)			
cv. Bell Boy	0.88	*B. cinerea*	Mercier *et al*., 2001
cv. Delphin	0.88		
Citrus aurantifolia (lime)			
cv. Tahiti	5.0	*Penicillium digitatum*	Ben-Yehoshua *et al*., 1992
Citrus cinensis (orange)			
cv. Biondo Comune	0.5 - 3.0	Not specified	D'hallewin *et al*., 1999
cv. Tarocco	0.5 – 1.5	as above	as above
cv. Valencia Late	0.5 – 1.5	Not specified	as above
cv. Washington Navel	0.5 - 3.0	as above	as above

Species and Cultivar	Optimum UV-C dose (kJ m^{-2})	Targeted Pathogen	Reference
Citrus cinensis (orange) continued			
cv. Shamouti	5.0	*P. digitatum*	Ben-Yehoshua *et al.*, 1992
cv. Valencia	5.0	as above	as above
Citrus limon (lemon)			
cv. Eureka	5.0	*P. digitatum*	Ben-Yehoshua *et al.*, 1992
Citrus paradisi (grapefruit)			
cv. Marsh Seedless	5.0	*P. digitatum*	Ben-Yehoshua *et al.*, 1992;
	1.6 – 8.0	as above	Droby *et al.*, 1993;
	2.2	as above	Stevens *et al.*, 1996;
	0.5	as above	D'hallewin *et al.*, 2000
Citrus reticulata (tangerine)			
cv. Dancy	0.84	*Alternaria citri*	Stevens *et al.*, 1996
	3.6	*Geotrichum candidum*	as above
	1.3	*P. digitatum*	as above
Cucurbita pepo (zucchini squash)			
cv. Tigress	4.93 – 9.86	Not specified	Erkan *et al.*, 2001

Species and Cultivar	Optimum UV-C dose (kJ m^{-2})	Targeted Pathogen	Reference
Daucus carota (carrot)			
cv. Caropak	4.4 – 8.8	*B. cinerea*	Mercier *et al.*, 1993a; 1993b
Ipomea batatas (sweet potato)			
cv. Jewel	4.8	*Fusarium spp.* and *Rhizopus spp.*	Stevens *et al.*, 1990
cv. Carver	4.8	as above	as above
cv. Georgia Jet	3.6	as above	as above
cv. Jewel	3.6	*Fusarium solani*	Stevens *et al.*, 1999
Fortunella margarita (kumquat)			
cv. Nagami	0.5	*P. digitatum*	Ben-Yehoshua *et al.*, 1992
	1.5	as above	Rodov *et al.*, 1992
Fragaria x ananassa (strawberry)			
cv. Pajaro	0.5 – 1.0	*B. cinerea*	Nigro *et al.*, 1998b; 2000
cv. Kent	0.25	as above	Baka *et al.*, 1999
cv. Elsanta	0.5 – 15.0	*B. cinerea*	Marquenie *et al.*, 2001; 2002

Species and Cultivar	Optimum UV-C dose (kJ m^{-2})	Targeted Pathogen	Reference
Lycopersicon esculentum (tomato)			
cv. Tuskegge 80-130	7.5	*Alternaria alternata* and *B. cinerea,*	Liu *et al.*, 1993
	3.6	*Rhizopus stolonifer*	as above
	3.7	*B. cinerea*	Charles *et al.*, 1999
Malus domestica (apple)			
cv. Golden Delicious	7.5	*Alternaria* sp. and *Monilinia* sp.	Lu *et al.*, 1991
cv. Golden Delicious	4.8	*Alternaria sp.*	Stevens *et al.*, 1996
	7.5	*Colletotrichum gleosporoides*	as above
cv. Empire	1.38	*B. cinerea* and *P. digitatum*	Wilson *et al.*, 1997a
Mangifera indica (mango)			
cv. Tommy Atkins	Not defined	Not specified	Gonzalez-Aguilar *et al.*, 2001
Prunus persica (peach)			
cv. Loring	20	*Monilinia fructicola*	Lu *et al.*, 1991
cv. Elberta	4.8	as above	as above
cv. Loring and Elberta	4.8 – 7.5	as above	Stevens *et al.*, 1996; 1998
Vitis vinifera (table grape)			
cv. Italia	0.125 – 0.5	*B. cinerea*	Nigro *et al.*, 1998a; 1998b

Most published research involving postharvest UV-C treatments have used 254 nm due to its commercial availability. The possibility of other wavelengths within the UV-C band (190-280 nm) enhancing NDR needs further investigation. In addition, effects of other light wavelengths on NDR are not yet fully explored. Saks and co-workers (1996) demonstrated that illumination with cool white florescent light reduced grey mould on strawberry cv. Dorit and Ofra fruit after 2h illumination.

2.4.2.3 *Biologically induced resistance*

Use of microbial antagonists for the control of postharvest fruit decay has been actively pursued (Peng and Sutton, 1991; Wilson and Wisniewski 1994; Ippolito and Nigro 2000). In strawberry, for example, grey mould development has been reduced using *Cladosporium herbarum* (Pers.) Link (Bhatt and Vaughan 1962), *Pseudomonas fluorescens*, *Bacillus pumilus* (Swadling and Jefferies 1996), *Ulocladium atrum* (Boff *et al.*, 1998), *Trichoderma* isolates (Tronsmo 1986; Pratella and Mari 1993; Tronsmo and Dennis 1997) and *Aureobasidium pullulans* (de Bary) Arnaud (Bhatt and Vaughan 1962; Lima *et al.* 1997; Adikaram *et al.*, 2002).

A number of antagonistic micro-organisms are capable of inducing defence reactions in host tissue (Droby and Chalutz, 1994). Concomitant with chemical and abiotic elicitors, some biotic inducers and biotic extracts, like yeast cell wall extracts (Adikaram *et al.*, 1992), and compost water extracts (Weltzein, 1989; Elad and Shtienberg, 1994) are able to boost general defence reactions in plants (Droby and Chalutz, 1994; Schneider and Ullrich, 1994). The use of avirulent or attenuated strains of otherwise pathogenic or saprophytic micro-organisms to induce SAR in vegetative host tissue has been relatively well researched (Deverall, 1995), including control of postharvest rots (Table 2.5).

Table 2.5: Examples of biological elicitors of host plant resistance in horticultural produce

Species	Elicitor	Pathogen	Experiment type	Reference
Ananas comosus (pineapple)	*Penicillium funiculosum* (non-pathogenic)	*P. funiculosum*	postharvest	Lim and Rohrbach (1980)
Capsicum annum (bell pepper)	*Glomerella cingulata*	*Botrytis cinerea*	postharvest	Adikaram *et al.*, 1982; 1988
Cucumis melo (cantaloupe melon)	*Colletotrichum lagenarium*	*C. lagenarium*	glasshouse	Caruso and Kuć, 1977
Citrullus lanatus (watermelon)	*Fusarium oxysporum f. sp. cucumerinum*	*C. lagenarium*	glasshouse	Caruso and Kuć, 1977 and Roby *et al.*, 1988
Daucus carota (carrot)	*B. cinerea*	*B. cinerea*	postharvest	Harding and Heale, 1980
	B. cinerea and *Sclerotinia sclerotiorum*	as above	as above	Mercier and Arul, 1993
	B. cinerea	as above	as above	Mercier *et al.*, 2001

Species	Elicitor	Pathogen	Experiment type	Reference
Fragaria ananassa (strawberry)	*Aureobasidium pullulans*	*B. cinerea*	field/postharvest	Lima *et al.*, 1997
	Colletotrichum musae	*C. musae*	postharvest	Hirai *et al.*, 2000
	A. pullulans	*B. cinerea*	field/postharvest and postharvest	Adikaram *et al.*, 2002
Malus domestica (apple)	Messanger™ (harpin)	*Penicillium expansum*	postharvest	De Capdeville *et al.*, 2000
Musa spp. (banana)	*Phyllosticta musarum*	*Colletotrichum musae*	postharvest	Abayasekara *et al.* 1998
Persea americana (avocado)	*Colletotrichum magna* (non-pathogenic)	*Colletotrichum gleosporioides*	postharvest	Prusky *et al.*, 1994

A new product, Messanger™, is based on the harpin protein derived from *Erwinia amylovora* (Wei *et al.*, 1992). The pathogen causes fire blight of pear, apple and related plants. Harpin has been shown to control *P. expansum* in apples after harvest (De Capdeville *et al.*, 2000). Another comparatively new plant activator is Milsana™. Milsana™ is the registered name given to an extract from leaves of the giant knotweed, *Reynoutria sachalinensis*. Although no published postharvest research has been carried out using this product, a number of reports have shown that Milsana™ can significantly reduce disease incidence of powdery mildew in cucumber through induction of localised resistance (Daayf *et al.*, 1997; Wurms *et al.*, 1999). Milsana™ is thought to induce plant defence responses, such as, enhanced expression of peroxidases, β-1,3-glucanases and phenolics with antifungal activity (Daarf *et al.*, 1997; Wurms *et al.*, 1999).

Mercier and Arul (1993) reported that SAR in carrots could be achieved by pre-inoculating carrot roots with mycelial *B. cinerea* plugs. When carrot crowns were subsequently challenged with *B. cinerea* 25 days later, the incidence of grey mould was significantly reduced. In a later study, pre-inoculation with *B. cinerea* was shown to elicit systemic accumulation of the phytoalexin, 6-methoxymellein (Mercier *et al.*, 2001). However, this systemic affect was not manifest in UV-C treated carrots where only local accumulation of phytoalexin was seen. This differential effect indicates that although the same defences are being induced, the responses are probably mediated differently (Mercier *et al.*, 2001).

2.4.3 Modulation of NDR through resource allocation

Plants have evolved adaptive mechanisms by which they respond to stressful environments in order to improve their probability of survival and/or production of progeny (Settler, 1990). Many of the mechanisms used in ameliorating environmental pressure involve changes in resource partitioning. Because plants have limited carbon to support physiological processes, environmental and ontogenetic constraints lead to trade-offs between growth, maintenance, storage, reproduction and defence (Herms and Mattson, 1992). Trade-offs occur between primary and secondary metabolism. During periods of augmented growth, defence metabolism involving carbon-based

secondary metabolites (CBSCs) (Koricheva *et al.*, 1998) may be substrate and/or energy limited. Thus, the allocation of carbon to secondary defence metabolism versus growth (i.e. protein synthesis) may influence NDR in horticultural produce.

Koricheva *et al.* (1998) proposed through analysis of published literature that carbon allocation to CBSCs is a function of five main hierarchical constraining factors (Fig. 2.10). Total carbon assimilated by a plant is determined by its photosynthetic rate (level 1). The availability of growth limiting nutrients, predominately nitrogen, determines the proportion of assimilated carbon that can be allocated to growth (level 2). Any surplus carbon not used in growth can be assigned to CBSC synthesis or storage (level 3) (Tuomi, 1988). Thus, in accordance with the carbon-nitrogen balance (CNB) hypothesis (Bryant, 1983) and growth differentiation balance hypothesis (GDB) (Loomis, 1932; 1953; Lorio, 1986) in resource rich environments, growth processes receive allocation priority for resources. This growth priority limits the proportional availability of carbon-based secondary defence compounds (Bazzak, 1987). Accordingly, nitrogen fertilisation will decrease foliar concentrations of CBSCs (Koricheva *et al.*, 1998). However, variable results regarding the effect of nitrogen fertilisation on disease have been reported for different crops grown in similar conditions (Goodman *et al.*, 1986). For instance, high fruit nitrogen has been associated with lower NDR in apple (Sharples, 1980) and pears (Sugar *et al.*, 1992). Verhoeff (1965; 1968) found a decrease in susceptibility to grey mould in tomato when it was grown in soil of high nitrogen content. Hobbs and Waters (1964) reported that under similar soil conditions chrysanthemum flowers suffered an increase in *B. cinerea* infection. Savary *et al.* (1995) argues that nitrogen fertilisation can indirectly lead to increase disease in rice by affecting density of foliage. This may provide a wetter microclimate within the canopy so as to favour infection (Savary *et al.*, 1995). Similarly, affects on microclimate due to increased vegetative growth may explain some of the contrasting results of nitrogen supply and postharvest disease in horticultural produce.

Environmental factors that impose sink limitations on growth more than photosynthesis will increase the carbon pool available for allocation to secondary metabolism (Herms and Watson, 1992). The carbon allocated to CBSCs is distributed into the shikimate and mevalonic acid pathways via pentose phosphate pathway and

glycolysis leading to phenylpropanoids, hydrolysable tannins and terpenoids (level 4) (Fig. 2.10). Each pathway then divides into additional branches to ultimately produce individual CBSCs (level 5) (Fig. 2.10).

A strong inverse relationship exists between the allocation of resources to growth and non-growth processes (Margna, 1977). Many amino acid precursors originating from the shikimate pathway, such as tryptophan and phenylalanine, give rise to proteins and carbon-based secondary compounds (Fig. 2.1) (Margna, 1977). Phenylalanine is typically the rate limiting precursor for phenylpropanoid synthesis. Thus, as plant growth is heavily dependant upon protein synthesis, phenolic synthesis competes with growth for this common substrate (Phillips and Henshaw, 1977; Margna *et al.*, 1989). Thus, any factor that limits growth will increase the availability of phenylalanine for phenylpropanoid based antifungal compounds. Given the importance phenylpropanoid based antifungal compounds have in horticultural produce (Table 2.1 and 2.2) it follows that pre-harvest environment and management factors that limit growth or yield will in effect increase NDR (Smedegaard-Petersen and Tolstrup, 1985).

Other CBSCs also compete with protein synthesis and consequently growth. Hydrolysed tannins derived from gallic acid may be synthesised from either phenylalanine or dehydroshikimic acid, an intermediate of the shikimate pathway (Gross, 1992). Terpenoids, derived from acetyl CoA which acts as a precursor to the mevalonic pathway (Gershenzon, 1984; Gray, 1987), also directly compete with protein synthesis. Acetyl CoA acts as the entry point into the Kreb's cycle. Likewise, alkaloids and other nitrogen containing compounds compete with protein synthesis (Lindsey and Yoeman, 1983).

Activation of phenylalanine ammonia-lyase (PAL), the key enzyme in the phenylpropanoid pathway, and synthesis of downstream products are important in restriction of pathogen growth (Benhamou and Nicole, 1999). Any factors that limit the synthesis of the phenylpropanoid and other CBSCs involved in constitutive or inducible defence will compromise the inherent NDR level of the plant. In fruit crops, the pattern of carbon resource allocation is a function of sink size and activity. Developing fruit are strong sinks. Thus, under conditions that favour fruit growth,

secondary metabolism and thus fruit defence may be jeopardised. Elicitors of plant defence responses, including systemic acquired resistance (SAR), may be ineffectual in boosting levels of constitutive and inducible carbon-based defence secondary compounds sufficiently to reduce disease incidence in resource rich environments. Thus, it is important that more research needs is conducted into how NDR and SAR may be manipulated in different environments.

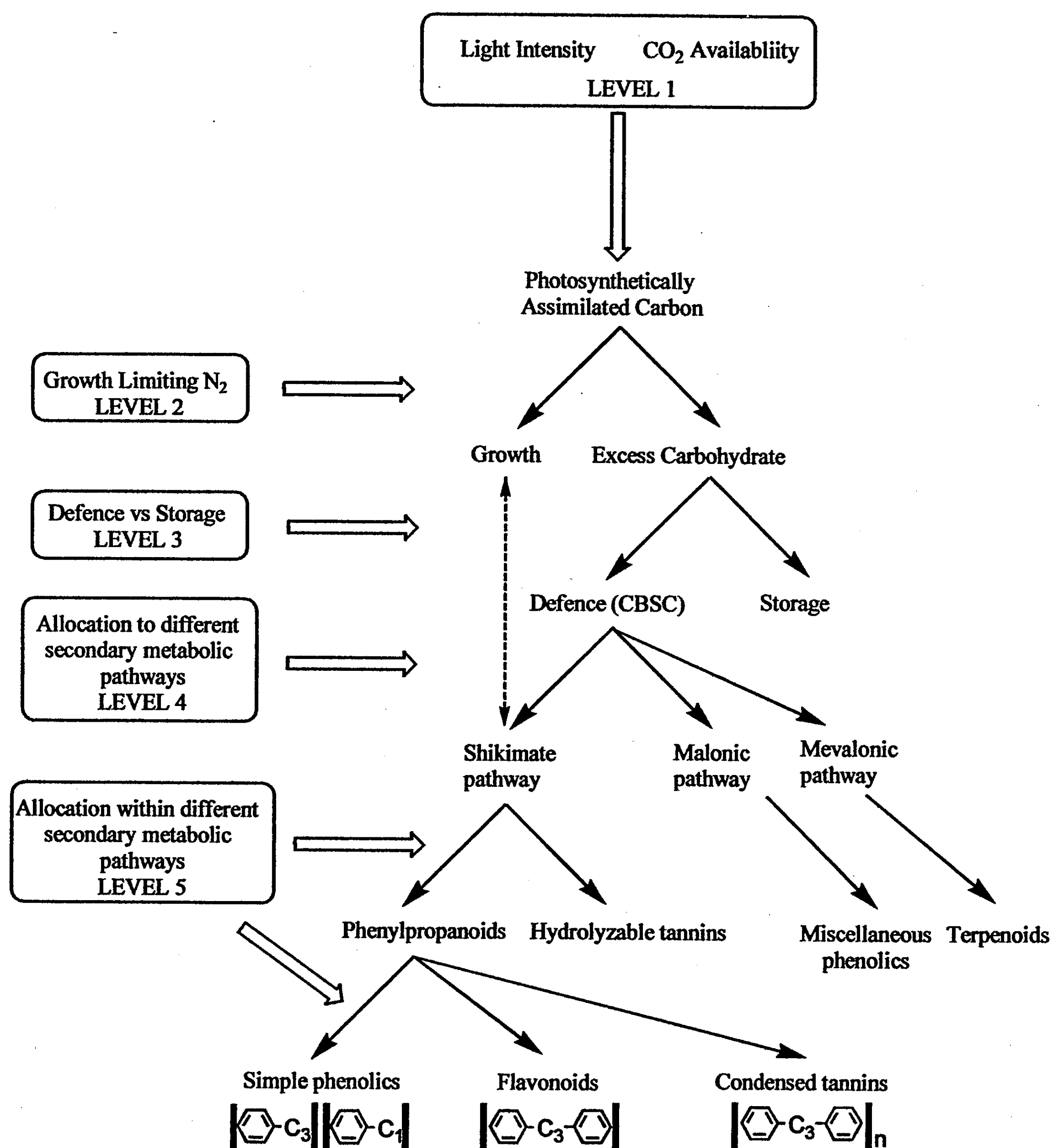


Figure 2.10: Simplified proposed hierarchical model of regulation of carbon secondary metabolism in plants (adapted from Koricheva *et al.* (1998)). The dashed arrow represents competition between protein synthesis (growth) and phenylalanine.

2.5 Conclusion

A decline in NDR correlates with the onset of many postharvest diseases. However, compared with fruit ripening, very little information is available on changes in the NDR of horticultural crops. Concerns are growing over the increasing loss of efficacy of conventional fungicides due to pathogen resistance. Moreover, there is a general lack of acceptability of fungicide usage by the general public and from environmental perspectives. These concerns favour the increasing adoption of IPM strategies. Exploitation of NDR is a potentially desirable strategy in achieving IPM (Wilson *et al.*, 1994). Thus, the prospects of disease suppression through modulation and/or elicitation of NDR mechanisms is attractive in comparison to conventional chemical control (Joyce and Johnson, 1999). Additionally, NDR may be enhanced through conventional and molecular plant improvement strategies (Joyce and Johnson, 1999). Other potential benefits of manipulating NDR include enhancement of antifungal compounds with reported health benefits (Cantos *et al.*, 2001). For example, trans-resveratrol has been shown to reduce tumour initiation, promotion and progression (Jang *et al.*, 1997). Cantos *et al.* (2001) suggested that enhancing NDR of table grapes using UV-C could be used a useful treatment to obtain 'functional fruit' as a dietary source of resveratrol. This said, manipulating NDR is not without its risks (Paiva, 2000). Selecting fruit and vegetables with too high concentrations preformed antifungal compounds can result in less palatable food (Paiva, 2000).

To realise maximum benefit of manipulating NDR in horticultural produce, considerable research is required. A better understanding of the role of preformed and inducible antifungals in harvested horticultural produce is needed. The influence of preharvest environment and management on resource partitioning is also essential. This fundamental knowledge will allow for greater efficacy in enhancing NDR in the pre-postharvest continuum and provide the basis for adopting IPM.

CHAPTER 3.

NATURAL RESISTANCE OF STRAWBERRY TO *BOTRYTIS CINEREA*

3.1 Introduction

The strawberry is grown in all temperate regions of the world, including North America, Mexico and Europe. Its increasing importance as a valuable horticultural world commodity is evident in that production has increased by 19% since 1990 (FAO Production Statistics, 2000). At present, over 3.05 million t are produced annually (FAO Production Statistics, 2000).

The strawberry (*Fragaria ananassa* Duch.) of commerce belongs to the Rosoideae order of the Rosaceae family (Darrow, 1966; Mabberely, 1987). It is a perennial herb with rooting runners that usually bears red berry fruit on maturity. The commercial strawberry is thought to derive from an interspecific hybrid cross between the two octaploid species, *F. chiloensis* (L.) Duch. and *F. virginiana* Duch. (Went, 1957; Darrow, 1966; Sistrunk and Morris, 1985; Hancock, 1999). Hundreds of different strawberry cultivars have been developed. The cultivar Elsanta predominates in North Western Europe (Rosati, 1990; Wilson, 1997).

3.1.1 Fruit physiology

The strawberry fruit is considered to be a 'false fruit' (Szczesniak and Smith, 1969; Perkins-Veazie, 1995) or aggregate fruit (Coombe, 1976), as it does not fit into the conventional botanical definition of the word. It does not originate from an augmentation of the ovary. Rather, the strawberry fruit develops through swelling of the parental receptacle. Esau (1977) describes the strawberry as an aggregate accessory fruit since, although it is formed from an apocarpous gynoecium with each carpel retaining its identity on maturity, the strawberry contains extracarpellary tissue.

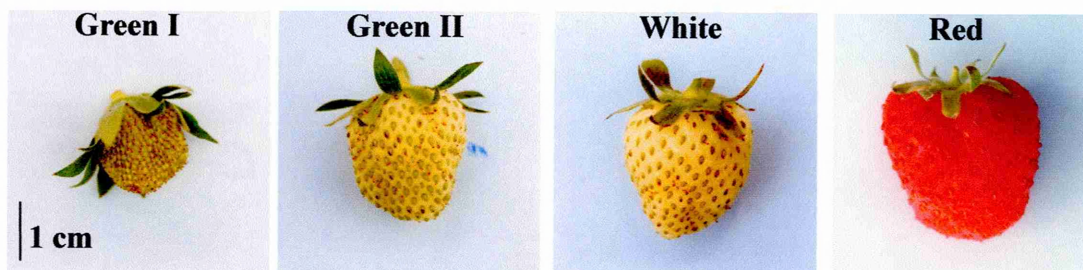
The central core of the swollen receptacle is pith. This core is girdled by corticular parenchyma and lightly waxed epidermal tissue (Harvis, 1943). The epidermis bears

a number of embedded one-seeded fruits called achenes. These 'seeds' are arranged in a spiral pattern (Abbott *et al.*, 1970). Achenes arise from the many ovaries that surround the receptacle (Darrow, 1966). They attach to the receptacle's vasculature by fibrovascular connections (Lis and Antoszewski, 1979; Manning, 1993). These connections are thought to be a pathway for inter-organ communication governing fruit growth and development (Strick and Proctor, 1988).

3.1.2 Fruit development

Strawberry fruit growth and development, including maturation and ripening, is characterised by changes in colour, texture and flavour (Manning, 1993). Four to five stages of berry growth and development have been described (Culpepper *et al.*, 1935; Spayd and Morris, 1981; Huber, 1984). In accordance with the expansion of non-ovarian receptacle tissue, these descriptive stages include green I, green II, white, and full red.

Plate 3.1.2.1: Developmental stages of strawberry cv. Elsanta fruit



Depending on environmental conditions, the strawberry plant bears full red fruit within approximately 30 to 40 days after anthesis (Dennis, 1984). Variation in time to reach maturity is associated with prevailing temperatures (Perkins-Veazie and Huber, 1987) and their effect on enzyme mediated metabolic rates (Manning, 1993).

Berry growth can be measured by changes in fresh and/or dry weight and/or changes in dimensions. Upon maturity, full red ripe stage fruit attain maximum fresh weight and size (Darrow, 1966; Perkins-Veazie, 1995). Whilst size is not directly related to

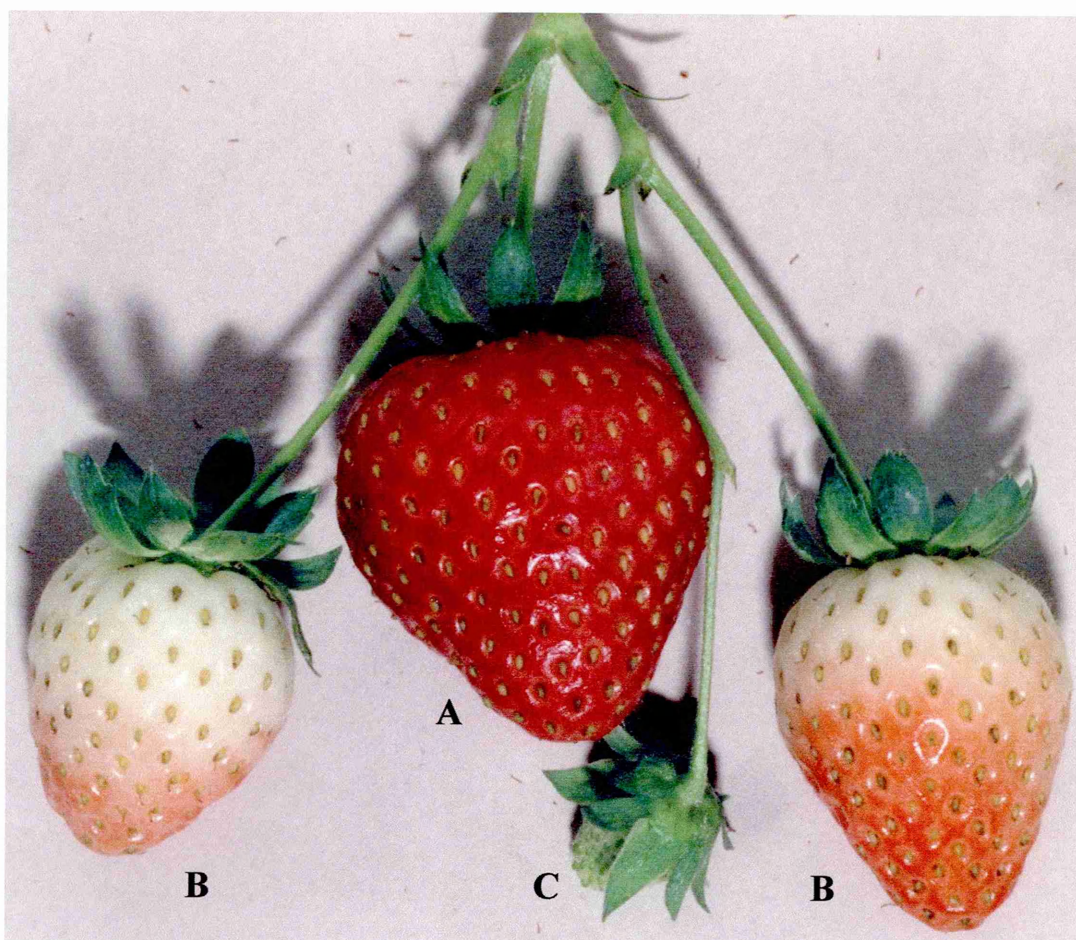
eating quality (Sistrunk and Morris, 1985), berry size is used for grading maturity in mechanically harvested strawberries (Morris *et al.*, 1978).

Strawberry fruit growth is often characterised by either a single sigmoid (Bollard, 1970; Woodward, 1972; Stutte and Darnell, 1987) or a double sigmoid (biphasic) (Thompson, 1969; Coombe, 1976; Miura *et al.*, 1990) growth curve. Different patterns of berry growth can be attributable to genetic variation among cultivars.

Large fruit size is an inherent trait (Darrow, 1966). Variations in berry size depend on the physiological resource allocation interactions between blossom position, number of developing achenes, inter-fruit competition and overall plant vigour (Janick and Eggert, 1968; Abbott *et al.*, 1970). Avigdori-Avidov (1986) suggests that the endogenous control of strawberry fruit growth also depends on innate differences in achene metabolic activity and receptacle plant hormone sensitivity. Final fruit size and shape are closely correlated with the number and size of fertile achenes on a fruit (Nitsch, 1955; Moore *et al.*, 1970; Sistrunk and Morris, 1985; Manning, 1993). In this respect, control of fruit growth is attributed to assimilate sink strength mediated by auxin secreted from developing achenes (Nitsch, 1950; Tukey, 1952; Manning, 1993).

The strawberry inflorescence, which is cymose, possesses a terminal primary inflorescence with secondary and tertiary inflorescences attached proximally to this primary bloom. Apical dominance within the cyme mediates inter-fruit competition for assimilates. Webb (1973) suggested that primary fruit possessed a shorter vascular system, and hence have a more efficient assimilate transport system. Primary fruit usually have the faster growth rate (Forney and Breen, 1986) and achieve larger size on maturity than secondary and tertiary fruit, respectively (Janick and Eggert, 1968; Moore *et al.*, 1970). This different fruit size phenomenon is physiologically determined and largely independent of environmental conditions (Stutte and Darnell, 1987). Removal of the primary bloom at anthesis results in an increase in secondary fruit weight (Stutte and Darnell, 1987).

Plate 3.1.2.2: Arrangement of strawberry cv. Andana fruit cluster. (A) primary fruit; (B) secondary fruit; (C) tertiary fruit.



3.1.3 Fruit ripening

The strawberry is a non-climacteric fruit (Coombe, 1976; Knee *et al*., 1977; Given *et al*., 1988a; Perkins-Veazie, 1988; Manning, 1993), exhibiting no respiratory increase during ripening (Biale and Young, 1981). However, distinct cellular and compositional changes occur during the ripening process.

3.1.3.1 *Cellular and compositional changes*

With the onset of ripening, physiochemical changes occur rapidly and lead to changes in texture, colour, soluble solids content, acidity and aroma. Strawberries soften

markedly between the green and white ripeness stages. Unexpanded cells of green fruit initially have dense cell walls. As fruit develop, cells expand and become vacuolated. Although not fully understood at the biochemical level, softening results from degradation of the middle lamella between cortical parenchyma cells (Abeles and Takeda, 1990). Hemicellulose and cellulose degradation may also contribute to softening (Barnes and Patchett, 1976; Knee *et al.*, 1977; Abeles and Takeda, 1990). Although strawberry cultivars vary in firmness, there is still no sound explanation for these differences (Perkins-Veazie, 1995). Specific enzymes responsible for strawberry fruit softening are not known. Little evidence for high endo- or exo-polygalacturanase activity has been found in strawberry (Nogata *et al.*, 1993).

Total soluble solid (TSS) contents of strawberry fruit increase steadily during development, from about 5% in green fruit to around 7.3% in overripe (dark red) fruit (Spayd and Morris, 1981). However, depending on environment and cultivar, the TSS of ripe fruit can vary between 4 to 12%. Titratable acidity, a function of cellular pH, is generally expressed as percent citric acid. According to Green (1971) citric acid represents 88% of the total organic acids in strawberry fruit. Upon full red colour development, acidity decreases on a per fruit basis (Woodward, 1972).

As strawberries ripen, an increase in anthocyanin content is accompanied by a decrease in chlorophyll content. Anthocyanins are synthesised from flavanoids, through the shikimic acid and phenylpropanoid pathways. The predominant anthocyanin in all red strawberry cultivars is pelargonidin 3-glucoside. Accumulation of anthocyanin coincides with the *de novo* induction of phenylalanine ammonia lyase (PAL) and uridine diphosphate glucose:flavonol O^3-D-glucotransferase (UDPGFT) (Given *et al.*, 1988b; 1988c; Cheng and Breen, 1991).

3.1.3.2 *Hormonal regulation of ripening*

Ripening in many fruits is associated with a decrease in auxin or an increase in ethylene production (Hobson, 1979). In strawberry, auxin and its derivatives play the primary role in controlling ripening. There is little evidence for involvement of ethylene in the ripening process. Nitsch (1950) first demonstrated that achenes,

believed to be the source of auxin, mediate growth and development of strawberry fruit. Removal of achenes in the early stages of growth dramatically retards the ripening process (Nitsch, 1950).

Nitsch (1950; 1955) demonstrated that topical application of synthetic auxins could restore growth of receptacles from which the achenes have been removed. Analysis of auxin contents have implicated achene activity and thus auxin as being the basis of ripening in strawberry fruit (Dreher and Poovaiah, 1982; Given *et al.*, 1988a; Manning, 1994). Auxin levels rise during early stages of fruit development and then decline (Archbold and Dennis, 1984). This is mediated by a decrease in auxin synthesis by achenes and also a reduction in the receptacle hormone sensitivity (Moore *et al.*, 1970).

Given and co-workers (1988c) confirmed that the achenes are of paramount importance in ripening. They showed that removing the achenes from one half of a detached large green fruit cv. Brighton accelerated ripening in the de-achened half. Given *et al.* (1988c) hypothesised that achene-derived auxin inhibits ripening in green fruit, such that ripening occurs only once free IAA in the receptacle falls below a critical threshold.

As strawberry is a non-climacteric fruit (Given, 1988a; Manning, 1993), ethylene is generally considered to have little or no effect on ripening (Iwata *et al.*, 1969; Knee *et al* 1977; Hoad and Williams, 1971; Perkins-Veazie, 1995). Exogenous application of ethylene (5 to 200 $\mu l\ l^{-1}$) was ineffective in initiating ripening in detached green stage strawberry fruit (Mason and Jarvis, 1970; Hoad and Williams, 1971; Janes *et al.*, 1978). Moreover, application of the aminocyclopropane-1-carboxylic acid (ACC) synthase inhibitor aminoethoxyvinylglycine and the ethylene binding inhibitors silver thiosulfate (STS) and norbornadiene (NBD) failed to block anthocyanin accumulation in developing strawberry fruit (Given *et al.*, 1988a). However, Tian *et al.* (1997) showed that application of the ethylene binding inhibitor diazocyclopentadiene (DACP) increased ethylene production, but not respiration, of ripe strawberry fruit. Enhanced ethylene production was explained as release from feedback inhibition on ACC synthesis.

In contrast, results of some other studies show that ethylene can have a role in strawberry fruit ripening. Curd (1988) showed that strawberries exposed to ethylene had more intense red colour than those stored in ethylene-free air. Tian *et al.* (2000) determined that exogenous ethylene induced secondary ripening processes (e.g. colour development, softening) in strawberry fruit. Wills and Kim (1995) found that use of the ethylene absorbent potassium permanganate extended the storage life of strawberry fruit. Thus, prevention of ethylene production and/or inhibition of ethylene action can extend postharvest longevity of ripe strawberry fruit.

El-Kazzaz *et al.* (1983) found that exogenous ethylene enhanced the growth of *B. cinerea* on strawberry and decrease fruit firmness. Ku *et al.* (1999) found that treatment with methylcyclopropene (1-MCP) extended the postharvest life of strawberry fruit cv. Selva, mainly through a delay in rotting. However, the beneficial response was obtained only over a limited concentration range (5-15 nl l^{-1}); 1-MCP at high concentrations (500 nl l^{-1}) increased the susceptibility of strawberry fruit to decay. This finding was also observed for cv. Everest (Jiang *et al.*, 2001). Treatment with high concentrations (500 and 1000 nl l^{-1}) 1-MCP inhibited phenylalanine ammonia-lyase activity, and lowered increases in phenolic content. Comparatively low levels of phenolics in fruit treated at the high 1-MCP concentration could account for decreased disease resistance (Jiang *et al.*, 2001).

3.1.4 Grey mould

Grey mould is the predominant post-harvest disease affecting strawberry fruit (Edney, 1964; Dennis and Mountford, 1975). *Botrytis cinerea* Pers. is the conidiaphore anamorph of *Botryotinia fuckeliana* (de Bary) Whetzel (Jarvis, 1977; Coley-Smith *et al.*, 1980; Bulit and Dubos, 1988). It attacks stems, leaves, influorescences and fruit of the strawberry plant. However, *B. cinerea* is most evident on ripe strawberry fruit.

B. cinerea can infect host tissue through natural openings and necrotic tissue or by direct penetration. However, *B. cinerea* is generally regarded as an opportunistic fungus that invades weak, damaged or senescent tissues (Haegermark, 1984; Elad and

Evensen, 1995). As with grape and raspberry fruits (Bulit and Dubos, 1988; Johnston *et al.*, 1994), grey mould of strawberry fruit tends to derive from pre-harvest infection of senescent or necrotic flower parts during or just after anthesis (Powelson, 1960; Bristow *et al.*, 1986). *B. cinerea* enters the receptacle through basal tissues of attached stamens and calyces (Powelson, 1960; Bristow *et al.*, 1986). Conidial germination and subsequent infection of petals, sepals and stamens of the inflorescence appears to be mediated by the stimulatory effect of pollen and pistillate parts (Chou and Preece, 1968; Borecka and Millikan, 1973). Removal of these parts immediately after flowering resulted in a marked reduction in the disease incidence on strawberry fruit (Powelson, 1960; Jarvis, 1962).

The infection of newly opened strawberry flowers, which are very sensitive to infection by *B. cinerea,* not only leads to blossom blight (Jarvis and Borecka, 1968), but also to quiescent infections of fruit (Davis and Dennis, 1979). Generally, grey mould is then only observed once quiescence is broken during storage. Thus, between these two phenological extremes of flowering and fruit senescence lies a period of relative resistance when epidemics are rare.

3.1.5 Constitutive resistance in strawberry

Strawberry fruit vary in their inherent susceptibility to *B. cinerea* according to their physiological status (Gilles, 1959) and genotype (Daugaard *et al.*, 1999). *B. cinerea* tends to infect inflorescences in the field, but extensive fruit decay is only usually seen after harvest when the fruit has reached full harvest maturity. Therefore, *B. cinerea* remains quiescent until physiological conditions imposed by the host fall beneath a threshold allowing the infection process to continue. Thus, the inherent natural disease resistance (NDR) of strawberry fruit declines during fruit development and storage. Moreover, the susceptibility of flowers increases with age to a maximum at petal-fall (Hennebert and Gilles, 1958)

Little work has been carried out to identify and characterise preformed and/or induced antifungal compounds in strawberry tissue (Table 3.1.5.1). Fillipone *et al.* (1999) isolated and purified a heat stable 316 Da preformed compound fragarin from immature strawberry cv. Chandler leaves. This compound was active against

Collectrichum actutatum, Collectrichum fragariae and Collectrichum gleosporioides, but its activity against *B. cinerea* was not tested. Vincent *et al.* (2000) detected and partly characterised three antifungal compounds in crude extracts of strawberry leaf tissue using thin layer chromatography (TLC) *C. fragariae* bioassay. These compounds at R_f = 0.32, 0.56 and 0.66 were undetectable by non-specific TLC reagent spray anisaldehyde and UV illumination. This indicates that these antifungal compounds inhibitory to conidia of *C. fragariae* were not phenols, propanoids, steroids or terpenes. However, two compounds (not specified) did react to iodine vapour (I_2). I_2 reacts non-specifically to natural products by binding with double bonds. Further characterisation was not attempted. TLC bioassays showed that the concentrations and presence of antifungal compounds varied between anthracnose resistant and susceptible cultivars. Sweet Charlie, an anthracnose-resistant cultivar, produced approximately 15 times more antifungal activity than the more susceptible cv. Chandler. Thus, Vincent *et al.* (1999) suggest that anthracnose resistance in strawberry leaves may depend on concentrations of preformed antifungals R_fs 0.56 and 0.66 and a third phytoalexin-like compound R_f 0.32 that was only induced in the resistant cv. Sweet Charlie. Lower anthracnose disease in older leaves (Smith, 1998) is consistent with higher concentrations of R_fs 0.56 and 0.66 (Vincent *et al.*, 1999).

Mussel and Staples (1971) discovered two phytoalexin-like compounds (A, R_f = 0.52; B, R_f = 0.39) from roots. These compounds were induced by challenging cv. Surecrop plants that were resistant to red stele disease with *Phytophthora fragariae.* Compounds A and B were detectable 24 - 48 h after inoculation. Inoculation of susceptible cv. Blakemore did not result in appearance of these compounds. However, another preformed antifungal compound (C, R_f = 0.84) was found in extracts of both cultivars regardless of whether or not they were inoculated. Using TLC bioassays both phytoalexin-like compounds (A, B) and preformed compound C were inhibitory *to Cladosporium cucumerinum.* Compounds A and B extracted from TLC plates and introduced into cultures of *Phytophthora fragariae* proved to be inhibitory to mycelial growth. Compound C was not inhibitory to *P. fragariae.* Identities of these three compounds were not determined. Phenylalanine-ammonia lyase (PAL) activity was determined in both healthy and infected strawberry roots, but did not change after infection in either resistant or susceptible cultivars. This constant

PAL activity suggests that the antifungal compounds A and B were not derived from the phenylpropanoid pathway.

Few authors have specifically looked at what role preformed antifungal compounds may have in determining strawberry fruit NDR and how such antifungal compounds may change during strawberry fruit development. According to Jersch and co-workers (1989), proanthocyanins (PA) are the basis for the temporary quiescence of *B. cinerea* in immature strawberry cv. Senga Sengana fruit. They suggested that quiescence in strawberry is directly related to PA concentration, such that the overall PA concentration of immature strawberry fruit is negatively correlated with mycelial development on inoculated fruit (Jersch *et al.*, 1989). PA inhibits polygalacturonase produced by *B. cinerea*. Polygalacturonase is a key enzyme in pathogenicity. Resistance of immature fruits to external infection was correlated with the pronounced deposition of PA in the epidermal layer. PA concentration was found to be higher in less susceptible strawberry cultivars. Harris and Dennis (1982) demonstrated that polygalacturonase produced by *B. cinerea* was rapidly inactivated by naturally occurring phenolics in infected strawberry cv. Cambridge Favourite fruit. Extracts of more resistant green stage strawberry fruit infected with *B. cinerea* showed approximately twice the endo-polygalacturonase activity of extracts of white and red stage fruit (Harris and Dennis, 1982).

Although Jersch *et al.* (1989) asserts that PA concentration governs *Botrytis* quiescence, aqueous extracts of green fruit did not indicate the presence of performed or inducible antifungal compounds when tested in Petri plate bioassays using *B. cinerea* or *Cladosporium herbarum*. This result contrasts findings by Herbert *et al.* (2001), who demonstrated, using a similar bioassay methodology, that aqueous extracts of immature strawberry cv. Chandler fruit did have direct antifungal activity against mycelial growth and germination of *B. cinerea*. Herbert *et al.* (2001) confirmed that increased resistance to *Botrytis* was positively correlated with PA concentration. Specifically, cultivars with higher concentrations of free and bound catechin, epicatechin and gallic acid were more resistant to *B. cinerea*.

Di Venere *et al.* (1998) also demonstrated that a decline in catechin content was correlated to differences in NDR to *B. cinerea* between fruit development stages and

to varying resistance of cultivars. A rapid decline in total hydrolyzable and condensed tannins was allied to increased susceptibility to *B. cinerea* during fruit development. Creasy *et al.* (1964) characterised through two-dimensional paper chromatography that strawberry leaf tissue contained detectable amounts of catechin. More recently, (+)-catechin was shown to be a preformed infection-inhibiting factor in strawberry leaves (Yamamoto *et al.*, 2000). This preformed antifungal compound also accumulated in response to inoculation with non-pathogenic spores of *A. alternata* (Yamamoto *et al.*, 2000). Catechin inhibited the formation of appressoria-derived hyphae, but allowed both spore germination and appressorial formation. Accumulation of catechin on a susceptible host by inoculation with pathogenic strain of *A. alternata* was not achieved. Enhanced synthesis of flavan-3-ols (catechins and proanthocyanidins) have also been reported to accumulate in necrotic boundary zone of strawberry cv. Hybride leaves infected with *Mycosphaerella fragariae* (Feucht *et al.*, 1992). In addition, the concentration catechol-based phenolics in leaf tissue was also negatively correlated with two spotted spider mite development (Luczynski *et al.*, 1990).

Preformed antifungal phenolic compounds, have been identified in strawberry roots (Nemec, 1973; 1976). Quercetin and salicylic acid (0.5 mg ml^{-1}) were the most inhibitory against *in-vitro* radial growth of *Phythium irregulare, Rhizoctonia solani* and *A. alternata* (Nemec, 1976). However, the growth of these strawberry root fungi was unaffected by gallic acid, chlorogenic acid, d-catechin, and arbutin. No correlation was apparent between the qualitative phenolic composition of cultivars tested and root disease resistance.

Work using aqueous extracts has shown that the achene extract from ripe cv. Chandler fruit inhibited radial growth of *B. cinerea*, but not of *Rhizopus stolonifer* (El Ghaouth *et al.*, 1991a). Extracts from receptacle tissue without achenes did not inhibit the growth of either fungus. Achenes appeared to be a stronger source of constitutive and potentially antifungal glucanohydrolases than receptacle tissue. Susceptibility of ripe strawberries to *B. cinerea* may be explained in part by low levels of chitinase, β-1,3-glucanase, and lysozyme activity in receptacle tissue. In fact, Gilles (1959) showed that sterilised strawberry juice markedly stimulated the germination of *B. cinerea*

conidia. It seems unlikely that achene derived glucanohydrolases contribute to the NDR of ripe fruit as their release may be hindered by the achene pericarp (El Ghaouth *et al.*, 1991a).

Grayer and Kokubun (2001) report that wide-ranging search for phytoalexins in strawberry failed to provide evidence of phytoalexin accumulation in strawberry leaves and fruit in either naturally or artificially infected tissue. In contrast, Hirai *et al.* (2000) identified three phytoalexin compounds from extracts of unripe strawberry cv. Houkouwase fruit wounded and challenged with *Colletotrichum musae*. These chemicals were identified through NMR spectral analysis as the triterpenes, euscaphic acid, tormentic acid and myrianthic acid (compounds 1-3). Such compounds are either biosynthesised *de-novo* from mevalonic acid and/or converted from non-hydroxylated triterpenes. Using TLC bioassays all three compounds were inhibitory to *C. musae*. It was suggested that compounds 1-3 might confer resistance to *Colletotrichum fragariae*. TLC analysis indicated that phytoalexin compounds 1 and 2 or 3 as found in green cv. Houkouwase fruit probably correspond to phytoalexin-like compounds A and B found in strawberry cv. Surecrop roots (Mussel and Staples, 1971).

The evidence presented above suggests that preformed and/or inducible antifungal compounds have a role in NDR of strawberry fruit. A series of experiments were performed to quantify NDR resistance in cv. Elsanta and determine the presence and identity of antifungal compounds during flower and fruit development. The location in different parts of the fruit of possible antifungals was also investigated.

Table 3.1.5.1: Preformed and inducible compounds found in strawberry tissues that demonstrate antifungal activity

Cultivar	Tissue	Chemical	Preformed or Inducible	Pathogen	Reference
Surecrop	Roots	N.I.	inducible	*Phytophthora fragaria; Cladosporium cucumerinum*	Mussel and Staples (1971)
Howard and Surecrop type varieties (n = 18)	Roots	Quercetin	preformed	*Phythium irregulare; Rhizoctonia solani; Alternaria alternata*	Nemec (1973); Nemec (1976)
Senga Sengana	Green fruit	Proanthocyanins	preformed	*Botrytis cinerea*	Jersch *et al.* (1989)
Chandler	Red fruit achenes	N.I.	preformed	*B. cinerea*	El Ghaouth *et al.* (1991a)
Clea and Pajaro	Various developmental stages	Proanthocyanins	preformed	*B. cinerea*	Di Venere *et al.* (1998)

Cultivar	Tissue	Chemical	Preformed or Inducible	Pathogen	Reference
Chandler	Leaves	Fragarin	preformed	*Collectrichum actutatum; C. fragariae; C. gleosporioides*	Fillipone *et al.* (1999)
Chandler	Leaves	N.I.	preformed and inducible	*C. fragariae*	Vincent *et al.* (1999)
Houkouwase	Green fruit	Triterpenes	inducible	*Colletotrichum musae*	Hirai *et al.* (2000)
Not in abstract	Leaves	Catechin	preformed	*Alternaria alternata*	Yamamoto *et al.* (2000)
Chandler and others	Various fruit development stages	Proanthocyanins (catechin, epicatechin, gallate)	preformed	*B. cinerea*	Herbert *et al.* (2001)
Elsanta	Green fruit	N.I	preformed and induced	*B. cinerea; Cladosporium cladosporioides*	Adikaram *et al.*, 2002

N.I. = not identified

3.2 Quantification of NDR *in-planta*

3.2.1 Materials and methods

3.2.1.1 *Plant material*

Cold-stored maiden-year A+ grade strawberry cv. Elsanta plants were supplied by KG Fruits Ltd. (Kent, UK). The plants were grown in a glasshouse in 1 litre capacity pots containing peat and expanded polystyrene (10 + 1, by volume). Total nitrogen content of peat was 8.88 g N kg^{-1}, as determined by the Kjeldahl method (Rowell, 1994). The plants received 4.4 ml l^{-1} of a general N, P, K, and Mg nutrient formulation (Tomarite™, Levington Horticulture Ltd., Ipswich, UK) twice weekly. Fertilisation started 2 weeks after planting and continued through flowering and fruiting. Plants were sprayed with 1.5 l chlorpyrifos ha^{-1} at two-week intervals, primarily to control spider mites (*Tetranychus* spp.). They also received a single spray of 1.5 l myclobutanil ha^{-1} 3 weeks after planting to control powdery mildew (*Sphaerotheca macularis* f. sp. *fragaria* Peries). Plants were not treated with any fungicide active against *B. cinerea*. Flowers were hand pollinated to minimise occurrence of misshapen fruit.

3.2.1.2 *Fruit sampling*

Twenty two fruit of green I, white and red stages of maturity with no signs of fungal infection were randomly harvested and weighed. The colour of each fruit was measured using a Minolta DP-100 colorimeter (Minolta Co. Ltd., Japan) with an 8 mm light path aperture. The instrument was calibrated with a Minolta standard white tile CR-200 (Y = 93.9, x = 0.3134, y = 0.3207). The mean of two readings around the equatorial surface of the fruit was recorded and the Hue angle (H°) calculated (Sacks and Shaw, 1994).

3.2.1.3 *Inocculum preparation*

A single-spored isolate of *B. cinerea* was recovered from naturally infected strawberry cv. Elsanta fruit. The isolate was cultured in 9 cm diameter Petri plates on ½ strength PDA (potato dextrose agar; 19.5 g PDA l^{-1} distilled water) (Oxoid Ltd, Basingstoke, UK) at 22

±1°C. Streptomycin (1.0 mg ml^{-1}) was also added to inhibit growth of bacteria. The isolate was obtained using a semi-mechanical method (Johnston and Booth, 1983). Briefly, a flamed, moistened loop was dipped into a series of spore suspensions diluted 10 fold. The suspensions were streaked across a marked line on PDA. After 12 h incubation at 22 ±1°C, single germinating single-spores were selected using a stereoscopic microscope. These were picked using a Borrowdale needle and each transferred to an individual agar plate. One *B. cinerea* isolate was selected and used in all experiments. Pathogenicity of this isolate was maintained by performing Koch's postulate every 3 months.

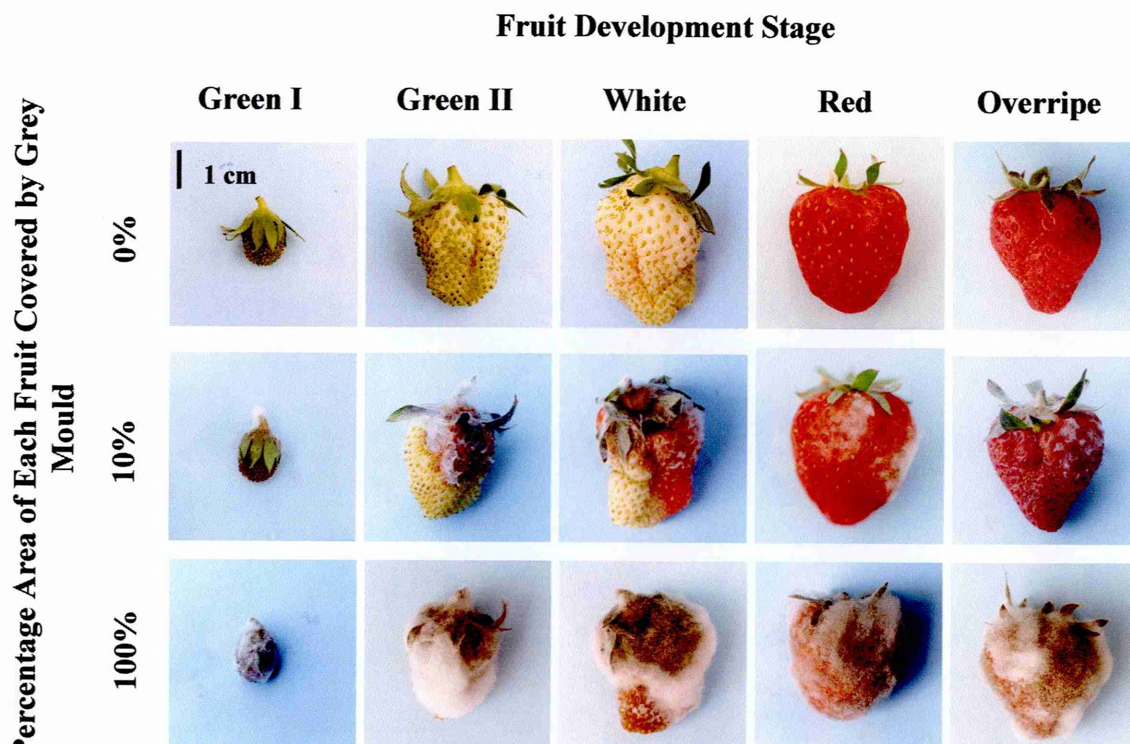
B. cinerea was subjected to diurnal (12 h d^{-1}) UV-A lighting (model number 10531, GE lighting, Surrey, UK) to induce sporulation (Leach, 1962). A conidial *B. cinerea* suspension was obtained from 4 day-old cultures by flooding with sterile distilled water containing 0.1% Tween 80 (El Ghaouth *et al.*, 1992) and scraping the mycelial surface with a sterile scalpel blade. The conidial suspension was filtered through glass wool to remove mycelial fragments. The spore concentration was adjusted using a haemocytometer with sterile distilled water to 2 x 10^4 conidia ml^{-1}.

3.2.1.4 *Fruit inoculation and disease assessment*

Fruit were wound inoculated by application of a 15 µl drop of a 4 day-old *B. cinerea* conidial suspension (2 x 10^4 conidia ml^{-1}) to the fruit shoulder. Non-inoculated fruit were controls. Fruit were held in the dark at 5°C and 95 to 100% relative humidity (RH) in individual closed but vented polystyrene containers (Plate 3.2.1.1) in a completely randomised design (CRD). The high RH was achieved by placing each fruit on a plastic mesh stand over distilled water (20 ml) and covering the transparent container (284 ml capacity) with perforated polypropylene film (15 µm thickness, 400 µm diameter holes, 5 holes per cm^2 [Cryovac 250 Y], Cryovac, UK) secured with an elastic band. Disease severity resulting from inoculation or natural infection was assessed as the percentage area of each fruit covered by grey mould, and was recorded daily (Plate 3.2.1.2). 10% disease severity score was defined as the first instance disease was visible on surface of fruit.

Plate 3.2.1.1: Transparent storage vessel used for disease severity assessment

Plate 3.2.1.2: Disease severity chart showing percentage areas of different fruit stages covered with grey mould disease



3.2.1.5 *Statistical analysis*

Fungal growth data were subject to general analysis of variance using Genstat Version 4.1. Where significance effects were obtained, least significant differences (LSDs) were calculated for mean separation at the 5% level.

3.2.2 Results and discussion

Fresh weight differed significantly ($P < 0.001$) with increasing fruit maturity (Table 3.2.2.1; Appendix 3.2). Conversely, H° decreased with increasing maturity. The most dramatic changes in weight and colour occurred between white and red stage fruit.

Table 3.2.2.1: Fresh weight and hue angle of strawberry fruit at different developmental stages (n = 22)

Colour Stage	Fresh Weight (g)[A]	Hue Angle (H°)[B]
Green I	3.03	111.54
White	6.55	109.30
Red	16.59	40.30

[A]Mean separation by LSD [P = 0.05] = 2.28

[B] Mean separation by LSD [P = 0.05] = 1.10

Time to 10% disease severity on green I fruit was significantly ($P < 0.001$) more than for white and red stages, respectively (Table 3.2.2.2; Appendix 3.2). Control and inoculated green I fruit were approximately 3 and 1.5 times more resistant than red fruit (Table 3.2.2.2). In contrast to white and red stage fruit, conidia were never produced on green I fruit (Plate 3.2.1.2). Rather a mass of white mycelium was observed. Thus, normal development of the fungus was apparently inhibited in green I fruit. Differences in time to disease severity score and growth habit of *B. cinerea* between different fruit development stages fruit may be due to resistance factors and/or availability of nutrients.

Table 3.2.2.2: Effect of fruit development stage and ± inoculation on grey mould disease (n = 11).

Colour stage	Time to 10% disease severity (days)	
	Inoculation[A]	Control[B]
Green I	25.9	32.1
White	19.6	27.4
Red	8.7	19.5
	18.3	*26.3*

[A]Mean separation by LSD [P = 0.05] = 2.9 d

[B]Mean separation by LSD [P = 0.05] = 2.4 d

Time to 10% disease severity at 5°C was significantly less ($P < 0.001$) for inoculated fruit (18.1 days) versus control fruit (26.3 days) (LSD [$P = 0.05$] = 2.4 d). There was no significant ($P > 0.05$) interaction between fruit stage and inoculation. Thus, both ± inoculation treatments gave similar trends (Table. 3.2.2.2). No difference was observed in morphology of grey mould disease between inoculated and control fruit of the same development stage.

The reducing delay in symptom expression with increasing fruit maturity confirms similar findings by Gilles (1959), Powelson (1960) and Jarvis (1962). Differences in symptom expression show that *B. cinerea* growth is suppressed in green fruit. This inhibition may be due to a defence mechanism(s) present in green I fruit but absent in white and red fruit. To help explain the apparent decline in NDR during strawberry fruit development, experiments were conducted to investigate a possible role for preformed antifungal compounds.

3.3 Preformed Antifungal Compounds in Strawberry

3.3.1 Materials and methods

3.3.1.1 *Plant material*

Cold-stored maiden-year cv. Elsanta strawberry plants supplied by KG Fruits Ltd. (Kent, UK) were grown as described in section 3.2.1.1.

3.3.1.2 *Thin layer chromatography*

Glass-backed thin layer chromatography (TLC) plates (20 x 10 or 20 x 20 cm) coated with silica gel 60 or silica gel 60 F_{254} (Merck, Darmstadt, Germany) were used. The plates were spotted (5-100 µl) with resuspended crude and sequential strawberry fruit extracts using a 5 or 10 µl micro-pipette. The protein synthesis inhibitor cycloheximide (0.5 mg ml^{-1}) and extracting solvents (99% v/v) were used as positive and negative controls, respectively. TLC plates were developed in either one or two dimensions at approximately 22°C in a TLC tank (20 x 20 x 10 cm) lined with filter paper (i.e. solvent saturated atmosphere). One-dimensional (1-D) TLCs were developed either in a running solvent of hexane: ethyl acetate: methanol (A = 60:40:1; B = 60:40:10; C = 60:40:20; D = 60:40:30 v/v/v; 100 ml) (Zanuri *et al.*, 2001) or in the organic phase of ethyl acetate: benzene: ethanol (4: 1: 1, v/v/v) (Mussel and Staples, 1971) or chloroform: methanol (90:10 v/v). Two-dimensional (2-D) TLCs (Wedge and Nagle., 2000) were developed in solvent system A (1-D) and then solvent system D (2-D). Developed 1-D and 2-D chromatograms were dried and either used for bioassay or sprayed with reagents.

3.3.1.3 *Inocculum preparation*

Single-spored isolates of the pathogen *B. cinerea* and the bioassay organism *C. cladosporioides* recovered from naturally infected strawberry cv. Elsanta fruit were cultured as described in section 3.2.1.3 at 22 ±1 and 20 ±1 °C, respectively. A single-

isolate each of both *B. cinerea* and *C. cladosporioides* was used in all experiments. The pathogenicity of each isolate was maintained by performing Koch's postulate every 3 months.

A conidial suspension of *B. cinerea* was prepared as described in section 3.2.1.3 and adjusted with Czapek Dox solution (Appendix 3.3.1) to 2 x 10^6 conidia ml^{-1}. A spore suspension of *C. cladosporioides* was obtained from 7-14 day-old cultures by flooding Petri plates with Czapek Dox solution (Adikaram and Ratnayake Bhandara, 1998). The solution was filtered through two layers of cheese cloth, and adjusted to 2 x 10^7 spores ml^{-1} (Adikaram *et al.*, 2002).

3.3.1.4 *TLC bioassay*

Developed chromatograms were sprayed with a spore suspension of *C. cladosporioides* or *B. cinerea* in Czapek Dox nutrient solution (Adikaram and Ratnayake Bhandara, 1998; Zanuri *et al.*, 2001; Appendix 3.3). Plates were incubated at 100% RH and 20°C for 3-6 days. Zones of fungal inhibition, where mycelial growth was absent, indicated the presence of antifungal activity (Klarman and Stanford 1968; Homans and Fuchs, 1970). Weak antifungal activity was defined as areas with lighter coloured mycelium from control. The R_f and area of inhibition were measured and recorded.

3.3.1.5 *Antifungal identification*

Detection procedures of compounds for 1 and 2-D TLCs included inspection under visible and ultraviolet light (254 and 336 nm) before and during fuming with 98% v/v ammonia. After examination, ammonia was removed from plates through air drying for 1 hour. Replicate plates were sprayed with 10% w/v phosphomolybdic acid (PMA) in ethanol, a general visualisation reagent for oxidizable compounds, and heated to about 100°C for 2 min. Similarly, more specific visualisation reagents were used for the detection of phenolics, alkaloids and terpenes. Phenolics were detected by spraying chromatograms with either 1.89 M sodium carbonate followed by using Folin Coicalteu reagent (1:3 v/v water) and fumigation with ammonia or with equal volumes of 0.94 M sodium carbonate and diazotized sulfanilic acid (Waterman and Mole, 1994). Dragendorff's reagent (0.11

M potassium iodide, 3.5 M acetic acid and 0.6 mM bismuth subnitrate; Sigma Chemicals Co., St Louis, USA) was used for detection of alkaloids and other nitrogen compounds. Ehlrich's reagent (0.007 M *p*-dimethylaminobenzaldehyde in 2-propanol) was used for detection of terpenes and sugars (results not shown). The Rf and colour of detected spots were recorded and compared to zones of fungal inhibition on duplicate bioassay chromatograms.

3.3.1.6 *Crude extraction of whole strawberry fruit at different development stages*

Whole green I, white and red stage strawberry fruit without calyxes (50 g fresh weight (FW)) (Plate 3.3.1) were harvested and immediately snap frozen in liquid nitrogen and stored at -18°C until use. Each sample was ground in liquid nitrogen and added to 99% (v/v) ethanol at -18°C (3 ml g^{-1} FW). The mixture was homogenised at 20, 500 rpm using an Ultra-Turrax T25 homogeniser (Janick and Kunkel, Stanfen, Germany) for 5 min. The homogenate was filtered under vacuum through Whatman No. 3, using a 5.5 cm diameter Buchner Funnel. The filtrate was concentrated in a rotary evaporator (Buchi Rotovapor, Büchi Labortechnik AG, Flawil, Switzerland) under vacuum (0.6 kPa) at 40°C to approximately 1/3rd of the original volume. The concentrated extract was then partitioned twice with equal volumes of 99% (v/v) dichloromethane using a separating funnel. The lower dichloromethane layers were pooled and dried by adding 6 g of anhydrous $MgSO_4$. This mixture was then filtered through Whatman No. 2 and evaporated to dryness in the rotary evaporator at 40°C. The organic extract was resuspended in 99% (v/v) ethanol (0.2 ml g^{-1} FW; Droby *et al.*, 1986) and stored at -18°C until use. The experiment was repeated.

3.3.1.7 *Sequential extraction of whole strawberry fruit at different development stages*

Green stage II, white and red fruit (Plate 3.3.1) were each snap frozen in liquid nitrogen, freeze dried, weighed and stored at -18°C until use. Each sample was added to hexane (99% v/v) at -18°C (3 ml g^{-1} FW) and homogenised and filtered as described in section 3.3.1.6. Samples were evaporated to dryness in a rotary evaporator at 35°C. The residue from filtration was then sequentially extracted with solvents of increasing polarity. These were, in turn, ethyl acetate, chloroform: methanol (1:1) and ethanol (80% v/v). These

extracts were each evaporated to dryness and then resuspended in extracting solvent (0.2 ml g^{-1} FW) and stored at -18°C.

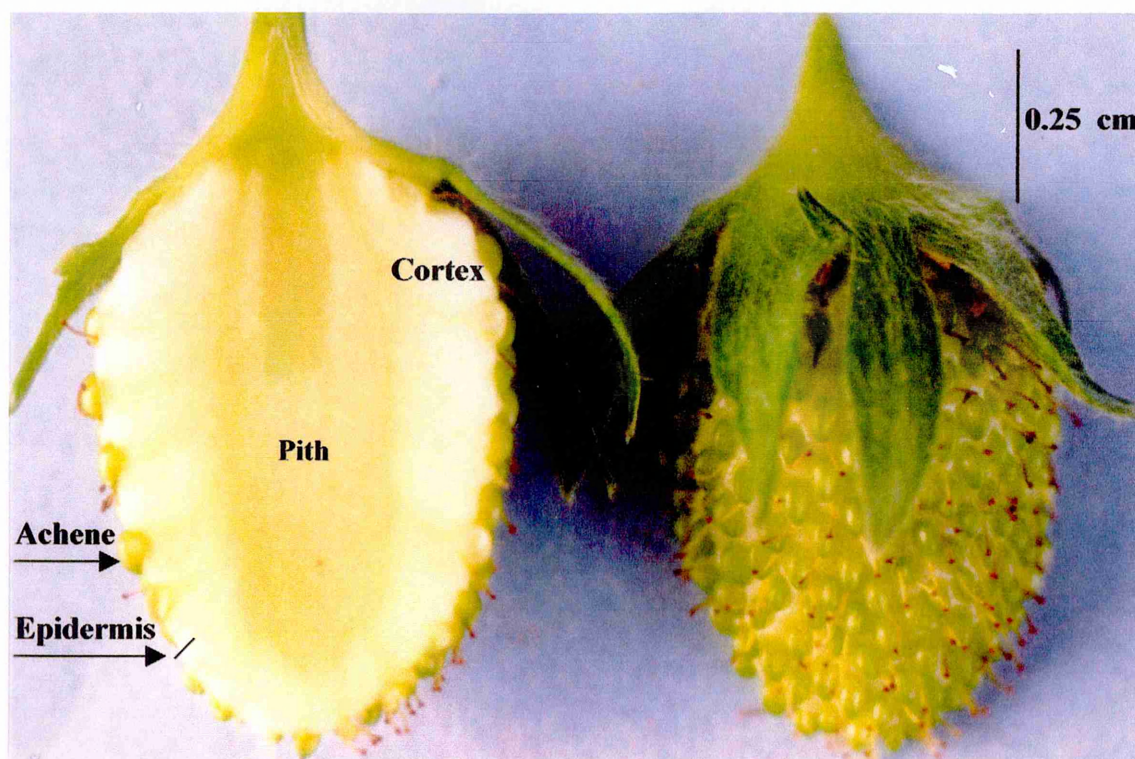
3.3.1.8 Crude extraction of different tissues in green stage I

Green stage I, white and red strawberry fruit were harvested, and dissected into pith tissue, corticular parenchyma tissue, epidermal tissue without achenes and achenes (Plate 3.3.1.1). Each tissue type (10 g FW) from each fruit stage and whole fruit were extracted as described in section 3.3.1.2.

3.3.1.9 *Sequential extraction of green stage I achenes*

Green stage I fruit were harvested and achenes removed. Achenes (10 g FW) were prepared and sequentially extracted in ethyl acetate and ethanol as described in section 3.3.2.3.

Plate 3.3.1.1: Transverse section (LHS) through green stage I strawberry cv. Elsanta fruit showing pith, cortex, epidermis and achenes.



3.3.1.10 *Sequential extraction and column chromatography of green stage I tissues*

Green stage I achenes (35 g FW; 10.12 g DW), whole fruit without achenes (75 g FW; 8.12 g DW) and whole fruit with achenes (125 g FW; 15.92 g DW) were sequentially extracted in hexane, ethyl acetate and ethanol (99% v/v) (3 ml g^{-1} FW) as described in section 3.3.2.4. 1-D TLCs were performed on each extract (0.2 ml g^{-1} FW) using PMA run in solvent system B (hexane; ethyl acetate; methanol; 60:40:10 v/v/v) as described in sections 3.3.1.2 and 3.3.1.5. On the basis of similar TLC behaviour to phosphomolybdic acid (PMA) hexane fractions of achenes (50 mg extract DW) and whole fruit with achenes (74 mg extract DW) were combined to increase the amount of material in preparation for purification. This was also done for ethyl acetate fractions (119; 83 mg extracts DW).

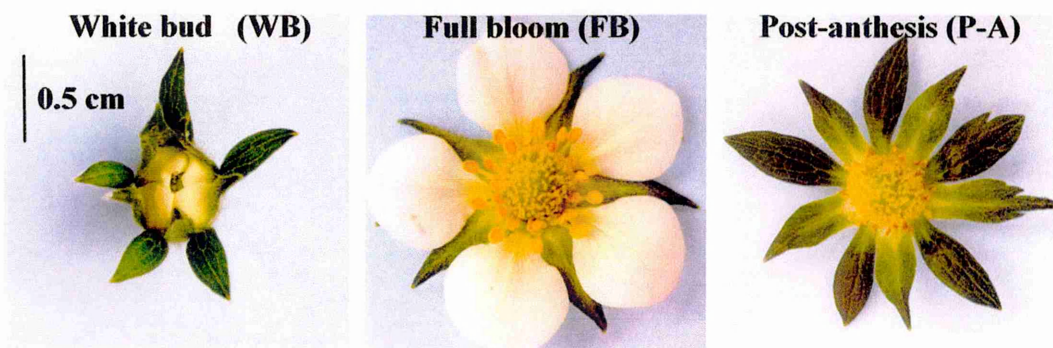
Hexane and ethyl acetate combined dry extracts (124 mg; 212 mg) were chromatographed in a glass column of length 7.5 cm and diameter 2 cm packed with Silica gel 60 (Fluka, St. Louis, USA) using three solvent combinations of increasing polarity. The extracts were first dissolved in the minimum volume of hexane: ethyl acetate (80: 20 v/v) and layered on top of the column pre-equilibrated with the same solvent. One thousand drop fractions of the column eluate were collected using a Pharmain LKB-Redifrac (Amersham BioSciences, Bucks., UK). The sequential solvent systems used (40 ml) for elution were hexane: ethyl acetate (60: 40 v/v), hexane: ethyl acetate: methanol 40:60:1 (v/v/v) and (20: 80: 1; v/v/v). Eighteen and 43 fractions were obtained from hexane and ethyl acetate combined extracts, respectively. These were reduced to 3 and 7 fractions by pooling on the basis of similar PMA and TLC behaviour run in solvent B (hexane: ethyl acetate: methanol; 60:40:10 (v/v/v). 1D-TLC bioassays were performed on pooled fractions run in solvent B.

3.3.1.11 *Sequential extraction of different strawberry flower development stages*

Strawberry flowers of white bud (WB), full bloom (FB) and post-anthesis (PA) developmental stages (Plate 3.3.1.2) were harvested and each snap frozen in liquid nitrogen, freeze dried, weighed and stored at -18°C until use. Besides whole flowers, receptacle tissue including pistils from 3 flower stages were selected and prepared as

above. Each sample (10 g FW) was sequentially extracted in ethyl acetate and ethanol (99% v/v) (3 ml g^{-1} FW) as described in section 3.3.2.7.

Plate 3.3.1.2: Development stages of strawberry cv. Elsanta flowers



3.3.2 Results and discussion

3.3.2.1 *Preformed antifungal compounds in strawberry fruit at different development stages*

Crude ethanol extracts partitioned with dichloromethane of green stage I, white and red stage strawberry fruit contain varying degrees of antifungal activity as shown by one-dimensional (1-D) TLC bioassay (Plate 3.3.2.1W and Table 3.3.2.1). Green stage I fruit contain at least two colourless preformed antifungal compounds (R_f = 0.44 and 0.37) that are not present in white and red stage fruit. Negative reactions to UV-C, phenolic reagent sprays (Folin Coicalteu and sulfanilic acid) and Dragendorff's reagent suggest that these compounds are not phenolics (Plate 3.3.2.1X-Z) or alkaloids. Less intense positive reactions of R_f = 0.37 to Ehrlich's reagent suggests that it may be a terpene. This preformed compound may correspond to one or more of the triterpenes phytoalexins previously reported by Hirai *et al.* (2000). However, plants grown in this study were not subjected to biotic or abiotic stress after harvest. It is also possible that compounds R_f = 0.44 and/or R_f = 0.37 may be similar to preformed antifungal compounds at R_f 0.66 and 0.56 (hexane: ethyl acetate; 80: 20 v/v) discovered in strawberry leaves (Vincent *et al.*,

1999). These compounds conferred resistance to *C. fragariae* in strawberry leaves, and were also undetectable by UV or anisaldehyde spray reagent. Anisaldehyde is used to detect a variety of natural products, including terpenes.

Plate 3.3.2.1: One-dimensional thin layer chromatographs of crude ethanol extract partitioned into an organic dichloromethane phase of green stage I, white and red cv. Elsanta fruit (100 μl spot; 0.2 ml g^{-1} FW) and run in hexane: ethyl acetate: methanol (60:40:1 v/v/v). W = bioassay (*Cladosporium cladosporioides*); X = UV 254 nm (silica gel 60 F_{254}); Y = Folin Coicalteu reagent spray; Z = diatozized sulfanilic acid reagent. Lane 1, green I fruit; lane 2, white fruit; lane 3, red fruit; lane 4, ethanol (99% v/v) (control). Dotted line = origin. Dashed line = solvent front.

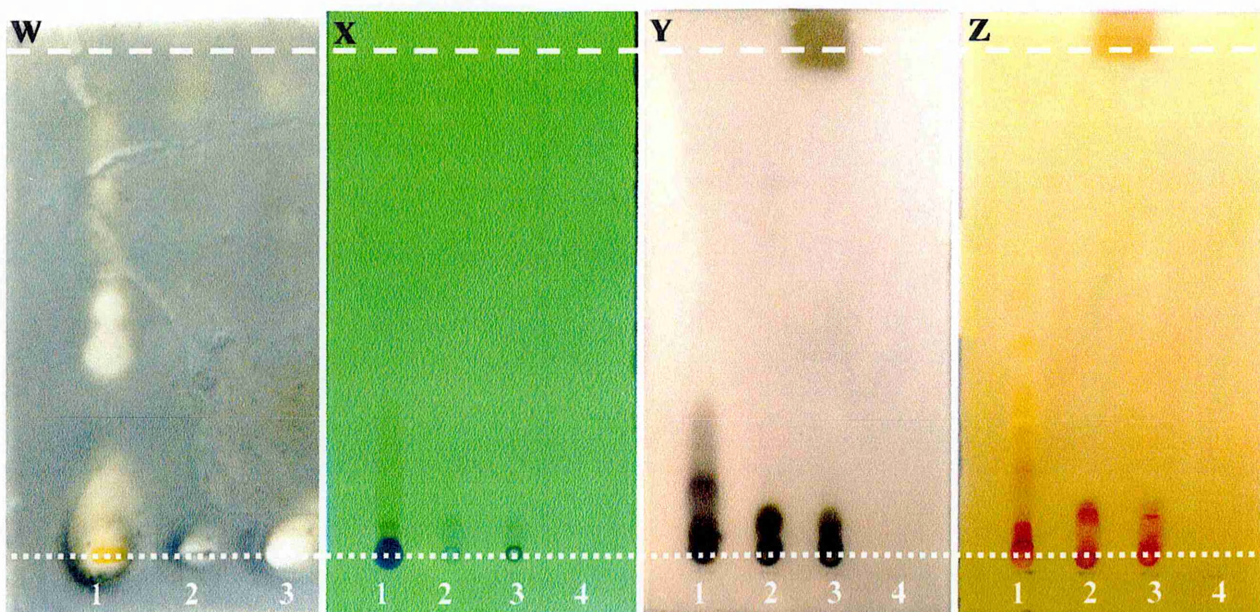


Table 3.3.2.1: Areas (cm^2) of fungal inhibition of one-dimensional thin layer chromatography bioassay (*Cladosporium cladosporioides*) of the crude ethanol extract partitioned into an organic dichloromethane phase of green I, white and red strawberry cv. Elsanta fruit extracts (100 µl spot; 0.2 ml g^{-1} FW) run in hexane: ethyl acetate: methanol (60:40:1 v/v/v).

R_f value	Fruit Development Stage		
	green I	white	red
0.86	---[A]	~1.26[B]	~1.26[B]
0.44	1.96	---	---
0.37	3.85	---	---
0.13-0.00	13.27	1.26 (R_f 0.00)	3.85 (R_f 0.00)
Total area	19.08	2.52	5.11

[A] = no inhibition zone

[B] = weak antifungal activity

Crude extracts of white and red fruit contain a yellow-coloured non-polar preformed antifungal compound (R_f = 0.86) of weak intensity that was not found in green I fruit. All fruit maturity stages showed antifungal activity at the origin of TLC plates. The approximate area of fungal inhibition at the origin in green stage I fruit extracts (R_f = 0.13-0.00) was 87 and 73% greater than in white and red fruit, respectively (Plate 3.3.2.1W; Table 3.3.2.1). Positive reactions to UV-C (dark), Folin Coicalteu (dark blue) and sulfanilic acid (brown) reagent sprays suggest that origin compounds contain phenolics (Plate 3.3.2.1X-Y).

Phenolic reagent sprays show that green I fruit extracts contain more phenolics with antifungal properties than white and red stage fruit. This observation is consistent with previous research showing that a decline in phenolics during fruit development is correlated to decreased NDR to *B. cinerea* (Jersch *et al.*, 1989; Di Venere *et al.*, 1998 and Herbert *et al.*, 2001). However, Jersch *et al.* (1989) failed to find evidence that green stage strawberry fruit contain preformed or inducible antifungal compounds. Jersch *et al.* (1989) suggested that the decline in proanthocyanins concentration during fruit development governs *B. cinerea* quiescence through inhibiting pathogen-derived

polygalacturonase. Conversely, Herbert *et al.* (2001) using a similar Petri plate bioassay to Jersch *et al.* (1989) reported a decline in phenolics with antifungal activity during fruit development. Thus, compounds at the origin found in this study may contain catechin, epicatechin and gallate reported by Herbert *et al.* (2001). Less intense positive reactions to Ehrlich's and Dragendorff's (orange) were also observed in Green I fruit, suggesting that terpenes and alkaloids may be also present at the origin.

Sequential extracts of green II, white and red stage fruit confirmed that later stages of fruit development contain less antifungal activity than green I fruit (Plate 3.3.2.2 and Plate 3.3.2.1). There was little apparent differences in antifungal activity between fruit stages (Plate 3.3.2.2 and Table 3.3.2.2). Most antifungal activity was found in chloroform: methanol (70: 30 v/v) fraction ($R_f = 0.00$). The sugars extracted in this solvent may have prevented fungal growth at the origin through osmotic inhibition. However, this result is consistent with previous findings observed in crude extracts of white and red stage fruit (Plate 3.3.2.1W). Compounds with weak antifungal activity were seen in hexane ($R_f = \sim 0.73$) and ethyl acetate fractions ($R_f = 0.27, 0.21$) run in hexane: ethyl acetate: methanol (60:40:10 v/v/v). The ethanol fraction (80 % v/v) gave no antifungal activity in all stages.

Plate 3.3.2.2: One-dimensional thin layer chromatography bioassays (*Cladosporium cladosporioides*) of green II, white and red stage strawberry cv. Elsanta fruit extracted in a series of solvents increasing in polarity (viz. A = hexane (99% v/v), B = ethyl acetate (99% v/v) (100 μl spots; 0.2 ml g^{-1} FW) and C = chloroform: methanol (70:30 v/v) (20 μl spot; 0.2 ml g^{-1} FW) run in hexane: ethyl acetate: methanol (60:40:10 v/v/v). Lanes 1 and 7, green fruit II; lanes 2 and 8, white fruit; lanes 3 and 9, red fruit; lane 4, corresponding extracting solvent (99% v/v); lane 5, cycloheximide (0.5 mg ml^{-1}) and lane 6, nothing. Dotted line = origin. Dashed line = solvent front.

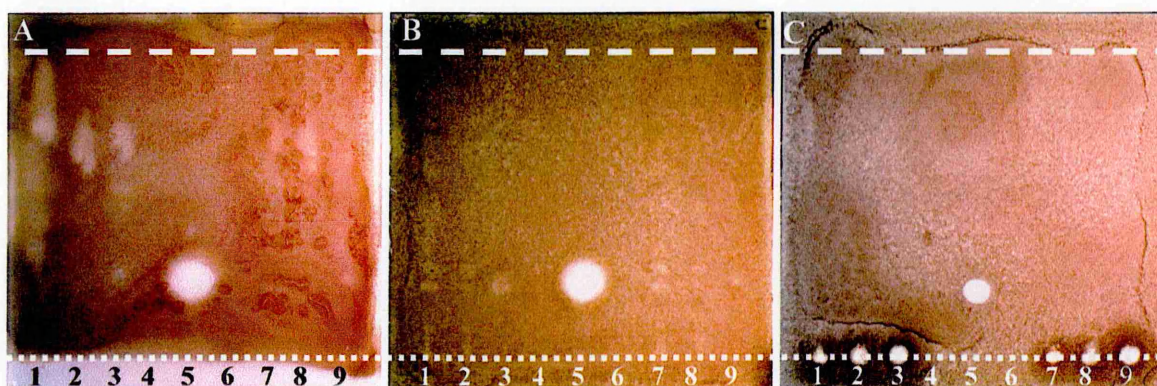


Table 3.3.2.2: One-dimensional thin layer chromatography bioassays (*Cladosporium cladosporioides*) R_f values of green II, white and red stage cv. Elsanta fruit sequentially extracted with hexane (A), ethyl acetate (B) (100 µl spot; 0.2 ml g^{-1} FW) and chloroform/methanol (C) (20 µl spot; 0.2 ml g^{-1} FW) run in hexane: ethyl acetate: methanol (60:40:10 v/v/v).

Extracting solvent	Fruit development stage		
	Green II	White	Red
Hexane	0.73-0.67	0.74-0.57	0.73-0.62
Ethyl acetate	0.27, 0.21	0.27, 0.21	0.21
Chloroform: methanol	0.00[A]	0.00[A]	0.00[A]

cycloheximide (0.5 mg ml^{-1}) $R_f = 0.24$

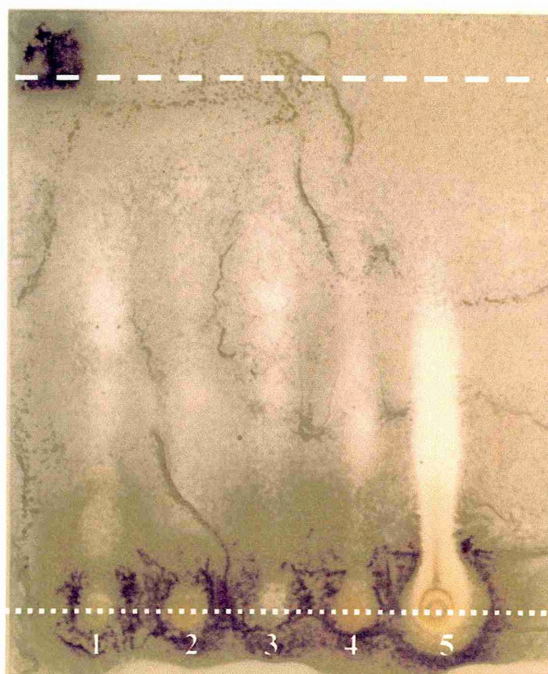
[A] = strongest antifungal activity

3.3.2.2 *Distribution of preformed antifungals in green stage I fruit tissue*

TLC bioassays of extracts from different green stage I tissues show that the majority of antifungal activity against both *C. cladosporioides* and *B. cinerea* was found in achenes (Plate 3.3.2.3 and 3.3.2.4). None-the-less, antifungal activity was found in all tissue types (viz. pith, cortex, epidermis) of green stage I fruit. The total area of fungal inhibition for green stage I achenes crude extracts was at least 40% greater than for other tissue types (Table 3.3.2.3A-E). All tissues showed similar antifungal profiles to those seen in whole green I fruit extracts (Plate 3.3.2.1W).

B. cinerea and *C. cladosporioides* were generally inhibited similarly by antifungal compounds separated in all running solvent systems (Plate 3.3.2.3 and Table 3.3.2.3A-E). Fungal growth inhibition by green stage I achene was also achieved at 20, 10 and 5 µl of crude extract (Plate 3.3.2.4; 3.3.2.6). The water content of achenes is almost one third that of other green stage I strawberry fruit tissue (Table 3.3.2.7). Thus, even on a dry weight basis, achenes contain more antifungal compounds compared to whole fruit, pith, cortex and epidermis. 1 and 2-D *C. cladosporioides* bioassays revealed that green I achenes may contain at least eight preformed antifungal compounds (Plate 3.3.2.4; 3.3.2.5).

Plate 3.3.2.3: One-dimensional thin layer chromatography bioassay (*Cladosporium cladosporioides*) of crude ethanol extract partitioned into an organic dichloromethane phase of green stage I strawberry cv. Elsanta fruit tissues (50 µl spots, 0.2 ml g^{-1} FW) run in hexane: ethyl acetate: methanol (60:40:10 v/v/v). Lane 1, whole fruit; lane 2, pith; lane 3, cortex; lane 4, epidermis; lane 5, achenes. Dotted line = origin. Dashed line = solvent front.



Little antifungal activity was observed in white and red stage achene tissue (Plate 3.3.2.5). This contrasts work by El Ghaouth *et al.* (1991) who reported that aqueous extracts of achenes from red fruit inhibited radial growth of *B. cinerea* in Petri plate bioassays.

Solvent system A (hexane: ethyl acetate: methanol; 60: 40:1 v/v/v) provided good separation of antifungal tissue extracts with two compounds at R_f = 0.42 and 0.34 (Table 3.3.2.4) corresponding to R_fs at 0.44 and 0.37 in whole green I extracts (Plates 3.3.2.1W and Table 3.3.2.1). However, when run in an organic phase of ethyl acetate; benzene; ethanol (50% v/v) (4:1:1) (Mussel and Staples, 1971), antifungal activity was observed at R_f = 0.76-0.58 (Plate 3.3.2.4B and Table 3.3.2.3B). Thus, one or more of these

compounds may be similar to phytoalexin-like compounds A and C (R_f = 0.84 and 0.52) discovered in strawberry roots (Mussel and Staples, 1971). Hirai *et al.* (2000) proposed through TLC analysis that inducible triterpenes found in unripe strawberry fruit which might confer resistance to *Colletotrichum fragariae* probably correspond to compounds reported by Mussel and Staples (1971). This suggests that the fruit may produce similar antifungal compounds to those in the roots. In this study, compounds at R_f = 0.42 and 0.34 may therefore be triterpenes identified by Hirai *et al.* (2000).

Plate 3.3.2.4: One-dimensional thin layer chromatography bioassays (*Cladosporium cladosporioides* or *Botrytis cinerea*) bioassays of crude ethanol extract partitioned into an organic dichloromethane phase of green stage I strawberry cv. Elsanta fruit tissues (20 μl spots, 0.2 ml g^{-1} FW) run in either hexane: ethyl acetate: methanol (60:40:1 v/v/v) (A), organic phase of ethyl acetate; benzene; ethanol (50% v/v) (4:1:1) (Mussel and Staples, 1971) (B, C) or chloroform: methanol (90:10 v/v) (D, E). A, C and E = *C. cladosporioides*; B and D = *B. cinerea.* Lane 1, whole fruit; lane 2, pith; lane 3, cortex; lane 4, epidermis; lane 5, achenes. Dotted line = origin. Dashed line = solvent front.

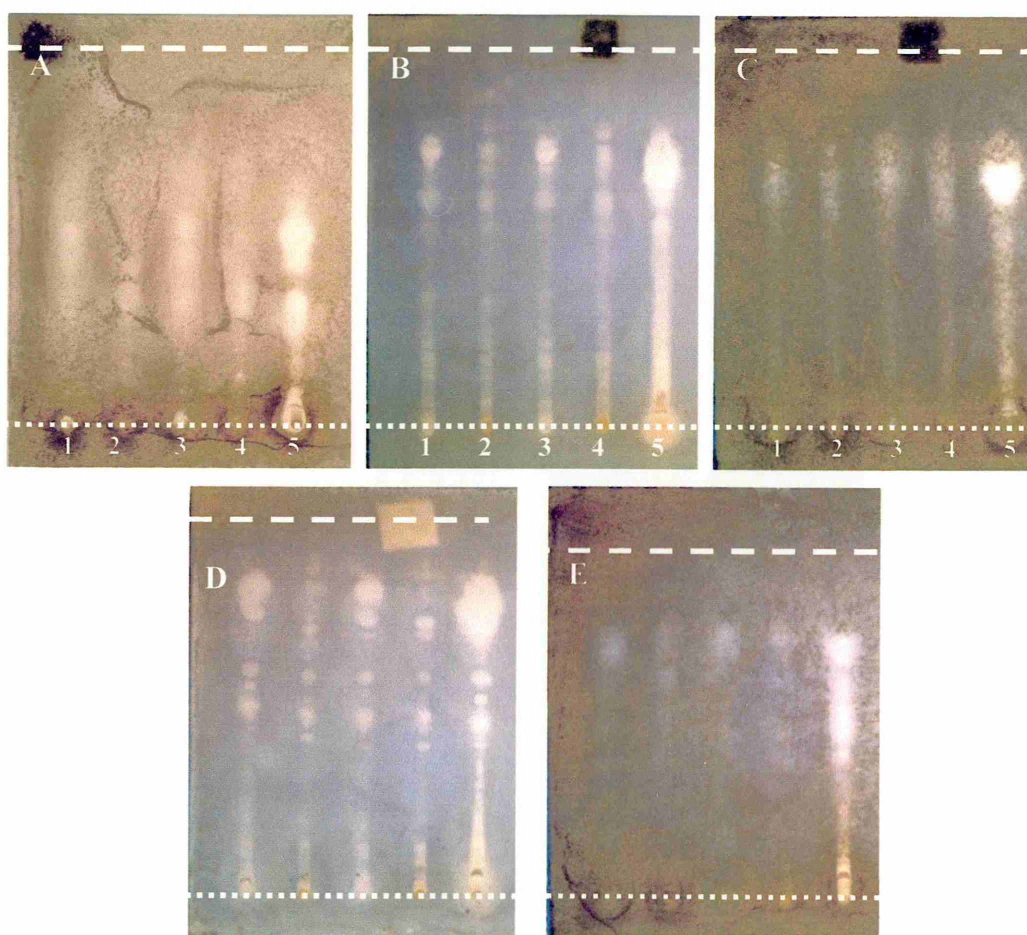


Table 3.3.2.3A: Areas (cm^2) of fungal inhibition of one-dimensional thin layer chromatography bioassay (*Cladosporium cladosporioides*) of crude ethanol extract partitioned into an organic dichloromethane phase of green stage I strawberry cv. Elsanta fruit tissues (viz. whole fruit, pith, cortex, epidermis, achene; 20 µl spots, 0.2 ml g^{-1} FW) run in hexane: ethyl acetate: methanol (60:40:1 v/v/v)

R_f value	Green I tissue type				
	whole	pith	cortex	epidermis	achenes
0.50	2.49	---	3.39	---	2.49
0.42	3.39	1.73	1.73	---	1.73
0.28	---[A]	2.49	1.73	4.42	4.99
0.10	---	---	---	---	0.62
0.00	0.62	0.62	1.11	1.11	3.39
Total area	6.50	4.84	7.96	5.53	13.22

---[A] = no inhibition zone

Table 3.3.2.3B: Areas (cm^2) of fungal inhibition of one-dimensional thin layer chromatography bioassay (*Botrytis cinerea*) of crude ethanol extract partitioned into an organic dichloromethane phase of green stage I strawberry cv. Elsanta fruit tissues (viz. whole fruit, pith, cortex, epidermis, achene; 20 µl spots, 0.2 ml g^{-1} FW) run in organic phase of ethyl acetate; benzene; ethanol (50% v/v) (4:1:1v/v/v)

R_f value	Green I tissue type				
	whole	pith	cortex	epidermis	achenes
0.70	1.65	1.06	1.06	1.06	7.98
0.58	1.06	1.06	1.06	1.06	---[B]
Total area	2.74	2.12	2.12	2.12	7.98

---[B] = overlapped R_f

Table 3.3.2.3C: Areas (cm^2) of fungal inhibition of one-dimensional thin layer chromatography bioassay (*Cladosporium cladosporioides*) of crude ethanol extract partitioned into an organic dichloromethane phase of green stage I strawberry cv. Elsanta fruit tissues (viz. whole fruit, pith, cortex, epidermis, achene; 20 μl spots, 0.2 ml g^{-1} FW) run in organic phase of ethyl acetate; benzene; ethanol (50% v/v) (4:1:1 v/v/v)

R_f value			Green I tissue type		
	whole	pith	cortex	epidermis	achenes
0.61	2.38	1.06	1.65	1.65	6.50
Total area	2.38	1.06	1.65	1.65	6.50

Table 3.3.2.3D: Areas (cm^2) of fungal inhibition of one-dimensional thin layer chromatography bioassay (*Botrytis cinerea*) of crude ethanol extract partitioned into an organic dichloromethane phase of green stage I strawberry cv. Elsanta fruit tissues (viz. whole fruit, pith, cortex, epidermis, achene; 20 μl spots, 0.2 ml g^{-1} FW) run in chloroform: methanol (90:10 v/v)

R_f value			Green I tissue type		
	whole	pith	cortex	epidermis	achenes
0.77	1.65	---^{A}	1.65	---^{A}	9.50
0.70	1.06	---^{A}	1.65	1.06	---^{B}
0.55	0.60	0.60	0.60	0.26	1.05
0.49	0.26	---^{A}	---^{A}	---^{A}	0.26
0.45	1.06	0.60	0.60	0.26	1.06
0.40	---^{A}	0.26	---^{A}	0.26	0.2
0.12	0.81	0.26	0.81	0.81	1.06
0.00	1.65	0.81	1.65	1.34	4.22
Total area	7.09	2.53	6.95	3.99	17.41

---^{A} = no inhibition zone

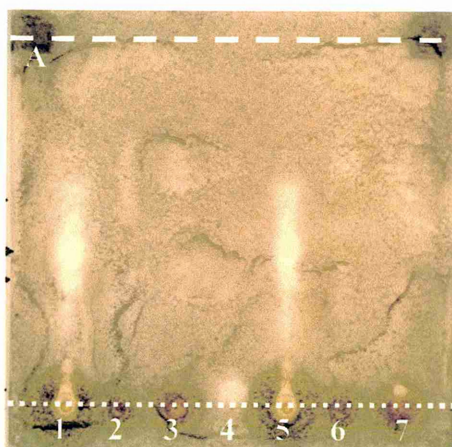
---^{B} = overlapped R_f

Table 3.3.2.3E: Areas (cm^2) of fungal inhibition of one-dimensional thin layer chromatography bioassay (*Cladosporium cladosporioides*) of crude ethanol extract partitioned into an organic dichloromethane phase of green stage I strawberry cv. Elsanta fruit tissues (viz. whole fruit, pith, cortex, epidermis, achene; 20 µl spots, 0.2 ml g^{-1} FW) run in chloroform: methanol (90:10 v/v)

R_f value			Green I tissue type		
	whole	pith	cortex	epidermis	achenes
0.75	2.37	1.06	1.65	1.05	2.37
0.64	--- [A]	0.59	---	---	1.65
0.54	---	---	---	---	1.65
0.48	---	---	---	0.26	0.26
0.44	---	---	---	---	0.26
0.24-0.00	---	---	---	---	1.32
Total area	2.37	1.65	1.65	1.31	7.51

---[A] = no inhibition zone

Plate 3.3.2.5: One-dimensional thin layer chromatography bioassays (*Cladosporium cladosporioides*) of crude ethanol extract partitioned into an organic dichloromethane phase of achenes of green stage I, white and red stage cv. Elsanta fruit (10 µl spot (A), 20 µl (B); 0.2 ml g^{-1} FW) run in hexane: ethyl acetate: methanol (60:40:1 v/v/v). Lane 1 and 5, green I achenes; lane 2 and 6, white achenes; lane 3 and 7, red fruit achenes; lane 4, cycloheximide (0.5 mg ml^{-1}). Dotted line = origin. Dashed line = solvent front.



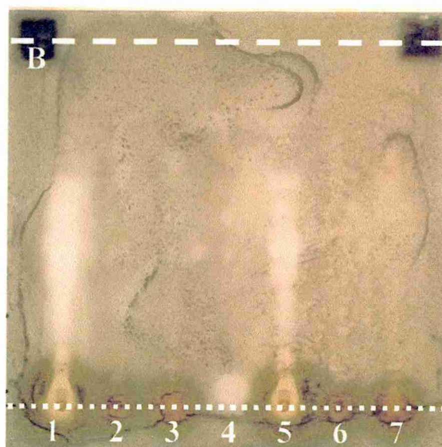


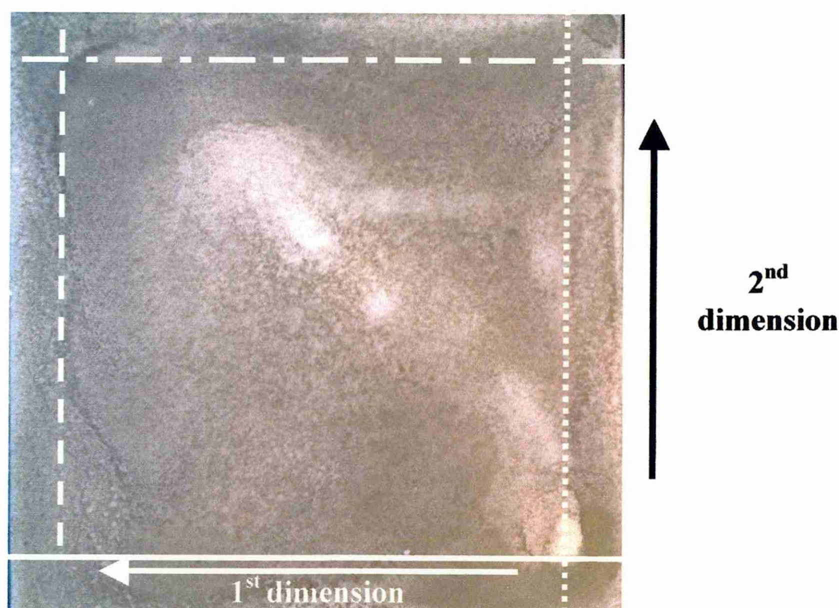
Table 3.3.2.4: Areas (cm^2) of fungal inhibition of one-dimensional thin layer chromatography bioassay (*Cladosporium cladosporioides*) of achenes of crude ethanol extract partitioned into an organic dichloromethane phase of green I, white and red stage cv. Elsanta fruit (20 μl spot; 0.2 ml g^{-1} FW) run in hexane: ethyl acetate: methanol (60:40:1 v/v/v).

Antifungal R_f	Fruit Development Stage		
	green I	white	red
0.57	0.34	---[A]	---
0.52	0.77	---	---
0.44	2.74	---	---
0.36	1.57	---	---
0.26	0.34	---	---
0.22	0.34	---	---
0.10	0.24	---	---
0.00	1.60	0.24	0.24
Total area	7.94	0.24	0.24

cycloheximide (0.5 mg ml^{-1}), R_f = 0.05 (1.36); ethanol (99% v/v) = no R_f

---[A] = no inhibition zone

Plate 3.3.2.6: Two-dimensional thin layer chromatography bioassay (*Cladosporium cladosporioides*) of crude ethanol extract partitioned into an organic dichloromethane phase (5 μl; 0.2 ml g^{-1} FW) of green stage I fruit achenes run in solvent A = (60:40:10 v/v/v) (one dimension, 1D) and solvent D = (60:40:30 v/v/v) (second dimension, 2D). Dotted line = first origin. Solid line = second origin. Dashed line = solvent front (1D). Dash-dot line = solvent front (2D).



Green I achenes (10 g FW; 2.89 g DW) sequentially extracted in ethyl acetate and ethanol displayed very different TLC behaviour to PMA reagent spray and bioassay (Plate 3.3.2.7). Ethyl acetate fraction (117 mg) showed similar but much less intense antifungal activity (R_fs = 0.54, 0.48, 0.28, 0.21 and 0.10) to previous achene extract bioassays run in solvent B (Plate 3.3.2.3) when applied at 30 µl (0.2 ml g^{-1} FW). In contrast to previous TLC bioassay results on achene extracts (Plate 3.3.2.5 and 3.3.2.6) strong inhibition was not seen at lower applications (10 and 20 µl). No antifungal activity in ethyl acetate fraction was observed at the origin. However, more polar phenolic compounds extracted in ethanol fraction (100 mg) showed antifungal activity at origin. Sequential extraction and column chromatography of green stage I tissues confirmed that the majority of antifungal activity is found in ethyl acetate and ethanol fractions (Table 3.3.2.7 and Plate 3.3.2.8). Four antifungal compounds with R_fs = 0.59, 0.49, 0.28, and 0.19 and previously observed in crude extracts (Plate 3.3.2.5 and Table 3.3.2.4) were eluted with solvent A during column chromatography (Table 3.3.3.8).

Plate 3.3.2.7: One-dimensional thin layer chromatography of green 1 achenes tissues (10-30 µl spots; 0.2 ml g^{-1} FW) sequentially extracted with ethyl acetate followed by ethanol sprayed with phosphomolybdic acid (A) or bioassay (*Cladosporium cladosporioides*) (B). 1-D TLC of sequentially extracted extracts sprayed with either (A) or bioassay (B) (*C. cladosporioides*) run in solvent B = (60:40:10 v/v/v). Lanes 1, 3, 5, ethyl acetate fraction at 10, 20 30 µl spot, respectively; lanes 2, 4, 6, ethanol fraction at 10, 20, 30 µl spot, respectively; lane 7, ethyl acetate (99% v/v) control. Dotted line = origin. Dashed line = solvent front.

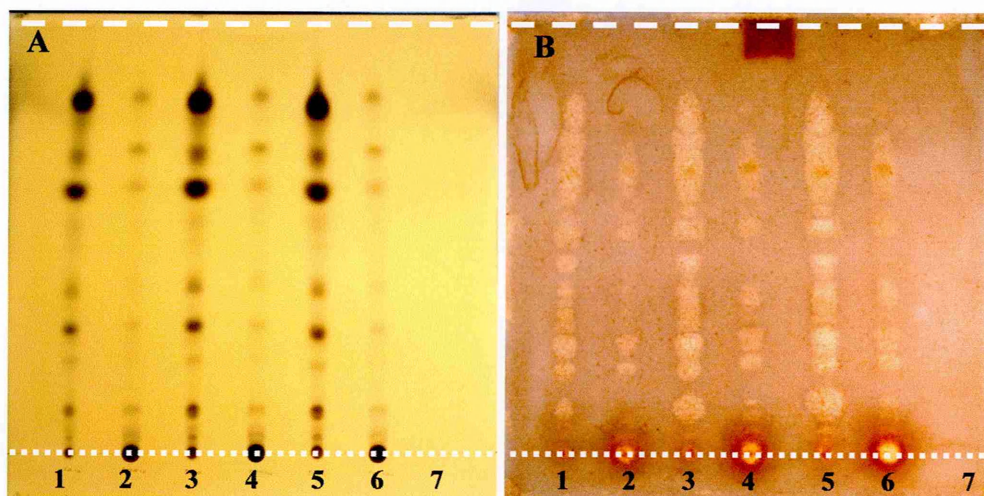


Table 3.3.2.5: R_f values from thin layer chromatographs sprayed with phosphomolybdic acid (PMA) of sequential extracts (30 µl; 0.2 ml g^{-1} FW) of green stage I strawberry cv. Elsanta fruit achenes run in hexane: ethyl acetate: methanol (60:40:10 v/v/v).

Ethyl acetate extract	Ethanol extract
0.81[C]	0.81
0.70[C]	0.70
0.61[C]	0.61
0.53[B]	---[A]
0.48[B]	---
0.37[C]	0.37
0.28[BC]	0.28
0.21[B]	---
0.10[BC]	0.10
0.05	0.05
0.00	0.00[B]

---[A] = no PMA reaction

[B] = compounds with antifungal activity against *Cladosporium cladosporioides*

[C] = most intense PMA reaction

Table 3.3.2.6: R_f values from thin layer chromatographs sprayed with phosphomolybdic acid of sequentially extracted green stage I strawberry cv. Elsanta fruit tissues

Achene (35 g FW; 10.12 g DW)			Fruit with achene (125 g FW; 15.92 g DW)			Fruit without achene (75 g FW; 8.12 g DW)		
Hex (50 mg)	EA (119 mg)	EtOH (525 mg)	Hex (74 mg)	EA (83 mg)	EtOH (1505 mg)	Hex (28 mg)	EA (163 mg)	EtOH (772 mg)
0.84	---	---	0.83	---	---	0.83	---	---
0.77	---	---	---	---	---	---	---	---
0.74	---	---	0.74	---	---	0.74	---	---
---[A]	0.72	---	---	0.72	---	---	0.72	---
---	0.68	---	---	0.68	---	---	0.68	---
0.66	---	---	0.64	---	---	0.64	---	---
---	0.44	---	---	0.42	---	---	0.42	---
---	0.38	---	---	---	---	---	---	---
---	0.34	---	---	0.35	---	---	0.35	---
---	0.30	---	---	0.30	---	---	---	---
---	0.16	---	---	0.16	---	---	0.16	---
---	0.13	---	---	0.13	---	---	0.13	---
---	0.10	---	---	0.10	---	---	0.10	---
---	0.10-0.00	0.00	---	0.10-0.00	0.00	---	0.02-0.00	0.00

---[A] = spot not present

Plate 3.3.2.8: One-dimensional thin layer chromatography bioassay (*Cladosporium cladosporioides*) of sequentially fractionated selected samples (5 µl spots; 0.2 ml g^{-1} FW; n = 12) extracts of combined green I achenes and whole fruit. Fractions 1-14 are hexane fractions and frc. 22-43 are ethyl acetate fractions. Lanes 1, fractions (frc.) 2-3; lane 2, frc. 7-9; lane 3, 16-18; lane 4, frc. 9-14; lane 5, frc. 22-23; lane 6, frc. 24-27; lane 7, frc. 28; lane 8, frc. 29-34; lane 9, frc. 36-39; lane 10, 40-43; lane 11, achene ethyl acetate; lane 12, achene ethanol extract. Dotted line = origin. Dashed line = solvent front.

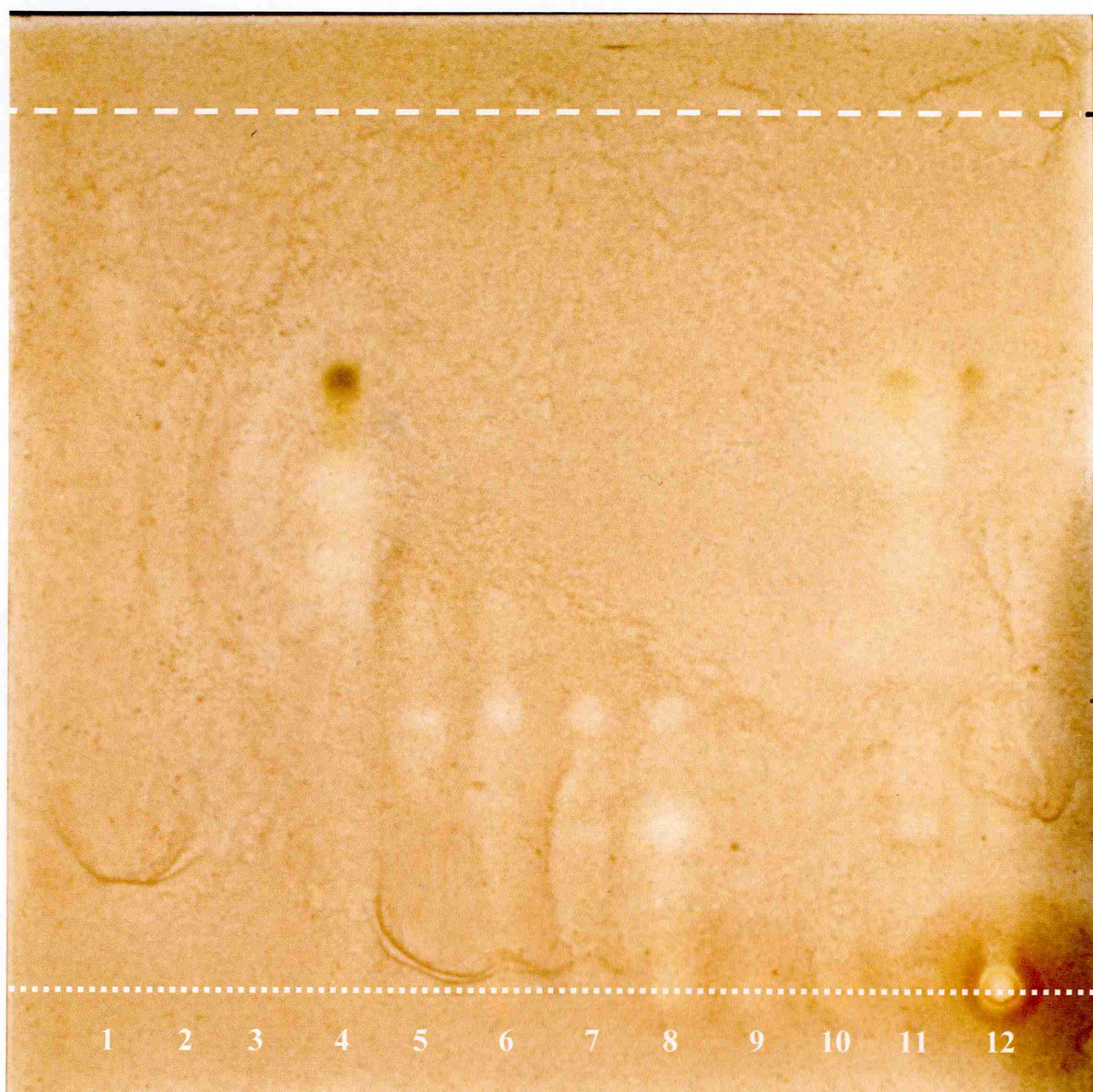


Table 3.3.2.7: One-dimensional thin layer chromatography bioassay (*Cladosporium cladosporioides*) and phosphomolybdic acid R_f values of sequentially fractionated combined green I tissues extracts (20 µl spots; 0.2 ml g^{-1} FW) run in hexane: ethyl acetate: methanol (60:40:10 v/v/v).

Pooled fractions	Extract dry weight (mg)	R_f values PMA	R_f values Antifungal
2,3[B]	71	0.81	---[A]
7-9[B]	37	0.64	---
9-14[C]	18	0.70, 0.63	0.59, 0.49
22-23[C]	3	0.30	0.28
24-27[C]	16	0.30, 0.36	0.28
28[C]	17	0.36, 0.30, 0.12, 0.09	0.28
29-34[C]	30	0.12	0.28, 0.19
36-39[C]	9	0.00	---
40-43[C]	98	0.00	0.00

[A] = no inhibition zone

[B]combined hexane extract (dry weight before and after purification = 124 and 116 mg)

[C]combined ethyl acetate extract (dry weight before and after purification = 202 and 191 mg)

3.3.2.3 *Preformed antifungal compounds in strawberry flower at different developmental stages*

White bud, flower stages sequentially extracted in ethyl acetate and ethanol (Tables 3.3.2.8 and 3.3.2.9) showed similar TLC behaviour to both PMA reagent spray and bioassay (Plates 3.3.2.9X-Z) as seen in sequentially extracted green I achenes (Plate 3.3.2.7). Whole flowers at post-anthesis (WFPA) displayed greater antifungal activity (R_fs = 0.38, 0.29, 0.21, 0.10, 0.00) than white bud and full bloom stages (Plate 3.3.2.9X). Whole flower ethyl acetate fractions had greater antifungal activity than pistils and receptacle tissue in all flower stages. There was no difference in the antifungal activity (R_f = 0.00) of ethanol fraction between whole flower stages and flower tissues (Plate 3.3.2.9Z). It is possible that these previously unreported antifungal compounds discovered in strawberry flowers in this study may play a role in initiating *B. cinerea* quiescence as *B. cinerea* tends to infect strawberries at flowering (Powelson, 1960).

However, more research is required to establish what exact role these compounds may have in influencing NDR in strawberry.

Table 3.3.2.8: Ethyl acetate and ethanol extract weights (mg 10 g^{-1} FW; mg 2 g^{-1} DW [in brackets]) of whole flower (WF) and pistils and receptacle tissue (PR) of white bud, full bloom and post-anthesis flower stages.

Flower stage	Ethyl acetate extract		Ethanol extract	
	WF	PR	WF	PR
White bud	159 [147]	181 [198]	268 [249]	305 [332]
Full bloom	94 [90.5]	69 [64]	573 [554]	145 [134]
Post-anthesis	100 [98]	132 [139]	547 [336]	444 [467]

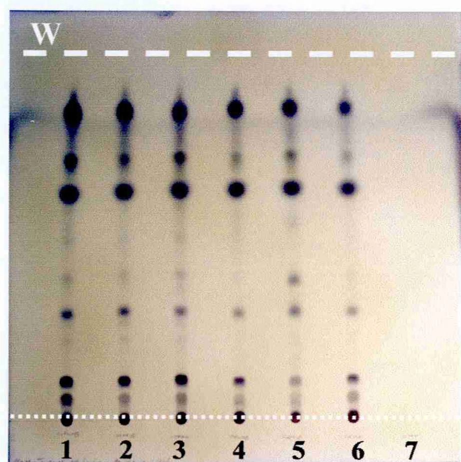
Table 3.3.2.9: R_f values from thin layer chromatographs sprayed with phosphomolybdic acid of sequential extracts (20 μl) of whole flower and pistils and receptacle tissue of white bud (WB), full bloom (FB) and post-anthesis (PA) flower stages run in hexane: ethyl acetate: methanol (60:40:10 v/v/v).

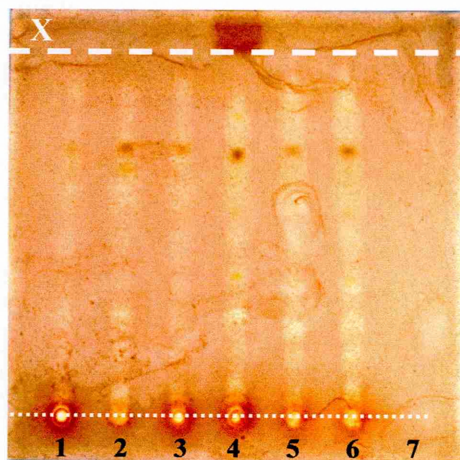
Ethyl acetate						Ethanol					
Whole flower			Pistils and receptacle			Whole flower			Pistils and receptacle		
WB	FB	PA	WB	FB	PA	WB	FB	PA	WB	FB	PA
0.83	0.83	0.83	0.83	0.83	0.83	0.83	0.83	0.83	0.83	0.83	0.83
0.70	0.70	0.70	0.70	0.70	0.70	0.68	0.68	0.68	0.68	0.68	0.68
0.60	0.60	0.60	0.60	0.60	0.60	0.58	0.58	0.58	0.58	0.58	0.58
0.49	0.49	0.49	---[A]	---	---	---	---	---	---	---	---
0.38[B]	0.38[B]	0.38[B]	---	0.38[B]	---	---	---	---	---	---	---
0.29[B]	0.29[B]	0.29[B]	0.29	0.29	0.29	---	---	---	---	---	---
0.26	0.26	0.26	---	---	---	---	---	---	---	---	---
0.21[B]	0.21[B]	0.21[B]	---	---	---	0.18	0.18	0.18	0.18	0.18	0.18
0.10[B]	0.10[B]	0.10[B]	0.10	0.10	0.10	---	---	---	---	---	---
0.05	0.05	0.05	0.05	0.05	0.05	0.04	0.04	0.04	0.04	0.04	0.04
0.00[B]	0.00[B]	0.00[B]	0.00[B]	0.00[B]	0.00[B]	0.00[B]	0.00[B]	0.00[B]	0.00[B]	0.00[B]	0.00[B]

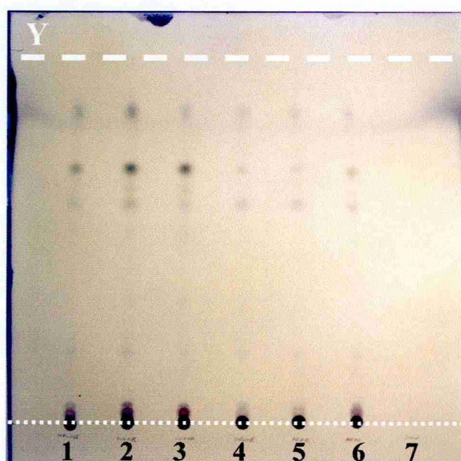
[A] = no inhibition zone

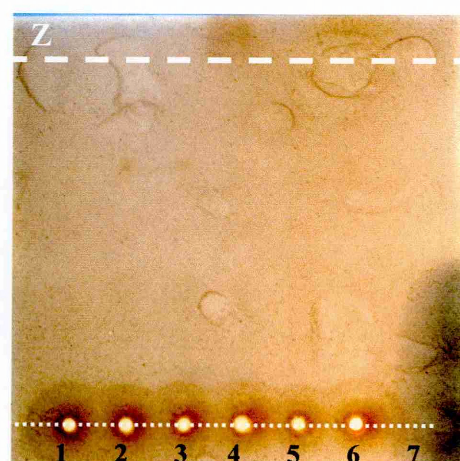
[B] = compounds with antifungal activity

Plate 3.3.2.9: One-dimensional thin layer chromatographs of different flower tissues (20 μl spots; 0.2 ml g^{-1} FW) (viz. whole flower (WF) and pistils/ receptacles (PA)) at three flower stages (viz. white bud (WB), full bloom (FB), post-anthesis (PA)) sequentially extracted with ethyl acetate (W, X) followed by ethanol (Y, Z) sprayed with phosphomolybdic acid (PMA) or bioassay (*Cladosporium cladosporioides*) run in hexane: ethyl acetate: methanol (60:40:10 v/v/v). PMA plates (W, Y) = Lane 1, WFWB; lane 2, WFFB; lane 3, WFPA; lane 4, PRWB; lane 5, PRFB; lane 6, PRPA; bioassay plates (X, Z) = lane 1, PRWB; lane 2, PRFB; lane 3, PRPA; Lane 4, WFWB; lane 5, WFFB; lane 6, WFPA. Lane 7, ethyl acetate or ethanol (99% v/v) control. Dotted line = origin. Dashed line = solvent front.









3.3 General Conclusion

Evidence from this study demonstrates that strawberry cv. Elsanta fruit and flowers contain at least eight previously unreported preformed antifungal compounds. These decline in number markedly during flower and fruit development. The greatest antifungal activity was found in green I fruit extracts, especially in achenes. This indicates that NDR to *B. cinerea* may depend on presence and decline of these preformed antifungal compounds during flower and fruit development. NDR in strawberry fruit declines with increasing fruit maturity (Gilles, 1959; Powelson, 1960; Jarvis, 1962). The presence of higher concentrations of preformed antifungal compounds in strawberry flower s and green I fruit is consistent with high NDR in these fruit to *B. cinerea.*

Many but not all of the preformed antifungal compounds reported in this study may be similar to compounds reported in strawberry fruit and other strawberry tissues (Mussel and Staples, 1971; Vincent *et al.*, 1999; Hirai *et al.*, 2000; Herbert *et al.*, 2001). However, full characterisation of the preformed antifungal compounds found in strawberry flower and fruit tissue and elucidation of pathways involved in their biosynthesis is required. This information will allow for precise definition of the role these compounds may play in strawberry fruit NDR against *B. cinerea.* In turn, such knowledge would enable strategies through preharvest treatment with elicitors to be implemented to enhance the levels of these compounds.

CHAPTER 4

INVESTIGATIONS INTO EFFECTS OF CHEMICAL, BIOLOGICAL AND PHYSICAL ELICITORS ON GREY MOULD OF STRAWBERRY FRUIT

4.0 Introduction

Grey mould disease caused by the fungal pathogen *Botrytis cinerea* Pers. is the most important disease affecting strawberry (*Fragaria ananassa* Duch.) fruit quality after harvest (Daugaard, 1999). Although this disease can be partially controlled by certain pre-harvest cultural methods and/or post-harvest packaging and storage techniques, the strawberry industry is still heavily reliant on chemical fungicide sprays applied during flowering and fruiting (Swadling and Jefferies, 1996). There are, however, concerns over increasing loss of efficacy of conventional fungicides due to pathogen resistance and general unacceptability of fungicide usage in terms of public and environmental risk (Howard, 1990; Sutton, 1990; Washington *et al.*, 1992). These concerns have favoured the introduction of integrated pest management (IPM) programmes (Jacobsen and Backman, 1993).

Exploitation of systemic acquired resistance (SAR) is a potentially desirable strategy in achieving IPM since it involves enhancing natural defence mechanisms in crops (Sticher *et al*, 1997; Lucas, 1999; Kuć, 2000). Certain biological, physical or chemical elicitors can be used to activate and/or boost natural disease resistance in non-infected plant tissue (Adikaram, 1990; Wilson *et al.*, 1994). Induced resistance through enhancement of plant defence metabolism has been shown to suppress development of a variety of crop diseases (Tally *et al.*, 2000), including *Peronospora tabacina* on tobacco, *Peronospora parasitica* on cabbage and *Fusarium* spp. on melon (Friedrich *et al.*, 1996; Godard *et al.*, 1999; Huang *et al.*, 2000). Induced resistance is particularly desirable for the control of grey mould on strawberry fruit. Strawberries are problematic with regard to timing of prophylactic sprays because of the long development sequence of the cymose inflorescence (Jarvis, 1994).

This chapter presents the results of an investigation into the potential of pre- and postharvest treatments of chemical (acibenzolar), biological (*Aureobasidium*

pullulans) and physical (UV-C) elicitors of induced resistance to suppress grey mould in harvested strawberry fruit.

PART A EFFECTS OF ACIBENZOLAR AND SALICYLIC ACID TREATMENTS ON STRAWBERRY GREY MOULD

4.1 Introduction

Amongst the range of natural and synthetic chemicals that activate SAR, acibenzolar (S-methyl benzo [1,2,3] thiadiazole-7-carbothioate; CGA 245704; benzothiadiazole or BTH, Actigard™) used as the 50% WG formulation Bion® is perhaps the most potent elicitor (Friedrich *et al.*, 1996). In tobacco, however, BTH-induced resistance was effective against *Peronospora tabacina*, *Phytophthora parasitica* and *Cercospora nicotianae*, but was not effective against *B. cinerea* (Friedrich *et al.*, 1996).

This study investigated the potential for postharvest and pre-harvest treatments with acibenzolar to suppress grey mould in harvested strawberry fruit. In addition, the effect of acibenzolar on *in-vitro* growth of *B. cinerea* was determined.

4.2 In-vitro Effects of Acibenzolar and Salicylic Acid on Mycelial Growth of *B. cinerea* and *Cladosporium cladosporioides*

4.2.1 Introduction

Various natural (e.g. salicylic acid) and synthetic (e.g. acibenzolar) substances are known activators of SAR. A chemical may only be considered an activator of SAR if it has three basic characteristics (Kessmann *et al.* 1994). These characteristics are "The compound/agent or its significant metabolites should not exhibit direct antimicrobial activity *in vitro* or *in planta*. The compound should induce resistance against the same spectrum of pathogens as in biologically activated SAR. The compound should induce the expression of the same marker genes as evident in pathogen activated SAR."

Although many chemicals have been reported as being potential activators of resistance, few have fulfilled the criteria above (Kessmann *et al.*, 1994). Acibenzolar has practically no inherent antifungal effect at 0.294 mg AI ml^{-1} (Friedrich *et al.*, 1996) and 0.75 mg AI ml^{-1} (Godard *et al.*, 1999), even though at these concentrations acibenzolar induces SAR in plant tissue. Acibenzolar acts downstream of salicylic acid (SA) (Vernooji *et al.*, 1994; Friedrich *et al.*, 1996). Treatment leads to the accumulation of the same SAR genes and pathogenesis related proteins as does SA and biologically induced SAR (Friedrich *et al.*, 1996). Kessmann *et al.* (1994) stated that neither SA nor its metabolites have significant antifungal activity.

The aim of this study was to confirm that, over a range of concentrations used in the present experiments, neither acibenzolar nor SA have direct inhibitory effect on *B. cinerea* (pathogen) or *Cladosporium cladosporiodes* (bioassay organism) mycelial and spore growth.

4.2.2 Materials and methods

4.2.2.1 *Media preparation*

Sterile solutions of either acibenzolar or salicylic acid (SA) were added to pre-autoclaved Potato Dextrose Agar (PDA) at 42°C to give concentrations ranging from 0 to 2.0 mg active ingredient (AI) ml^{-1} in ½ strength PDA. Streptomycin at 1.0 mg ml^{-1} was also added to the amended media to inhibit growth of bacteria. The media was poured into 9 cm Petri plates and stored at 5°C until used.

4.2.2.2 *Isolate preparation*

Single-spored isolates of *B. cinerea* and *C. cladosporioides* were cultured as described in section 3.3.1.3.

Four mm diameter agar discs were taken from the advancing margin of 4 day-old *B. cinerea* and 1 week-old *C. cladosporioides* cultures. These discs were positioned centrally on the ½ PDA plates containing either acibenzolar or SA. The 9 cm Petri

plates were then closed with Nescofilm® and held at 22 ±1°C. Mean radial growth rate of the fungus was determined daily by measuring the diameter of the colony in two directions at right angles daily until the untreated control cultures had reached the plate periphery. A completely randomised design was adopted for four replicate plates per treatment.

4.2.2.3 *TLC bioassay*

TLC bioassays (n = 2) using *C. cladosporioides* (as described in section 3.3.1.2 and 3.3.1.4) and the running solvent hexane: ethyl acetate: methanol (60:40:10 v/v/v) were performed for acibenzolar (2.0 mg AI ml^{-1}) and SA (2.0 mg ml^{-1}) which were resuspended in ethanol (99% v/v). Cycloheximide, a protein inhibitor, (0.5 mg ml^{-1}) and ethanol were used as positive and negative controls, respectively.

4.2.2.4 *Statistical analysis*

Relationship between means and standard errors for radial growth (y) and acibenzolar concentration (x) were described by negative exponential regressions using the curve-fitting package in SigmaPlot Version 5.0.

4.2.3 Results and discussion

Results of the *in-vitro* experiments contrast with those of others. Radial mycelial growth of both *B. cinerea* and *C. cladosporioides* was progressively reduced with increasing acibenzolar concentrations from 0.05 to 2.0 mg AI ml^{-1} (Fig. 4.2.3.1). Radial growth of *B. cinerea* and *C. cladosporioides* was also progressively reduced by increasing SA concentrations, with complete inhibition at 0.5 mg ml^{-1} (Fig 4.2.3.2).

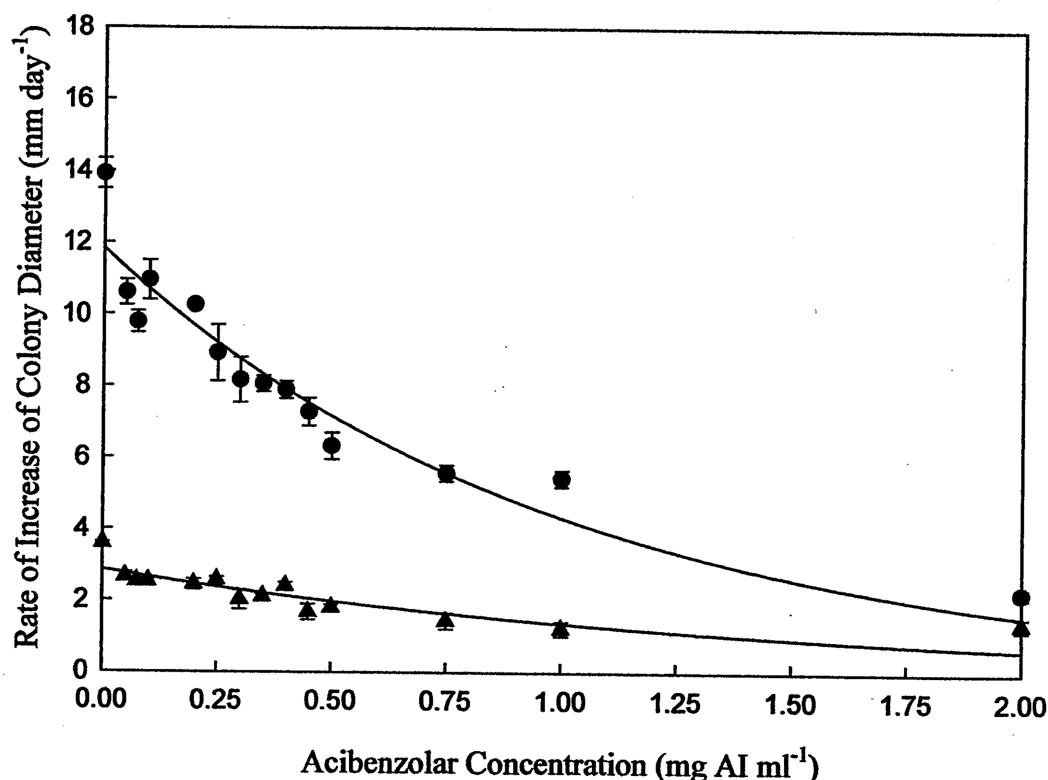


Figure 4.2.3.1: Effect of acibenzolar concentration on mycelial growth rate of *B. cinerea* (●) and *C. cladosporioides* (▲) grown on acibenzolar amended ½ PDA at 22 ±1°C. Data presented are treatment means ±s.e. (n = 4). Where standard errors (s.e.) are not shown, the symbols are bigger than s.e. The relationships between radial growth (y) and acibenzolar concentration (x) are described by the negative exponential regressions: $y = 11.841 * \exp^{(-1.006x)}$, n = 14, $r^2 = 0.912$ for *B. cinerea* and $y = 2.867 * \exp^{(-0.739x)}$, n = 14, $r^2 = 0.830$ for *C. cladosporioides*.

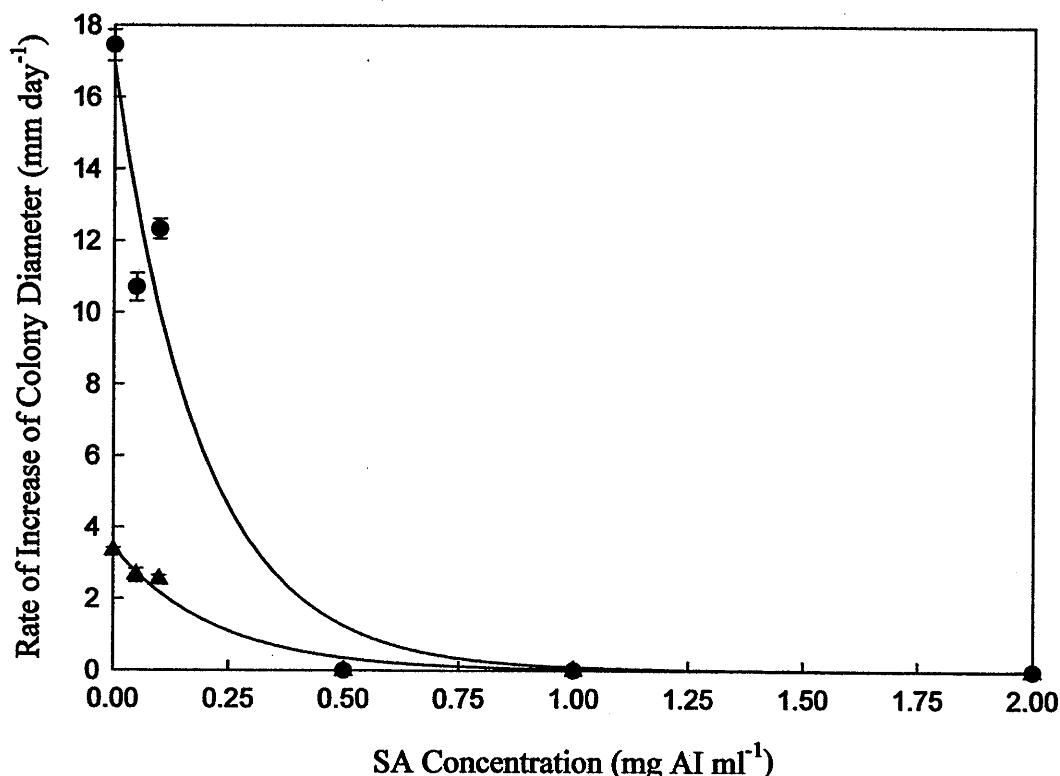
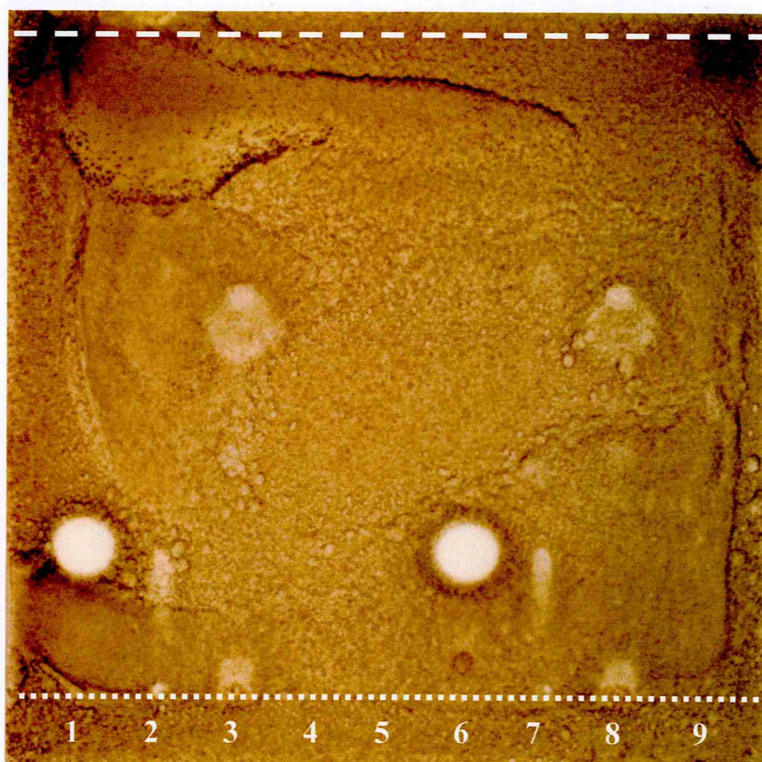


Figure 4.2.3.2: Effect of SA concentration on mycelial growth rate of *B. cinerea* (●) and *C. cladosporioides* (▲) grown on SA amended ½ PDA at 22 ±1°C. Data presented are treatment means ±s.e. (n = 4). Where standard errors are not shown symbols are bigger than s.e. Relationship between radial growth (y) and acibenzolar concentration (x) is described by the negative exponential regressions: $y = 16.912 * \exp^{(-5.215x)}$, n = 5, $r^2 = 0.958$ for *B. cinerea* and $y = 3.453 * \exp^{(-4.561x)}$, n = 5, $r^2 = 0.979$ for *C. cladosporioides.*

The TLC bioassays of SA at 2.0 mg ml^{-1} and acibenzolar at 2.0 mg AI ml^{-1} revealed inhibition zones at R_f 0.20 and at 0.60, 0.55, 0.35 and 0.00, respectively (Plate 4.2.3.1). The positive control, cycloheximide applied at 0.5 mg ml^{-1}, and the negative control, 99% ethanol (v/v), gave inhibition zones at R_f 0.22 and no inhibition zone, respectively.

Plate 4.2.3.1: One-dimensional thin layer chromatography bioassay (*Cladosporium cladosporioides*) of acibenzolar and salicylic acid (50 µl spots) run in solvent mix of hexane: ethyl acetate: methanol (60:40:10 v/v/v). Lanes 1 and 6 = cycloheximide (0.5 mg ml^{-1}); lanes 2 and 7 = SA 2.0 mg ml^{-1}); lanes 3 and 8 = acibenzolar (2.0 mg AI ml^{-1}); lanes 4 and 9 = ethanol (99% v/v); lane 5 = blank. Dotted line = origin. Dashed line = solvent front.



According to these results, acibenzolar or SA cannot be classed as an activator of SAR, at least when tested against the fungal species used in this study. TLC bioassay (Plate 4.2.3.1) and mycelial growth bioassays (Fig. 4.2.3.1) both clearly demonstrate that acibenzolar is antifungal against *B. cinerea* and *C. cladosporioides* at concentrations ranging from 0.05 to 2.0 and mg AI ml^{-1}. According to Kessmann *et al.* (1994), a true chemical activator of SAR should not exhibit direct microbial activity. When tested against *B. cinerea* and *C. cladosporioides* at concentrations ≤ 0.002 mg AI ml^{-1}, acibenzolar fulfilled the three criteria set out by Kessmann *et al.*

(1994) (see section 4.2.1). Similarly, Friedrich *et al.* (1996) found that the mycelial radial growth of a range of plant pathogens, including *B. cinerea*, was not inhibited when grown on acibenzolar amended agar at a final concentration of 1.4 mM acibenzolar (0.294 mg AI ml^{-1}). Ryals *et al.* (1996) and Godard *et al.* (1999) confirmed that neither 0.057 nor 0.075 mg AI ml^{-1} of acibenzolar had any direct activity against a range of fungi and bacteria. However, where higher concentrations of 0.15 to 0.45 (Godard *et al.* 1999) and 0.5 mg AI ml^{-1} (Cole, 1999) have been shown to induce resistance *in-planta*, the direct antifungal activity of acibenzolar has not been tested. Again, where higher concentrations of acibenzolar (0.375 mg AI ml^{-1}) were shown to induce SAR in-planta, the direct antifungal activity was not tested (Dann *et al.*, 1998). However, Dann *et al.* (1998) demonstrated that 0.065 mg AI ml^{-1} had no direct antifungal activity against *in-vitro* growth of *Sclerotinia sclerotiorum*. Ishii *et al.* (1999) reported similar results that acibenzolar amended PDA did not have any inhibitory effect on the mycelial growth of *Cladosporium cucumerinum* or *B. cinerea*. Using a similar method to Ishii *et al.* (1999), Huang *et al.* (2000) demonstrated that acibenzolar had no direct antifungal action against isolates of *Fusarium* sp. and *Alternaria* sp. Acibenzolar was, however, inhibitory to *Didymella bryoniae* at 0.002 mg AI ml^{-1} (Ishii *et al.*, 1999). Similar results showing direct antifungal effect of natural activators of SAR were reported by Meir *et al.* (1998). These workers found that methyl jasmonate had a direct inhibitory effect on *in-vitro* spore germination of *B. cinerea*. Likewise, methyl jasmonate and jasmonic acid have both been reported to reduce *in-vitro* spore germination and mycelial growth of *Phytophthora infestans* (Cohen *et al.*, 1994b).

Both the TLC bioassay (Plate 4.2.3.1) and the mycelial growth bioassays (Fig. 4.2.3.2) clearly demonstrate that SA is strongly antifungal at 0.05 and 0.1 mg ml^{-1}, and gives complete inhibition of *B. cinerea* and *C. cladosporioides* at concentrations ranging from 0.5 to 2.0 mg ml^{-1}. Beasley (2001) showed that *B. cinerea* mycelial growth was retarded, but not arrested, on PDA amended with SA at 1.0 and 2.0 mg ml^{-1}. However, mycelial growth was not affected at 0.1 and 0.5 mg ml^{-1}. These differences in the degree of antifungal activity may be explained by variations in tolerances of particular *B. cinerea* isolates to SA. Beasley (2001) also found mycelial growth of *Cladosporium* sp. was not inhibited at 2.0 mg ml^{-1} using TLC bioassays (agar overlay method) (Rios *et al.*, 1988; Rahalison *et al.*, 1991; Stirling, 1995). In

the present study, where a direct TLC bioassay (Klarman and Stanford 1968; Homans and Fuchs, 1970) was used, *C. cladosporioides* was inhibited at 2.0 mg ml^{-1}. It is possible that Beasley (2001) observed contrasting results because SA in the silica gel 60 was unable to diffuse into the agar overlaying the plate. Thus, the fungus was perhaps not in direct contact with SA. SA (0.5 mg ml^{-1}) was inhibitory against *in-vitro* radial growth of *Phythium irregulare*, *Rhizoctonia solnai* and *Alternaria alternata* (Nemec, 1976). However, Coquoz *et al.* (1995) reported that mycelial growth of *Phytophthora infestans* was not affected when grown on 1.38 mg SA ml^{-1} amended V8 agar. Likewise, mycelial growth of *Alternaria solani* was not effected when grown on 0.028 mg SA ml^{-1} amended MCM agar (Spletzer and Enyedi, 1999).

The contrasting results presented here suggest that differences may exist in the tolerance of various fungal isolates to elicitors of SAR. Although direct anti-microbial effects of acibenzolar and SA in the plant cannot be discounted, the disease suppression response observed with elicitors of SAR *in-planta* is inherently different in nature to the progressive suppression response *in-vitro* (Tally *et al.*, 2000).

4.3 Postharvest Acibenzolar Treatment

4.3.1 Introduction

Most research using acibenzolar to induce SAR has been carried out with a view to controlling pre-harvest diseases (Tally *et al.*, 2000). In this study, the efficacy of a postharvest acibenzolar treatment against *B. cinerea* on harvested strawberry fruit was tested.

4.3.2 Materials and methods

4.3.2.1 *Fruit source and sampling*

Commercially grown strawberry cv. Camarosa fruit imported from Spain were supplied by KG Fruits Ltd. (Kent, UK). Fruit were packed and pre-cooled within 6 h

of harvest and transported at ca. 2°C to the UK within 2 days. A total of 108 fruit showing no signs of infection were blocked into sets of 36 according to their size. Fruit were surface sterilised by dipping in a solution of 5% sodium hypochloite and then air dried on galvanised wire racks. The colour of each fruit was measured as described in section 3.2.1.2.

4.3.2.2 *Acibenzolar treatment*

A completely randomised block design was used for six acibenzolar concentrations and three inoculation treatments (3 blocks x 36 treatments = 108). Fruit (n = 18) were fully immersed in acibenzolar solutions at 0, 0.1, 0.2, 0.3, 0.4 and 0.5 mg AI ml^{-1} for 30 sec and then allowed to air dry for 1 h at 20°C on galvanised steel mesh racks. They were then held at 5°C as described in section 3.2.1.4 for 2 days until inoculation.

4.3.2.3 *Fruit inoculation and disease assessment*

Fruit (n = 6) were either fully immersed in *B. cinerea* conidial suspensions of 2×10^4 conidia ml^{-1} or 2×10^6 conidia ml^{-1}. Control fruit were dipped in sterile distilled water. After inoculation, fruit were air dried at 20°C for 2 h and then held in the dark at either 5 or 20°C and 95 to 100% RH as described in 3.2.1.4. Disease severity resulting from natural and artificial infection was recorded daily as described in section 3.2.1.4.

4.3.3 Results and discussion

Postharvest treatments with acibenzolar at 0.1 - 0.5 mg AI ml^{-1} were not effective in suppressing grey mould on strawberry cv. Camarosa fruit after harvest at either 5 or 20°C storage (Table 4.3.3.1; Appendix 4.3). Fruit stored at 5°C and 20°C showed grey mould disease symptoms at 7.26 and 2.48 days, respectively. Time to 100% disease severity score at 5 and 20°C was 15.76 and 5.98 days, respectively. Inoculation had no significant effect on time to either 10 or 100% disease severity. Since fruit were not wound inoculated, this result supports earlier work by Jarvis

(1962) that showed that grey mould derived through direct penetration is very rare after harvest. Similarly, Powelson (1960) found no evidence of primary infections originating from direct infection of the fruit through their epidermis.

Table 4.3.3.1: Effect of postharvest acibenzolar treatments on times to 10 and 100% disease severity score for harvested strawberry cv. Elsanta fruit held at 5°C.

Acibenzolar concentration (mg AI ml^{-1})	Time to disease severity score (days)	
	10% disease severity	100% disease severity
0	5.0	13.7
0.1	7.9	15.7
0.2	9.2	16.8
0.3	7.9	17.1
0.4	5.7	15.4
0.5	7.9	15.9

Table 4.3.3.2: Effect of postharvest acibenzolar treatment on time to 10 and 100% disease severity score on harvested strawberry cv. Elsanta fruit held at 20°C.

Acibenzolar concentration (mg AI ml^{-1})	Time to disease severity score (days)	
	10% disease severity	100% disease severity
0	2.0	6.4
0.1	2.3	5.4
0.2	2.4	5.6
0.3	3.3	7.0
0.4	2.7	5.9
0.5	2.1	5.6

The ineffectiveness of postharvest acibenzolar treatment in suppressing *B. cinerea* contrasts with a similar trial on grey mould of carrots. Reddy and Arul (1999)

reported that postharvest application of acibenzolar protected carrots from postharvest decay by *B. cinerea* at both 4 and 13°C. Although, acibenzolar (0.025 mg AI ml^{-1}) was also effective as a postharvest treatment of potato disks against dry rot caused by *Fusarium semitechtum*, but was not possible to induce resistance by dipping potato tubers (Bokshi *et al.*, 2000). Negative results for postharvest treatment of strawberry fruit with acibenzolar may have resulted because the time from acibenzolar treatment to disease incidence was just ca. 2 days at 20°C. Induction time required to induce the maximal resistance responses in plants treated with acibenzolar is typically between 2 and 4 days (Kessman *et al.*, 1994; Tally *et al.*, 2000). Acibenzolar may have been ineffective for strawberries stored at 5°C because the metabolic rate will have been reduced . Respiration rate of strawberries at 5 and 20°C is 28 and 127 ml CO_2 kg^{-1} h^{-1}, respectively (Robinson *et al.*, 1975). A reduction in metabolic rate may not have allowed for sufficient signal transduction and establishment of SAR. In addition, acibenzolar is more effective when used as a preventative prophylactic treatment (Tally *et al.*, 2000). As grey mould in strawberry fruit tends to be derived from pre-harvest infection of inflorescences (Powelson, 1960), acibenzolar may not be able to control *B. cinerea* once a quiescent infection is established. Conversely, *B. cinerea* infects carrots during storage, and so does not go through a quiescent phase. This difference in mode of infection may explain the difference in efficacy of postharvest acibenzolar treatment between strawberry and carrot (Reddy and Arul, 1999).

4.4 Preharvest Acibenzolar Treatment

4.4.1 Introduction

As reported earlier, most research using acibenzolar to induce SAR has been carried out to control pre-harvest diseases (Tally *et al.*, 2000). A series of seven glasshouse experiments were conducted between 1999 and 2001 to investigate the potential for pre-harvest use of acibenzolar to suppress grey mould in harvested strawberry fruit. Successive experiments allowed evaluation of potentially variable efficacy of acibenzolar throughout each growing season.

4.4.2 General materials and methods

4.4.2.1 *Plant material*

Cold-stored maiden-year A+ grade strawberry cvs. Elsanta or Andana plants supplied by KG Fruits Ltd. were grown as described in section 3.2.1.1 unless otherwise stated.

4.4.2.2 *Acibenzolar treatments*

Acibenzolar was dissolved in distilled water to give spray solutions containing 0.002 to 2.0 mg AI ml^{-1}. These concentrations were applied to incipient runoff using approximately 10 ml $plant^{-1}$. Seven experiments were conducted during summer and winter from 1999-2001 using various acibenzolar concentrations and spray regimes.

Experiment 1: July - August 1999

A completely randomised design (CRD) was used for five acibenzolar concentrations. There were 10 samples plants per acibenzolar treatment. Elsanta plants received a single spray of 0, 0.2, 0.3, 0.5 or 1.0 mg AI ml^{-1} when the majority of tertiary flowers were at anthesis. Primary and secondary flowers were at green stage and post-anthesis, respectively. Anthesis of primary flowers commenced 6 weeks after planting which equated to 2 weeks prior to application.

Experiments 2 and 3: September - December 1999

A 10 block randomised complete block design (RCBD) was used for six acibenzolar concentrations. There were four sample plants per acibenzolar treatment. Andana plants received weekly sprays of 0, 0.25, 0.5, 0.75, 1.0 or 2.0 mg AI ml^{-1}. Spraying started 4 weeks after planting and continued for 9 weeks through flowering and fruiting. Anthesis of primary flowers commenced 2 weeks after the first application.

In Experiment 3, a randomised complete block design (RCBD) was used with five acibenzolar concentrations (6 blocks x 5 treatments = 30). There were three sample plants per acibenzolar treatment. Elsanta plants received a single spray of 0, 0.25, 0.5, 1.0 or 2.0 mg AI ml^{-1} when the majority of primary flowers were at anthesis.

Experiment 4: April - July 2000

A RCBD was adopted for two acibenzolar concentrations (4 blocks x 2 treatments = 8). Elsanta plants received weekly sprays 0 or 0.2 mg AI ml^{-1}. There were five sample plants per acibenzolar treatment. Spraying started 1 week after planting and continued for 3 weeks until primary anthesis.

Experiment 5: July – August 2000

A split plot block design was adopted with each of 3 blocks divided in half for cv. Elsanta plants receiving either a conidial suspension of *B. cinerea* (2×10^5 conidia ml^{-1}) (section 3.2.1.3) or distilled water 4 weeks after planting when the majority of primary, secondary and tertiary flowers were at green stage 1, anthesis and white bud stage, respectively. Within plots, a CRD was adopted with five acibenzolar concentrations of 0, 0.002, 0.02, 0.2 and 2.0 mg AI ml^{-1}, three different fungicide treatments and four acibenzolar spraying regimes (A, B, C, D) (3 blocks x 120 treatments = 360) (Plate 4.4.2.1).

Plants were sprayed once, 4 weeks after planting, with either 2 ml Scala l^{-1} (0.8 mg pyrimethanil ml^{-1}) (Taylor *et al.*, 1999), 1 ml Rovral l^{-1} (0.5 mg iprodione ml^{-1}) or distilled water when the majority of primary, secondary and tertiary flowers were at post-anthesis, anthesis or white bud stage, respectively. For acibenzolar spray regimes, plants received (A) a single spray 2 weeks after planting where plants were 1 week prior to primary anthesis, (B) 3-weekly interval sprays starting 2 weeks after planting and continuing through primary and secondary anthesis, (C) a single spray 5 weeks after planting when primary and secondary fruit were at red and green 2 stage, respectively, and (D) a single spray 6 weeks after planting when primary fruit had been harvested and secondary and tertiary fruit were at red and white stages, respectively.

Plate 4.4.2.1: Picture of Experiment 5 layout of strawberry cv. Elsanta plants arranged in 3 blocks.

Experiments 6 and 7: February –May; June – July 2001, respectively

In Experiments 6 and 7, a split-plot block design was adopted with each of 4 blocks divided in half for cv. Elsanta plants receiving either high or low nitrogen (Section 4.4.2.4). Within plots, a CRD was adopted with two acibenzolar concentrations of 0 or 0.25 mg AI ml^{-1}. There were 16 sample plants per treatment (4 blocks x 4 treatments = 16). Plants received 2-weekly interval sprays during flowering and fruiting.

Plate 4.4.2.2: Picture of Experiment 6 layout of strawberry cv. Elsanta plants grown in a glasshouse and arranged in 4 blocks

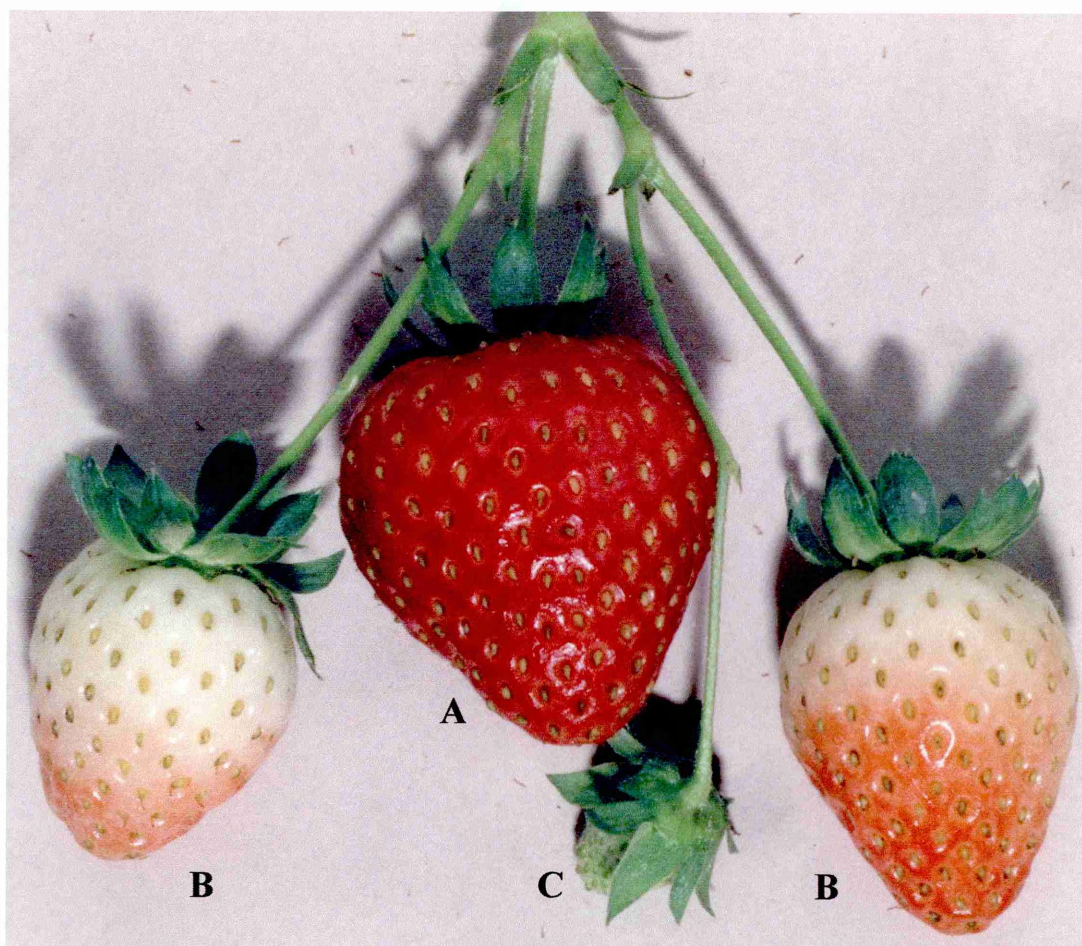
4.4.2.3 *Fruit and disease assessment*

In Experiments 1 to 3 and 5 to 7, ripe fruit from each treatment were harvested at least twice weekly during the fruiting season unless otherwise stated. In Experiment 4, fruit were harvested according to development stage; viz. 7, 14, 21, 28, 35 days after

anthesis (DAA) (Plate 3.2.1.2). DAA was monitored by tagging flowers at anthesis in experiments 4, 6 and 7. Fruit weights at harvest were recorded for all experiments. In all experiments, harvested fruit were held in the dark at 5°C and 95 to 100% RH in individual closed but vented containers (Plate 3.2.1.1). Disease severity resulting from natural and/or artificial infection was assessed (section 3.2.1.4).

For Experiments 1, 2 and 3, no distinction was made between primary, secondary and tertiary fruit (Plate 4.4.2.3). For Experiments 4 and 5, each fruit was assessed for disease during postharvest storage. In Experiments 6 and 7, only secondary fruit were assessed for disease. In all experiments, fruit were harvested from the primary truss only.

Plate 4.4.2.3: Arrangement of a strawberry fruit cluster (cyme) showing (A) primary, (B) secondary, and (C) tertiary fruit.



4.4.2.4 *Nitrogen treatments*

In Experiments 6 and 7, the split plots received either high nitrogen (1 times the standard N in full strength Hoagland's solution) (Appendix 4.4.6 a; b; c) (Hoagland and Arnon, 1950; Norman *et al.*, 1996) or low nitrogen (0 times N in full strength modified Hoagland's solution). Where low nitrogen was used, $Ca(NO_3)_2$ and KNO_3 were replaced with equivalent $CaCl_2$ and KCl concentrations, respectively (P. Blamey, 2001 pers. comm.). The risk of chloride accumulation and potential toxicity was minimised by watering every day throughout the experiment with distilled water until leachate started dripping from the pots. Drainage was of volumes from ca. 50 to 100 ml. Nutrient supplementation started 3 (experiment 6) and 1 week (experiment 7) after planting and was continued through flowering and fruiting. Plants were fertigated once per week with 50-100 ml of Hoagland's solution (Norman *et al.*, 1996). This frequency was increased to twice per week on emergence of trusses, which occurred 5 (experiment 6) and 2 (experiment 7) weeks after planting. No signs of plant water deficit stress were observed. Electrical conductivity of leachate (ECe) from 16 plants per treatment was measured 4, 8 and 12 weeks after planting. EC (mS cm^{-1}) was monitored using a Mettler Toledo MPC 227 pH/Conductivity meter fitted with a InLab 730 probe (Mettler-Toledo Ltd, Leicester, UK).

4.4.2.5 *Environmental monitoring*

In Experiments 6 and 7, temperatures within the glasshouse were recorded throughout the experiments at hourly intervals using a Delta-T datalogger (Cambridge, UK). Temperature was measured using two calibrated type-T (copper-constantan) thermocouple probes positioned at canopy level in each block. One thermocouple measured conditions 1m above the middle of each block. Each thermocouple was shielded from solar radiation by a polystyrene cup.

4.4.2.6 *Inoculum load*

In Experiments 6 and 7, the *B. cinerea* inoculum load in each block was monitored according to Kritzman and Netzer (1978) and Kerssies (1990) using a supplemented

selective basal media as a spore trap. The basal medium contained 1.0 $NaNO_3$, 1.2 K_2HPO_4, 0.2 $MgSO_4$ $7H_20$, 0.15 KCl, 20.0 glucose, and 25.0 agar (g l^{-1} distilled water). The medium was autoclaved and then supplemented with 0.015 Terrachlor (PCNB; quintozene; pentochloronitrobenzene 75% WP), 0.02 Maneb (manganese ethylene bisdithiocarbamate), 0.05 chloramphenicol, 2.2 $CuSO_4$, 0.1 Rubigan (fenarimol), and 5.0 tannic acid (g l^{-1} basal medium). These supplements were added at 60 ±1°C and pH was adjusted to 4.5 with 5.0 N NaOH. This selective media allowed normal growth of *B. cinerea*. Spore traps consisted of four 9 cm diameter Petri plates positioned at right angles to one another (Kerssies, 1990). Plates were fixed to a plant pot using Velcro™. Each plate contained 20-25 ml of selective media (Kerssies, 1990).

Two traps were positioned at canopy level in each block adjacent to each thermocouple probe. Another single trap was positioned 1 m above each block. Traps were placed for one day in the glasshouse for 8 hours, between 09.00 and 17.00, when the majority of secondary inflorescences were at the white bud stage. Traps were then removed and incubated at 22 ±2°C for 7 days. Dark brown spots, which occur due to the reaction between pathogen derived polyphenol oxidase (PPO) and tannic acid, were counted as colony forming units of *B. cinerea* (Kerssies, 1990). Conidia were produced by *Botrytis* colonies after 14 days. The pathogenicity of conidia derived from each plate was tested by inoculating ripe fruit with 15 μl of a conidial suspension (2×10^4 conidia ml^{-1}) derived from each plate. Inoculation was into a 2 mm diameter superficial wound made by piercing the shoulder of the fruit with a needle (Adikaram *et al.*, 2002). Disease incidence was recorded.

4.4.2.7 *Fruit quality attributes*

In Experiments 6 and 7, primary red fruit from each plant were used to assess firmness using a AFG 500N Advanced Force Gauge (Mecmesin Ltd., West Sussex, UK) fitted with a 8 mm diameter flat probe (Civello *et al.*, 1997). This was mounted onto the crosshead of a conventional Model 1122 Instron Universal Testing Machine (High Wycombe, UK). Head speed was set at 20 mm min^{-1} (Civello et *al.*, 1997; Baka *et al.*, 1999). The fruit were cut in half longitudinally so they could be placed

firmly on a flat surface. Firmness was expressed as the maximum force (N) required until tissue failure (i.e. bio-yield point). The firmness of each fruit was measured twice; i.e. on both opposite sides. Juice extracted from these fruit by squeezing tissue through muslin was used to determine total soluble solids (TSS) using an Atago PR-1 (Tokyo, Japan) digital refractometer.

4.4.2.8 *Statistical analysis*

All data were subjected to analysis of variance using Genstat Version 4.1. LSDs were calculated for mean separation at the 5 % level.

4.4.3 Results and discussion

4.4.3.1 *Phytotoxicity and fruit weight*

Acibenzolar treatments had no apparent phytotoxic effects in any experiment on either strawberry fruit or plants. Phytotoxicity was not expected, since reports of detrimental acibenzolar effects on plant growth or yield are rare. None-the-less, Godard *et al.* (1999) found that acibenzolar at 0.0015 to 0.25 mg AI ml^{-1} reduced growth of cauliflower (*Brassica oleracea*) seedlings in a dose-dependent fashion. Similarly, acibenzolar at 0.588 mg AI ml^{-1} resulted in mild phytotoxic symptoms in cucumber (*Cucumis sativus*) (Wurms *et al.*, 1999). Symptoms were leaf yellowing (chlorosis), with veins remaining green).

There was no significant difference in fruit weights at harvest between control and acibenzolar treatments in all seven experiments (e.g. Table 4.4.3.1; Appendix 4.4). The only exception was in Experiment 5, where the control treatment fruit had significantly less weight than all acibenzolar treatments.

Table 4.4.3.1: Effects of acibenzolar treatments on harvest weights of strawberry cv. Elsanta fruit from plants sprayed at anthesis of tertiary flowers (Experiment 1), cv. Andana fruit from plants sprayed at weekly intervals for 9 weeks (Experiment 2), and cv. Elsanta fruit from plants sprayed at anthesis of primary flowers (Experiment 3).

Acibenzolar concentration (mg AI ml^{-1})	Mean fruit weight (g)		
	Experiment 1[A]	Experiment 2[B]	Experiment 3[C]
0.00	12.23	9.19	12.15
0.20	14.28	---	---
0.25	---[D]	9.41	12.57
0.30	12.21	---	---
0.50	13.35	8.72	11.56
0.75	---	8.38	---
1.00	12.44	8.73	11.87
2.00	---	8.39	12.03

[A] Mean separation by LSD [P = 0.05] = 2.79
[B] Mean separation by LSD [P = 0.05] = 0.95
[C] Mean separation by LSD [P = 0.05] = 1.38
[D] Concentration not included in experiment

4.4.3.2 *Effects of acibenzolar on grey mould disease severity*

Experiment 1: July-August 1999

In summer, pre-harvest treatments with acibenzolar at 0.2 - 1.0 mg AI ml^{-1} and sprayed when the majority of tertiary flowers were at anthesis were not effective in suppressing grey mould on strawberry fruit after harvest (Appendix 4.4.1). This result was somewhat surprising, as acibenzolar induces SAR in a number of other dicotyledonous plants, including banana, cucumber and tomato (Gorlach *et al.*, 1996; Sticher *et al.*, 1997; Tally *et al.*, 2000). None-the-less, in tobacco (*Nicotiana tabacum*), acibenzolar (0.294 mg AI ml^{-1}) was ineffective against *B. cinerea*, although

it was effective against *Peronospora tabacina*, *Phytophthora parasitica* and *Cercospora nicotianae* (Friedrich *et al.*, 1996). It was not clear why acibenzolar treatment was ineffective against *B. cinerea*. Moreover, acibenzolar at 0.588 mg AI ml^{-1} was also ineffective in controlling powdery mildew (*Sphaerotheca fuliginea*) on cucumber regardless of whether it was applied either before or after artificial inoculation (Wurms *et al.*, 1999). Acibenzolar (0.5 mg AI ml^{-1}) was also not effective against flower blight in Camellia, caused by *Ciborinia camelliae*, when applied before flowering, during flowering or both before and during flowering (Van Toor *et al.*, 2001). Preharvest application to potato plants 60 days after sprouting of acibenzolar (0.025 – 0.1 mg AI ml^{-1}) did not induce resistance in harvest tubers against *Fusarium* spp. (Bokshi *et al.*, 2000). However, acibenzolar treatments did control leaf disease caused by *Alternaria solani*. An early treatment (0.05 mg AI ml^{-1}) of potato plants at 30 days after sprouting induced resistance in tubers against *F. semitectum* (Bokshi *et al.*, 2000). This result suggests that the timing of acibenzolar treatment and developmental stage of plant are probably important in determining its efficacy.

Experiment 2 and 3: September-December 1999

In later plantings in 1999 season, the effects of acibenzolar on grey mould suppression as a function of concentration were positive. Moreover, effects were similar for both Andana sprayed weekly (Fig. 4.3.1A) and Elsanta given a single spray (Fig. 4.3.1B) (Appendix 4.4.2 and 4.4.3). In contrast to experiment 1, grey mould disease on harvested strawberry fruit held at 5°C was suppressed by all preharvest acibenzolar treatments. This effect seemed to be independent of the number of acibenzolar treatments (cf. Experiments 2 and 3). This result contrasts with some other work showing that multiple applications of acibenzolar or other plant activators (e.g. 2,6-dichloroisonicotinic acid; INA) are necessary for a decrease in disease severity to be observed (Nielsen *et al.*, 1994; Kessman *et al.*, 1995; Ruess *et al.*, 1995; Dann *et al.*, 1998). On the other hand, one application of acibenzolar was sufficient to induce disease resistance in both monocotyledonous (e.g. wheat) (Gorlach *et al.*, 1996) and dicotyledonous plants (e.g. potato and melon) (Bokshi *et al.*, 2000; Huang *et al.*, 2000). It is not known why these different observations as to acibenzolar treatment efficacy occur (Dann *et al.*, 1998). However, efficacy may also influenced by

developmental age of plant at time of spraying (Bokshi *et al.*, 2000). Strawberry plants in Experiments 2 and 3 were sprayed pre-anthesis and/or at anthesis, rather than after anthesis as in experiment 1. This result suggests that acibenzolar is only effective if sprayed prior and/or at primary anthesis. Similar results showing that earlier acibenzolar treatment are more effective than later treatments have been shown in potatoes (Bokshi *et al.*, 2000). Huang *et al.* (2000) also demonstrated that acibenzolar was effective as a pre-flowering foliar spray.

Averaging across the five acibenzolar treatment concentrations, treated Andana fruit expressed 10% disease severity 3.9 days after control fruit (LSD [P=0.05] = 1.74 days). The corresponding delay for 100% disease severity was 3.3 days (LSD [P=0.05] = 1.72 days). For Elsanta fruit, and averaged across four acibenzolar concentrations, times to 10 and 100% disease severity were increased to 3.2 days (LSD [P=0.05] = 1.79 days) and 2.0 days (LSD [P=0.05] = 2.02 days), respectively, as compared to corresponding controls. This delay in disease symptom expression of about 2 days, as compared with untreated control fruit was equivalent to a 15 to 20% increase in fruit storage life.

The degree of disease suppression judged as time to 10 or 100% disease severity was similar for all acibenzolar concentrations. For Andana fruit (Experiment 2), linear regression excluding control treatments for time to 10% disease severity (y) against acibenzolar concentration (x) was $y = 18.314 + (0.946\ x)$, n = 5, $r^2 = 0.700$ (Fig. 4.4.3.1A). The corresponding regression for 100% disease severity was $y = 22.118\ (0.982\ x)$, n = 5, $r^2 = 0.814$ (Fig. 4.4.3.1A). For Elsanta fruit (Experiment 3), the linear regressions excluding control fruit for 10 and 100% disease severity were $y = 17.934 + (0.533\ x)$, n = 4, $r^2 = 0.518$ and $y = 22.667 + (0.597\ x)$, n = 4, $r^2 = 0.224$, respectively (Fig. 4.4.3.1B). The linear nature of these relationships and their shallow slopes suggest that the response to acibenzolar was on/off in nature. That is, the effect as compared to controls, was not strongly dose-dependant over the range of concentrations tested.

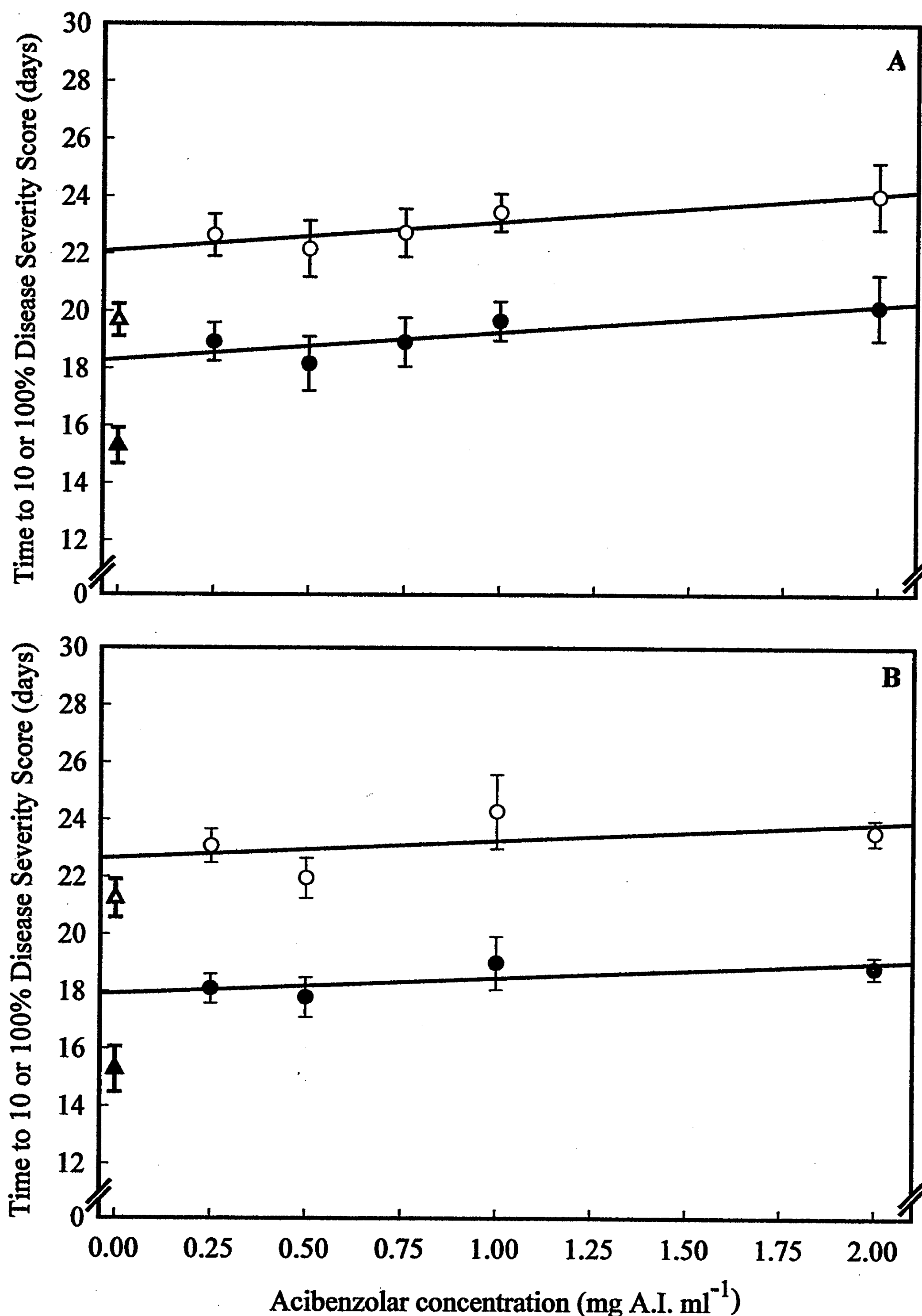


Figure 4.4.3.1: Effect of acibenzolar treatments on grey mould disease of strawberry fruits. (A) cv. Andana plants sprayed at weekly intervals over nine weeks (Experiment 2). (B) cv. Elsanta plants sprayed once at anthesis of primary flowers (Experiment 3). Triangles represent data for untreated and circles for treated plants, respectively. Solid symbols represent time to 10% disease severity. Hollow symbols represent time to 100% disease severity.

As noted above, and in contrast to the *in-vitro* results described in section 4.2, disease was not progressively suppressed *in-vivo* with increasing acibenzolar concentrations. Although direct anti-microbial effects of acibenzolar cannot be discounted, the on/off suppression response *in-vivo* (Fig 4.4.3.1A and B) is inherently different in nature to the progressive suppression response *in-vitro* (Fig. 4.2.3.1). The on/off suppression response suggests that an induced disease resistance mechanism is operative. This non dose-dependent trend is in agreement with Huang *et al.* (2000).

Induced resistance has been observed in strawberry leaves treated with salicylic acid (SA) (Malolepsza *et al.*, 1994). Acibenzolar is an 'functional analogue' of SA. Preharvest application to strawberry cv. Dallas plants of SA (1.26 mg ml^{-1}) 1 month after planting substantially increased concentrations of phenolic compounds (Malolepsza *et al.*, 1994). This increase was especially evident 24 and 48 h after treatment. A smaller increase in active oxygen species (superoxide anion and superoxide dismutase) was also determined. Subsequent infection with *B. cinerea* lead to reduced disease symptoms on leaves of SA pre-treated plants.

Only one other report has demonstrated that a preharvest acibenzolar treatment can reduce postharvest disease severity in fruit. Foliar sprays of acibenzolar at either 0.05 or 0.025 mg AI ml^{-1} prior to blossom was effective in reducing *Alternaria* spp. and *Fusarium* spp. disease in both cvs. Eldorado and South Cross rock melons and cv. Early Yellow hami melons (Huang *et al.*, 2000). In contrast with this study, acibenzolar had no activity against *in-vitro* mycelial growth of the postharvest pathogens tested (Huang *et al.*, 2000). Huang *et al.* (2000) suggested two hypotheses as to how a preharvest acibenzolar may suppress disease severity in melons harvested 7-8 weeks later. Firstly, acibenzolar may reduce postharvest disease levels through decreasing the inoculum load of foliar pathogens through SAR. However, melons get infected largely via their ground spot. Alternatively, acibenzolar affects the flower so as to induce a long-lasting change in the fruit. Both hypotheses may explain how acibenzolar suppressed grey mould of strawberry fruits in experiments 2 and 3.

It is likely that induced resistance was expressed in the strawberry fruit as only time to disease severity score was affected by acibenzolar treatment. Disease incidence was not affected, as eventually both control and acibenzolar treated strawberry fruit

showed symptoms of grey mould. Harvested strawberry fruit are infected with *B. cinerea* at flowering (Powelson, 1960), with grey mould only being expressed once quiescence has broken. Thus, it follows that acibenzolar treatment induced a physiological change in strawberry fruit that extended the period of quiescence. Work by Malolepsza *et al* (1994) demonstrated that elicitor compounds increase levels of phenolic compounds in leaf tissue with a concomitant reduction in disease incidence. The concentration of phenolics in strawberry have been implicated with natural disease resistance in strawberry by other authors (Nemec, 1976; Herbert *et al.*, 2001; Jiang *et al.*, 2001). Further investigation is necessary to show whether acibenzolar increased phenolic compounds in leaf or fruit tissue.

Experiment 4: April - May 2000

In contrast to Experiments 2 and 3, in Experiment 4 pre-harvest treatment with acibenzolar (0.2 mg AI ml^{-1}) was ineffective in suppressing grey mould on strawberry fruit after harvest (Fig. 4.4.3.2A; Appendix 4.4.4)). However, although not significant, acibenzolar treated fruit, at various stages of development, consistently developed grey mould after control fruit (Fig. 4.4.3.2A). This result indicates that acibenzolar treatment may have induced a resistance response. Time to 10 and 100% disease severity decreased with increasing fruit development (Fig. 4.4.3.2B). This results confirms findings described in section 3.2.

Variation in the efficacy of acibenzolar was also reported by Huang *et al.* (2000). Although acibenzolar was effective in reducing disease in most experiments conducted by Huang *et al.* (2000), it was not effective in all situations. In a repeat trial, acibenzolar at 0.025 – 0.05 mg AI ml^{-1} did not significantly ($P > 0.05$) decrease disease incidence of cv. Eldorado rock melons. In contrast, acibenzolar treatment did reduce disease severity, especially of *Alternaria* spp. When preharvest acibenzolar treatment was combined with a postharvest fungicide (0.5 and 0.25 mg guazatine ml^{-1}), disease incidence was reduced significantly ($P < 0.05$) more than by fungicide treatment alone (Huang *et al.*, 2000).

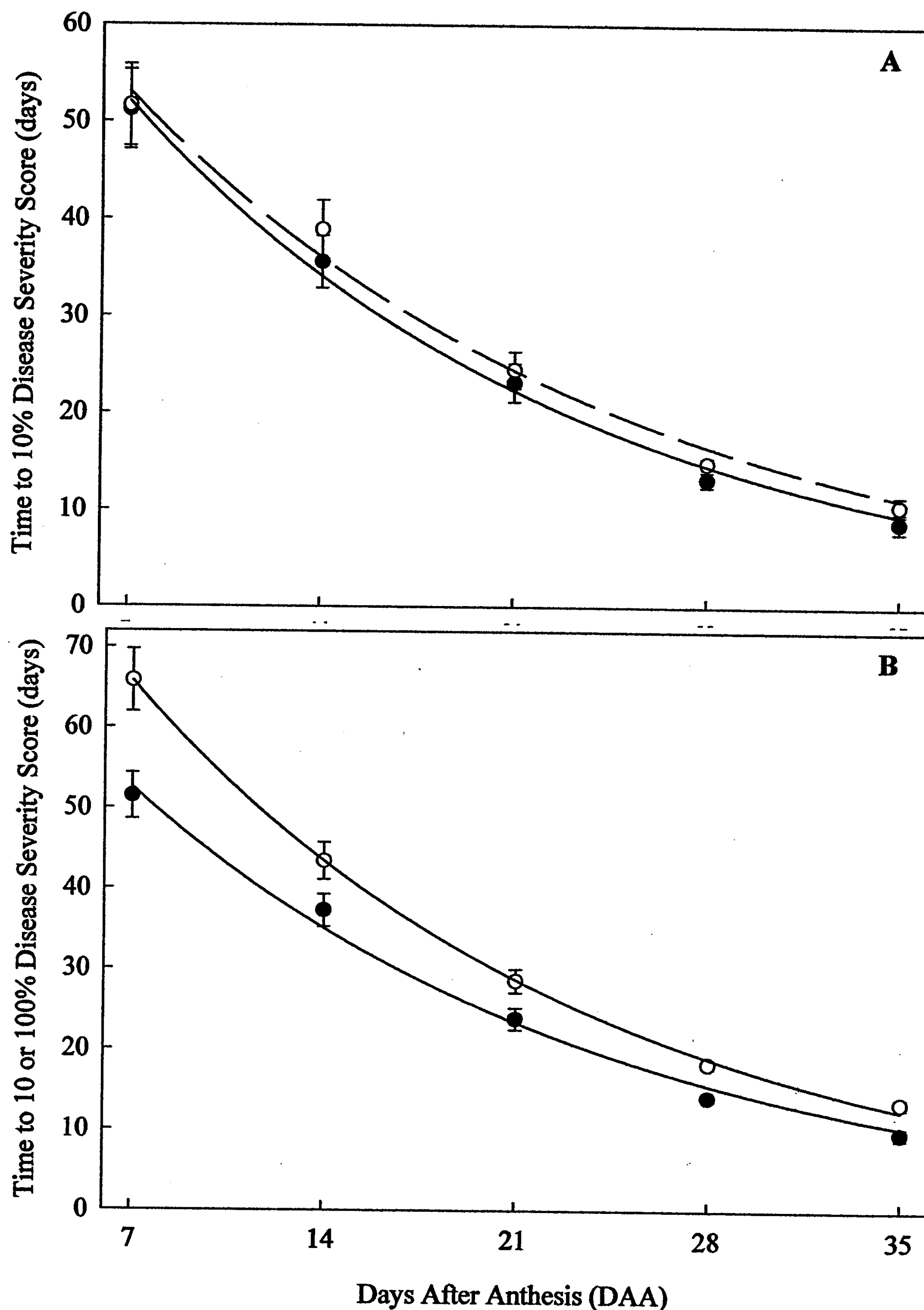


Figure 4.4.3.2 A: Effect of acibenzolar on grey mould disease on strawberry fruit harvested at different development stage in Experiment 4. ● control; ○ acibenzolar treatment.

Figure 4.4.3.2 B: Effect of fruit development stage on grey mould disease on strawberry fruit in Experiment 4. ■ Time to 10% disease severity; □ Time to 100% disease severity presented are treatment means ±s.e. (n = 12). Where standard errors are not shown symbols are bigger than s.e.

Experiment 5: July – August 2000

In contrast to winter trials (Experiments 2 and 3), and in line with the negative results of summer trials (Experiments 1 and 4), acibenzolar (0.002-2.0 mg AI ml^{-1}) was, in this second summer trial of 2000 ineffective in suppressing grey mould on strawberry fruit after harvest (Table 4.4.3.3; Appendix 4.4.5). Timing and number of acibenzolar applications had no significant ($P > 0.05$) effect on time to 10% and 100% disease severity scores for primary, secondary or tertiary fruit. Further work is required to understand more complex interactions between treatments in Experiment 5 as four and five order interactions are not discussed.

Table 4.4.3.3: Effect of acibenzolar treatments on time to 10% and 100% disease severity score on harvested strawberry cv. Elsanta fruit in Experiment 5.

Acibenzolar concentration (mg AI ml^{-1})	Time to disease severity score (days)	
	10% disease severity[A]	100% disease severity[B]
0	20.1	26.6
0.002	19.4	24.9
0.02	18.3	24.0
0.2	20.0	26.0
2.0	19.8	25.1

[A]Mean separation by LSD [P = 0.05] = 1.05 d

[A]Mean separation by LSD [P = 0.05] = 1.40 d

All acibenzolar treatments in combination with either a single application at secondary anthesis of 2 ml Scala l^{-1} (0.8 mg pyrimethanil ml^{-1}) (Taylor *et al.*, 1999) or 1 ml Rovral l^{-1} (0.5 mg iprodione ml^{-1}) were not significantly ($P > 0.05$) different from fungicide treatments alone. This result contrasts with findings that increasing acibenzolar concentration in combination with postharvest fungicide treatment 'synergistically' decreased disease incidence in cv. Eldorado rock melons, even when acibenzolar alone was ineffective in significantly ($P > 0.05$) reducing disease incidence (Huang *et al.*, 2000). Similarly, treatment of wild-type *Arabidopsis* with

acibenzolar combined with either metalaxyl, fosetyl or $Cu(OH)_2$ resulted in a 'synergistic' effect on pathogen resistance against *Peronospora parasitica* (Molina *et al.*, 1998).

Time to 10 and 100% disease severity scores of harvested fruit from plants treated with Rovral were not significantly different ($P > 0.05$) from control fruit (Table 4.4.3.4). This was the case regardless of fruit position (Table 4.4.3.4). This result suggests a degree of pathogen resistance to iprodione. Resistance of *B. cinerea* in strawberry to iprodione has been reported previously (Howard, 1990; Washington *et al.*, 1992; Berrie *et al.*, 1998). However, it is rather surprising that iprodione resistant *B. cinerea* strains may have developed during experiment 5, as plants only received one application of the fungicide. *B. cinerea* readily develops resistance to iprodione when more than two sprays are used successively in strawberries (Berrie *et al.*, 1998). Similarly, Blacharski *et al.* (2001) showed that two applications of iprodione combined with captan or thiram did not consistently reduce postharvest incidence of grey mould on strawberry.

Scala provided the best control against *B. cinerea*, and increased time to 10% and 100% disease severity score by 25-30% compared to control (Table 4.3.3.4). Pyrimethanil is an anilinopyrimidine fungicide that suppresses *B. cinerea* by directly inhibiting methionine biosynthesis (Fritz *et al.*, 1997). Scala has been shown to be an effective control against *B. cinerea* in other crops including Geraldton waxflower (Taylor *et al.*, 1999).

Primary fruit inoculated at green stage I were more resistant to subsequent development of grey mould symptoms after harvest than either secondary or tertiary fruit. At the time of ± inoculation, the majority of primary, secondary and tertiary flowers were at green stage I, anthesis and white bud stage, respectively. Primary and tertiary fruit derived from cv. Elsanta plants inoculated with *B. cinerea* (2×10^5 conidia ml^{-1}) 4 weeks after planting showed symptoms of grey mould significantly earlier than control fruit (Table 4.4.3.5). However, only tertiary fruit showed a significant difference in time to 100% disease severity score (Table 4.4.3.5). There was no significant difference in time to 10 or 100% disease severity for secondary

fruit (Table 4.4.3.5). These results differ from those found by Jarvis and Borecka (1968). According to Jarvis and Borecka (1968), the open-flower stage (the petals fully reflexed at around anthesis) was the most susceptible and green bud stage the least susceptible to the establishment of latent infection leading eventually to grey mould disease on fruit. White buds proved to be relatively resistant in this respect (Jarvis and Borecka, 1968). This pattern in disease resistance of various flower stages was similar in six cultivars tested (Jarvis and Borecka, 1968). However, these experiments were conducted in the field rather than under glass as in Experiment 5.

Table 4.4.3.4: Effect of fungicide treatment on time to 10% and 100% disease severity score (days) of harvest primary, secondary and tertiary strawberry cv. Elsanta fruit in Experiment 5. Plants were sprayed 4 weeks after planting with either distilled water (control) or a single application at secondary anthesis of 2 ml Scala l^{-1} (0.8 mg pyrimethanil ml^{-1}) or 1 ml Rovral l^{-1} (0.5 mg iprodione ml^{-1}).

Inoculation	10% disease severity[A]			100% disease severity[B]		
	primary	secondary	tertiary	primary	secondary	tertiary
Control	17.6	16.1	16.9	24.0	21.9	22.2
Rovral	18.3	16.4	17.1	25.2	22.6	22.4
Scala	28.8	23.6	20.8	34.2	28.7	26.8

[A]Mean separation by LSD [P = 0.05] = 1.27 d

[B]Mean separation by LSD [P = 0.05] = 1.35 d

Table 4.4.3.5: Effect of ± *Botrytis cinerea* inoculation on time to 10% and 100% disease severity score (days) of harvested primary, secondary and tertiary strawberry cv. Elsanta fruit in Experiment 5. Plants were sprayed with either a conidial suspension of *B. cinerea* (2 x 10^5 conidia ml^{-1}) or distilled water 4 weeks after planting when the majority of primary, secondary and tertiary flowers were at green stage 1, anthesis and white bud stage, respectively.

Inoculation	10% disease severity[A]			100% disease severity[B]		
	primary	secondary	tertiary	primary	secondary	tertiary
Control	22.0	19.1	19.8	28.1	24.6	25.4
B. cinerea	21.2	18.3	16.8	27.5	24.2	22.2

[A]Mean separation by LSD [P = 0.05] = 0.81 d

[B]Mean separation by LSD [P = 0.05] = 1.14 d

Experiments 6 and 7: February – May; June – July 2001, respectively

Pre-harvest treatment of acibenzolar (0.25 mg AI ml^{-1}) was effective at suppressing grey mould on secondary fruit after harvest in experiment 6 (Table 4.4.3.6; Appendix 4.4.6). Elsanta fruit treated with acibenzolar expressed 10% disease severity 1.6 days after control fruit. The corresponding delay for 100% disease severity was 1.5 days. The delay in symptom expression of about 1.5 days compared with untreated control fruit was equivalent to only a 5% increase in storage life of the fruit. The degree of disease suppression, judged as time to 10 or 100% disease severity was much less than for the other positive results of in experiments 2 and 3. In a replicate study of Experiment 6, conducted in summer between June and July 2001 (experiment 7), acibenzolar was ineffective in suppressing grey mould (Table 4.4.3.8; Appendix 4.4.7). This result suggests that the efficacy of acibenzolar may dependant on time of planting and environmental conditions during flowering and fruiting.

Table 4.4.3.6: Effect of acibenzolar on time to 10 and 100% disease severity score caused by *B. cinerea* of secondary strawberry cv. Elsanta fruit grown between winter and early summer (February and May, 2001; Experiment 6) and summer 2001 (July and July, 2001; Experiment 7)

Acibenzolar concentration	Experiment 6 (February – May)		Experiment 7 (June – July)	
(mg AI ml^{-1})	10% disease severity[A]	100% disease severity[B]	10% disease severity	100% disease severity
0.00	19.3	29.2	11.7	17.5
0.25	20.9	30.7	12.0	17.6

[A]Mean separation by LSD [P = 0.05] = 1.08 d

[B]Mean separation by LSD [P = 0.05] = 1.25 d

Temperatures during Experiment 6 were greatly lower than for Experiment 7 (Figs. 4.4.3.3 and 4.4.3.4). Temperatures during Experiment 6 ranged from –0.7 to 39.9°C with a mean temperature of 13.0°C (Fig. 4.4.3.3). Temperatures for Experiment 7 ranged from 7.1 to 48.6°C with a mean temperature of 21.6°C (Fig. 4.4.3.4). Degree

days (°C days) for Experiments 6 and 7 were 1389.6 and 1015.2, respectively. These differences in temperature between the two replicate experiments resulted in different fruit development rates (Tables 4.4.3.7) Primary and secondary fruit took over twice as long to reach harvest maturity from planting and anthesis in Experiment 6 as compared to Experiment 7 (Tables 4.4.3.7). Mean fruit weight was also very different with Experiment 6 fruit being up to 3 times heavier than Experiment 7 fruit (Table 4.4.2.8). In addition, mean TSS (n = 256) of primary fruit was 9.65 and 9.02 °Brix in experiments 6 and 7, respectively. Extreme temperatures (35-40°C) are known to reduce photosynthetic rate, fresh weight and TSS in strawberries (Hellman and Travis, 1988; Wang and Camp, 2000). Maximum temperatures for Experiment 7 frequently exceeded 35°C over the whole growing period (Fig. 4.4.3.4).

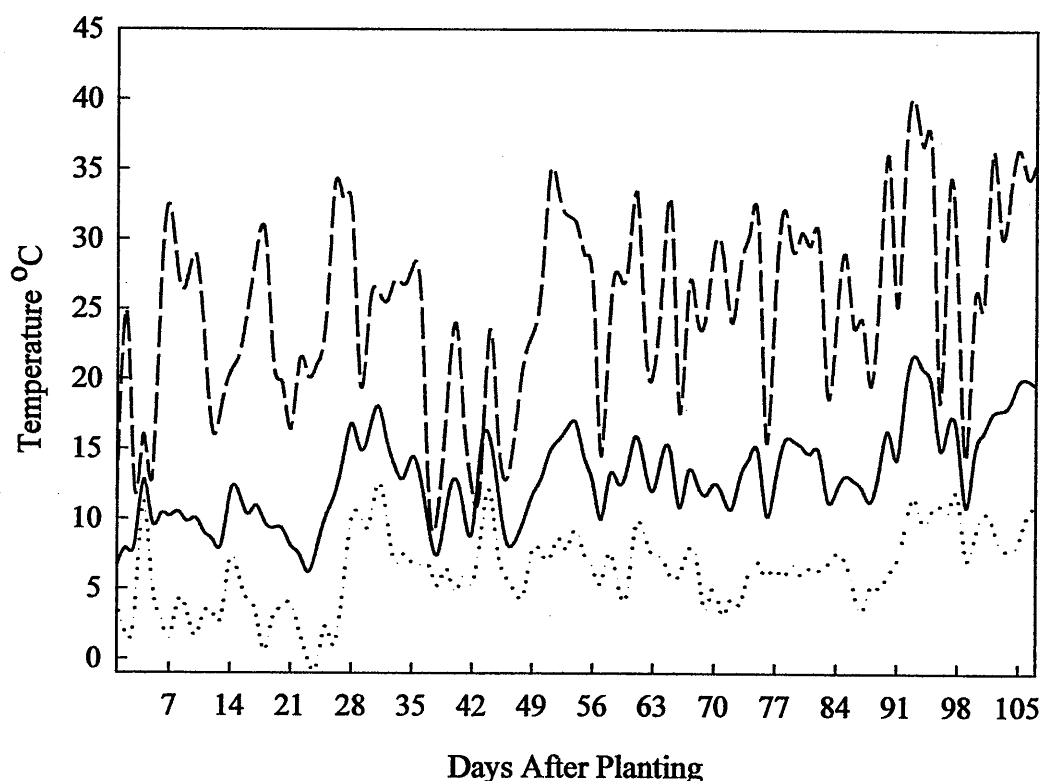


Figure 4.4.3.3: Daily maximum (- - -), minimum (······) and mean (——) glasshouse temperatures (n = 288) for strawberry cv. Elsanta plants grown in Experiment 6 in winter – early summer from February 8th to May 25rd 2001.

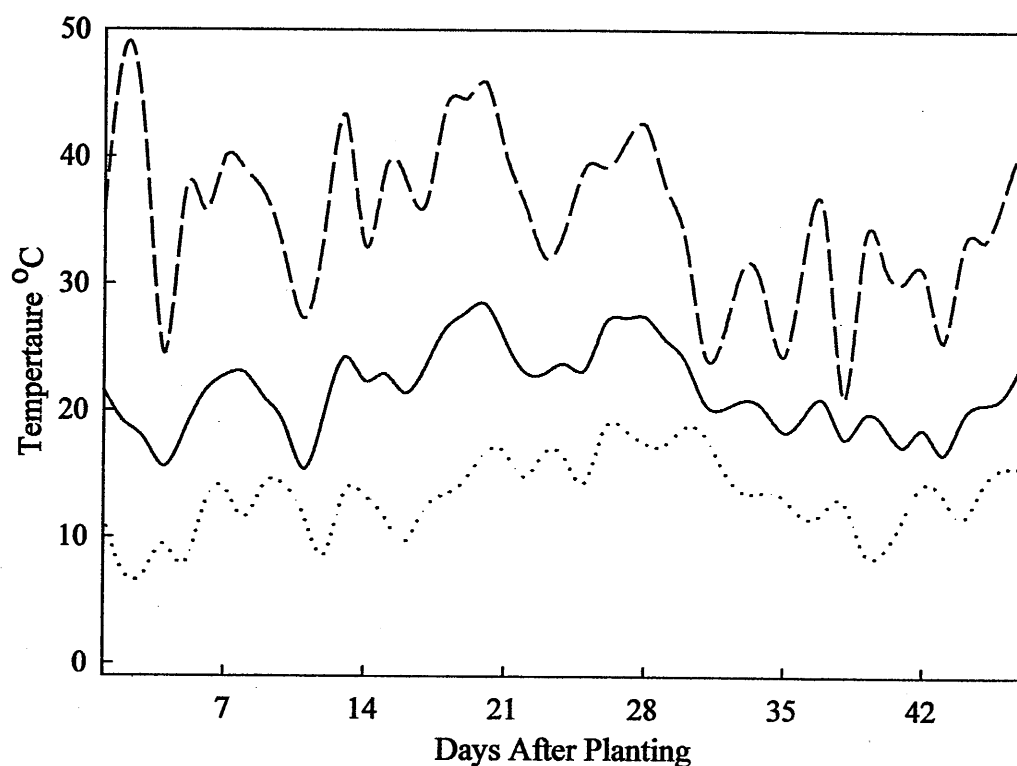


Figure 4.4.3.4: Daily maximum (---), minimum (······) and mean (——) glasshouse temperatures (n = 288) for strawberry cv. Elsanta plants grown in Experiment 7 in summer from June 7th to July 23rd 2001.

Table 4.4.3.7: Mean days after planting (DAP) and days after anthesis (DAA) until harvest for secondary and primary fruit grown between winter and early summer (February and May) 2001 (Experiment 6) and summer 2001 (July and July) (Experiment 7).

Fruit Position	Experiment 6 (February – June)		Experiment 7 (June – August)	
	DAP	DAA	DAP	DAA
Primary	94.1	41.5	40.4	21.3
Secondary	95.0	41.1	42.0	21.9

Table 4.4.3.8: Mean fruit weight (g) for secondary and primary grown between Winter and early summer (February and May) 2001 (experiment 6) and summer 2001 (July and July)

Fruit Position	Mean fruit weight (g)	
	Experiment 6	Experiment 7
Primary	33.71	14.53
Secondary	26.33	9.62

Days to 10% disease severity for control fruit from Experiment 6 was 19.3 days as compared to 11.7 days for fruit from experiment 7 (Table 4.4.3.6). However, the inoculum load at secondary anthesis was very similar in both experiments. Selective media spore traps for *B. cinerea* (Kerssies, 1990, section 4.4.2.6) demonstrated that mean colony forming units (cfu) per plate (Kerssies, 1990) (n = 48) for experiment 6 and 7 were 8.2 (s.e. ±0.433) and 8.1 (s.e. ±0.393) , respectively. Position of spore trap at either canopy level or 1m above canopy level had no effect on number of cfu recorded. Thus, disease severity data suggests that fruit grown in experiment 6 had an inherently higher mean natural disease resistance level than fruit grown in experiment 7. This difference in NDR may explain why different authors have reported that the relative NDR of Elsanta is high or low (Daugaard, 1999).

Whilst high nitrogen fertilisation increased secondary fruit weight and TSS content in primary fruit in Experiment 6, it had no effect on primary fruit weight and firmness in either of Experiments 6 and 7. However, Saxena and Locascio (1968) reported that strawberries fertilised with N increased shear resistance and thus fruit firmness. High nitrogen fertilisation had no effect on either grey mould disease severity in secondary fruit, and the period between 10 and 100% disease severity scores. This result contrasts other work that showed that whilst high nitrogen can increase yield in strawberries it can also decrease shelf life due to increase in grey mould severity (Kolbe, 1979; Lyons *et al.*, 1995). One reason for the contradictory results between in Experiments 6 and 7 and proceeding published work may be that the nitrogen content of the peat mixture (8.9 g N kg^{-1}) used in the present work was too high to allow large differences in disease severity between low and high nitrogen treatments. In addition,

ECe levels in both experiments 6 and 7 were comparatively high at between 2.2-2.6 mS cm^{-1} (Table 4.3.3.9) as compared to ideal levels of ca. 1.7 mS cm^{-1} (Wilson, 1997). This may have also obscured any large effects of nitrogen treatment as EC can provide a useful measure of feed strength. There were no significant differences between ECe levels (mS cm^{-1}) between treatments in either Experiments 6 or 7.

High nitrogen treatment may also have not affected time to disease severity in strawberry fruit as nitrogen source used in this study was Hoagland's solution. Hoagland's solution is nitrate rather than ammonium based. Sol (1967) found that *Vicia faba* plants fertilised with a nitrate source of nitrogen were more resistant to *Botrytis cinerea* than plants fertilised with an ammonium source. Thus, if an ammonium based nutrient solution was used rather than Hoagland's differences in disease severity may perhaps have been seen between treatments. In contrast to Experiment 6, nitrogen treatments in Experiment 7 had no significant effect ($P > 0.05$) on primary fruit quality judged by weight, TSS and firmness.

Table 4.4.3.9: Monthly electrical conductivity levels of leachate (ECe; mS cm^{-1}) from plants ($n = 64$) grown between Winter and early summer (February and May) 2001 (experiment 6) and summer 2001 (July and July).

Weeks after planting	Experiment 6	Experiment 7
4	2.283	2.642
8	2.642	2.303
12	2.303	---[A]

[A]reading not taken as after last harvest

Koricheva *et al.* (1998) proposed through meta-analysis of past literature that in resource rich environments, growth processes (e.g. protein synthesis) receive allocation priority for carbon resource. This limits the proportionate availability of carbon for phenypropanoid derived and other carbon-based secondary defence compounds (CBSC). Trade–offs are inevitable between primary and secondary metabolism since during periods of augmented growth, defence metabolism involving carbon based secondary metabolites may be substrate and/or energy limited. It

follows, therefore, that lower fruit NDR observed in Experiment 7 was probably because factors that limit the synthesis of defence related CBSCs will compromise the inherent NDR of the fruit. Thus, under conditions that favour growth, secondary metabolism may be jeopardised. Acibenzolar may have been ineffectual in boosting levels of constitutive or inducible carbon-based secondary defence compounds in fast growing strawberry fruit sufficiently to reduce disease severity in Experiment 7.

4.4.4. Conclusion

Most published data demonstrating chemical activation of SAR are concerned with control of pre-harvest diseases in arable crops (Sticher *et al.*, 1997). Relatively little attention has been paid to the potential for controlling postharvest fruit diseases through enhancing plant natural disease resistance (Joyce and Johnson, 1999; Huang *et al.*, 2000). Moreover, the effect of the environment and nitrogen fertigation on acibenzolar efficacy have not been researched. This work demonstrates that the pre-harvest applications of the chemical plant activator acibenzolar was ineffective in suppressing grey mould on strawberry after harvest for plants grown during the summer months. However, acibenzolar was effective in suppressing grey mould on strawberry fruit when plants were grown during the winter period. This suggests that acibenzolar could be valuable to the strawberry industry in helping to control grey mould disease on fruit for early cropping plantings. More work is required, however, to optimise suppression of *B. cinerea* and to elucidate the SAR mechansim(s) involved.

PART B EFFECTS OF *AUREOBASIDIUM PULLULANS* AND UV-C TREATMENTS ON STRAWBERRY GREY MOULD

4.5 Effects of Preharvest *Aureobasidium pullulans* Treatment on Grey Mould Disease on Strawberry Fruit

4.5.1 Introduction

Development of microbial antagonists for the control of postharvest decay of fruit has been pursued actively (Wilson and Wisniewski 1994; Ippolito and Nigro 2000; Peng and Sutton, 1991; Sutton, 1995). In strawberry, grey mould development has been reduced using *Cladosporium herbarum* (Pers.) Link (Bhatt and Vaughan 1962), *Pseudomonas fluorescens*, *Bacillus pumilus* (Swadling and Jefferies 1996), *Ulocladium atrum* (Boff et al., 1998), *Trichoderma* isolates (Tronsmo 1986; Pratella and Mari 1993; Tronsmo and Dennis 1997) and *Aureobasidium pullulans* (de Bary) Arnaud (Bhatt and Vaughan 1962; Lima *et al.* 1997; Adikaram *et al.*, 2002). Yeasts and yeast-like fungi are very effective antagonists of important postharvest pathogens such as *B. cinerea* and *Rhizopus stolonifer* (Ehrenb.: Fr.) Vuill. on strawberry (Lima *et al.* 1997). They compete for nutrients, interact directly with pathogens and induce host defence reactions (Droby and Chalutz 1994; Ippolito and Nigro 2000; Castoria *et al.* 2001). These mechanisms make such organisms acceptable to consumers as biocontrol agents, provided that antibiotic production is not involved (Lima *et al.* 1997). However, *in vitro* studies show that at least one *A. pullulans* isolate can secrete antibiotics (McCormack *et al.* 1994).

A. pullulans is one of the most widespread and well-adapted saprophytes in the phyllosphere (Blakeman and Fokkema 1982; Andrews *et al.* 1994). *A. pullulans* is reported as a biocontrol agent for several postharvest diseases, including grey mould (Lima *et al.*, 1997; Schena *et al.* 1999; Ippolito *et al.* 2000; Ippolito and Nigro 2000). It has also been isolated from the surface of strawberry fruit by Buhagiar and Barnett (1971) and Lima *et al.* (1997). *A. pullulans* effectively controlled grey mould on ripe strawberry fruit when applied at flowering (Lima *et al.*, 1997) and when applied as a preharvest dip to green fruit still attached to the plant (Adikaram *et al.*, 2002).

Competition for nutrients and enhanced natural disease resistance have been proposed as important modes of action (Lima *et al.* 1997; Adikaram *et al.*, 2002). For instance, treatment of detached green stage strawberry fruit with *A. pullulans* isolate (GRA1-2) reduced grey mould disease by increasing levels of preformed antifungal compounds and the synthesis of new phytoalexin-like substances. The ability of *A. pullulans* to induce host resistance in apples by increasing the activities of β-1,3 glucanase, chitinase and peroxidase has also been suggested as a basis for of activity against *B. cinerea* and *Penicillium expansum* in fruit (Ippolito *et al.* 2000).

This study investigated whether our *A. pullulans* isolate, as reported by Adikaram *et al.* (2002) (Appendix) to control grey mould, could be effective when applied at flowering in accordance with work carried out by Lima *et al.* (1997).

4.5.2 Materials and methods

4.5.2.1 *Plant material*

Cold-stored maiden-year A+ grade strawberry plants cv. Elsanta supplied by KG Fruits Ltd. were grown in a glasshouse as described in section 3.2.1.1.

4.5.2.2 *Isolate preparation*

A single-spored isolate of *A. pullulans* (GRA1-2) used by us earlier (Adikaram *et al.*, 2002) was recovered from naturally infected green stage strawberry fruit cv. Elsanta and maintained on ½ PDA (1.0 mg streptomycin ml^{-1}) at 20 ±1°C. A cell suspension of 14-day-old *A. pullulans* was prepared according to Ippolito *et al.* (2000). Cells were pelleted by centrifugation at 3000 g for 20 min, re-suspending in de-ionised water and centrifuged again. Resulting pellets were dispersed in de-ionised water and adjusted to 5×10^7 cfu ml^{-1} using a haemocytometer (Lima *et al.*, 1997). A conidial suspension of *B. cinerea* (2×10^5 conidia ml^{-1}) was prepared as described in section 3.2.1.3.

4.5.2.3 A. pullulans *and* B. cinerea *inoculation*

A completely randomised design was adopted with 48 plants. Flowers on the primary truss from each plant were sprayed either with a cell suspension of *A. pullulans* or de-ionised water using a TLC sprayer until run-off. Spraying commenced 8 weeks after planting when the majority of primary, secondary and tertiary flowers on the primary truss were at post-anthesis, anthesis and white bud stages, respectively (Plate 3.3.1.2). Two days later, each flower received an additional spray of either 2 x 10^5 *B. cinerea* conidia ml^{-1} or de-ionised water (Jarvis and Borecka, 1968). Each plant received approximately 1 ml of the ± *A. pullulans* and ± *B. cinerea* inocculum. Flowers were not hand-pollinated in order to minimise cross contamination between fungal treatments.

4.5.2.4 *Natural infection load*

The inocculum load of *B. cinerea* conidia in glasshouse during anthesis of secondary flowers was determined according to Kritzman *et al.* (1978) and Kerssies (1990) as described in section 4.4.2.6.

4.5.2.5 *Fruit and disease assessment*

Ripe fruit from each treatment were harvested daily during the fruiting season. Fruit weight and days after anthesis (DAA) were recorded. Fruit were held in the dark at 5°C and 95 to 100% RH in individual closed but vented polystyrene containers as described in section 3.2.1.4. Disease severity after harvest resulting from natural and artificial infection was assessed as described in section 3.2.1.4.

4.5.2.6 Statistical analysis

Missing values resulting from an increase in misshapen fruit due to lack of complete uniform pollination were accounted for by subjecting data to analysis of variance using Genstat Version 4.1. Where significant differences between the treatments was determined LSDs were calculated for mean separations at the 5% level.

4.5.3 Results and discussion

Pre-treatment of strawberry flowers with ± *A. pullulans* and ± *B. cinerea* had no significant effect ($P > 0.05$) on harvested fruit weight or days to anthesis (DAA) (Appendix 4.5). Primary fruit had significantly higher fresh weight at harvest than secondary and tertiary fruit, respectively (Table 4.5.3.1). Days after anthesis (DAA) also decreased with lesser infructescence position (Table 4.5.3.1).

Table 4.5.3.1: Effect of ± *Aureobasidium pullulans* (5×10^7 cfu ml^{-1}) and ± *Botrytis cinerea* (2×10^5 conidia ml^{-1}) sprayed at flowering on harvested fruit weight and days after anthesis.

Parameter	Infructescence position		
	Primary	Secondary	Tertiary
Fruit Weight (g) [A]	23.94	20.74	13.59
DAA [B]	44.1	41.9	42.6

[A] Mean separation by LSD [P = 0.05] =2.79

[B] Mean separation by LSD [P = 0.05] = 1.56 d

Pre-treatment of strawberry flowers with *A. pullulans* was not effective in delaying the development of grey mould disease on harvested strawberry fruit held at 5°C. This result contrasts work by Lima *et al.* (1997). They showed that a different isolate of *A. pullulans* was an effective biocontrol agent when sprayed at flowering. Moreover, we found (Adikaram *et al.*, 2002), using the same GRA1-2 isolate as in this experiment, that dip-inoculation of unwounded green strawberry fruit with *A. pullulans* when still attached to the plant delayed the development of grey mould after harvest. A postharvest *A. pullulans* treatment was also shown to reduced grey mould disease in detached green stage strawberry fruit. It is not clear why similar results were not seen in this later experiment. One possibility is that the inocculum load in the glasshouse may have been of such a high level that any effects of *A. pullulans* were overridden. However, inocculum load at secondary anthesis was recorded as

only 8.8 (s.e. ±0.63) colony forming units per plate (n = 16) This is comparatively low when compared to other studies (Jordan, 1978).

Inoculation with *B. cinerea* 2 days after ± *A. pullulans* significantly ($P < 0.001$) reduced the time to 10 and 100% disease severity by about 3 days (Table 4.5.3.2). This result was equivalent to a 15-20% reduction in shelf life. There was no significant difference in time from 10 and 100% disease severity for *B. cinerea* and control treatment. This suggests that, although, a higher inoculation load of *B. cinerea* at flowering reduces shelf life it does not affect disease spread once quiescence has been broken.

Table 4.5.3.2: Effect of ± *Aureobasidium pullulans* (5×10^7 cfu ml^{-1}) and ± *Botrytis cinerea* (2×10^5 conidia ml^{-1}) sprayed at flowering on grey mould disease

Treatment[A]	Time to disease severity score (days)	
	10%[B]	100%[C]
- Ap and – Bc	14.43	22.68
+Ap and – Bc	15.68	22.93
-Ap and + Bc	12.35	19.69
+Ap and + Bc	12.15	19.32

[A] Ap = *A. pullulans*; Bc = *B. cinerea*

[B] Mean separation by LSD [P = 0.05] =1.90d

[C] Mean separation by LSD [P = 0.05] = 1.83d

Primary and secondary fruit after harvest developed grey mould disease at 12.79, 13.21 days which was significantly ($P = 0.026$; LSD [P=0.05] = 1.65) more quickly than tertiary fruit at 14.95 days. However, there was no significant interaction between fruit position and inoculation treatment. Thus, there was no difference in susceptibility between post-anthesis, full bloom and white bud flower stages. Jarvis and Borecka (1968) showed that full bloom and white bud stages were the most susceptible flower stages to blossom blight. Grainger (1956) first reported that first-formed berries in the strawberry (cv. Auchincruive climax) inflorescence are more susceptible to grey mould than successively later berries on the same inflorescence.

These results were, however, based on recording grey mould in the field from randomly picked ripe strawberry fruit rather than specifically looking at the effect of fruit position on postharvest disease severity.

4.6 Effect of Postharvest UV-C Treatment on Grey Mould Disease on Strawberry Fruit

4.6.1 Introduction

Low doses of short-wave ultraviolet light (UV-C, 190-280 nm wavelength) have been used to control storage rots in fruit and vegetables (Table 2.4). In strawberries, application of UV-C at doses ranging from 0.125-2.0 kJ m^{-2} were effective in controlling grey mould and extending the shelf life of ripe strawberries (Nigro *et al.*, 1998b; 2000; Baka *et al.*, 1999). Nigro *et al.* (1998b) reported that the extension in shelf life in strawberry cv. Pajaro fruit was correlated with an increase in phenylalanine ammonia lyase (PAL) activity. Baka *et al.* (1999) demonstrated that UV-C treated (0.25 kJ m^{-2}) strawberry cv. Kent fruit also had a lower respiration rate, higher titratable acidity, and higher anthocyanin content and were firmer than control fruit.

This preliminary study investigated whether UV-C treatment could be effective in controlling grey mould on strawberry cv. Elsanta fruit.

4.6.2 Materials and methods

4.6.2.1 *Plant material*

Cold-stored maiden-year A+ grade strawberry cv. Elsanta plants supplied by KG Fruit Ltd. were grown in a glasshouse as described in section 3.2.1.1.

4.6.2.2 *UV-C treatment*

One hundred and twenty red stage fruit were harvested and irradiated using the method of Nigro *et al.*, (1998a). Fruit were irradiated using three germicidal low-pressure vapour UV lamps (Osram HNS OFR). Each lamp (2.5 cm tube diameter; 88 cm length) had a nominal power output of 30W and peak wavelength emission of 253.7 nm. The lamps were assembled 15 cm apart and 25 cm above fruit. The UV-C field area under the lamps was 60 x 100 cm. Irradiance (W m^{-2}) was measured using a Multi-Sense 100 Optical Radiometer fitted with a 254 nm UV-C light sensor (Ultra-violet Products, Cambridge, UK). Fruit were arranged in plastic trays in a single layer and turned over on their longitudinal access to expose opposite sides of the fruit to UV-C treatment at 15°C. Each fruit side either received a dose of 0.25 kJ m^{-2} or 0 kJ m^{-2} (control).

4.6.2.3 *Fruit inoculation*

Thirty UV-C treated and control fruit were inoculated by applying 15 μl of a 7 day-old *B. cinerea* conidial suspension (2 x 10^4 conidia ml^{-1}) on a 2 mm superficial wound, made by piercing the shoulder of the fruit with a needle (Adikaram *et al.*, 2002). Fruit were either inoculated at 0h and 48h after UV-C treatment. Non-inoculated with wound and non-inoculated fruit without wound were used as controls. Fifteen minutes after irradiation and control treatments was considered as being 0 hours (Nigro *et al.*, 1998a). After ±UV-C treatment at 15°C fruit were arranged in a CRD and held in the dark at 5°C and 95 to 100% RH in individual closed but vented polystyrene containers as described in section 3.2.1.4.

4.6.2.4 *Fruit disease assessment*

Disease severity resulting from natural and artificial infection was assessed as described in section 3.2.1.4.

4.6.2.5 *Statistical analysis*

Data was subjected to analysis of variance using Genstat Version 4.1 as described in section 3.2.1.5.

4.6.3 Results and discussion

UV-C treatment at 0.25 kJ m^{-2} was not effective in delaying the development of grey mould disease on harvested strawberry fruit held at 5°C (Appendix 4.6). This result contrasts work by Nigro *et al.* (1997) and Baka *et al.* (1999) which showed that 0.5 and 0.25 kJ m^{-2} were the most effective doses for reducing disease incidence in strawberry fruit, respectively. Nigro *et al.* (1997) reported that at 0.25 and 0.5 kJ m^{-2} the effect of UV-C treatment on grey mould disease incidence was less for both inoculated and non-inoculated cv. Pajaro fruit stored at 3°C for 10 days. It is possible that differences between the present results and those obtained by Nigro *et al.* (1997) might be explained by a difference in cultivars. Recent unpublished work by Marquenie *et al.* (2001) has shown that higher UV-C doses of +0.5 kJ m^{-2} were required for effective grey mould control in strawberry cv. Elsanta fruit.

Table 4.6.3.1: The effect of wounding and inoculation with *Botrytis cinerea* on grey mould disease

Treatment	Time to disease severity score (days)	
	10% [A]	100% [B]
Non-wounded control	12.67	20.30
Wounded – *B. cinerea*	12.27	19.30
Wounded + *B. cinerea* 0h	8.97	15.77
Wounded + *B. cinerea* 48h	11.87	18.23

[A] Mean separation by LSD [P = 0.05] = 0.62 d

[B] Mean separation by LSD [P = 0.05] = 0.89 d

Wound inoculation with *B. cinerea* significantly ($P > 0.001$) reduced the time to 10 and 100% disease severity compared to non-wounded control (Table 4.6.3.1). There was no significant ($P < 0.05$) effect of wounding alone on time to 10% disease

severity as compared to non-wounded control fruit. Fruit inoculated 48 h after wounding had delayed the development of grey mould disease compared to fruit inoculated at 0h after wounding. Wounding after harvest has been reported to elicit an induced resistance response in other fruit (Ismail and Brown, 1979).

CHAPTER 5

PREFORMED ANTIFUNGAL COMPOUNDS IN GERALDTON WAXFLOWER

5.1 Introduction

Geraldton waxflower (*Chamelaucium uncinatum* Schauer) is an Australian woody perennial shrub belonging to Myrtaceae (Plate 5.1.1). Of the 12 native *Chamelaucium* spp. from Western Australia (Mabberely, 1987; Manning *et al.*, 1996), *C. uncinatum* is the most economically important native cut flower export. Its small attractive flowers make it particularly suitable as a filler in floral bouquets. Recent waxflower research has concentrated on understanding and reducing postharvest flower abscission. This inherent problem results in low prices and reduced consumer confidence (Joyce, 1993).

Plate 5.1.1: Sprig of Geraldton waxflower (*C. uncinatum*) 'Mid Pink' (K. Young, 2001 pers. comm.).

5.1.1 Cultivation

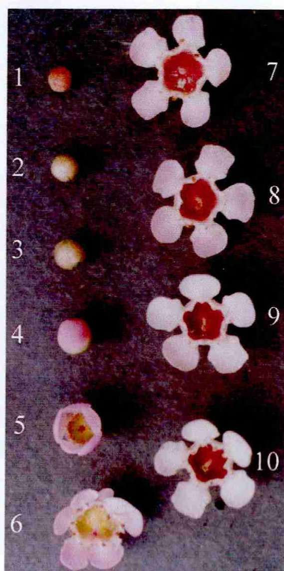
Commercial plantings of waxflower can be found throughout Australia and also overseas in Israel, California, Peru, and Thailand (Wearing and Joyce, 1994). During

harvesting about 50% of the stems and foliage is left, as only the top flowering stems are of cut flower value (D. Joyce, 2001 pers. comm.). Following harvest, the remaining plant material is cut down to a basal level as a 'platform' for stem growth in the following season. This harvested waste (foliage and woody stems) is burnt, as currently it has no use (A. Macnish, 2001 pers. comm.).

5.1.2 Flower abscission

Postharvest floral organ abscission usually occurs at the abscission zone at the base of the calyx tube (Joyce, 1993). Separation can occur in mature (non-nectiferous hypanthium) and immature (nectiferous hypanthium) flowers as well as at the bud stages (Plate 5.1.2). Abscission results from mechanical damage, high storage temperatures, water deficit stress, exposure to exogenous ethylene and/or infection by pathogenic fungi such as *Botrytis cinerea* (Joyce, 1992; Tomas, 1995).

Plate 5.1.2: Geraldton waxflower tissue 'Mid Pink' floral developmental stages showing nectiferous (6) and non-nectiferous (7-10) flowers (after Olley *et al.*, 1996).



Ensuring that the postharvest cold chain is maintained is fundamental to reducing floral abscission (Joyce, 1989; Taylor *et al.*, 2001). A reduction in storage temperature from just 4 to 0°C can limit flower fall (Joyce, 1993), as low temperature

during storage decreases respiration rate, delays the onset of disease and thus limits pathogen-induced abscission. In practice, however, temperature abuse during the cold chain is common. Accordingly, additional postharvest measures such as pulsing with silver thiosulphate (STS) (Joyce, 1988; 1989; 1993; Taylor *et al.*, 1996) and/or 1-methylcyclopropane (1-MCP) (Serek *et al.*, 1995; Macnish *et al.*, 2000) treatments are employed to control abscission.

5.1.3 Pathogen-induced abscission

Infection of Geraldton waxflower by *B. cinerea* can lead to unacceptable levels of flower abscission after harvest (Joyce, 1993; Tomas *et al.*, 1995; Taylor *et al*, 1999). Endogenous ethylene produced as a result of pathogen infection can cause floral abscission (Joyce, 1993). Ethylene production in infected flowers is much higher (7.71 µl kg^{-1} h^{-1}) than in healthy flowers (0.05 µl kg^{-1} h^{-1}). Thus, if infection is extensive and the storage environment confined, ethylene could accumulate and induce floral abscission (Joyce, 1993). Directly controlling *B. cinerea* through employing fungicides reduces endogenous ethylene levels and thereby flower abscission.

B. cinerea can be currently controlled by using either Rovral (50 mg iprodione l^{-1}) or Scala (80 mg pyrimethanil l^{-1}) as a postharvest dip and/or pre-harvest spray (Joyce, 1988; Taylor *et al.*, 1999; A. Macnish, 2001 pers. comm.). Other fungicides used on waxflower include iprodione (a dicarboximide), benomyl (benzimidazole) and mancozeb (dithiocarbamate) (Taylor *et al.*, 1999). There are, however, concerns over increasing loss in efficacy of conventional fungicides due to pathogen resistance and general unacceptability of fungicide usage in terms of environmental risk. Resistant strains of *B. cinerea* have been isolated from Geraldton waxflower in Australia (Joyce and Wearing, 1996; Taylor *et al.*, 1996). Consequently, finding alternative treatments effective against *B. cinerea* for the waxflower industry is desirable.

5.1.4 Constitutive defence in Geraldton waxflower

There has been very little research on the plant-pathogen interaction between Geraldton waxflower and *B. cinerea*. Beasley (2001) conducted preliminary studies on the relationship between waxflower maturity, susceptibility to flower abscission and constitutive defence relating to endogenous antifungal compounds. Initial results using thin layer chromatography (TLC) bioassays with *Cladosporium* sp. in an agar overlay method (Rahalison *et al.*, 1991; Stirling, 1995) showed that crude extracts from Geraldton waxflower flowers gave a zone of antifungal activity (no R_f stated). The antifungal compound(s) were not identified. There were no discernible differences in inherent constitutive resistance between different waxflower flower development stages. In addition, the organic phase of crude ethanol waxflower flower extracts partitioned with dichloromethane did not inhibit *Alternaria* sp., *B. cinerea* or *Epicoccum* sp. in a Petri plate bioassay (Beasley, 2001). Antifungal activity in leaf tissue was not tested.

Crude essential oil extracts from Geraldton waxflower flower tissue obtained by steam distillation had antibacterial activity against *Mycobacterium phlei* (Atkinson, 1949). Moreover, growth of *Staphylococcus aureus* was inhibited at a dilution of 1:500 in nutrient broth. Leaf extracts had no antibacterial activity against these important bacteria which can affect humans. The active chemicals in flower tissue was obtained through fractional distillation under vacuum in nitrogen with the essential oils being boiled off at 46-59°C at 13 mm of mercury pressure (1.73 kPa). Most of the inactive material, which represented approximately 90% of the crude extract, was boiled off at lower temperatures (Atkinson, 1949). The antibacterial compound(s) were not identified. It was, however, concluded that cineole was unlikely to be the active component of Geraldton waxflower oil, as Atkinson (1949) was unable to obtain crystalline material when treated -60°C. Atkinson and Brice (1955) also demonstrated with agar dilution bioassays that only 1 of 6 essential oils extracted from flowers and leaves showed strong antibacterial activity against *Mycobacterium phlei*. Complete growth inhibition was seen at 0.4 µl oil ml medium^{-1}. Antibacterial titres were performed by emulsifying the oils in 0.5% Tween 80 so

that they could be added to culture medium. Antifungal activity of crude oil extracts was not evaluated.

In earlier investigations, α-pinene was identified as the major compound in waxflower essential oil (Watson, 1944; Bowyer, 1957 both cited in Egerton-Warburton and Ghisalberti, 1995). A more recent unpublished study by Dunlop and co-workers (1989) (Egerton-Warburton and Ghisalberti, 1995) showed that the major constituents of waxflower 'CWA Pink' oil were α-pinene (44%), β-pinene (16%), citronellal (15%) and limonene (4%). Egerton-Warburton and Ghisalberti (1995) identified α-pinene (36.3%), limonene (18%), citronellal (16.1%), linalool (4.2%), α-terpinyl acetate (3.9%), geraniol (3.3%), globulol (2.9%), β-pinene (2.8%) and 1,8 cineole (2%) to be the major constituents of essential oils of bulked waxflower leaf tissue. However, there was considerable variation in oil composition between different *C. uncinatum* genotypes (Egerton-Warburton *et al.,* 1998). Antifungal activity of the individual essential oil extracts was not tested.

This study extends the preliminary work by Beasley (2001). A series of three experiments were conducted to investigate the nature and identities of constitutive antifungal compounds in Geraldton waxflower flower tissue. Moreover, the antifungal profile of leaf tissue was also investigated with a view to finding a possible use for this currently wasted vegetative material.

5.2 Materials and Methods

5.2.1 Plant material

Imported cut Geraldton waxflower (*Chamelaucium uncinatum* Schauer) cvs. 'Snowflake' and 'Mid Pink' and the hybrid cross *C. floriferum* x C. *uncinatum* (FxU) were sourced from Zwettloots and Son Ltd. (Sandy, UK). Sprigs were harvested and imported from Israel to UK in three to four days and maintained at 4°C during transport (Zwettloots and Son Ltd, 2001 Pers. Comm.). Sprigs were taken back to the laboratory within 1 h of purchase, immediately put into water and used within 30 min. Various cultivars were used due to importer and seasonal availability.

5.2.2 Experiment I

5.2.2.1 *Crude extraction*

Two 20 g FW samples each of Geraldton waxflower 'Snowflake' leaf, nectiferous and non-nectiferous flower tissues (Plate 5.1.2) were each ground in liquid nitrogen and extracted following the extraction procedure for strawberry described in section 3.3.1.6. Briefly, waxflower tissues were ground in liquid nitrogen and homogenised in ethanol (99% v/v) at -18°C (3 ml g^{-1} fresh weight (FW)). The homogenate was filtered, concentrated and partitioned twice with dichloromethane (99% v/v). The lower organic layers were combined, evaporated to dryness and resuspended in ethanol (0.2 ml g^{-1} FW) and stored at -18°C until use (Droby *et al.*, 1986; Zanuri *et al.*, 2001).

5.2.2.2 *Antifungal activity of crude tissue extracts*

One-dimensional (1-D) Thin Layer Chromatographs (TLC) bioassays using *C. cladosporioides* or *B. cinerea* were performed as described in sections 3.3.1.2 and 3.3.1.4 on crude ethanol extract partitioned into an organic dichloromethane phase of leaf and nectiferous and non-nectiferous flower tissues (50 μl spot; 0.2 ml g^{-1} FW) run in hexane: ethyl acetate: methanol (60:40:1 v/v/v). Additional TLC bioassays were performed on crude extracts (5 or 10 μl spots; 0.2 ml g^{-1} FW) using four solvent systems of increasing polarity. These were A = hexane: ethyl acetate: methanol (60:40:1 v/v/v), B = (60:40:10), C = (60:40:20) and D = (60:40:30). Cycloheximide (0.5 mg ml^{-1}) and ethanol (99% v/v) were used as positive and negative controls, respectively. Two-dimensional (2-D) TLC bioassays (Wedge and Nagle., 2000) using *C. cladosporioides* were performed as described in section 3.3.1.2 on each crude extracts of waxflower tissue (5 μl spot; 0.2 ml g^{-1} FW) run in solvent system A (one-dimension) and solvent system D (second dimension). R_fs of antifungal zones were recorded after 4 days incubation at 20°C and 100% RH. These zones were characterised by a lack of aerial mycelium which indicates the presence of antifungal activity (Klarman and Stanford 1968; Homans and Fuchs, 1970; Adikaram and Ratnayake Bhandara, 1998). Each plate was replicated once.

5.2.2.3 *Preliminary identification of antifungal compounds*

Replicate plates for 1-D and 2-D TLCs were performed and visualised under visible, UV-A (366 nm) and UV-C (254 nm) light using UV-C florescent TLC plates (Silica gel 60 F_{254}, Merck). 2-D TLCs were also sprayed with the phenolic reagent sprays Folin-Coicalteu and diazotized sulfanilic reagent as described in section 3.4.1.5. Carvacrol (2-Methyl-5-(1-methylethyl)phenol) was used as a positive control for phenolic reagent sprays. The colour of reacting spots corresponding to R_fs with antifungal activity was recorded.

5.2.3 Experiment II

5.2.3.1 *Sequential extraction*

Geraldton waxflower 'Mid Pink' leaf (330 g FW; 86.9 g dry weight (DW)) and combined nectiferous and non-nectiferous flower (240 g FW; 79.4 g DW) tissue were freeze dried, powdered in liquid nitrogen and sequentially extracted with ethyl acetate (99% v/v) and ethanol (99% v/v) as described in section 3.3.1.7 (Fig 5.2.3). 1-D TLC bioassays were performed on leaf and flower ethyl acetate and ethanol extracts. On the basis of TLC bioassay behaviour, the antifungal ethyl acetate fraction was partitioned three times with hexane and ethanol (80% v/v). 1-D TLC *C. cladosporioides* bioassays were performed on hexane and ethanol fractions (5 µl; 0.2 ml g^{-1} DW) run in solvent system D (hexane: ethyl acetate: methanol; 60:40:30 v/v/v). Replicate plates were visualised under UV-A and UV-C light and either sprayed with Folin-Ciocalteu, sulfanilic acid (Waterman and Mole, 1994) or Dragendorff's reagents as described in section 3.3.1.5. The colours of reacting spots corresponding to R_fs with antifungal activity were recorded.

5.2.3.2 *Co-chromatography*

1-D TLCs were performed on hexane and ethanol partitioned extracts (5 µl spot; 0.2 ml g^{-1} DW) and co-chromatographed with standard terpenes (99% v/v; 0.5 µl spot) previously reported to be in Geraldton waxflower oil extracts (Egerton-Warburton and

Ghisalberti, 1995). These included α-pinene (2,6,6-Trimethylbicyclo [3.1.1] hept-2-ene), limonene (1-Methyl-4-(1-methylethenyl) cylcohexene), linalool (3,7-Dimethyl-1,6-octadien-3-ol), geraniol ((E)-3,7-Dimethyl-2,6-octadien-1-ol) and 1,8-cineole. Plates were developed in solvent systems hexane: ethyl acetate: methanol B = (60:40:10 v/v/v), C = (60:40:20 v/v/v) and D = (60:40:30 v/v/v) and sprayed with phosphomolybdic acid (10% w/v ethanol; PMA) as described in section 3.4.10. to visualise organic compounds.

5.2.3.3 *Column chromatographic separation of antifungal compounds*

The more antifungal 80% (v/v) ethanol fractions obtained from sequentially extracted and partitioned ethyl acetate waxflower extracts were individually chromatographed in a glass column of length 7.5 cm and diameter 2 cm packed with Silica gel 60 (Fluka, St. Louis, USA) using four solvent combinations of increasing polarity. The leaf and flower extracts were first dissolved in the minimum volume of methanol and a slurry was prepared with silica gel 60. This was applied to the column pre-equilibrated with hexane: ethyl acetate (80:20 v/v). One thousand-drop fractions were collected using a Pharmain LKB-Redifrac fraction collector (Amersham BioSciences, Bucks., UK). The sequential solvent systems used were E = hexane: ethyl acetate (60:40 v/v), and B = hexane: ethyl acetate: methanol (60:40:10 v/v/v), C = (60:40:20 v/v/v) and D = (60:40:30 v/v/v). TLCs were performed on aliquots (5 µl spot) of each eluted fraction and developed in solvent system D. Plates were sprayed either with bioassay using *C. cladosporioides* or PMA. Thirty-eight fractions obtained from flower and leaf tissue were both reduced to six fractions. by pooling on the basis of similar PMA and antifungal TLC behaviour (Fig. 5.2.3). Two fractions, WFF-A and WFF-B from flower and one fraction, WFL-A, from leaf extracts were further purified.

5.2.3.4 *Purification of antifungal WFF-A*

Fraction WFF-A (89 mg) was subjected to preparative TLC (silica gel GF plate; 1 mm thick coating) run in solvent system C and visualised under UV-C. Six resulting bands were scraped from the plates, eluted with diethyl ether : methanol (7:3 v/v) and

tested for antifungal activity in solvent system D (Fig. 5.2.3). From TLC behaviour, two fractions (WFF-A1 (22 mg) and WFF-A2 (14 mg)) were prepared for NMR and HPLC analysis.

5.2.3.5 *Purification of antifungal WFF-B*

Fraction WFF-B (190 mg) was further fractionated using column chromatography (CC) using solvent system C as described in section 5.1.3.3. The last four fractions. were eluted with ethyl acetate: methanol (1:1). Thirty-eight fractions were again obtained. These were reduced to 9 fractions by pooling on the basis of similar TLC behaviour using PMA (Fig. 5.2.3). They were all evaporated to dryness, purged with N_2 and stored briefly at –18°C. Nine fractions were resuspended in 99% v/v ethanol (0.2 ml g^{-1} DW) and tested for antifungal activity. On the basis of TLC bioassay behaviour one fraction was further purified. This was named WFF-Be.

5.2.3.6 *Purification of antifungal WFF-Be*

Reverse phase TLC plates (RP-18 F_{254s}; 5 x 10 cm; Merck, Germany) were performed on fraction WFF-Be (1 µl) resuspended in acetonitrile and developed in solvent system F = acetonitrile: water (9:1 v/v). On the basis of TLC behaviour using UV-A and UV-C to visualise compounds, WFF-Be (50 mg) was dissolved in acetonitrile and Reverse Phase (RP) chromatographed in a column, length 14 cm; diameter 1 cm, using the solvent system acetonitrile: water (85:15 v/v) (Fig. 5.2.3). The column was packed with silica 60 RP-18 and covered with acid washed sand. Twenty-four 3 ml fractions were obtained. Each fraction (1 µl spot) was developed on RP-18 TLC in solvent system F. RP-TLC plates were visualised under UV-A and UV-C light and tested for antifungal activity using a modified RP-TLC bioassay methodology. Briefly, developed RP-TLCs were sprayed with *C. cladosporoides* (2 x 10^7 ml^{-1}) in Czapek Dox nutrient solution (Klarman and Stanford, 1968; Homans and Fuchs, 1970) with 1% Tween 80. A high concentration of Tween 80 was used to enable the spore suspension to stick to the hydrophobic RP plate. This concentration of Tween 80 did not inhibit growth of bioassay. The first 2 eluted fractions, WFF-Be1 (20 mg) and WFF-Be2 (41 mg), were prepared for NMR and RP-HPLC analysis.

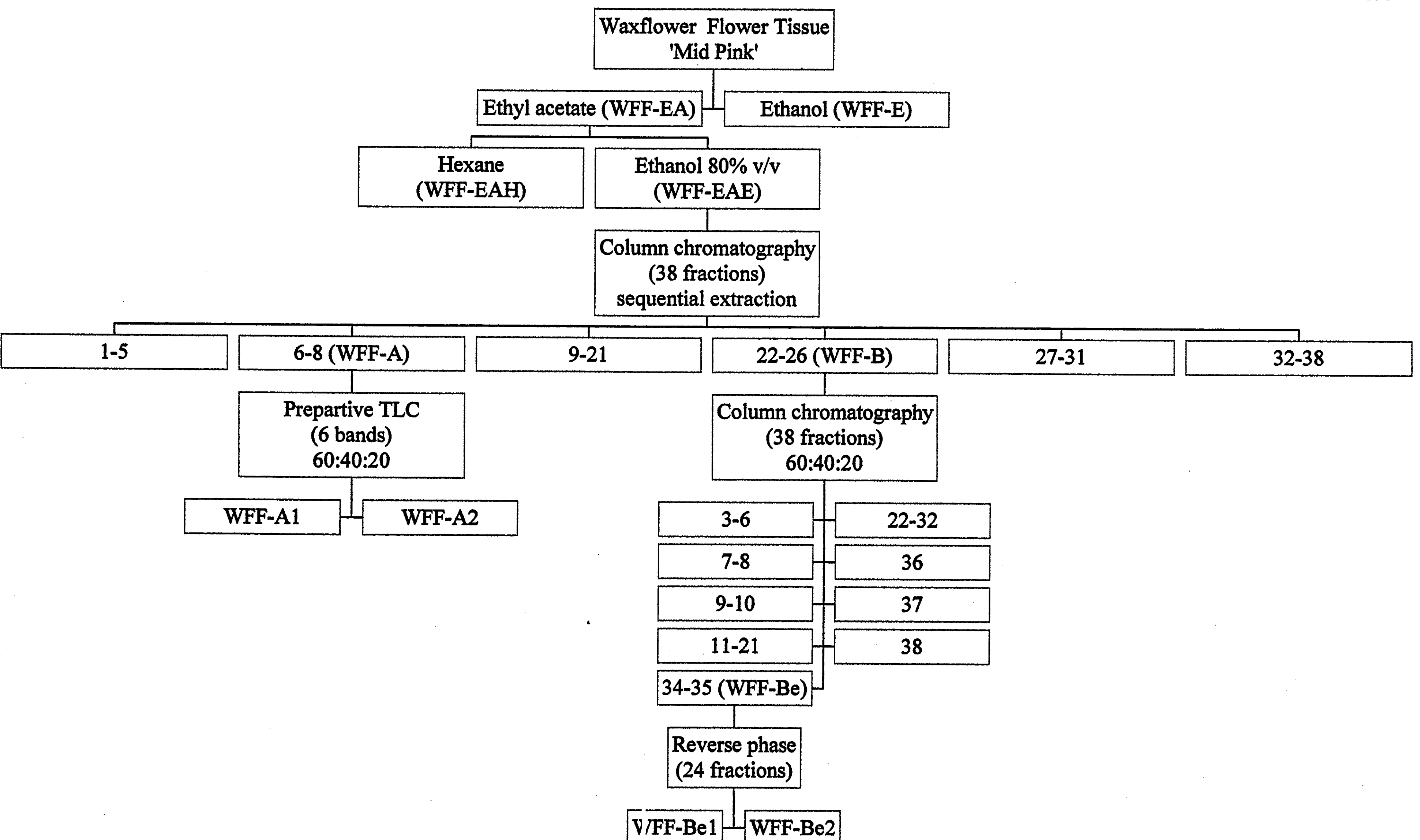


Figure 5.2.3.1: Flow chart of fractionation of Gerladton waxflower flower tissue in Experiment II.

5.2.3.7 *Purification of antifungal WFL-A*

Fraction WFL-A (38 mg) was subjected to preparative TLC (silica gel GF; 1 mm) run in solvent system hexane: ethyl acetate: methanol (60:40:5 v/v/v) and visualised under UV-A. Five resulting bands were scraped from the silica, eluted with diethyl ether: methanol (7:3 v/v) and tested for antifungal activity in solvent system D (hexane: ethyl acetate: methanol; 60:40:30 v/v/v). From TLC behaviour 1 fraction (WFL-A1; 8 mg) was prepared for NMR and RP-HPLC analysis.

5.2.3.8 *Spectral analysis*

^{1}H and ^{13}C NMR (Nuclear Magnetic Resonance) spectra were recorded at IACR Rothamsted on a JEOL GX400 spectrometer (Maryland, USA), operating at 400 and 100 MHz, respectively. Deuterochloroform or deutromethanol were used as solvents.

RP-HPLC (Reverse Phase High Performance Liquid Chromatography) was performed using Gilson equipment and an Aqua 5 µl C18, 4.6 x 250 mm (Phenomenex) HPLC column. Peaks were monitored using a UV detector set at 254 nm. Acetonitrile: water, 9:1 v/v) was used at a flow rate of 1 ml min^{-1}.

5.2.4 Experiment III

5.2.4.1 *Sequential extraction*

Geraldton waxflower (*C. floriferum x C. uncinatum*; FxU) hybrid leaf (300 g FW; 78.9 g DW) and combined flower (105 g FW; 34.6 g DW) tissues was prepared and sequentially extracted as described in section 5.1.3.1. 1-D TLCs bioassay using *C. cladosporioides* or *B. cinerea* were performed on leaf and flower ethyl acetate and ethanol extracts (5 µl; 0.2 ml g^{-1} DW). On the basis of TLC behaviour the ethyl acetate fraction was partitioned as described in section 5.2.3.1. 1-D TLCs were performed on partitioned extracts run in solvent systems C (hexane: ethyl acetate: methanol; 60:40:20 v/v/v) either for antifungal activity or PMA visualisation and

prepared for NMR and RP-HPLC analysis. These partitioned extracts were compared to NMR and RP-HPLC spectra for 'Mid Pink' purified extracts as described in experiment 2.

5.3 Results

5.3.1 Experiment I 'Snowflake'

5.3.1.1 *Crude ethanol extraction*

Geraldton waxflower 'Snowflake' leaf, nectiferous and non-nectiferous flower tissues produced varying degrees of antifungal activity on 1-D TLC bioassays using *C. cladosporioides*. Fungal inhibition was achieved when either 50, 10 or 5 μl of extracts (0.2 ml g^{-1} FW) were applied to TLCs bioassays. Leaf tissue contained fewer antifungal compounds than either flower tissue samples (Plates 5.3.1.1 and 5.3.1.2) with 4 and 5 antifungal zones, respectively (solvent system D; hexane: ethyl acetate: methanol, 60: 40:30 v/v/v). The intensity of fungal inhibition in leaf tissue was much less than corresponding antifungal activity found in flower tissue. Solvent systems C and D provided the greatest separation of antifungal compounds in tissue extracts. Non-nectiferous flower tissue demonstrated a similar antifungal 'fingerprint' to nectiferous flower tissue, with R_f 0.49 and R_f 0.37-0.21 showing the greatest activity in solvent system D (Tables 5.3.1.1 and 5.3.1.3). Positive reactions to UV light suggested that R_f 0.37-0.21 is a phenolic. Negative reactions to UV light suggested that R_fs 0.69, 0.59, 0.55 and 0.49 are not phenolics (Table 5.3.1.2).

Two-dimensional TLC bioassays using *C. cladosporioides* confirmed there to be at least 13, 15 and 2 preformed antifungal compounds in nectiferous and non-nectiferous flowers and leaf tissues, respectively (Plate 5.3.1.3; Table 5.3.1.4). R_f 1/ R_f 2 at 0.60, 0.82; 0.48, 0.81; 0.09, 0.57; 0.02, 0.44; 0.02, 0.28 and 0.06, 0.19 showed the greatest antifungal activity. Positive reactions to diazotized sulphanilic acid reagent spray suggest that compounds at R_f 1/ R_f 2 0.60, 0.82; 0.02, 0.28; 0.06, 0.25; 0.26 0.24 and 0.06, 0.19 are phenolics (Table 5.3.1.5).

Plate 5.3.1.1: One-dimensional thin layer chromatography bioassays (*Cladosporium cladosporioides*) of the organic phase from crude ethanol (99% v/v) extracts partitioned with dichloromethane of Geraldton waxflower 'Snowflake' tissues of (5 µl spots; 0.2 ml g^{-1} FW) run in solvent systems of increasing polarity (hexane: ethyl acetate: methanol (v/v/v); A = 60:40:1; B = 60:40:10; C = 60:40:20 and, D = 60:40:30). Lanes 1 and 7, leaf extracts; lanes 2 and 8, nectiferous flower extracts; lanes 3 and 9, non-nectiferous flower extracts; lane 4, ethanol (99% v/v); lane 5, nothing and lane 6, cycloheximide (0.5 mg ml^{-1}). Dotted line = origin. Dashed line = solvent front.

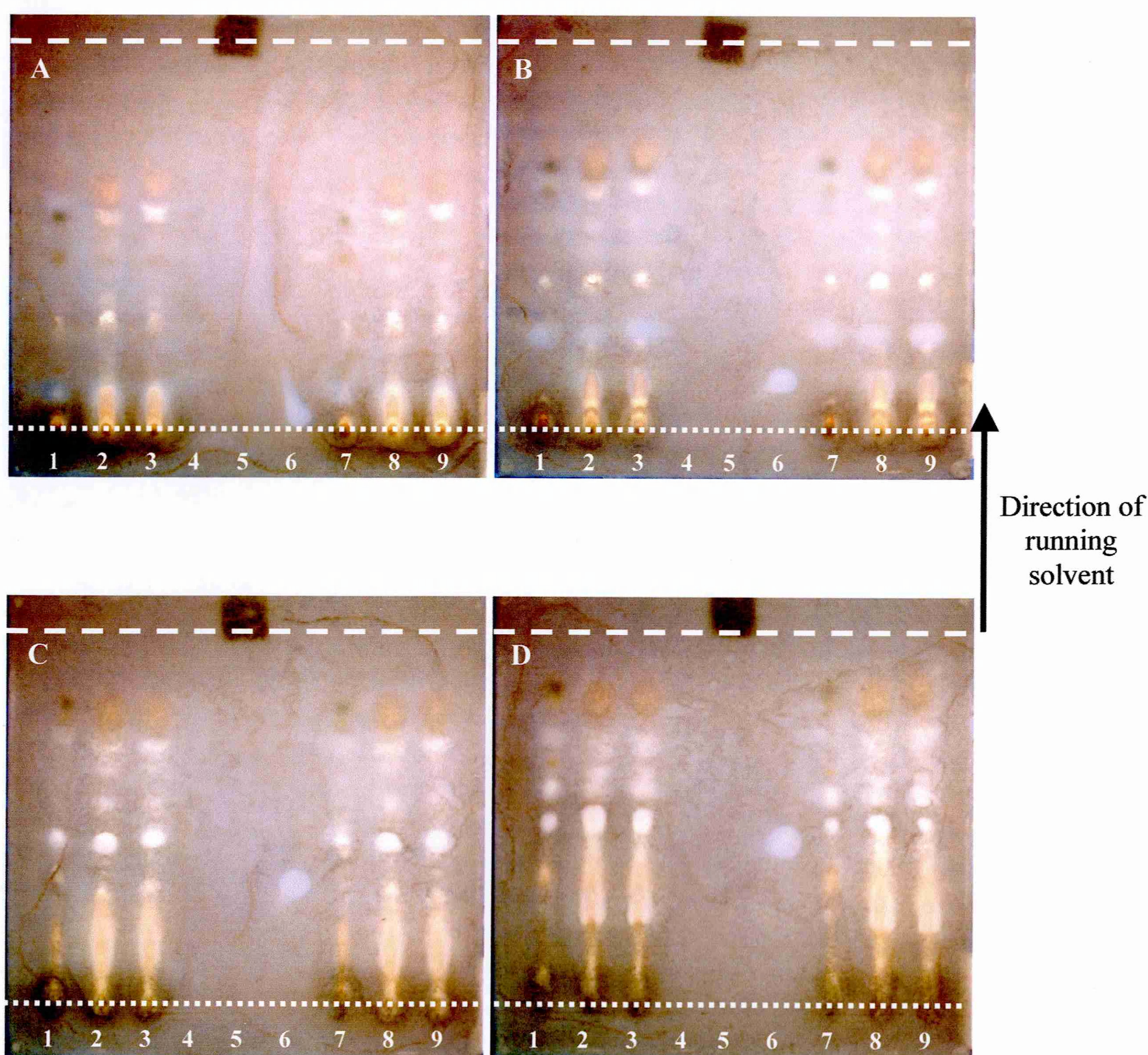


Table 5.3.1.1: One-dimensional thin layer chromatography bioassay (*Cladosporium cladosporioides*) R_f values of crude ethanol extract partitioned into an organic dichloromethane phase of Geraldton waxflower 'Snowflake' tissues (5 µl spots; 0.2 ml g^{-1} FW) (viz. Leaf (L), nectiferous (N), non-nectiferous (N-N)) run in solvent systems of increasing polarity solvent systems of increasing polarity (hexane: ethyl acetate: methanol (v/v/v); A = 60:40:1; B = 60:40:10; C = 60:40:20 and, D = 60:40:30).

Solvent system A			Solvent system B			Solvent system C			Solvent system D		
L	N	N-N	L	N	N-N	L	N	N-N	L	N	N-N
---[A]	0.55	0.55	---	0.61	0.61	---	0.67	0.67	0.69	0.69	0.69
---	---	---	---	0.52	0.52	---	0.61	0.61	---	0.59	0.59
0.26	0.26	0.26	0.39	0.39	0.39	0.54	0.54	0.54	0.55	0.55	0.55
---	---	---	0.24	0.24	0.24	0.44	0.44	0.44	0.49	0.49	0.49
0.02	0.12-0.00	0.12-0.00	0.06	0.15-0.00	0.15-0.00	0.18	0.26-0.01	0.26-0.01	0.33	0.37-0.21	0.37-0.21

cycloheximide (0.5 mg ml^{-1}; positive control) R_f = 0.04 (A), 0.12 (B), 0.33 (C), 0.43 (D); ethanol (99% v/v; negative control) = no R_f

[A] = no inhibition zone

Table 5.3.1.2: Reaction of antifungal spots on one-dimensional thin layer chromatography bioassay (*Cladosporium cladosporioides*) of the organic phase of crude ethanol extracts partitioned with dichloromethane of Geraldton waxflower 'Snowflake' run in solvent system C (hexane: ethyl acetate: methanol; 60:40:20 v/v/v) to light detection methods.

Detection procedure	Appearance	Zones reacting (R_f value)
Visible light	yellow	0.54
	red/brown	0.44, 0.26 - 0.01
UV lamp 254 nm	blue	26 – 0.01
UV lamp 366 nm	florescent	26 – 0.01

Cavarcrol (0.25 µl) $R_f = 0.64$ (positive reaction to UV-C and phenolic reagent sprays)

Table 5.3.1.3: One-dimensional thin layer chromatography bioassay (*Cladosporium cladosporioides*) R_f values of the organic phase of crude ethanol extracts partitioned with dichloromethane of Geraldton waxflower 'Snowflake' tissues (10 µl spots; 0.2 ml g^{-1} FW) (viz. Leaf (L), nectiferous (N), non-nectiferous (N-N)) run in solvent systems of increasing polarity (hexane: ethyl acetate: methanol, v/v/v; A = 60:40:1; B = 60:40:10; C = 60:40:20 and, D = 60:40:30).

Solvent system A			Solvent system B			Solvent system C			Solvent system D		
L	N	N-N	L	N	N-N	L	N	N-N	L	N	N-N
---[A]	0.47	0.56	---	0.54	0.61	---	0.64	0.69	---	0.66	0.66
---	0.4	0.46	---	0.51	0.55	---	0.56	0.60	---	0.62	0.62
---	0.31	0.37	0.39	0.39	0.39	0.46	0.46	0.46	0.57	0.57	0.57
0.16	0.18	0.19	---	0.30	---	---	0.34	0.34	0.51	0.51	0.51
0.08-0.0	0.16-0.00	0.15-0.00	0.14-0.04	0.23-0.00	0.23-0.00	0.19	0.31-0.10	0.31-0.10	0.27	0.47-0.22	0.47-0.22

Cycloheximide (0.5 mg ml^{-1}; positive control) $R_f = 0.10$ (A), 0.23 (B), 0.31 (C), 0.48 (D); ethanol (99% v/v; negative control) = no R_f

Plate 5.3.1.2: One-dimensional thin layer chromatography bioassays (*Cladosporium cladosporioides*) of organic phase of crude ethanol (99% v/v) extracts partitioned with dichloromethane of Geraldton waxflower 'Snowflake' tissues (10 µl spots; 0.2 ml g^{-1} FW) run in solvent systems of increasing polarity (hexane: ethyl acetate: methanol, v/v/v; A = 60:40:1; B = 60:40:10; C = 60:40:20 and, D = 60:40:30). For plate A lanes 1 and 5, leaf extracts; lanes 2 and 6, nectiferous flower extracts; lanes 3 and 7, non-nectiferous flower extracts and lane 4, cycloheximide (0.5 mg ml^{-1}). For plates B-D lanes 1 and 7, leaf extracts; lanes 2 and 8, nectiferous flower extracts; lanes 3 and 9, non-nectiferous flower extracts; lane 4, ethanol (99% v/v); lane 5, nothing and lane 6, cycloheximide. Dotted line = origin. Dashed line = solvent front.

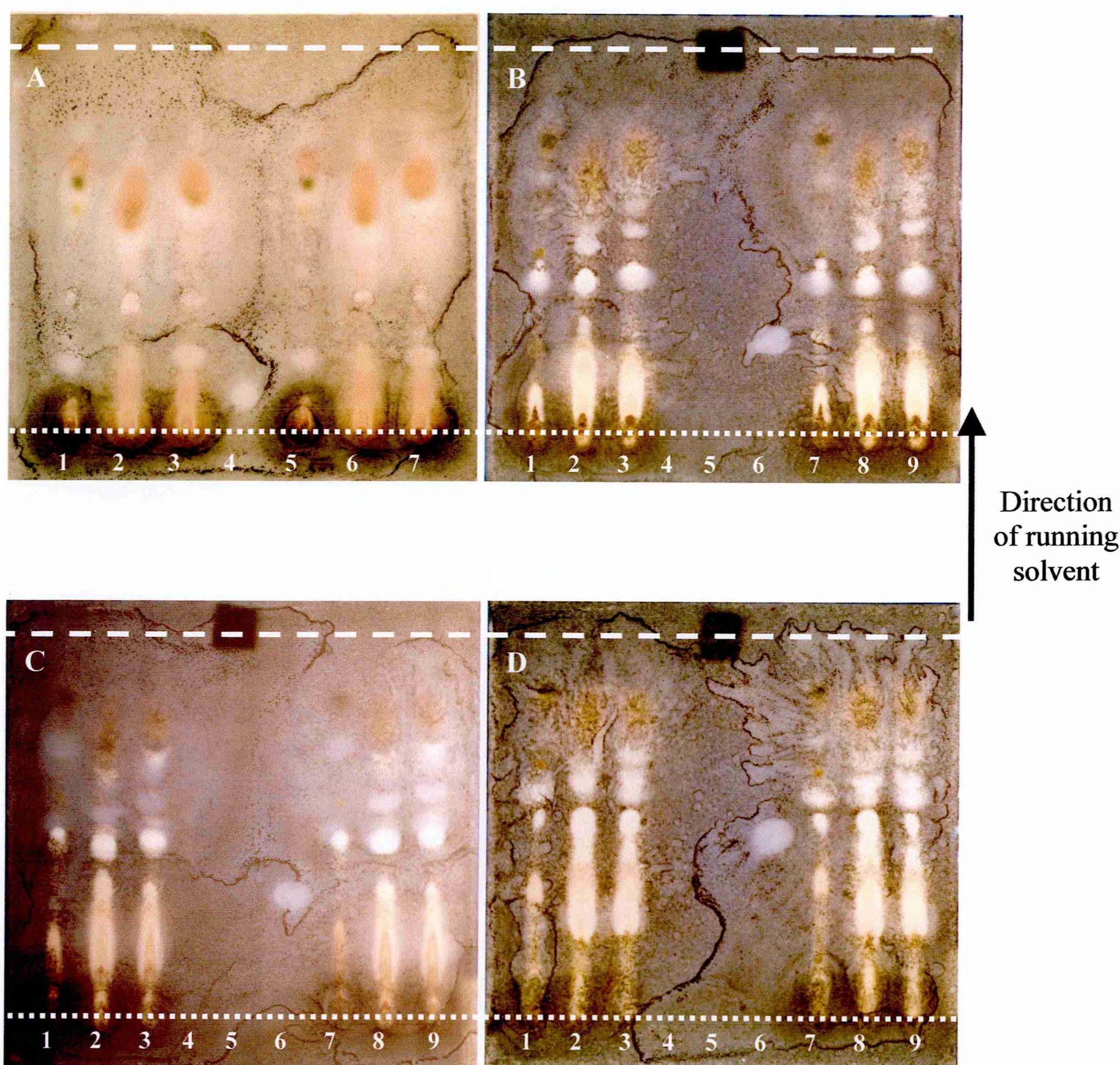


Plate 5.3.1.3: Two-dimensional thin layer chromatography bioassay (*Cladosporium cladosporioides*) of crude extracts partitioned with dichloromethane of tissues (5 µl spots; 0.2 ml g^{-1} FW) of X = leaf, Y = nectiferous flowers and Z = non-nectiferous flowers of Geraldton Waxflower 'Snowflake' run in a solvent system A of hexane: ethyl acetate: methanol, v/v/v (60:40:1 (one dimension, 1-D)); solvent system D (60:40:30 (second dimension, 2-D)). Dotted line = first origin. Solid line = second origin. Dashed line = solvent front (1-D). Dash-dot line = solvent front (2-D).

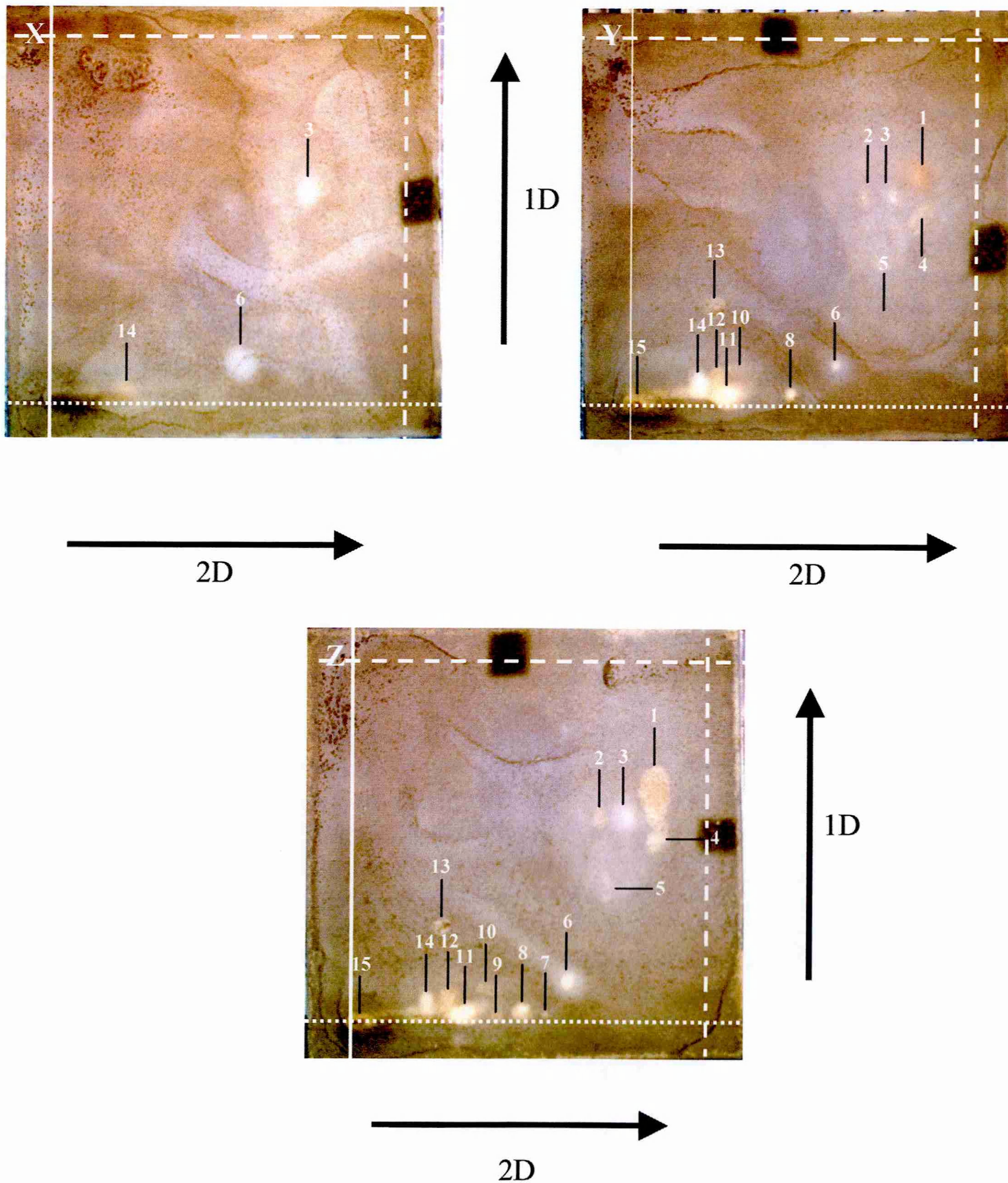


Table 5.3.1.4: Two-dimensional thin layer chromatography bioassay (*Cladosporium cladosporioides*) antifungal R_f values of crude extracts (5 μl spots; 0.2 ml g^{-1} FW) of leaf, nectiferous flowers and non-nectiferous flowers of Geraldton Waxflower 'Snowflake' run in solvent systems A (1-D) and D (2-D).

Leaf		Nectiferous flower		Non-nectiferous flower	
R_f 1	R_f 2	R_f 1	R_f 2	R_f 1	R_f 2
---[A]	---	0.61	0.81	0.60	0.82
---	---	0.55	0.73	0.60	0.73
0.60	0.66	0.48	0.80	0.54	0.67
---	---	0.55	0.66	0.48	0.81
---	---	0.43	0.70	0.39	0.67
0.09	0.51	0.09	0.57	0.01	0.57
---	---	---	---	0.00	0.51
---	---	0.01	0.45	0.02	0.44
---	---	---	---	0.00	0.38
---	---	0.07	0.34	0.06	0.35
---	---	0.01	0.28	0.02	0.28
---	---	0.07	0.24	0.06	0.25
---	---	0.26	0.24	0.26	0.24
		0.02	0.20	0.06	0.19
---	---	0.00	0.05	0.00	0.05

[A] = no inhibition zone

Table 5.3.1.5: Reaction of antifungal spots on two-dimensional thin layer chromatograph run in solvent systems A (1-D) and D (2-D) to detection methods.

Detection procedure	Appearance	Spots reacting (R_f1/R_f2)
Visible light	green	0.61/0.81
	yellow	0.48/0.81, 0.01/0.28, 0.02/0.20
UV –C[AB]	dark	0.60/0.82; 0.02/0.28; 0.06/0.25; 0.06/0.19
Diazotized sulfanilic acid[B]	brown	0.60/0.82; 0.02/0.28; 0.06/0.25; 0.26/0.24; 0.06/0.19

[A] = extracts run on Silica gel 60 F_{254}. [B] = phenolics indicator

5.3.2 Experiment II 'Mid Pink'

5.3.2.1 *Sequential extraction*

Antifungal activity was mainly observed in the ethyl acetate (WF-EA) fraction of sequentially extracted leaf and flower tissues 'Mid Pink'. When partitioned with hexane and ethanol (80 %v/v) the later fraction (WF-EAE) demonstrated the greatest antifungal activity (Plate 5.3.2.1). The hexane fraction had no antifungal activity (WF-EAH) (Plate 5.3.2.1) but contained far more polar material, including pigments, than WF-EAE as shown by the PMA reagent spray and under visible light.

Two major antifungal zones at R_f 0.50 and 0.24 in WF-EAE were visualised by TLC bioassay (solvent D) and were assigned the names WF-A and WF-B, respectively (Plate 5.3.2.1). WF-EAE leaf extract (5 µl; 0.2 ml g^{-1} DW) showed less antifungal activity but had the same antifungal profile as flower tissue (Plate 5.3.2.1; Table 5.3.2.1A). The areas of fungal inhibition of WF-A and WF-B in leaf ethanol fraction were 77% and 30% smaller than in flower ethanol fraction (Table 5.3.2.1A). Positive reactions to Folin-Ciocalteu, diazotized sulfanilic acid and UV-C suggested that compound(s) WF-B is a phenolic (Table 5.3.2.1B). Negative reactions to phenolic reagent sprays and UV-C indicated that compound(s) WF-A is not a phenolic (Table 5.3.2.1B). Positive reactions to Dragendorff's reagent suggest that WF-B may also include an alkaloid(s) or quaternary nitrogen compound(s) (Table 5.3.2.1B). However, because WF-B is yellow-brown in visible light, this may be a false result.

5.3.2.2 *Co-chromatography*

Co-chromatography showed that WF-A and WF-B had different Rfs to some of the major terpenes previously found in waxflower essential oil (Egerton-Warburton and Ghisalberti, 1995) (Table 5.3.2.2). This suggested that WF-A and WF-B are not α-pinene, limonene, linalool, geraniol or 1,8 cineole.

Plate 5.3.2.1: One-dimensional thin layer chromatography bioassays (*Cladosporium cladosporioides*) of partitioned ethyl acetate extracts of Geraldton waxflower 'Mid Pink' leaf and flower tissue (5μl spots; 0.2 ml g^{-1} DW) run in solvent system D (hexane: ethyl acetate: methanol (60:40:30 v/v/v)). Lanes 1 and 5, leaf hexane fraction; lanes 2 and 6, leaf ethanol (80% v/v) fraction; lanes 3 and 7, flower hexane fraction; lanes 4 and 8, flower ethanol (80% v/v) fraction; lane 9, ethanol (99% v/v) (control). Dotted line = origin. Dashed line = solvent front.

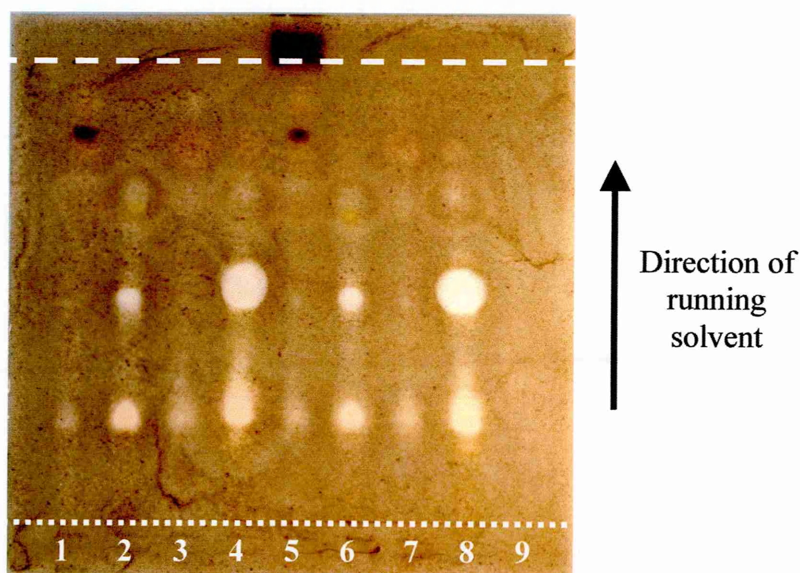


Table 5.3.2.1A: One-dimensional thin layer chromatography bioassay (*Cladosporium cladosporioides*) antifungal R_f values and approximate area of antifungal activity of ethanol (80% v/v) fraction of partitioned ethyl acetate extracts (WF-EAE) of Geraldton waxflower 'Mid Pink' leaf and flower tissue (5 μl spots; 0.2 ml g^{-1} DW) run in solvent system D (hexane: ethyl acetate: methanol (60:40:30 v/v/v)).

	Flower		Leaf	
Zone	R_f	Area (cm^2)	R_f	Area (cm^2)
WF-A	0.50	2.53	0.50	0.58
WF-B	0.24	1.24	0.24	0.87

Table 5.3.2.1B: Reaction of antifungal compounds WF-A and WFB (5 µl spots; 0.2 ml g^{-1} DW) on one-dimensional thin layer chromatograph run in solvent system D (hexane: ethyl acetate: methanol (60:40:30 v/v/v)) to various detection methods.

Detection procedure	Appearance	
Reagent	WF-A	WF-B
Visible light	yellow	yellow brown
UV-A[B]	---[A]	florescent
UV-C*[B]	---	black
Folin-Coicalteu[B]	---	blue-black
Diazotized sulphanilic acid[B]	---	brown
Phosphomolybdic acid	black	black
Dragendorff's[C]	---	Orange?

* = extracts run on Silica gel 60 F_{254}

---[A] = no reaction.

[B] = phenolics indicator

[C] = alkaloids indicator

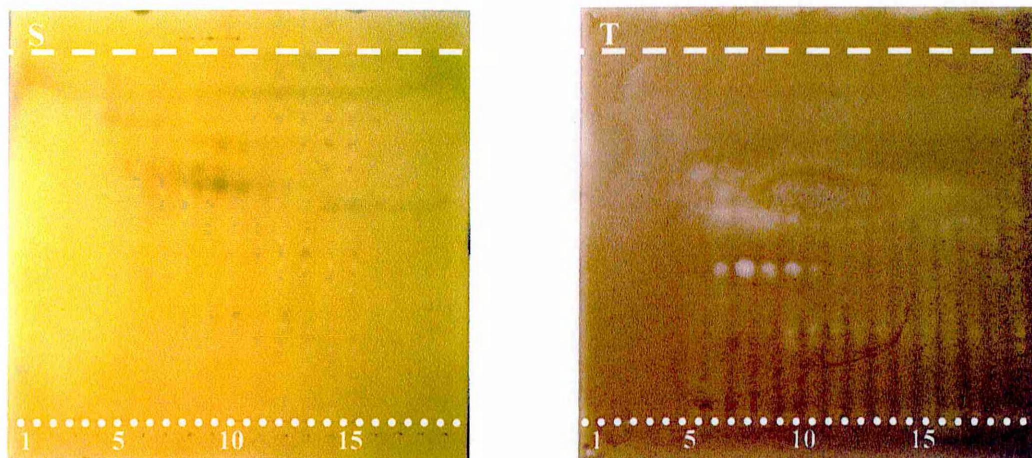
Table 5.3.2.2: One-dimensional thin layer chromatograph R_f values of various terpene standards (0.25 µl spot) co-chromatographed with antifungals WF-A and WF-B (5 µl spots; 0.2 ml g^{-1} DW) extracts sprayed with phosphomolybdic acid run in solvent systems (hexane: ethyl acetate: methanol, v/v/v; B = 60:40:10; C = 60:40:20 and, D = 60:40:30).

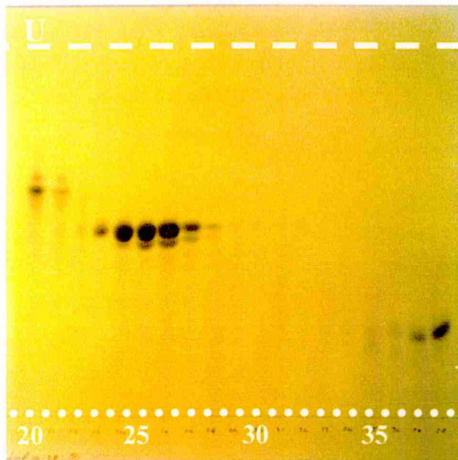
Controls	Solvent B	Solvent C	Solvent D
α-pinene	0.54	0.60	0.62
limonene	0.54	0.60	0.64
linalool	0.61	0.64	0.66
geraniol	0.52	0.56	0.61
cineole	0.70	0.73	0.73
WF-A	0.39	0.51	0.49
WF-B	0.08	0.20	0.28

5.3.2.3 Column chromatography of antifungal compounds

WF-EAE flower and leaf extracts (517 mg; 460 mg) were further purified using normal phase column chromatography (CC) and four solvent combinations of increasing polarity (viz. E = hexane: ethyl acetate (60:40 v/v), B = hexane: ethyl acetate: methanol (60:40:10 v/v/v), C = (60:40:20 v/v/v), and D = (60:40:30 v/v/v). For both tissue samples, 38 fractions were collected (Fig. 5.2.3). These were reduced to six fractions by pooling on the basis of similar TLC bioassay and PMA behaviour for both flower (fractions 1-5, 6-8, 9-21, 22-26, 27-31 and 32-38) (Plates 5.3.2.2E-H) and leaf extracts (fractions 1-6, 7-13, 14-19, 20-22, 23-29 and 30-38) (Plates 5.3.2.2A-D). Flower extracts had significantly more antifungal activity than leaf tissue as shown by TLC bioassay, (Plates 5.3.2.2A-F; Table 5.2.2.3AB). Two fractions from flower extracts fractions 6-8 (89 mg; WFF-A) and fractions. 22-26 (190 mg; WFF-B) and one fraction from leaf fractions 20-22 (38 mg; WFL-A) were further characterised. These pooled fractions corresponded to either WF-A or WF-B found in both flower and leaf tissue (Plate 5.3.2.1) and were eluted by solvent B and C during column chromatography (as described in section 3.1.3.3), respectively.

Plate 5.3.2.2: One-dimensional thin layer chromatographs of sequentially fractionated samples (n = 38) from partitioned ethyl acetate leaf (S -V) and flower (W – Z) extracts (EtOH 80% v/v fraction) sprayed with either phosphomolybdic acid (S, U, W, Y) or bioassay (T, V, X, Z) run in Solvent D (hexane: ethyl acetate: methanol (60:40:30 v/v/v)). Dotted line = origin. Dashed line = solvent front.

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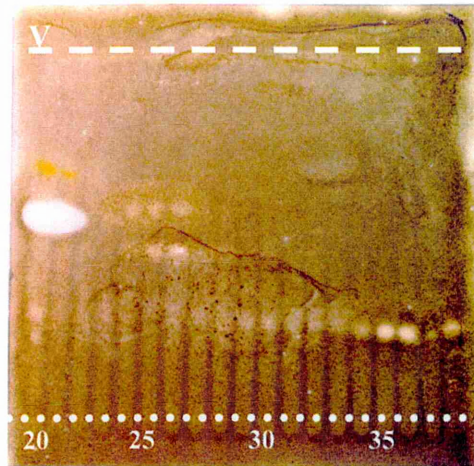
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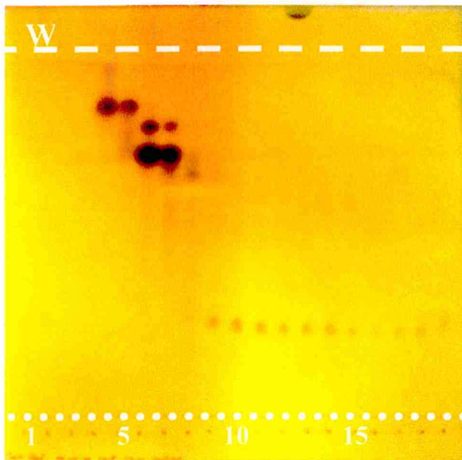
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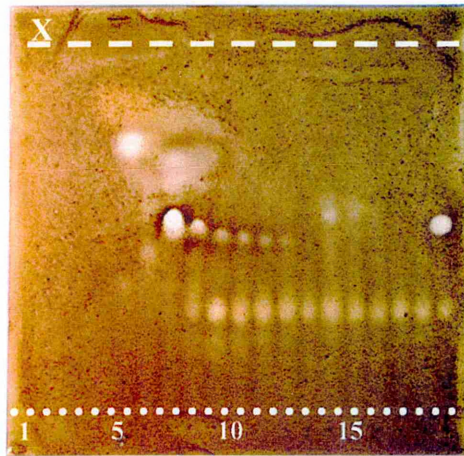
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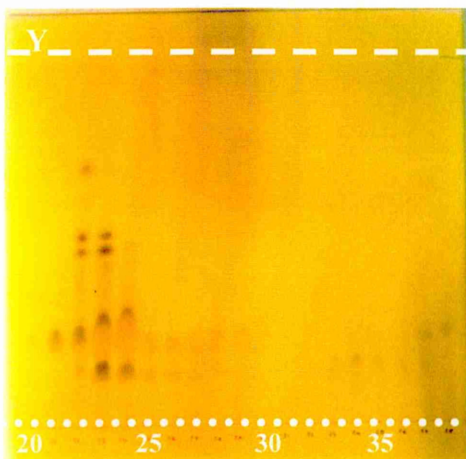
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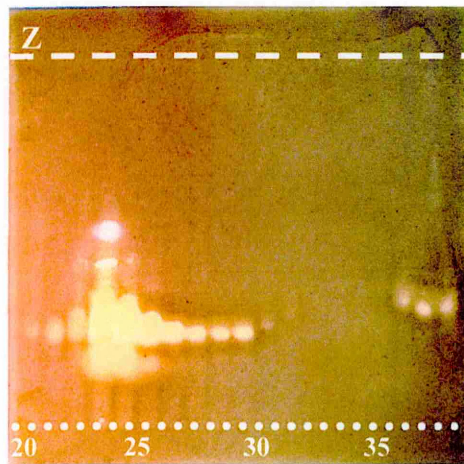
Z
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Table 5.3.2.3A: One-dimensional thin layer chromatography bioassay (*Cladosporium cladosporioides*) and phosphomolybdic acid R_f values of sequentially fractionated pooled fractions (n = 6) from WF-EAE leaf extracts of Geraldton waxflower 'Mid Pink' (1 µl spots; 0.2 ml g^{-1} DW) run in solvent system D (hexane: ethyl acetate: methanol (60:40:30 v/v/v)).

Pooled Fractions	Extract weight (mg)[B]	TLC-R_f	
		PMA	Antifungal
1-6	21	0.82, 0.79, 0.70	0.68, 0.56, 0.41
7-13	63	0.79, 0.70, 0.63, 0.23	0.68, 0.56, 0.41, 0.24
14-19	24	0.23	0.24
20-22[A]	38	0.65, 0.61, 0.50	0.54
23-29	140	0.50, 0.47, 0.23	0.54, 0.45, 0.23
30-38	100	0.23, 0.18	0.25

[A] fraction further characterised (WFL-A)

[B] total weight of extract = 386 mg (460 mg, weight before purification)

Table 5.3.2.3B: One-dimensional thin layer chromatography bioassay (*Cladosporium cladosporioides*) and phosphomolybdic acid R_f values of sequentially fractionated pooled fractions (n = 6) from WF-EAE flower extracts of Geraldton waxflower 'Mid Pink' (1 µl spots; 0.2 ml g^{-1} DW) run in solvent system D (hexane: ethyl acetate: methanol (60:40:30 v/v/v)).

Pooled Fractions	Extract weight (mg)[C]	TLC-Rf (no. of spots in spots)	
		PMA	Antifungal
1-5	54	0.80	0.80
6-8[A]	89	0.80, 0.74, 0.64	0.65, 0.56
9-21	85	0.74, 0.64. 0.56, 0.27	0.51, 0.56, 0.27
22-26[B]	190	0.71, 0.53, 0.49, 0.42, 0.37, 0.32-0.22, 0.13	0.53, 0.44, 0.35-0.25, 0.15
27-31	16	0.26, 0.22, 0.13	0.24
32-38	58	0.26, 0.13	0.31

[A] fraction further characterised (WFF-A)

[B] fraction further characterised (WFF-B)

[C] total weight of extract = 492 mg (517 mg, weight before purification)

5.3.2.4 *Identification of antifungal compound(s) WFF-A*

Preparative TLC of WFF-A revealed six bands. Bioassay directed fractionation with *C. cladosporioides* run in solvent D revealed that two compounds $R_f = 0.73$ (22 mg) and $R_f = 0.54$ (14 mg) had antifungal activity (Plate 5.3.2.3). NMR and MS indicted that $R_f = 0.73$ (WFF-A1) is the sesquiterpene globulol (Fig. 5.3.2.1 and Fig. 5.3.2.3-4). However, MS spectral analysis revealed WFF-A1 as a mixture of globulol and aromadendrene (Fig. 5.2.3.1). Aromadendrene may be an artefact produced by dehydration of globulol during isolation procedure (B. Khambay, 2001 pers. comm.). $R_f = 0.54$ (WFF-A2) was confirmed as the monoterpene, grandinol as spectral data matched that reported in literature (Chiba *et al.*, 1998) (Fig. 5.3.2.2; and Fig. 5.3.2.5-6).

5.3.2.5 *Purification of antifungal compound WFF-B*

Column chromatography (CC) of WFF-B produced 38 fractions. These were pooled on the basis of TLC behaviour using PMA into eight fractions (3-6, 7-8, 9-10, 11-21, 22-33, 34-35, 36-37, 38). TLC bioassay with *C. cladosporioides* run in solvent D demonstrated that fractions 7-8 (13 mg; $R_f = 0.54$), 11-21 (41 mg; $R_f = 0.24$), 22-32 (40 mg; $R_f = 0.32 - 0.19$), 34-36 (85 mg; $R_f = 0.24$) and 38 (30 mg; $R_f = 0.24$) all showed antifungal activity (Plate 5.3.2.3).

5.3.2.6 *Identification of antifungal compound WFF-Be*

Further purification through reverse phase CC of fractions 34-36 (WFF-Be) produced 24 fractions. RP-TLC bioassay run in solvent E demonstrated that only fractions 1 (24 mg; R_fs = 0.72, 0.42; WFF-Be1), 2 (41 mg; $R_f = 0.44$; WFF-Be2) and 3-4 (14 mg; $R_f = 0.44$) showed antifungal activity. The compound(s) at ~$R_f = 0.44$ was/were visible under UV-A and UV-C light. Compound at $R_f = 0.72$ was visible under UV-C but not under UV-A. Normal phase TLC bioassay and PMA run in solvent D demonstrated that WFF-Be-1 showed two antifungal compounds (R_fs = 0.24, 0.19) and WFF-Be2 one ($R_f = 0.24$) (Plate 5.3.2.3). Preliminary RP-HPLC and NMR, however, indicted that both WFF-Be1 and WFF-Be2 contain two compounds with different activity. These compounds are likely to be phenolics as their parent fraction

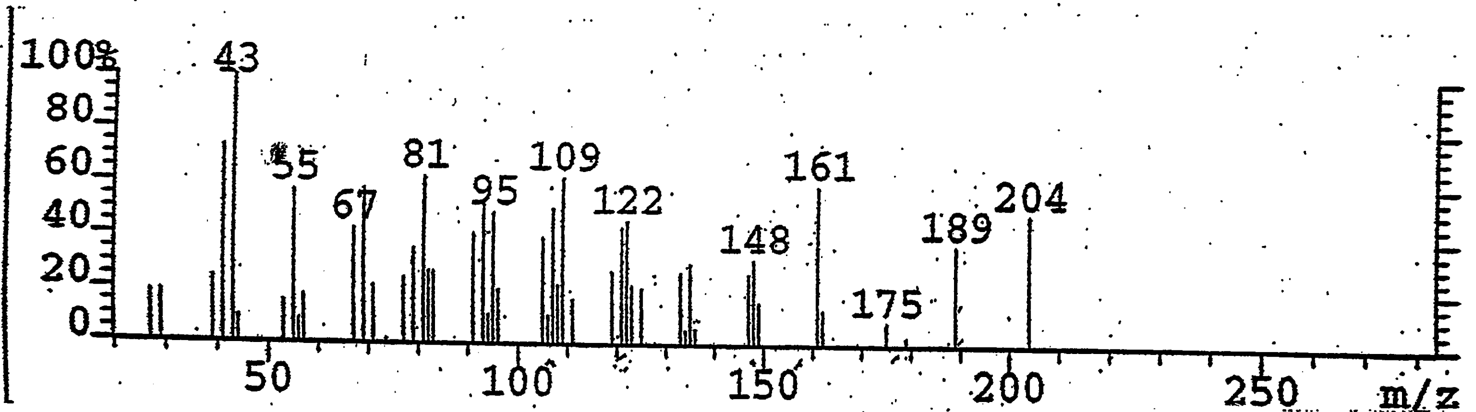


Figure 5.4.2.1: Mass spectrum of WFF-A1 (globulol/aromadendrene)

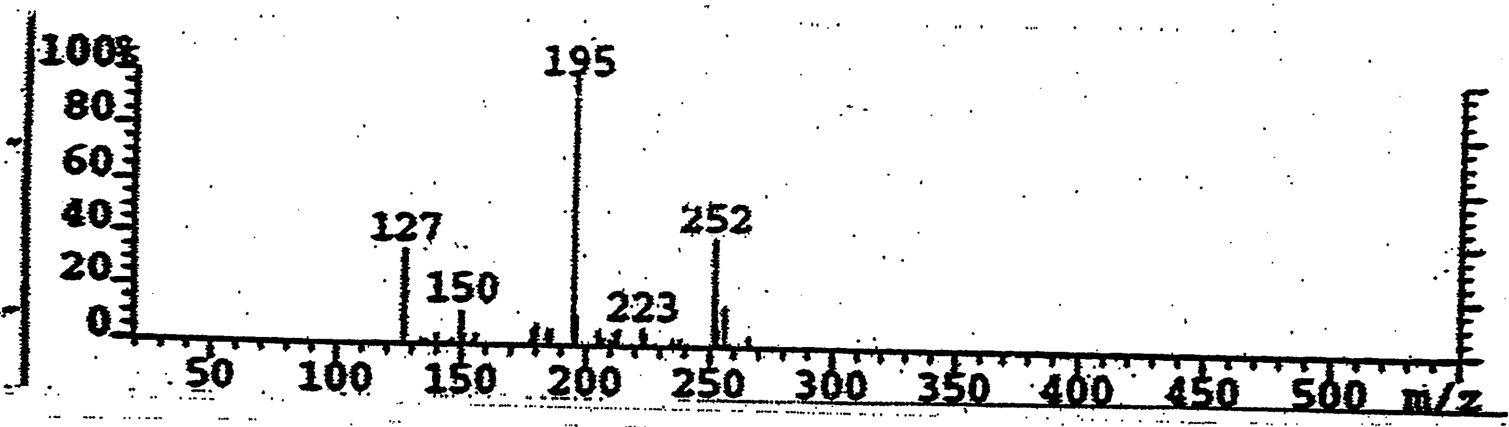


Figure 5.4.2.2: Mass spectrum of WFF-A2 (grandinol)

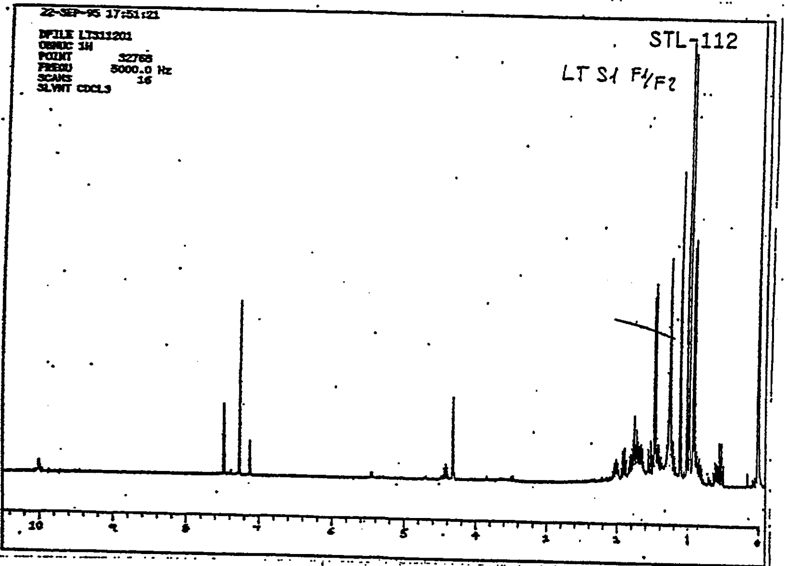


Figure 5.4.2.3: ^{1}H-NMR spectrum of WFF-A1

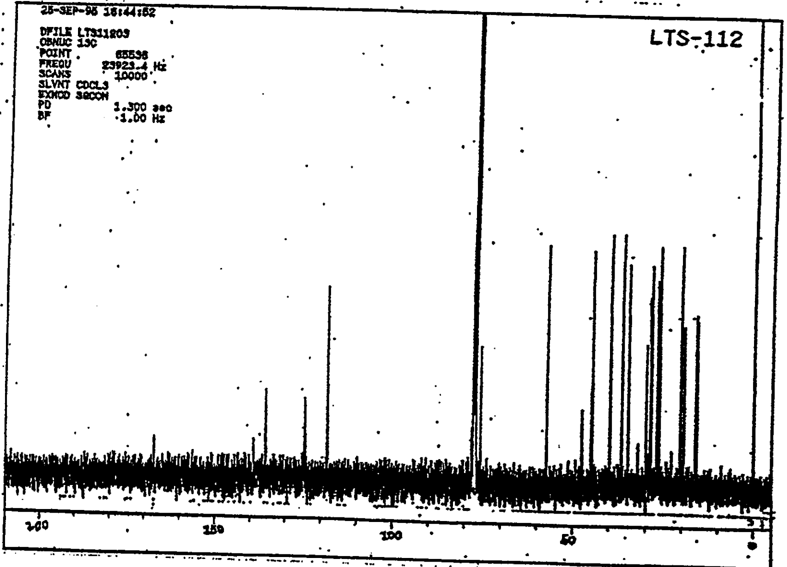


Figure 5.4.2.4: ^{13}C-NMR spectrum of WFF-A1

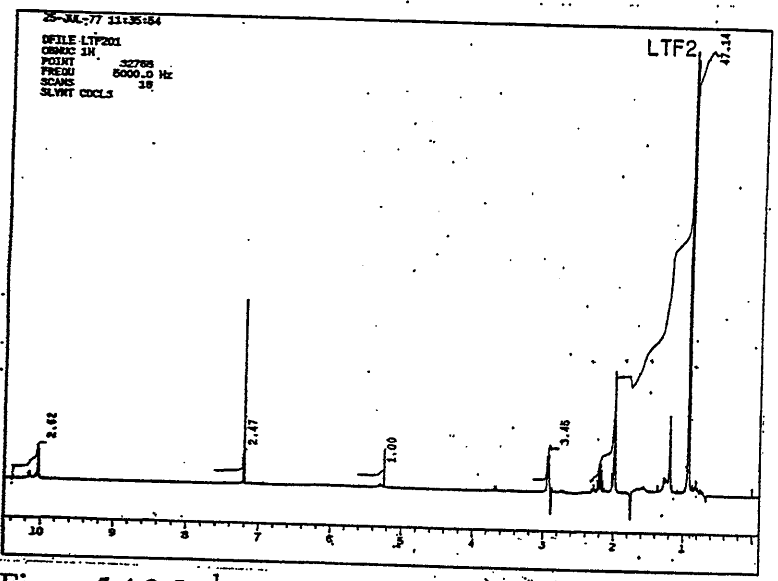


Figure 5.4.2.5: ^{1}H-NMR spectrum of WFF-A2

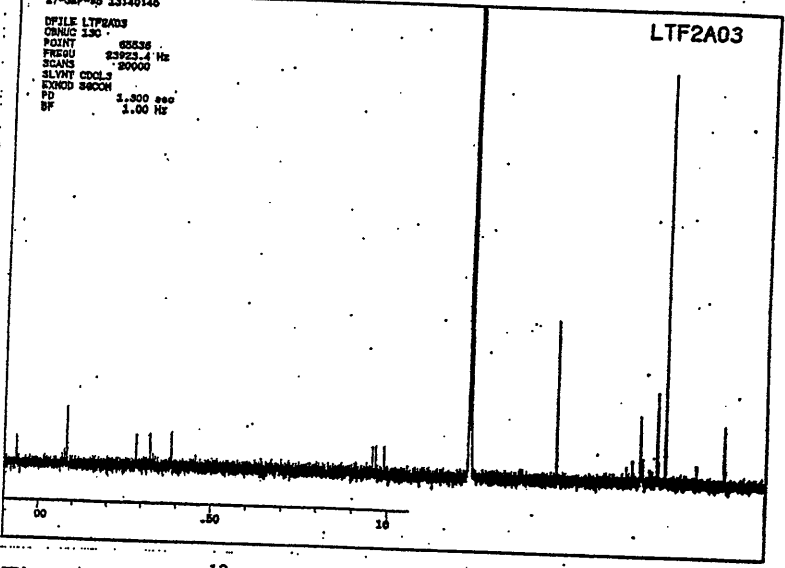


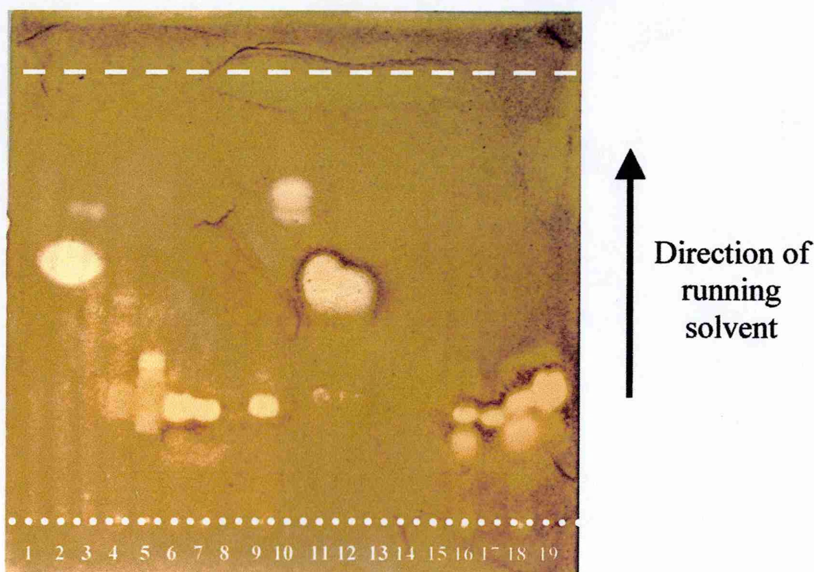
Figure 5.4.2.6: ^{13}C-NMR spectrum of WFF-A2

WF-B (R_f = 0.24) reacted positively to phenolic reagent sprays (Table 5.3.2.1B). However, further purification is necessary before they can be fully characterised.

5.3.2.7 *Identification of antifungal compound WFL-A*

Preparative TLC of WFL-A revealed six bands. A TLC bioassay with *C. cladosporioides* run in solvent system D demonstrated that one compound at R_f = 0.54 (3 mg; pale yellow; WFL-A1) showed antifungal activity and was the same as WFF-A2 found in flower tissue. WFL-A1 was confirmed as grandiol by NMR and GCMS.

Plate 5.3.2.3: One-dimensional thin layer chromatography bioassay (*Cladosporium cladosporioides*) of sequentially fractionated selected samples (n = 17) run in solvent system D (hexane: ethyl acetate: methanol (60:40:30 v/v/v)). Lanes 1, fractions 3-6; lane 2, frc. 7-8; lane 3, frc. 9-10; lane 4, frc. 11-21; lane 5, frc. 22-33; lane 6, frc. 34-36 (WFF-Be); lane, 7 frc. 36; lane 8, frc. 37; lane 9, frc. 38; lane 10, WFF-A1; lane 11, WFF-A2; lane 12, WFF-A3; lane 13, WFF-A4; lane 14, WFF-A5; lane, 15 WFF-A6; lane 16 and 18, WFF-Be1; lane 17 and 19, WFF-Be2. Dotted line = origin. Dashed line = solvent front.



5.3.3 Experiment III

Leaf and flower extracts of the hybrid waxflower (FxU) showed similar TLC antifungal profiles against both *B. cinerea C. cladosporioides* and to cvs. 'Snowflake and 'Mid Pink' waxflower (Table 5.3.3.1 and 5.3.3.2). Likewise, 'FxU' leaf tissue

contained less antifungal compounds than flower tissue. The majority of antifungal compounds were present in the ethyl acetate fraction partitioned with ethanol 80% (v/v) compared to the hexane partition. The intensity of inhibition of antifungal compounds in leaf tissue was also much less than similar compounds found in flower tissue. It is likely that R_fs = 0.52, 0.46 and 0.25-0.07 in 'FxU' TLC bioassay (Table 5.3.3.1) are the same antifungal compound(s) as R_fs = 0.54, 0.44 and 0.26-0.01 in 'Snowflake' TLC bioassay run in solvent C (Table 5.3.1.1).

Table 5.3.3.1: One-dimensional thin layer chromatography bioassay (*Cladosporium cladosporioides*) R_f values of Geraldton waxflower 'FxU' flower and leaf tissues (5 μl spots; 0.2 ml g^{-1} DW) extracts (viz. ethyl acetate (EtAc); ethanol (EtOH) and ethyl acetate partitioned with hexane (EA-H) and ethanol 80% (v/v) (EA-E)) run in solvent system C (hexane: ethyl acetate: methanol (60:40:20 v/v/v)).

Leaf				Flower			
EtAc	EtOH	EA-H	EA-E	EtAc	EtOH	EA-H	EA-E
0.52	---	---	0.52	0.52	---	---	0.52
0.46	---	---	0.46	0.46	---	---	0.46
---	---	---	---	0.32	---	0.32	0.32
0.22- 0.07	---	---	0.25- 0.09	0.25- 0.07	---	0.19- 0.12	0.30- 0.07

Preliminary RP-HPLC and NMR analysis of partitioned extracts showed at least three active fractions. Grandinol, globulol and compounds similar to WFF-Be1/2 were clearly identified in WF-EAE leaf and flower extracts. It is likely that these correspond to R_fs 0.52, 0.46, 0.24-0.08, respectively (Tables 5.3.3.1 and 5.3.3.2). However, some differences in the NMR and HPLC spectral profile of antifungal compounds did exist between 'Mid Pink' and 'FXU' WF-EAE extracts indicating that more work is necessary to elucidate the different antifungal profiles in these varieties.

Table 5.3.3.2: One-dimensional thin layer chromatography bioassay (*Botrytis cinerea*) R_f values of Geraldton waxflower 'FxU' flower and leaf tissues (5 µl spots; 0.2 ml g^{-1} DW) extracts (viz. ethyl acetate (EtAc); ethanol (EtOH) and ethyl acetate partitioned with hexane (EA-H) and ethanol 80% (v/v) (EA-E)) run in solvent system C (hexane: ethyl acetate: methanol (60:40:20 v/v/v)).

Leaf				Flower			
EtAc	EtOH	EA-H	EA-E	EtAc	EtOH	EA-H	EA-E
---	---	---	---	---	---	---	0.56
0.46	---	---	0.46	0.46	---	---	0.46
---	---	---	---	0.32	---	0.33	0.33
0.18- 0.09	---	---	0.23- 0.13	0.24- 0.08	---	0.13- 0.10	0.29- 0.09

5.4 Discussion and Conclusion

This is the first report of clear evidence that Geraldton waxflower leaf and flower tissue contain constitutive antifungal compounds active against *C. cladosporioides* and *B. cinerea*. Some of these compounds are common to different waxflower cultivars. For example, WFF-A1 (globulol + aromadendrene), WFF-A2 (grandinol) and WFF-Be1/2 (unidentified phenolics) observed in 'Mid Pink' purified WF-EAE flower and leaf extracts show similar TLC behaviour to R_fs 0.54, 0.44 and 0.26-0.01 in crude extracts of 'Snowflake' and TLC and NMR spectra of antifungal compounds in 'FxU' WF-EAE extracts run in solvent C.

Notwithstanding similarities in antifungal profiles, it was also evident that significant differences exist between waxflower cvs. Snowflake, Mid Pink and FxU. Previous research on waxflower (Watson, 1944; Bowyer, 1957; Egerton-Warburton and Ghisalberti, 1995) did not identify both aromadendrene, grandinol or phenolic antifungal compounds found in this study. Globulol was previously identified in waxflower leaf oil (Egerton-Warburton and Ghisalberti, 1995). However, Egerton-Warburton *et al.* (1998) found that there was considerable intraspecific variation in volatile leaf oil composition in Geraldton waxflower. It is not clear whether the

differences in antifungal profile and specific antifungal compounds in waxflower tissues seen in this study have any significant impact on pathogen-host interactions.

Leaf tissue in the three cvs. examined had considerably less antifungal activity against both *C. cladosporioides* and *B. cinerea* than flower tissue either in terms of number or the intensity of those antifungal compounds present. Nonetheless, leaf tissue still contained preformed compounds with antifungal activity. Aromadendrene, found in this study is also a waste product left over after steam distillation of desired monoterpenes such as α-pinene, limonene and geraniol from *Eucalyptus globulus* (Lamers *et al.*, 2001) and some other members of the Myrtaceae (Brophy *et al.*, 2000). This compound may have use as a chiral starting material for synthesis of fragrances and crop protection agents (Lamers *et al.*, 2001). Aqueous extract (6.25% dilution) of *Eucalyptus australiana* was reported to have *in-vitro* antifungal activity against conidial germination of *B. cinerea* (Wilson *et al.*, 1997b). In addition, limonene, cineole, α-pinene and β-pinene were all found to be antifungal against *B. cinerea* (Wilson *et al.*, 1997b).

Although it is recognised that crude extracts of certain plants, including horticultural crops, demonstrate antifungal activity (Wilson *et al.*, 1997b; Mari and Guizzardi, 1998), as yet few plant-derived antifungal compounds have been commercialised into alternatives to synthetic fungicides (Wilson *et al.*, 1997b). Natural products are potential alternatives to synthetic fungicides (Wilson and Wisniewski, 1989). For instance, essential oils from Thyme (*Thymus vulgaris*) were shown to control *B. cinerea* and *Rhizopus stolonifer* in strawberry fruits (Bhaskara Reddy *et al.*, 1998). A volatile oil, Hinokitiol (β-thujaplicin), derived from the roots of the Japanese cypress tree inhibited *in-vitro* spore germination and mycelial growth of *B. cinerea, Alternaria alternata* and *R. stolonifer* (Fallik and Grinberg, 1992). This compound controlled postharvest disease in eggplant and pepper fruit when applied as a postharvest dip (Fallik and Grinberg, 1992). Similarly, the foliage wasted after harvesting of Geraldton waxflower may provide the ecological and financial incentive to develop another viable prophylactic treatment.

Future work will look at the spectrum of antifungal activity of waxflower extracts against various pathogens including isolates *B. cinerea* and *A. alternata* (Taylor *et al.*, 1998) with a view to understanding the role of these compounds in pathogen-host interactions and pathogen-induced floral abscission. Work will also ascertain whether the sesquiterpenes and/or phenolics in leaf and even flower tissues can be used as a viable prophylactic treatment against pathogenic fungi.

CHAPTER 6 SUMMARY AND GENERAL DISCUSSION

Concerns are growing over the increasing loss of efficacy of conventional fungicides due to pathogen resistance. Moreover, there is a general lack of acceptability of fungicide usage by the general public and from environmental perspectives. These concerns favour the increasing adoption of IPM strategies. Exploitation of NDR is a potentially desirable strategy in achieving IPM (Adikaram, 1990; Wilson *et al.*, 1994; Joyce and Johnson, 1999). Thus, the prospects of disease suppression through modulation and/or elicitation of NDR mechanisms is attractive in comparison to conventional chemical control (Joyce and Johnson, 1999). To realise maximum benefit of manipulating NDR in horticultural produce, considerable research is required. In contrast to inducible biochemical defences, limited research has been conducted on characterising the preformed antifungal compounds that occur in horticultural produce (Mercier, 1997; Morrissey and Osbourn, 1999).

The possibility that preformed antifungal compounds have a role in influencing *B. cinerea* quiescence in strawberry fruit has been researched by a number of authors (Jersch *et al.*, 1989; El Ghaouth *et al.*, 1991a; Herbert *et al.*, 2001). In this present study, the preformed antifungal profile in strawberry cv. Elsanta fruit and flowers at different stages of development was studied. Antifungal activity against both *B. cinerea* and *C. cladosporioides* declined with increasing fruit maturity as shown by TLC bioassay. The presence of higher concentrations of preformed antifungal compounds in immature fruit (green stage I) was shown to be consistent with high NDR in these fruit to *B. cinerea.* The majority of antifungal activity was found in achenes of green stage I fruit. None-the-less, antifungal activity was found in all tissue types (viz. pith, cortex, epidermis) of green stage I fruit. Green stage I fruit contain at least two previously unreported preformed antifungal compounds (R_f = 0.44 and 0.37) that are not present in white and red stage fruit. TLC reagent sprays showed that both of these compounds were neither phenolics nor alkaloids. Less intense positive reactions at R_f = 0.37 to Ehrlich's reagent suggests that this compound may be a terpene. On the basis of similar TLC behaviour it is possible that this preformed compound (R_f = 0.37) may be similar to inducible antifungal compounds reported in green stage fruit (Hirai *et al.*, 2000), strawberry leaves (Vincent *et al.*, 1999) and roots (Mussel and Staples, 1971). Achene extracts of green

stage I fruit contained at least eight antifungal compounds that were not found in white and red stage fruit. Many of these compounds were also found in strawberry flower tissue. TLC bioassays showed that all fruit maturity stages showed antifungal activity at the origin. The approximate area of fungal inhibition at origin the in green stage I fruit extracts (Rf = 0.13-0.00) was 90 and 70% greater than in white and red fruit, respectively. TLC reagent sprays confirmed that antifungal compounds at origin include phenolics. Some of these phenolic compounds may be similar to those previously reported in strawberry fruit and that are inhibitory to *B. cinerea* (Di Venere *et al.*, 1998; Herbert *et al.*, 2001).

This report provides preliminary evidence on the partial identity and distribution of preformed antifungal compounds in strawberry fruit and flowers. Further work is required to fully characterise the preformed antifungals compounds found in strawberry flower and fruit tissues and to elucidate pathways involved in their biosynthesis. Such information will allow precise definition of the roles that these compounds may play in suppression of floral infection and in strawberry fruit NDR against *B. cinerea*. In turn, this knowledge should enable IPM strategies that enhance levels of these compounds.

The strawberry industry is still heavily reliant on fungicides applied during flowering fruiting (Swaldling and Jefferies, 1996). The search for less toxic alternatives to control *B. cinerea* promoted an investigation into the potential of enhancing NDR in strawberry using different elicitor treatments. This study investigated the efficacy of pre- and postharvest treatments of the chemical plant activator acibenzolar in suppressing grey mould on strawberry. However, acibenzolar was ineffective as a postharvest treatment. Similarly, acibenzolar was also ineffective as a preharvest treatment in suppressing grey mould development on strawberry fruit harvested from summer grown plants. Conversely, preharvest applications of acibenzolar (0.25 – 2.0 mg AI ml^{-1}) were effective in suppressing grey mould on strawberry fruit harvested from winter grown plants. Where acibenzolar was effective, disease development was reduced by as much as 2 days. This delay was equivalent to a 15-20% increase in storage life of the fruit. This finding suggests that acibenzolar and perhaps other plant activators could prove valuable in the commercial management of grey mould on strawberry fruit. If SAR can be implemented in unison with existing controls as part

of a IPM strategy then it is likely that a more environmentally sound method of improved *B. cinerea* control on strawberry will be achieved. However, for there to be improved disease control more research is required on how the environment and management practices affect the efficacy of elicitors such as acibenzolar.

Geraldton waxflower is the most economically important Australian cut flower export. Recent waxflower research has concentrated on understanding and reducing postharvest flower abscission. This inherent problem results in low prices and reduced consumer confidence (Joyce, 1993). Infection of Geraldton waxflower by *B. cinerea* can lead to unacceptable levels of flower abscission after harvest. Thus, an investigation was conducted into the nature and identities of constitutive antifungal compounds in imported Geraldton waxflower flower and leaf tissues. Antifungal activity against *B. cinerea* and *C. cladosporioides* was observed in both Geraldton waxflower leaf and flower tissue. Leaf tissue contained considerably less antifungal activity than flower tissue. Some antifungal compounds were common to the three different waxflower cultivars studied. Through TLC reagent sprays and NMR GC-MS spectra, these antifungal compounds were identified as globulol and grandinol. There were also at least two unidentified phenolics. Notwithstanding similarities in antifungal profiles, it was also evident from TLC bioassays that significant variations exist between different waxflower cultivars. It is not clear whether these differences in antifungal profile and specific antifungal compounds in waxflower tissues have significant impact on host-pathogen interactions. Further work is also required to look at the spectrum of antifungal activity of waxflower extracts with a view to ascertaining whether the sesquiterpenes and/or phenolics in leaf tissue, much of which is wasted during harvesting, can be used as a viable prophylactic treatment.

Grey mould disease caused by the fungal pathogen *Botrytis cinerea* is the most important disease affecting quality of strawberry fruit (Daugaard, 1999) and Geraldton waxflower after harvest (Joyce, 1993). This study has provided valuable insights into the nature of preformed antifungal compounds in both of these two different horticultural crops. However, more research is needed on the detailed role that each antifungal compound plays in specific host-pathogen relationships. Improved understanding of the host-pathogen interaction for strawberry or Geraldton waxflower and *B. cinerea* will contribute to the development of improved control

strategies against grey mould. Better understanding of the role of preformed and inducible antifungals may allow for greater efficacy in enhancing NDR of harvested horticultural produce.

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APPENDICES

Appendix 3.2:

Quantification of NDR *in-planta*

3.2.1: Fresh weight of different strawberry fruit development stages

Treatment	Fruit Development Stage			
	Green	White	Red	
- inoculation.	2.75	6.31	17.74	*8.93*
+ inoculation.	3.30	6.78	15.43	*8.50*
	3.03	*6.55*	*16.59*	

3.2.2: ANOVA of fresh weight of different strawberry fruit development stages

Source of Variation	d.f. (m.v)	s.s	m.s	v.r.	F pr.
Fruit Stage	2	2178.41	1089.21	75.97	<0.001
Inoculation	1	3.07	3.07	0.21	0.645
Fruit Stage x Inoculation	2	29.30	14.65	1.02	0.366
Residual	60	860.22	14.34		
Total	65	3071.00			

3.2.4: H* values of different strawberry fruit development stages

Treatment	Fruit Development Stage			
	Green	White	Red	
- inoculation	111.98	108.86	40.35	*87.06*
+ inoculation	111.20	109.19	40.24	*86.85*
	111.54	*109.03*	*40.30*	

3.2.5: ANOVA of H* values of different strawberry fruit development stages

Source of Variation	d.f. (m.v)	s.s	m.s	v.r.	F pr.
Fruit Stage	2	719009.90	35954.95	$1.1x10^4$	<0.001
Inoculation	1	0.787	0.787	0.23	0.632
Fruit Stage x Inoculation	2	4.093	2.047	0.60	0.550
Residual	60	203.517	3.392		
Total	65	72118.30			

3.2.6: Effect of strawberry fruit development stage and ± inoculation with *B. cinerea* on time to 10% disease severity

Treatment	Fruit Development Stage			
	Green	White	Red	
- inoculation	32.09	27.36	19.45	*26.30*
+ inoculation	25.91	19.55	8.73	*18.06*
	29.00	*23.45*	*14.09*	

3.2.7: ANOVA of Effect of strawberry fruit development stage and ± inoculation with *B. cinerea* on time to 10% disease severity

Source of Variation	d.f. (m.v)	s.s	m.s	v.r.	F pr.
Fruit Stage	2	2498.55	1249.27	53.39	<0.001
Inoculation	1	1120.97	1120.97	47.90	<0.001
Fruit Stage x Inoculation	2	58.20	29.15	1.25	0.295
Residual	60	1404.00	23.40		
Total	65	5081.82			

Appendix 3.3

Preformed Antifungal Compounds in Strawberry

3.3.1: Composition of Czapek (Dox) media used for TLC bioassays (Johnston and Booth, 1983)

Sodium nitrate ($NaNO_3$)	2.0 g
Potassium dihydrogen phosphate (KH_2PO_4)	1.0 g
Magnesium sulfate ($MgSO_4.7H_2O$)	0.5 g
Potassium chloride (KCl)	0.5 g
Ferrous sulfate ($FeSO_4.7H_2O$)	0.01 g
Zinc sulfate ($ZnSO_4.7H_2O$)	0.01 g
Copper sulfate ($CuSO_4.5H_2O$)	0.005 g
Sucrose	30.0 g
Glass distilled water	1.0 l

Autoclave at 15 p.s.i. for 20 min

Appendix 4.3

Postharvest Acibenzolar Treatment

4.3.1a: Effect of postharvest acibenzolar treatment and on time to 10% disease severity stored at 5°C

Acibenzolar (mg AI ml^{-1})	Inoculation method		
	2×10^4	2×10^6	Control
0.00	4.33	4.67	6.00
0.10	6.00	10.67	7.00
0.20	9.33	11.67	6.67
0.30	6.00	5.67	12.00
0.40	4.33	5.33	7.33
0.50	8.67	7.67	7.33

4.3.1b: Effect of postharvest acibenzolar treatment and on time to 10% disease severity stored at 20°C

Acibenzolar (mg AI ml^{-1})	Inoculation method		
	2 x 10^4	2 x 10^6	Control
0.00	2.67	1.67	1.67
0.10	2.33	2.67	2.00
0.20	1.67	3.00	2.67
0.30	4.67	2.67	2.67
0.40	2.00	2.67	3.33
0.50	2.00	2.00	2.33

4.3.2: ANOVA of effect of postharvest acibenzolar treatment on time to 10% disease severity

Source of Variation	d.f. (m.v)	s.s	m.s	v.r.	F pr.
Block stratum	2	20.074	10.037	1.22	
Temperature A	1	616.333	616.333	74.74	<0.001
Acibenzolar conc. B	5	70.630	14.126	1.71	0.143
Inoculation C	2	7.463	3.731	0.45	0.638
A x B	5	53.889	10.778	1.31	0.271
A X C	2	10.722	5.361	0.65	0.525
B X C	10	84.426	8.443	1.02	0.433
A X B X C	10	85.389	8.539	1.04	0.424
Residual	70	577.259	8.247		
Total	107	1526.185			

4.3.3a: Effect of postharvest acibenzolar treatment and on time to 10% disease severity stored at 5°C

Acibenzolar (mg AI ml^{-1})	Inoculation method		
	2 x 10^4	2 x 10^6	Control
0.00	17.33	12.33	11.33
0.10	13.00	17.67	16.33
0.20	17.00	17.00	16.33
0.30	12.00	19.00	20.33
0.40	15.33	16.33	14.67
0.50	14.67	16.33	16.67

4.3.3b: Effect of postharvest acibenzolar treatment and on time to 10% disease severity stored at 20°C

Acibenzolar (mg AI ml^{-1})	Inoculation method		
	2 x 10^4	2 x 10^6	Control
0.00	8.33	5.67	5.33
0.10	6.33	5.33	4.67
0.20	5.00	6.33	5.33
0.30	9.67	6.00	5.33
0.40	6.00	5.00	6.67
0.50	6.00	4.67	6.00

4.3.4: ANOVA of effect of postharvest acibenzolar treatment on time to 100% disease severity

Source of Variation	d.f. (m.v)	s.s	m.s	v.r.	F pr.
Block stratum	2	46.352	23.176	3.04	
Temperature A	1	2581.333	2581.333	338.18	<0.001
Acibenzolar conc. B	5	41.741	8.348	1.09	0.372
Inoculation C	2	0.907	0.454	0.06	0.942
A x B	5	41.778	8.356	1.09	0.371
A X C	2	44.056	22.028	2.89	0.062
B X C	10	99.537	9.954	1.30	0.246
A X B X C	10	148.167	14.817	1.94	0.054
Residual	70	534.315	7.633		
Total	107	3538.185			

4.3.5a: Effect of postharvest acibenzolar treatment on time between of 10% and 100% disease severity stored at 5°C

Acibenzolar (mg AI ml-1)	Inoculation method		
	2 x 10^4	2 x 10^6	Control
0.00	13.00	7.67	5.33
0.10	7.00	7.00	9.33
0.20	7.67	5.33	9.67
0.30	6.00	13.33	8.33
0.40	11.00	11.00	7.33
0.50	6.00	8.67	9.33

4.3.5b: Effect of postharvest acibenzolar treatment on time between of 10% and 100% disease severity stored at 20°C

Acibenzolar (mg AI ml-1)	Inoculation method		
	2 x 10^4	2 x 10^6	Control
0.00	5.67	4.00	3.67
0.10	4.00	2.67	2.67
0.20	3.33	3.33	2.67
0.30	5.00	3.33	2.67
0.40	4.00	2.33	3.33
0.50	4.00	2.67	3.67

4.3.6: ANOVA of effect of postharvest acibenzolar treatment on time between of 10% and 100% disease severity

Source of Variation	d.f. (m.v)	s.s	m.s	v.r.	F pr.
Block stratum	2	11.722	5.861	1.00	
Temperature A	1	675.000	675.000	115.73	<0.001
Acibenzolar conc. B	5	28.556	5.711	0.98	0.437
Inoculation C	2	9.556	4.778	0.82	0.445
A x B	5	17.778	3.556	0.61	0.693
A X C	2	12.667	6.333	1.09	0.343
B X C	10	132.889	13.289	2.28	0.022
A X B X C	10	133.556	13.356	2.29	0.022
Residual	70	408.278	5.833		
Total	107	1430.000			

Appendix 4.4

PART A: Preharvest Acibenzolar Treatment

Experiment 1

4.4.1.1 ANOVA of effect of preharvest acibenzolar treatment on fruit weight

Source of Variation	d.f. (m.v)	s.s	m.s	v.r.	F pr.
Acibenzolar concentration	4	105.14	26.29	0.78	0.538
Residual	166	5575.46	33.59		
Total	170	5680.60			

4.4.1.2: ANOVA of effect of preharvest acibenzolar treatment on time to 10% disease severity

Source of Variation	d.f. (m.v)	s.s	m.s	v.r.	F pr.
Acibenzolar concentration	4	79.042	19.761	2.15	0.077
Residual	166	1527.461	9.202		
Total	170	1606.503			

4.4.1.3: ANOVA of effect of preharvest acibenzolar treatment on time to 100% disease severity

Source of Variation	d.f. (m.v)	s.s	m.s	v.r.	F pr.
Acibenzolar concentration	4	123.30	30.83	2.36	0.056
Residual	166	2168.71	13.06		
Total	170	2292.01			

Experiment 2

4.4.2.1: ANOVA of effect of preharvest acibenzolar treatment on fruit weight

Source of Variation	d.f. (m.v)	s.s	m.s	v.r.	F pr.
Acibenzolar concentration	5	50.343	10.069	1.51	0.185
Residual	347	2309.474	6.656		
Total	352	2359.818			

4.4.2.2: ANOVA of effect of preharvest acibenzolar treatment on time to 10% disease severity

Source of Variation	d.f. (m.v)	s.s	m.s	v.r.	F pr.
Acibenzolar concentration	5	875.50	175.10	7.88	<0.001
Residual	199	4420.46	22.21		
Total	204	4973.92			

4.4.2.3: ANOVA of effect of preharvest acibenzolar treatment on time to 100% disease severity

Source of Variation	d.f. (m.v)	s.s	m.s	v.r.	F pr.
Acibenzolar concentration	5	668.34	133.67	6.14	<0.001
Residual	199	4332.43	21.77		
Total	204	4753.00			

Experiment 3

4.4.3.1: ANOVA of effect of preharvest acibenzolar treatment on fruit weight

Source of Variation	d.f. (m.v)	s.s	m.s	v.r.	F pr.
Acibenzolar concentration (A)	4	29.22	7.31	0.58	0.681
Residual	253	3214.27	12.70		
Total	257	3243.50			

4.4.3.2: ANOVA of effect of preharvest acibenzolar treatment on time to 10% disease severity

Source of Variation	d.f. (m.v)	s.s	m.s	v.r.	F pr.
Acibenzolar concentration (A)	4	234.62	58.66	4.91	<0.001
Residual	154	1841.31	11.96		
Total	158	2075.94			

4.4.3.3: ANOVA of effect of preharvest acibenzolar treatment on time to 100% disease severity

Source of Variation	d.f. (m.v)	s.s	m.s	v.r.	F pr.
Acibenzolar concentration (A)	4	150.83	37.71	2.49	0.046
Residual	154	2333.56	15.15		
Total	158	2484.39			

Experiment 4

4.4.4.1: Effect of preharvest control acibenzolar treatment (0.00 mg AI ml^{-1}), development stage and fruit position on fruit weight

DAA	Fruit Position			
	Primary	Secondary	Tertiary	
7	1.54	1.14	1.12	*1.27*
14	4.13	2.75	2.23	*3.03*
21	10.79	8.76	5.36	*8.30*
28	15.70	9.52	8.48	*11.23*
35	17.21	10.00	8.55	*11.92*
	9.87	*6.43*	*5.15*	

4.4.4.2: Effect of preharvest acibenzolar treatment (0.25 mg AI ml^{-1}), development stage and fruit position on fruit weight

DAA	Fruit Position			
	Primary	Secondary	Tertiary	
7	1.56	0.92	0.88	*1.12*
14	5.43	3.69	3.26	*4.13*
21	13.28	5.58	6.59	*8.49*
28	20.30	11.24	8.34	*13.29*
35	16.54	9.71	6.80	*11.02*
	11.42	*6.23*	*5.17*	

4.4.4.3: ANOVA of effect of preharvest acibenzolar treatment, development stage and fruit position on fruit weight

Source of Variation	d.f. (m.v)	s.s	m.s	v.r.	F pr.
Block stratum	3	70.214	23.405	2.80	
Acibenzolar concentration (A)	1	6.279	6.279	0.75	0.388
DAA (B)	4	2264.087	566.022	67.71	<0.001
Fruit position (C)	2	688.392	334.196	39.98	<0.001
A x B	4	31.675	7.919	0.95	0.441
A x C	2	18.187	9.093	1.09	0.341
B x C	8	296.455	37.057	4.43	<0.001
A x B x C	8	42.714	5.339	0.64	0.743
Residual	87	727.261	8.359		
Total	119	4125.262			

4.4.4.4: Effect of preharvest control acibenzolar treatment (0.00 mg AI ml^{-1}), development stage and fruit position on time to 10% disease severity

DAA	Fruit Position			
	Primary	Secondary	Tertiary	
7	54.75	52.25	46.75	51.25
14	33.25	38.50	35.00	35.58
21	24.00	19.75	25.75	23.17
28	12.25	11.25	16.25	13.25
35	6.50	10.75	9.25	8.83
	26.15	26.50	26.60	

4.4.4.5: Effect of preharvest control acibenzolar treatment (0.25 mg AI ml^{-1}), development stage and fruit position on time to 10% disease severity

DAA	Fruit Position			
	Primary	Secondary	Tertiary	
7	58.50	55.50	41.00	*51.67*
14	29.50	43.25	44.00	*38.92*
21	24.00	25.75	23.75	*24.50*
28	13.50	15.25	16.00	*14.92*
35	11.50	8.75	11.50	*10.58*
	27.40	*29.70*	*27.25*	

4.4.4.6: ANOVA of effect of preharvest acibenzolar treatment, development stage and fruit position time to 10% disease severity

Source of Variation	d.f. (m.v)	s.s	m.s	v.r.	F pr.
Block stratum	3	251.53	83.84	1.18	
Acibenzolar concentration (A)	1	86.70	86.70	1.22	0.273
DAA (B)	4	28290.88	7072.72	99.14	<0.001
Fruit position (C)	2	42.12	21.06	0.30	0.745
A x B	4	26.72	6.68	0.09	0.984
A x C	2	35.55	17.77	0.25	0.780
B x C	8	1174.47	146.81	2.06	0.049
A x B x C	8	385.03	48.13	0.67	0.712
Residual	87	6206.47	71.34		
Total	119	36499.47			

4.4.4.7: Effect of preharvest control acibenzolar treatment (0.00 mg AI ml^{-1}), development stage and fruit position on time to 100% disease severity

DAA	Fruit Position			
	Primary	Secondary	Tertiary	
7	65.50	65.75	60.25	*63.83*
14	39.75	46.50	38.25	*41.50*
21	28.25	23.50	32.50	*28.08*
28	17.00	14.75	19.75	*17.17*
35	11.50	13.25	13.50	*12.75*
	32.40	*32.75*	*32.85*	

4.4.4.8: Effect of preharvest control acibenzolar treatment (0.25 mg AI ml^{-1}), development stage and fruit position on time to 100% disease severity

DAA	Fruit Position			
	Primary	Secondary	Tertiary	
7	78.00	74.25	51.25	*67.83*
14	35.00	48.00	53.00	*45.33*
21	29.00	29.50	28.50	*29.00*
28	19.25	19.50	19.25	*19.33*
35	14.50	13.25	14.75	*14.17*
	35.15	*36.90*	*33.35*	

4.4.4.9: ANOVA of effect of preharvest acibenzolar treatment, development stage and fruit position time to 100% disease severity

Source of Variation	d.f. (m.v)	s.s	m.s	v.r.	F pr.
Block stratum	3	369.0	123.0	1.13	
Acibenzolar concentration (A)	1	182.5	182.5	1.68	0.198
DAA (B)	4	43243.2	10810.8	99.72	<0.001
Fruit position (C)	2	60.4	30.2	0.28	0.757
A x B	4	46.9	11.7	0.11	0.979
A x C	2	67.8	33.9	0.31	0.732
B x C	8	1712.9	214.1	1.98	0.059
A x B x C	8	988.5	123.6	1.14	0.345
Residual	87	9431.5	108.4		
Total	119	56102.8			

Experiment 5

4.4.5.1: ANOVA of effect of ± inoculation with *B. cinerea*, acibenzolar concentration, spray interval, botryticide and fruit positions on fruit weight

Source of Variation	d.f. (m.v)	s.s	m.s	v.r.	F pr.
Block stratum	2	251.714	125.857	4.56	
Inoculation.	1	9.269	9.269	0.34	0.621
Residual	2	55.213	27.607	2.52	
Acibenzolar Concentration (A)	4	218.393	54.598	4.98	<0.001
Spray interval (B)	2	168.345	84.172	7.67	<0.001
Botryticide (C)	2	68.055	34.027	3.10	0.047

Inoculation x A	4	79.798	19.949	1.82	0.127
Inoculation x B	2	20.274	10.137	0.92	0.399
A x B	8	86.704	10.838	0.99	0.447
Inoculation x C	2	46.952	23.476	2.14	0.121
A x C	8	343.557	42.945	3.91	<0.001
B x C	4	94.762	23.691	2.16	0.075
Inoculation x A x B	8	243.981	30.498	2.78	0.006
Inoculation x A x C	8	44.561	5.570	0.51	0.849
Inoculation x B x C	4	17.747	4.437	0.40	0.805
A x B x C	16	181.672	11.354	1.03	0.422
Inoculation x A x B x C	16	696.825	43.552	3.97	<0.001
Residual	176	1930.831	10.971	1.47	

Source of Variation	d.f. (m.v)	s.s	m.s	v.r.	F pr.
Fruit position (E)	2	13335.776	6667.888	890.58	<0.001
Inoculation x E	2	56.257	28.129	3.76	0.024
A x E	8	177.075	22.134	2.96	0.003
B x E	4	139.155	34.789	4.65	0.001
C x E	4	112.315	28.079	3.75	0.005
Inoculation x A x E	8	89.190	11.149	1.49	0.160
Inoculation x B x E	4	18.590	4.648	0.62	0.648
Inoculation x C x E	16	200.927	12.558	1.68	0.049
Inoculation x C x E	4	100.603	25.151	3.36	0.010
A x C x E	16	297.770	18.611	2.49	0.001
B x C x E	8	51.591	6.449	0.86	0.549
Inoculation x A x B x E	16	220.058	13.754	1.84	0.025
Inoculation x A x C x E	16	149.812	9.363	1.25	0.227
Inoculation x B x C x E	8	80.244	10.030	1.34	0.222
A x B x C x E	32	256.524	8.016	1.07	0.368
Inoculation x A x B x C x E	32	556.279	17.384	2.32	<0.001
Residual	359	2695.355	7.487		
Total	808	23096.173			

4.4.5.2: ANOVA of effect of ± inoculation with *B. cinerea*, acibenzolar concentration, spray interval, botryticide and fruit positions on time to 10% disease severity

Source of Variation	d.f. (m.v)	s.s	m.s	v.r.	F pr.
Block stratum	2	70.15	35.008	18.06	
Inoculation.	1	489.26	489.26	251.86	0.004
Residual	2	3.89	1.94	0.08	
Acibenzolar Concentration (A)	4	341.48	85.37	3.72	0.006
Spray interval (B)	2	54.07	27.04	1.18	0.310
Botryticide (C)	2	9758.54	4879.27	212.57	<.001
Inoculation x A	4	171.58	42.9	1.87	0.118
Inoculation x B	2	53.22	26.61	1.16	0.316
A x B	8	200.12	25.02	1.09	0.372
Inoculation x C	2	62.83	31.41	1.37	0.257
A x C	8	173.33	21.67	0.94	0.482
B x C	4	31.23	7.81	0.34	0.851
Inoculation x A x B	8	302.38	37.80	1.65	0.115
Inoculation x A x C	8	378.21	47.28	2.06	0.042
Inoculation x B x C	4	54.68	13.67	0.60	0.666
A x B x C	16	465.96	29.12	1.27	0.222
Inoculation x A x B x C	16	468.62	29.29	1.28	0.217
Residual	176	4039.81	22.95	1.39	

Source of Variation	d.f. (m.v)	s.s	m.s	v.r.	F pr.
Fruit position (E)	2	1746.80	873.4	52.81	<0.001
Inoculation x E	2	213.94	106.97	6.47	0.002
A x E	8	154.31	19.29	1.17	0.319
B x E	4	334.05	83.51	5.05	<0.001
C x E	4	1488.86	372.22	22.50	<0.001
Inoculation x A x E	8	153.53	19.19	1.16	0.322
Inoculation x B x E	4	380.38	95.09	5.75	<0.001
Inoculation x C x E	16	365.16	22.82	1.38	0.148
Inoculation x C x E	4	38.365	9.59	0.58	0.677
A x C x E	16	205.09	12.82	0.78	0.714
B x C x E	8	416.66	52.08	3.15	0.002
Inoculation x A x B x E	16	314.4	19.65	1.19	0.275
Inoculation x A x C x E	16	630.22	39.39	2.38	0.002
Inoculation x B x C x E	8	621.89	77.74	4.70	<0.001
A x B x C x E	32	618.46	19.33	1.17	0.248

Inoculation x A x B x C x E	32	907.70	28.37	1.72	0.011
Residual	359 (1)	5937.67	16.54		
Total	808 (1)	31645.91			

4.4.5.3: ANOVA of effect of ± inoculation with *B. cinerea*, acibenzolar concentration, spray interval, botryticide and fruit positions on time to 100% disease severity

Source of Variation	d.f. (m.v)	s.s	m.s	v.r.	F pr.
Block stratum	2	252.00	126.00	5.04	
Inoculation.	1	384.96	384.96	15.39	0.059
Residual	2	50.02	25.01	0.62	
Acibenzolar Concentration (A)	4	684.42	171.10	4.21	0.003
Spray interval (B)	2	3.56	1.78	0.04	0.957
Botryticide (C)	2	8512.33	4256.16	104.79	<0.001
Inoculation x A	4	249.13	62.28	1.53	0.194
Inoculation x B	2	185.23	92.61	2.28	0.105
A x B	8	398.94	49.87	1.23	0.285
Inoculation x C	2	283.90	141.95	3.50	0.032
A x C	8	905.11	113.14	2.79	0.006
B x C	4	58.81	14.70	0.36	0.835
Inoculation x A x B	8	524.95	65.62	1.62	0.123
Inoculation x A x C	8	405.94	50.74	1.25	0.273
Inoculation x B x C	4	226.83	56.71	1.40	0.237
A x B x C	16	1034.89	64.68	1.59	0.075
Inoculation x A x B x C	16	1035.10	64.69	1.59	0.075
Residual	175 (1)	7107.53	40.61	3.54	

Source of Variation	d.f. (m.v)	s.s	m.s	v.r.	F pr.
Fruit position (E)	2	2508.98	1254.49	109.26	<0.001
Inoculation x E	2	310.26	155.13	13.51	<0.001
A x E	8	325.73	40.72	3.55	<0.001
B x E	4	605.17	151.29	13.18	<0.001
C x E	4	857.82	214.45	18.68	<0.001
Inoculation x A x E	8	843.76	105.47	9.19	<0.001
Inoculation x B x E	4	390.43	97.61	8.50	<0.001
Inoculation x C x E	16	401.71	25.11	2.19	0.006
Inoculation x C x E	4	303.94	75.98	6.62	<0.001
A x C x E	16	325.86	20.37	1.77	0.034
B x C x E	8	685.17	85.65	7.46	<0.001

Inoculation x A x B x E	16	322.56	20.16	1.76	0.036
Inoculation x A x C x E	16	1560.52	97.53	8.49	<0.001
Inoculation x B x C x E	8	616.75	77.09	6.71	<0.001
A x B x C x E	32	1323.51	41.36	3.60	<0.001
Inoculation x A x B x C x E	32	578.04	22.23	1.94	0.005
Residual	303 (57)	3478.89	11.48		
Total	745 (64)	23787.63			

4.4.5.4: ANOVA of effect of ± inoculation with *B. cinerea*, acibenzolar concentration, spray interval, botryticide on final truss number per plant

Source of Variation	d.f. (m.v)	s.s	m.s	v.r.	F pr.
Block stratum	2	2.022	1.011	4.79	
Inoculation.	1	0.044	0.044	0.21	0.691
Residual	2	0.042	0.211	0.26	
Acibenzolar Concentration (A)	4	4.178	1.044	1.28	0.279
Spray interval (B)	2	0.956	0.478	0.59	0.558
Botryticide (C)	2	0.289	0.144	0.18	0.838
Inoculation x A	4	6.400	1.600	1.96	0.102
Inoculation x B	2	0.956	0.478	0.59	0.558
A x B	8	6.489	0.811	0.99	0.442
Inoculation x C	2	2.289	1.144	1.40	0.249
A x C	8	5.489	0.686	0.84	0.568
B x C	4	4.644	1.161	1.42	0.228
Inoculation x A x B	8	6.267	0.783	0.96	0.469
Inoculation x A x C	8	2.600	0.325	0.40	0.920
Inoculation x B x C	4	6.111	1.528	1.87	0.117
A x B x C	16	13.244	0.828	1.01	0.443
Inoculation x A x B x C	16	17.333	1.083	1.33	0.184
Residual	176	143.556	0.816		

4.4.5.5: ANOVA of effect of ± inoculation with *B. cinerea*, acibenzolar concentration, spray interval, botryticide on final leaf number per plant

Source of Variation	d.f. (m.v)	s.s	m.s	v.r.	F pr.
Block stratum	2	3.756	1.878	0.23	
Inoculation.	1	10.000	10.000	1.21	0.385
Residual	2	16.467	8.233	1.47	

Acibenzolar Concentration (A)	4	43.044	10.761	1.92	0.110
Spray interval (B)	2	17.222	8.611	1.53	0.218
Botryticide (C)	2	13.889	6.944	1.24	0.293
Inoculation x A	4	49.222	12.306	2.19	0.072
Inoculation x B	2	2.067	1.033	0.18	0.832
A x B	8	35.889	4.486	0.80	0.604
Inoculation x C	2	2.067	1.033	0.18	0.832
A x C	8	32.889	4.111	0.73	0.663
B x C	4	76.178	19.044	3.39	0.011
Inoculation x A x B	8	71.711	8.964	1.60	0.129
Inoculation x A x C	8	19.378	2.422	0.43	0.901
Inoculation x B x C	4	6.667	1.667	0.30	0.880
A x B x C	16	73.378	4.586	0.82	0.665
Inoculation x A x B x C	16	110.889	6.931	1.23	0.246
Residual	176	987.778	5.612		

4.4.5.6: ANOVA of effect of ± inoculation with *B. cinerea*, spray interval, botryticide on secondary and tertiary fruit sprayed with different acibenzolar concentrations when the majority of plants were at were at red and white stages, respectively on time to 10% disease severity

Source of Variation	d.f. (m.v)	s.s	m.s	v.r.	F pr.
Block stratum	2	156.33	78.46	4.54	
Inoculation.	1	235.59	235.59	13.62	0.066
Residual	2	34.59	17.29	0.41	
Acibenzolar Concentration (A)	4	83.21	20.80	0.49	0.742
Botryticide (C)	2	2224.60	1112.30	26.25	<0.001
Inoculation x A	4	62.83	15.71	0.37	0.828
A x B	2	26.39	13.20	0.31	0.734
Inoculation x B	8	263.61	32.95	0.78	0.624
Inoculation x A x B	8	370.64	46.33	1.09	0.382
Residual	54 (2)	2287.78	42.37	2.08	

Source of Variation	d.f. (m.v)	s.s	m.s	v.r.	F pr.
Fruit position (E)	1	81.84	81.84	4.01	0.050
Inoculation x E	1	15.81	15.81	0.77	0.382
A x E	4	142.40	35.60	1.75	0.153
B x E	2	387.82	35.60	9.51	<0.001
Inoculation x A x E	4	29.31	193.91	0.36	0.837
Inoculation x B x E	2	10.98	7.33	0.27	0.765

A x B x E	8	160.26	5.49	0.98	0.460
Residual	56 (12)	1142.38	20.03		
Total	165 (14)	6126.85	20.40		

4.4.5.7: ANOVA of effect of ± inoculation with *B. cinerea*, spray interval, botryticide on secondary and tertiary fruit sprayed with different acibenzolar concentrations when the majority of plants were at were at red and white stages, respectively on fruit weight

Source of Variation	d.f. (m.v)	s.s	m.s	v.r.	F pr.
Block stratum	2	62.794	31.397	2.91	
Inoculation.	1	11.309	11.309	1.05	0.413
Residual	2	21.557	10.778	1.80	
Acibenzolar Concentration (A)	4	24.117	30.29	1.01	0.412
Botryticide (C)	2	2.099	1.049	0.18	0.840
Inoculation x A	4	25.001	6.250	1.04	0.393
A x B	2	0.691	0.346	0.06	0.944
Inoculation x B	8	44.947	5.618	0.94	0.493
Inoculation x A x B	8	90.319	11.290	1.89	0.081
Residual	54 (2)	323.383	5.989	1.19	

Source of Variation	d.f. (m.v)	s.s	m.s	v.r.	F pr.
Fruit position (E)	1	1370.113	1370.113	273.26	<0.001
Inoculation x E	1	11.299	11.299	2.25	0.139
A x E	4	2.895	0.724	0.14	0.965
B x E	2	0.880	0.440	0.09	0.916
Inoculation x A x E	4	25.897	6.474	1.29	0.284
Inoculation x B x E	2	29.937	14.969	2.99	0.058
A x B x E	8	26.800	3.350	0.67	0.717
Residual	56 (12)	285.799	5.014		
Total	165 (14)	2046.279			

4.4.5.8: ANOVA of effect of ± inoculation with *B. cinerea*, spray interval, botryticide on secondary and tertiary fruit sprayed with different acibenzolar concentrations when the majority of plants were at were at red and white stages, respectively on final truss number

Source of Variation	d.f. (m.v)	s.s	m.s	v.r.	F pr.
Block stratum	2	3.600	1.800	0.55	
Inoculation.	1	0.200	0.200	0.06	0.828
Residual	2	6.533	3.267	0.85	
Acibenzolar Concentration (A)	4	27.422	1.267	1.78	0.145
Botryticide (C)	2	2.533	1.811	0.33	0.721
Inoculation x A	4	7.244	4.067	0.47	0.757
A x B	2	8.133	3.406	1.06	0.354
Inoculation x B	8	27.244	3.761	0.89	0.534
Inoculation x A x B	8	30.089	3.843	0.98	0.462
Residual	56	215.200			

4.4.5.9: ANOVA of effect of ± inoculation with *B. cinerea*, spray interval, botryticide on secondary and tertiary fruit sprayed with different acibenzolar concentrations when the majority of plants were at were at red and white stages, respectively on final truss number

Source of Variation	d.f. (m.v)	s.s	m.s	v.r.	F pr.
Block stratum	2	1.644	0.822	0.25	
Inoculation.	1	0.556	0.556	0.17	0.723
Residual	2	6.711	3.356	6.20	
Acibenzolar Concentration (A)	4	2.533	0.633	1.17	0.334
Botryticide (C)	2	3.511	1.756	3.24	0.046
Inoculation x A	4	3.333	0.833	1.54	0.203
A x B	2	2.178	1.089	2.01	0.143
Inoculation x B	8	5.600	0.700	1.29	0.266
Inoculation x A x B	8	2.933	0.367	0.68	0.709
Residual	56	30.311	0.541		

Experiment 6

4.4.6a: Initial concentrations (μ moles l^{-1}) of individual elements in full strength Hoagland's solution

Nitrogen	15000
Potassium	6000
Calcium	5000
Magnesium	2000
Phosphorous	1000
Sulfur	2000
Chlorine	18
Iron	25
Boron	46
Manganese	9
Zinc	0.8
Copper	0.3
Molybdenum	0.1

4.4.6b: Composition of 1 times standard nitrogen in full strength Hoagland's solution for plants grown in peat/polystyrene (10:1 v/v).

Compound	ml l^{-1} of nutrient solution
1 M KH_2PO_4 (potassium acid phosphate	2
1 M KNO_3 (potassium nitrate)	3
1 M $Ca(NO_3)_2$ (calcium nitrate)	4
1 M $MgSO_4$ $7H_2O$ (magnesium sulfate)	2
1M ferric citrate	
Micronutrients	All added to 1 L, then 1 ml taken per L of solution
H_3BO_3 (boric acid) 2.86 g	
MnCl $4H_2O$ (manganese chloride) 1.81 g	
$ZnSO_4$ $7H_2O$ (zinc sulfate) 0.22 g	
$CuSO_4$ $5H_20$ (copper sulfate) 0.08 g	

4.4.6c: Composition of 0 times standard nitrogen in full strength Hoagland's solution for plants grown in peat/polystyrene (10:1 v/v).

Compound	ml l^{-1} of nutrient solution
1 M KH_2PO_4 (potassium acid phosphate	2
1 M KCl (potassium chloride)	3
1 M $CaCl_2$ (calcium choride)	4
1 M $MgSO_4$ $7H_2O$ (magnesium sulfate)	2
1M ferric citrate	
Micronutrients	All added to 1 L, then 1 ml taken per L of solution
H_3BO_3 (boric acid) 2.86 g	
MnCl $4H_2O$ (manganese chloride) 1.81 g	
$ZnSO_4$ $7H_2O$ (zinc sulfate) 0.22 g	
$CuSO_4$ $5H_20$ (copper sulfate) 0.08 g	

4.4.6.1: ANOVA of effect of acibenzolar and nitrogen on day from planting to harvest of primary fruit

Source of Variation	d.f. (m.v)	s.s	m.s	v.r.	F pr.
Block stratum	3	158.215	52.738	12.59	
Block. Wholeplot stratum					
Nitrogen	1	0.428	0.428	0.1	0.770
Residual	3	12.564	4.188	1.4	
Block.whole-plot units stratum					
Acibenzolar	1	0.005	0.005	0.00	0.968
Acibenzolar.N	1	16.251	15.251	5.44	0.021
Residual	225 (21)	671.542	2.985		
Total	234 (21)	847.464			

4.4.6.2: ANOVA of effect of acibenzolar and nitrogen on day from anthesis to harvest of primary fruit

Source of Variation	d.f. (m.v)	s.s	m.s	v.r.	F pr.
Block stratum	3	55.75	18.58	1.23	
Block. Wholeplot stratum					
Nitrogen	1	16.81	16.81	1.11	0.369
Residual	3	45.42	15.14	0.63	
Block.whole-pl,units stratum					
Acibenzolar	1	56.52	56.52	2.37	0.125
Acibenzolar.N	1	0.13	0.13	0.01	0.941
Residual	225 (21)	5374.76	23.89		
Total	234 (21)	5526.3			

4.4.6.3: ANOVA of effect of acibenzolar and nitrogen on primary fruit weight

Source of Variation	d.f. (m.v)	s.s	m.s	v.r.	F pr.
Block stratum	3	284.94	94.98	1.15	
Block. Wholeplot stratum					
Nitrogen	1	608.87	608.87	7.38	0.073
Residual	3	247.61	82.54	1.32	
Block.whole-pl,units stratum					
Acibenzolar	1	32.58	32.58	0.52	0.471
Acibenzolar.N	1	21.06	21.06	0.34	0.562
Residual	225 (21)	14073.59	62.55		
Total	234 (21)	15133.5			

4.4.6.4: ANOVA of effect of acibenzolar and nitrogen on mean firmness of primary fruit

Source of Variation	d.f. (m.v)	s.s	m.s	v.r.	F pr.
Block stratum	3	25.211	8.404	3.56	
Block. Wholeplot stratum					
Nitrogen	1	0.540	0.540	0.23	0.665
Residual	3	7.074	2.358	2.08	
Block.whole-pl,units stratum					
Acibenzolar	1	1.564	1.564	1.38	0.242
Acibenzolar.N	1	0.788	0.788	0.69	0.406
Residual	223 (23)	253.206	1.135		
Total	232 (23)	284.568			

4.4.6.5: ANOVA of effect of acibenzolar and nitrogen on TSS of primary fruit

Source of Variation	d.f. (m.v)	s.s	m.s	v.r.	F pr.
Block stratum	3	7.146	2.382	1.43	
Block. Wholeplot stratum					
Nitrogen	1	28.666	28.666	17.16	0.026
Residual	3	5.013	1.671	1.12	
Block.whole-pl,units stratum					
Acibenzolar	1	6.903	6.903	4.64	0.032
Acibenzolar.N	1	0.723	0.723	0.49	0.486
Residual	223 (23)	331.595	1.487		
Total	223 (23)	375.864			

4.4.6.6: ANOVA of effect of acibenzolar and nitrogen on day from planting to harvest of secondary fruit

Source of Variation	d.f. (m.v)	s.s	m.s	v.r.	F pr.
Block stratum	3	343.965	114.655	3.79	
Block. Wholeplot stratum					
Nitrogen	1	17.511	17.511	0.58	0.502
Residual	3	90.702	30.234	4.73	
Block.whole-pl,units stratum					
Acibenzolar	1	0.146	0.146	0.02	0.880
Acibenzolar.N	1	5.219	5.219	0.82	0.367
Residual	220 (26)	1405.590	6.389		
Total	220 (26)	1791.722			

4.4.6.7: ANOVA of effect of acibenzolar and nitrogen on day from anthesis to harvest of secondary fruit

Source of Variation	d.f. (m.v)	s.s	m.s	v.r.	F pr.
Block stratum	3	27.323	9.108	0.94	
Block. Wholeplot stratum					
Nitrogen	1	8.463	8.463	0.87	0.419
Residual	3	29.080	9.693	2.01	
Block.whole-pl,units stratum					
Acibenzolar	1	0.495	0.495	0.10	0.749
Acibenzolar.N	1	2.886	2.886	0.60	0.440
Residual	220 (26)	1063.196	4.833		
Total	220 (26)	1122.591			

4.4.6.8: ANOVA of effect of acibenzolar and nitrogen on secondary fruit weight

Source of Variation	d.f. (m.v)	s.s	m.s	v.r.	F pr.
Block stratum	3	499.91	166.64	12.02	
Block. Wholeplot stratum					
Nitrogen	1	149.03	149.03	10.75	0.046
Residual	3	41.59	13.86	0.34	
Block.whole-pl,units stratum					
Acibenzolar	1	67.51	67.51	1.66	0.199
Acibenzolar.N	1	22.40	22.40	0.55	0.459
Residual	220 (26)	8967.61	40.76		
Total	229 (26)	9635.46			

4.4.6.9: ANOVA of effect of acibenzolar and nitrogen on time to 10% disease severity of secondary fruit

Source of Variation	d.f. (m.v)	s.s	m.s	v.r.	F pr.
Block stratum	3	27.21	9.07	0.19	
Block. Wholeplot stratum					
Nitrogen	1	63.76	63.76	1.31	0.336
Residual	3	146.51	48.84	2.56	
Block.whole-pl,units stratum					
Acibenzolar	1	163.14	163.14	8.54	0.004
Acibenzolar.N	1	0.54	0.54	0.03	0.866
Residual	220 (26)	4201.90	19.10		
Total	229 (26)	4562.26			

4.4.6.10: ANOVA of effect of acibenzolar and nitrogen on time to 100% disease severity of secondary fruit

Source of Variation	d.f. (m.v)	s.s	m.s	v.r.	F pr.
Block stratum	3	84.28	28.09	0.78	
Block. Wholeplot stratum					
Nitrogen	1	85.30	85.30	2.37	0.221
Residual	3	107.80	35.93	1.39	
Block.whole-pl,units stratum					
Acibenzolar	1	151.18	151.18	5.87	0.016
Acibenzolar.N	1	0.00	0.00	0.00	0.990
Residual	220 (26)	5667.76	25.76		
Total	229 (26)	6056.65			

4.4.6.11: ANOVA of effect of acibenzolar and nitrogen on time between 10% and 100% disease severity of secondary fruit

Source of Variation	d.f. (m.v)	s.s	m.s	v.r.	F pr.
Block stratum	3	29.845	9.948	4.95	
Block. Wholeplot stratum					
Nitrogen	1	1.565	1.565	0.78	0.443
Residual	3	6.035	2.012	0.29	
Block.whole-pl,units stratum					
Acibenzolar	1	0.228	0.228	0.03	0.857
Acibenzolar.N	1	0.641	0.641	0.09	0.763
Residual	220 (26)	1548.622	7.039		
Total	229 (29)	1583.343			

4.4.6.12: ANOVA of daily temperatures recorded over growing period from February to May 2001

Source of Variation	d.f. (m.v)	s.s	m.s	v.r.	F pr.
Block stratum	3	2104.74	701.58	27.00	
Position	2	1980.76	990.38	38.12	<0.001
Date	106	339638.50	3204.14	123.32	<0.001
Position. Date	212	2201.93	10.39	0.40	1.000
Residual	30312	787591.85	25.98		
Total	30635	1133517.28			

4.4.6.13: ANOVA of monthly EC levels recorded over growing season from February to May 2001

Source of Variation	d.f. (m.v)	s.s	m.s	v.r.	F pr.
Block stratum	3	3.0084	1.0028	12.19	
Block. Wholeplot stratum					
Nitrogen	1	0.0984	0.0984	1.20	0.354
Residual	3	0.2469	0.0823	0.75	
Block.whole-pl,units stratum					0.756
Acibenzolar	1	0.108	0.108	0.10	0.345
Acibenzolar.N	1	0.1000	0.1000	0.91	
Residual	54	5.9548	0.1103		
Total	63	9.4193			

Experiment 7

4.4.7.1: ANOVA of effect of acibenzolar and nitrogen on day from planting to harvest of primary fruit

Source of Variation	d.f. (m.v)	s.s	m.s	v.r.	F pr.
Block stratum	3	179.523	59.841	22.66	
Block. Wholeplot stratum					
Nitrogen	1	0.262	0.262	0.10	0.773
Residual	3	7.921	2.640	0.83	
Block.whole-pl,units stratum					
Acibenzolar	1	0.007	0.007	0.00	0.962
Acibenzolar.N	1	0.742	0.742	0.23	0.630
Residual	154 (92)	490.158	3.183		
Total	163 (92)	611.970			

4.4.7.2: ANOVA of effect of acibenzolar and nitrogen on day from anthesis to harvest of primary fruit

Source of Variation	d.f. (m.v)	s.s	m.s	v.r.	F pr.
Block stratum	3	27.419	9.140	2.65	
Block. Wholeplot stratum					
Nitrogen	1	0.009	0.009	0.00	0.962
Residual	3	10.356	3.452	1.27	
Block.whole-pl,units stratum					
Acibenzolar	1	1.023	1.023	0.38	0.540
Acibenzolar.N	1	0.009	0.009	0.00	0.955
Residual	154 (92)	417.635	2.712		
Total	163 (92)	443.140			

4.4.7.3: ANOVA of effect of acibenzolar and nitrogen on primary fruit weight

Source of Variation	d.f. (m.v)	s.s	m.s	v.r.	F pr.
Block stratum	3	128.21	42.74	0.62	
Block. Wholeplot stratum					
Nitrogen	1	27.49	27.49	0.40	0.571
Residual	3	205.40	68.47	3.54	
Block.whole-pl,units stratum					
Acibenzolar	1	28.10	28.10	1.45	0.230
Acibenzolar.N	1	28.12	28.12	1.46	0.229
Residual	154 (92)	2974.49	19.31		
Total	163 (92)	3222.84			

4.4.7.4: ANOVA of effect of acibenzolar and nitrogen on mean firmness of primary fruit

Source of Variation	d.f. (m.v)	s.s	m.s	v.r.	F pr.
Block stratum	3	2.7450	0.9150	3.71	
Block. Wholeplot stratum					
Nitrogen	1	0.0163	0.0163	0.07	0.813
Residual	3	0.7391	0.2464	0.90	
Block.whole-pl,units stratum					
Acibenzolar	1	0.0258	0.0258	0.09	0.759
Acibenzolar.N	1	0.0897	0.0897	0.33	0.567
Residual	154 (92)	42.0079			
Total	163 (92)	44.4598			

4.4.7.5: ANOVA of effect of acibenzolar and nitrogen on TSS of primary fruit

Source of Variation	d.f. (m.v)	s.s	m.s	v.r.	F pr.
Block stratum	3	28.498	9.499	6.10	
Block. Wholeplot stratum					
Nitrogen	1	10.642	10.642	6.83	0.079
Residual	3	4.675	1.558	0.75	
Block.whole-pl,units stratum					
Acibenzolar	1	1.480	1.480	0.71	0.400
Acibenzolar.N	1	0.529	0.529	0.26	0.614
Residual	154 (92)	319.492	2.075		
Total	163 (92)	349.380			

4.4.7.6: ANOVA of effect of acibenzolar and nitrogen on day from planting to harvest of secondary fruit

Source of Variation	d.f. (m.v)	s.s	m.s	v.r.	F pr.
Block stratum	3	22.125	7.375	1.	
Block. Wholeplot stratum					
Nitrogen	1	0.099	0.099	0.02	0.889
Residual	3	12.908	4.303	3.09	
Block.whole-pl,units stratum					
Acibenzolar	1	0.035	0.035	0.02	0.875
Acibenzolar.N	1	0.368	0.368	0.26	0.607
Residual	215 (31)	298.962	1.391		
Total	224 (31)	324.560			

4.4.7.7: ANOVA of effect of acibenzolar and nitrogen on day from anthesis to harvest of secondary fruit

Source of Variation	d.f. (m.v)	s.s	m.s	v.r.	F pr.
Block stratum	3	13.653	4.551	3.35	
Block. Wholeplot stratum					
Nitrogen	1	0.406	0.406	0.30	0.623
Residual	3	4.080	1.360	0.76	
Block.whole-pl,units stratum					
Acibenzolar	1	0.261	0.261	0.15	0.702
Acibenzolar.N	1	0.377	0.377	0.21	0.646
Residual	215 (31)	382.593			
Total	224 (31)	399.396			

4.4.7.8: ANOVA of effect of acibenzolar and nitrogen on secondary fruit weight

Source of Variation	d.f. (m.v)	s.s	m.s	v.r.	F pr.
Block stratum	3	59.639	19.880	1.45	
Block. Wholeplot stratum					
Nitrogen	1	15.527	15.527	1.13	0.366
Residual	3	41.219	13.740	2.94	
Block.whole-pl,units stratum					
ACIBENZOLAR	1	16.561	16.561	3.54	0.061
ACIBENZOLAR.N	1	0.192	0.192	0.04	0.840
Residual	215 (31)	1005.033	4.675		
Total	224 (31)	1124.430			

4.4.7.9: ANOVA of effect of acibenzolar and nitrogen on time to 10% disease severity of secondary fruit

Source of Variation	d.f. (m.v)	s.s	m.s	v.r.	F pr.
Block stratum	3	78.585	26.195	3.98	
Block. Wholeplot stratum					
Nitrogen	1	0.398	0.398	0.06	0.822
Residual	3	19.736	6.579	2.24	
Block.whole-pl,units stratum					
Acibenzolar	1	9.312	9.312	3.17	0.076
Acibenzolar.N	1	1.185	1.185	0.40	0.526
Residual	215 (31)	631.729	2.938		
Total	224 (31)	725.449			

4.4.7.10: ANOVA of effect of acibenzolar and nitrogen on time to 100% disease severity of secondary fruit

Source of Variation	d.f. (m.v)	s.s	m.s	v.r.	F pr.
Block stratum	3	141.670	47.223	4.05	
Block. Wholeplot stratum					
Nitrogen	1	11.807	11.807	1.01	0.388
Residual	3	34.993	11.664	2.77	
Block.whole-pl,units stratum					
Acibenzolar	1	0.364	0.364	0.09	0.769
Acibenzolar.N	1	0.001	0.001	0.00	0.991
Residual	215 (31)	906.778	4.218		
Total	224 (31)	1078.196			

4.4.7.11: **ANOVA of effect of acibenzolar and nitrogen on time between 10% and 100% disease severity of secondary fruit**

Source of Variation	d.f. (m.v)	s.s	m.s	v.r.	F pr.
Block stratum	3	27.403	9.134	1.12	
Block. Wholeplot stratum					
Nitrogen	1	0.890	0.890	0.11	0.763
Residual	3	24.404	8.135	2.58	
Block.whole-pl,units stratum					
Acibenzolar	1	5.556	5.556	1.76	0.186
Acibenzolar.N	1	1.334	1.334	0.42	0.516
Residual	215 (31)	690.437	3.153		
Total	224 (31)	744.454			

4.4.7.12: **ANOVA of daily temperatures recorded over growing period from June to July 2001**

Source of Variation	d.f. (m.v)	s.s	m.s	v.r.	F pr.
Block stratum	3	2885.56	961.85	24.62	
Position	2	71.93	35.97	0.92	0.398
Date	46	142081.51	3088.73	79.07	<0.001
Position. Date	92	867.09	9.42	0.24	1.000
Residual	13128	512823.66	39.06		
Total	13271	658729.75			

4.4.7.13: **ANOVA of monthly EC recorded over growing period from June to July 2001**

Source of Variation	d.f. (m.v)	s.s	m.s	v.r.	F pr.
Block stratum	3	6.2598	2.0866	6.93	
Block. Wholeplot stratum					
Nitrogen	1	0.908	0.0908	0.30	0.621
Residual	3	0.9038	0.3013	1.89	
Block.whole-pl,units stratum					0.888
Acibenzolar	1	0.0032	0.0032	0.02	0.114
Acibenzolar.N	1	0.4112	0.4112	2.58	
Residual	54	8.6094	0.1594		
Total	63	16.2781			

Appendix 4.5

PART B: Effects of Preharvest *Aureobasidium pullulans* Treatment on Grey Mould Disease on Strawberry Fruit

4.5.1: Effect of *A. pullulans* and *B. cinerea* sprayed at flowering on time from anthesis to harvest of different fruit positions

Treatment	Fruit			
	Primary	Secondary	Tertiary	
-Ap and –Bc	43.80	42.13	43.00	*42.97*
+Ap and –Bc	46.19	42.33	40.38	*42.96*
-Ap and +Bc	43.62	41.00	42.22	*42.28*
+Ap and +Bc	42.83	42.10	44.86	*43.26*
	44.11	*41.89*	*42.61*	

4.5.2: ANOVA of effect of *A. pullulans* and *B. cinerea* sprayed at flowering on time from anthesis to harvest of different fruit positions

Source of Variation	d.f. (m.v)	s.s	m.s	v.r.	F pr.
Trt ^{A}	1	17.16	5.72	0.42	0.739
Fruit ^{B}	3	112.93	56.47	4.15	0.019
A x B	3	177.56	29.59	2.17	0.053
Residual	84 (36)	1143.17	13.61		
Total	95 (36)	1335.41			

4.5.3: Effect of *A. pullulans* and *B. cinerea* sprayed at flowering on harvested fruit weight of different fruit positions

Treatment	Fruit			
	Primary	Secondary	Tertiary	
-Ap and –Bc	24.61	21.32	14.22	*20.05*
+Ap and –Bc	23.21	19.32	15.52	*19.35*
-Ap and +Bc	23.68	22.68	13.97	*20.11*
+Ap and +Bc	24.26	19.64	10.66	*18.19*
	23.94	*20.74*	*13.59*	

4.5.4: ANOVA of effect of *A. pullulans* and *B. cinerea* sprayed at flowering on harvested fruit weight of different fruit positions

Source of Variation	d.f. (m.v)	s.s	m.s	v.r.	F pr.
Trt [A]	3	79.07	26.36	0.61	0.612
Fruit [B]	2	2468.70	1234.35	28.47	<0.001
A x B	6	155.26	25.88	0.60	0.732
Residual	84 (36)	3642.30	43.36		
Total	95 (36)	5511.12			

4.5.5: Effect of *A. pullulans* and *B. cinerea* sprayed at flowering on time to 10% disease severity of different fruit positions

Treatment		Fruit		
	Primary	Secondary	Tertiary	
-Ap and –Bc	13.67	15.00	14.29	*14.32*
+Ap and –Bc	12.78	14.00	19.25	*15.34*
-Ap and +Bc	11.38	12.56	13.13	*12.35*
+Ap and +Bc	13.26	11.30	13:14	*12.57*
	12.77	*13.21*	*14.95*	

4.5.6: ANOVA of effect of *A. pullulans* and *B. cinerea* sprayed at flowering on time to 10% disease severity of different fruit positions

Source of Variation	d.f. (m.v)	s.s	m.s	v.r.	F pr.
Trt [A]	3	203.41	67.80	4.45	0.006
Fruit [B]	2	116.91	58.45	3.83	0.026
A x B	6	197.24	32.87	2.16	0.056
Residual	79 (41)	1204.36	15.25		
Total	90 (41)	1589.03			

4.5.7: Effect of *A. pullulans* and *B. cinerea* sprayed at flowering on time to 100% disease severity of different fruit positions

Treatment		Fruit		
	Primary	Secondary	Tertiary	
-Ap and –Bc	21.89	23.75	22.33	*22.66*
+Ap and –Bc	21.33	21.25	26.00	*22.86*
-Ap and +Bc	18.50	20.56	19.83	*19.63*
+Ap and +Bc	20.58	18.70	19.57	*19.62*
	20.57	*21.06*	*21.93*	

4.5.8: ANOVA of effect of *A. pullulans* and *B. cinerea* sprayed at flowering on time to 100% disease severity of different fruit positions

Source of Variation	d.f. (m.v)	s.s	m.s	v.r.	F pr.
Trt [A]	3	325.18	108.39	7.47	<0.001
Fruit [B]	2	41.82	20.91	1.44	0.244
A x B	6	185.03	30.84	2.12	0.061
Residual	79 (41)	1045.27	14.52		
Total	90 (41)	1448.95			

4.5.9: Effect of *A. pullulans* and *B. cinerea* sprayed at flowering on time to between 10% and 100% disease severity of different fruit positions

Treatment	Fruit			
	Primary	Secondary	Tertiary	
-Ap and –Bc	8.22	8.75	8.33	*8.44*
+Ap and –Bc	7.98	7.86	6.75	*7.53*
-Ap and +Bc	7.13	8.00	7.00	*7.38*
+Ap and +Bc	7.95	7.40	6.43	*7.26*
	7.82	*8.00*	*7.13*	

4.5.10: ANOVA of effect of *A. pullulans* and *B. cinerea* sprayed at flowering on time to between 10% and 100% disease severity of different fruit positions

Source of Variation	d.f. (m.v)	s.s	m.s	v.r.	F pr.
Trt [A]	3	28.349	9.450	1.36	0.261
Fruit [B]	2	18.708	9.354	1.35	0.266
A x B	6	12.702	2.117	0.31	0.932
Residual	79 (41)	491.743	6.926		
Total	90 (41)	531.422			

Appendix 4.6

Effect of Postharvest UV-C Treatment on Grey Mould Disease on Strawberry Fruit

4.6.1: Effect of UV-C on time to 10% disease severity

UV-C	Time to 10% disease severity				
	NW-Bc	W-Bc	W+Bc	W+Bc 48h	
0.00 kJ m^{-2}	12.33	12.47	8.73	11.60	*11.28*
0.25 kJ m^{-2}	13.00	12.07	9.20	12.13	*11.60*
	12.67	*12.27*	*8.97*	*11.87*	

W = wound

NW = no wound

Bc = *B. cinerea*

4.6.2: ANOVA of effect of UV-C on time to 10% disease severity

Source of Variation	d.f. (m.v)	s.s	m.s	v.r.	F pr.
UV-C [A]	1	3.008	3.008	2.02	0.158
Inoculation trt [B]	3	254.625	84.875	57.04	<0.001
A x B	3	5.292	1.764	1.19	0.319
Residual	112	166.667	1.488		
Total	119	429.592			

4.6.3: Effect of UV-C on time to 100% disease severity

UV-C	Time to 100% disease severity				
	NW-Bc	W-Bc	W+Bc	W+Bc 48h	
0.00 kJ m^{-2}	20.20	20.07	15.93	18.07	*18.57*
0.25 kJ m^{-2}	20.40	18.53	15.60	18.40	*18.23*
	20.30	*19.30*	*15.77*	*18.23*	

4.6.4: ANOVA of effect of UV-C on time to 100% disease severity

Source of Variation	d.f. (m.v)	s.s	m.s	v.r.	F pr.
UV-C [A]	1	3.333	3.333	1.09	0.298
Inoculation trt [B]	3	341.467	113.822	37.30	<0.001
A x B	3	16.267	5.422	1.78	0.156
Residual	112	341.733	3.051		
Total	119	702.800			

4.6.5: Effect of UV-C on time between 10% and 100% disease severity

UV-C	Time to 10% disease severity				
	NW-Bc	W-Bc	W+Bc	W+Bc 48h	
0.00 kJ m^{-2}	7.87	7.60	7.20	6.47	*7.28*
0.25 kJ m^{-2}	7.40	6.47	6.40	6.27	*6.63*
	7.63	*7.03*	*6.80*	*6.37*	

4.6.6: ANOVA of effect of UV-C on time between 10% and 100% disease severity

Source of Variation	d.f. (m.v)	s.s	m.s	v.r.	F pr.
UV-C [A]	1	12.675	12.675	4.65	0.033
Inoculation trt [B]	3	25.092	8.364	3.07	0.031
A x B	3	3.692	1.231	0.45	0.717
Residual	112	305.333	2.726		
Total	119	346.762			

Suppression of grey mould on strawberry fruit with the chemical plant activator acibenzolar†

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Abstract: Grey mould caused by *Botrytis cinerea* is the most important post-harvest disease affecting strawberry fruit. This disease is normally controlled by application of fungicides. Increasing public concern over the use of conventional pesticides prompted an investigation as to whether induced systemic acquired resistance (SAR) might be used to help suppress *B cinerea* on strawberry fruit. Acibenzolar (*S*-methyl benzo[1,2,3]thiadiazole-7-carbothioate) is a chemical activator of SAR. When applied to strawberry plants at 0.25–2.0 mg AI ml^{-1}, acibenzolar delayed by about 2 days the development of grey mould disease on harvested strawberry fruit held at 5 °C. This delay was equivalent to a 15–20% increase in storage life of the fruit. This preliminary finding suggests that acibenzolar, or perhaps other chemical plant activators, could prove valuable in the commercial management of grey mould on strawberry fruit.
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Keywords: acibenzolar; benzothiadiazole; *Botrytis cinerea*; induced resistance; strawberry; systemic acquired resistance

1 INTRODUCTION

Grey mould caused by the fungal pathogen *Botrytis cinerea* Pers is the most important disease affecting strawberry (*Fragaria ananassa* Duch) fruit quality after harvest.[1] Although this disease can be controlled partially by certain pre-harvest cultural methods and post-harvest storage techniques, the strawberry industry is still heavily reliant on chemical fungicide sprays applied during flowering and fruiting.[2] There are, however, concerns over increasing loss of efficacy of conventional fungicides due to pathogen resistance and general unacceptability of fungicide usage in terms of public and environmental risk.[3–5] These concerns have favoured the introduction of integrated pest management (IPM) programmes.[6]

Exploitation of systemic acquired resistance (SAR) is a potentially desirable strategy in achieving IPM since it involves enhancing natural defence mechanisms in crops.[7,8] Certain biological, physical or chemical elicitors can be used to activate and/or boost natural disease resistance in non-infected plant tissue. Induced resistance through enhancement of plant defence metabolism has been shown to suppress development of a variety of crop diseases.[8–10] Amongst the range of natural and synthetic chemicals that activate SAR, acibenzolar (*S*-methyl benzo[1,2,3]thiadiazole-7-carbothioate; BTH) is perhaps the most potent elicitor.[10] However, in tobacco, BTH-induced resistance was not effective against *B cinerea*.[10]

This preliminary study investigated the potential for pre-harvest use of acibenzolar to suppress grey mould in harvested ripe strawberry fruit and the effect of acibenzolar on growth of *B cinerea* in vitro.

2 MATERIALS AND METHODS

2.1 Plant material

Cold-stored maiden-year strawberry plants, cvs Elsanta and Andana, were grown in a glasshouse at 20 °C in 1-litre capacity pots containing peat + polystyrene (10 + 1, by volume). They received a general N, P, K, Mg nutrient formulation twice weekly. Plants were sprayed with chlorpyrifos (1.5 litre ha^{-1}) at 2-week intervals, primarily to control spider mites (*Tetranychus* spp). They also received a single spray of myclobutanil (1.5 litre ha^{-1}) 3 weeks after planting to control powdery mildew (*Sphaerotheca macularis* Jacz f sp *fragaria* Peries). Sprays were applied to runoff. Plants received no fungicide active against *B cinerea*. Flowers were hand pollinated to avoid misshapen fruit.

2.2 Acibenzolar treatments

Acibenzolar 500 g kg^{-1} WG (Bion®; Novartis Crop

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Protection) was dispersed in distilled water to give spray solutions containing 0.25–2.0 mg AI ml^{-1}. These were applied to incipient runoff using approximately 10 ml per plant. In Experiment 1, a randomised complete block design was used for six chemical treatments (10 blocks × 6 treatments = 60). There were four sample plants per treatment. Andana plants received weekly sprays of 0, 0.25, 0.5, 0.75, 1.0 or 2.0 mg AI ml^{-1}. Spraying started 4 weeks after planting and continued for 9 weeks through flowering and fruiting. Anthesis of primary flowers commenced 2 weeks after the first application. Experiment 2 was a randomised complete block design with five chemical treatments (6 blocks × 5 treatments = 30). There were three sample plants per treatment. Elsanta plants received a single spray of 0, 0.25, 0.5, 1.0 or 2.0 mg AI ml^{-1} when the majority of primary flowers were at anthesis.

2.3 Fruit and disease assessment

Ripe fruit from each treatment were harvested at least twice a week during the fruiting season. Their weights were recorded. They were held in the dark at 5 °C and 95–100% RH in individual closed but vented containers. Disease severity resulting from natural infection was assessed as the percentage area of each fruit covered by grey mould, and was recorded daily.

2.4 Effect of acibenzolar on *Botrytis cinerea* growth *in vitro*

Solutions of acibenzolar were added to Potato Dextrose Agar (PDA) at 42 °C to give concentrations ranging from 0 to 2.0 mg AI ml^{-1}. Streptomycin (1.0 mg ml^{-1}) was also added to inhibit bacteria. Plates were kept at 5 °C until used.

A single-spored *B cinerea* isolate from a naturally infected Elsanta fruit was cultured on half-strength PDA at 22(±1) °C. Agar discs (4 mm diameter) were taken from the advancing margin of 4-day-old *B cinerea* cultures. These were positioned centrally on half-strength PDA plates containing acibenzolar. The plates were then closed with Nescofilm® and held at 22(±1) °C. The mean radial growth of the fungus was determined daily by measuring the diameter of the colony in two directions at right angles until the untreated control cultures had reached the plate periphery. There were four replicate plates per treatment.

3 RESULTS AND DISCUSSION

Acibenzolar treatments had no apparent phytotoxic effects on either fruits or plants. There was no significant difference in fruit weight between the control and acibenzolar treatments in either of the two experiments (Table 1). Absence of phytotoxicity was to be expected since reports of detrimental effects of acibenzolar on plant growth or yield are rare. Godard *et al*[11], however, found that acibenzolar at 0.0015–0.25 mg AI ml^{-1} reduced growth of cauliflower (*Brassica oleracea* L) seedlings in a dose-dependent fashion.

Table 1. Effect of acibenzolar treatment on weight of strawberry cv Andana fruit from plants sprayed at weekly intervals for 9 weeks (experiment 1) and cv Elsanta fruit from plants sprayed at anthesis of primary flowers (experiment 2).

Acibenzolar concentration (mg AI ml^{-1})	*Mean fruit weight (g)*	
	Experiment 1[a]	*Experiment 2*[b]
0.00	9.19	12.15
0.25	9.41	12.57
0.50	8.72	11.56
0.75	8.38	—[c]
1.00	8.73	11.87
2.00	8.39	12.03

[a] Experiment 1: SED = 0.483, 347 df, LSD (P = 0.05) = 0.95.
[b] Experiment 2: SED = 0.700, 253 df, LSD (P = 0.05) = 1.38.
[c] Concentration not included in experiment 2.

Effects on fruit disease as a function of acibenzolar treatment concentration were similar for both Andana (Experiment 1; sprayed weekly; Fig 1A) and Elsanta (Experiment 2; single spray; Fig 1B). Grey mould

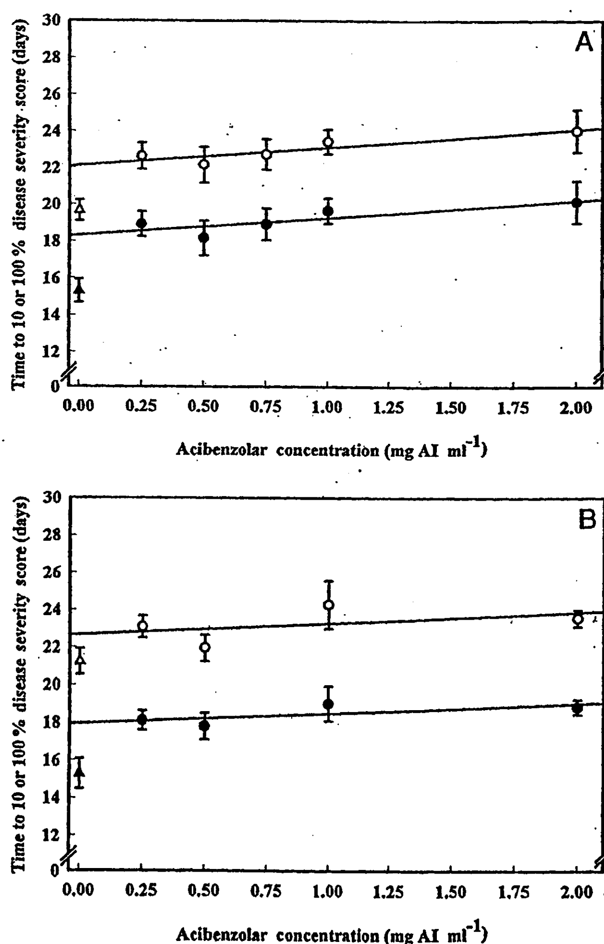

Figure 1. Effect of acibenzolar treatments on grey mould disease on strawberry fruits. A. cv Andana plants sprayed at weekly intervals over 9 weeks. B. cv Elsanta plants sprayed once at anthesis of primary flowers. (△ ▲) Untreated, and (○ ●) treated plants; (▲, ●) time to 10% disease severity; (△, ○) time to 100% disease severity.

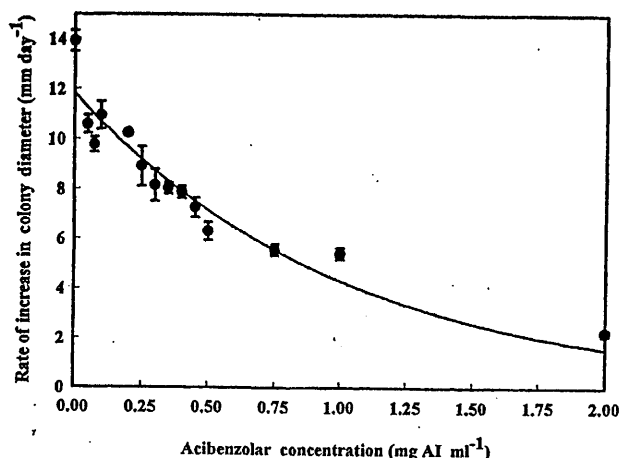

Figure 2. Effect of acibenzolar concentration on mycelial growth of *Botrytis cinerea in vitro*.

disease on harvested strawberry fruit held at 5 °C was suppressed by all pre-harvest acibenzolar treatments. Based on averaging across the five acibenzolar treatment concentrations, treated Andana fruit expressed 10% disease severity 3.9 days after control fruit (LSD [P=0.05]=1.7 days). The corresponding delay for 100% disease severity was 3.3 days (LSD [P=0.05]=1.7 days). For Elsanta fruit, and across four acibenzolar concentrations, times to 10 and 100% disease severity were increased to 3.2 days (LSD [P=0.05]=1.8 days) and 2.0 days (LSD [P=0.05]=2.0 days), respectively, compared with corresponding controls. The delay in symptom expression of about 2 days compared with untreated control fruit was equivalent to a 15–20% increase in storage life of the fruit.

The degree of disease suppression, judged as time to 10 or 100% disease severity was similar for all acibenzolar concentrations. With Andana fruit, linear regression, excluding control treatments, for time to 10% disease severity (y) against acibenzolar concentration (x) was

$$y = 18.314 + 0.946x, n = 5, r^2 = 0.700$$

(Fig 1A) The regression for 100% disease severity was

$$y = 22.118 + 0.982x, n = 5, r^2 = 0.814$$

(Fig 1A) With Elsanta fruit, the linear regressions excluding control fruit for 10 and 100% disease severity were

$$y = 17.934 + 0.533x, n = 4, r^2 = 0.518$$

and

$$y = 22.667 + 0.597x, n = 4, r^2 = 0.224$$

respectively (Fig 1B). The linear nature of these relationships and their shallow slopes suggest that the response to acibenzolar was on/off in nature. That is, the effect was not strongly dose-dependant over the range of concentrations tested.

Data from the *in vitro* experiment show that radial mycelial growth of *B cinerea* was progressively reduced with increasing acibenzolar concentrations from 0.05 to 2.0 mg AI ml^{-1} (Fig 2). The relationship between mycelial growth (y) and acibenzolar concentration (x) was described by the negative exponential relationship.

$$y = 11.841 \times \exp^{(-1.006x)}, n = 14, r^2 = 0.912$$

According to Kessman *et al*,[12] a true chemical activator of SAR should not exhibit direct antimicrobial activity. Friedrich *et al*[10] found that radial mycelial growth of a broad spectrum of fungi, including *B cinerea*, was not inhibited when they were grown on agar at a final concentration of 1.4 mM acibenzolar (0.294 mg AI ml^{-1}).

As noted above, and in contrast to the *in vitro* results, disease was not progressively suppressed *in vivo* with increasing acibenzolar concentrations. Although direct anti-microbial effects of acibenzolar in the plant cannot be discounted, the on/off suppression response *in planta* (Figs 1A and B) is inherently different in nature to the progressive suppression response *in vitro* (Fig 2). The on/off suppression response *in vivo* suggests that an induced disease resistance mechanism is operative.

4 CONCLUSION

Most published data demonstrating chemical activation of SAR are concerned with control of pre-harvest diseases of arable crops.[8] Relatively little attention has been paid to the potential for controlling post-harvest fruit diseases through enhancing plant natural disease resistance.[13] These two preliminary experiments suggest that the chemical plant activator acibenzolar could be valuable to the strawberry industry in helping to control grey mould disease on fruit. Our ongoing work with this material on strawberries aims to optimise suppression of *B cinerea* and to elucidate the SAR mechanism(s) involved.

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