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**THE MODE OF ACTION OF
SODIUM BICARBONATE / ADDITIVE
MIXTURES AGAINST CUCUMBER
POWDERY MILDEW DISEASE**

A thesis submitted in partial fulfilment of the requirements for the degree of
Master of Applied Science in Plant Pathology, Massey University.

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ABSTRACT

The host-parasite-complex of cucumber (*Cucumis sativus* L.) and *Sphaerotheca fuliginea* (Schlechtendal ex Fr.) Pollacci, the causal agent of cucumber powdery mildew, was studied to determine whether stimulation of host defences was a mode of action of sodium bicarbonate/additive mixtures. Additives included shellspray mineral oil and sunlight dishwashing liquid. The roles in cucumber defence of phenolic compounds, enzymes: peroxidase (PO) and phenylalanine ammonia-lyase (PAL), and induced structural barriers, papillae, were investigated. Comparisons were made between the effects of bicarbonate/additive mixtures and the plant extract Milsana on these host defences, as related to three levels (cultivars) of host resistance to disease: susceptible, disease-tolerant and resistant.

Long and short duration glasshouse studies found disease incidence highest on less resistant cultivars and lowest on the most resistant cultivar, 'Slice King' ('SK'). Applied alone at 2 g/litre, sodium bicarbonate failed as a protectant. When combined with either additive, the fungicidal activity of bicarbonate was enhanced. Shellspray oil and bicarbonate-oil mixtures were the most effective treatments for less resistant cultivars, 'Lebanese' ('Leb') and 'Slicemaster' ('SM'). These treatments and Milsana provided comparable control against disease affecting 'SK'.

The role of phenolic compounds was investigated in thin-layer chromatogram bioassays for antifungal phenolics in leaf extracts. This revealed much variability in free state phenolic patterns between host resistance/treatment combinations, in the presence and absence of disease. Glycosidically-bound phenolics hydrolysed into their free form, aglycones, were more abundant than free phenolics. Because of their frequency and arbitrary concentrations, it was unclear whether stimulation of aglycone production had occurred in infected leaves, as a result of any specific treatment or level of host resistance. The presence of these fungitoxic substances could not solely account for differences in disease severity. Hence, data did not support the concept of stimulation of host materials by either mildew infection or the treatments applied.

The responses of PO and PAL to treatment and infection were assessed by spectrophotometric measurements of activity in leaf samples. Higher enzymic-activities in healthy leaves were coupled with increasing levels of host resistance to disease. During the 14 days post-treatment, significant increases were detected in healthy and inoculated leaves treated with bicarbonate and Milsana; levels varied between cultivars. General enzymic-activity was higher in inoculated than noninoculated 'Leb' and 'SM', and of a comparable level in 'SK' controls and leaves treated with materials other than shellspray oil and bicarbonate-oil mixtures. For these apparent "normal" increases in plants treated with other materials occurred during this time.

Fluorescence and bright-field microscopy revealed similar numbers of conidia germinated 24 h after inoculation, irrespective of treatment and cultivar, although, significantly fewer conidia germinated on leaves treated with oil and bicarbonate-oil mixtures. With increasing host resistance the number of multiple germ tubes produced 72 h post-infection was less, papillae deposited in epidermal cells had increased, and the number of haustoria less, 120 h post-infection. Shellspray oil and bicarbonate-oil mixtures provided the most significant reduction in multiple germ tubes and haustoria, and the least apparent stimulation of papillae production.

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1. INTRODUCTION

1.1 PATHOGEN BIOLOGY

1.1.1 Taxonomy And Host Range

Sphaerotheca fuliginea (Schlechtendal ex Fr.) Pollacci is an ascomycete fungus classified in the Family Erysiphaceae (Alexopoulos & Mims 1979), and has been known as the primary causal agent of powdery mildew disease on members of the plant family Cucurbitaceae Juss. (cucurbits) since the 1800's (Zitter et al. 1996). Powdery mildew is perhaps the major factor limiting field and glasshouse production of cucurbits in most areas of the world (Hammett 1977; Cohen et al. 1996; Reuveni et al. 1996). All cucurbits, including the cucumber (*Cucumis sativus* L.), are susceptible regardless of the growing environment.

1.1.2 Symptoms Of Disease

Given favourable environmental conditions, disease can occur under glass throughout the year (Hammett 1977). The first symptoms of infection appear as small, dusty-white, circular to irregular, somewhat superficial areas on stems (Kapoor 1967), petioles and abaxial (upper) surfaces of older leaves and shaded lower leaves (Sitterly 1978; Zitter et al. 1996). These lesions increase in number and coalesce, eventually covering entire leaves (Agrios 1988). Figures 1 and 2 contrast healthy with infected leaves. Severely infected young leaves may turn brown and become shrivelled. Localised areas of chlorosis that later become necrotic, and death of an organ, may ensue.

Small pin-head size fruiting bodies (cleistothecia), at first white, later brown, and finally black when mature, may be present in older areas of infection (Agrios 1988). Roots are not affected but flowers are often colonised. Cucumber fruit are usually free of visible infection (Hammett 1977), even when foliage is heavily infested. However, they are likely to be fewer in number, suffer sun damage (sunscald), or ripen prematurely or incompletely, due to premature senescence of infected leaves (McGrath 1996). Such fruit have lower soluble solids and consequently poor flavour (Dougherty 1980). Late developing fruit often fail to mature and appear small and misshapen (Sitterly 1978). In addition, powdery mildew



Figure 1.1 Healthy leaves of cucumber (*Cucumis sativus* L.) 'Lebanese'



Figure 1.2 Lesions on leaves of cucumber 'Lebanese' characteristic of powdery mildew infection caused by *Sphaerotheca fuliginea* (Schlechtendal ex Fr.) Pollacci

infection predisposes plants to gummy stem blight (*Didymella bryoniae*) (Bergstrom et al. 1982). Thus infection can result in a reduction in yield and market quality, which appears to occur in proportion to timing and severity of disease development (Sitterly 1978).

1.1.3 Disease Cycle And Epidemiology

The primary source of inoculum consists of short-lived unicellular conidia, which mature in long chains on short euoidium-type conidiophores (Blumer 1967) (Figure 1.3). These arise at right angles from a network of mycelium on the leaf surface (Atkinson et al 1956), which is predominantly hyaline (translucent) in colour, darkening to brown with age (Kapoor 1967; Alexopoulos & Mims 1979) (Figure 1.4). Conidia are also hyaline, appearing smooth-surfaced (Hammett 1977), ellipsoid or barrel-shaped, and roughly 25-37 x 14-25 μm in size (Kapoor 1967). They contain an average of 15 distinct watery vacuoles and well-developed cell inclusions called fibrosin bodies (Hammett 1977), that can be clearly seen when fresh conidia are dusted onto clean glass slides or mounted in 3% aqueous KOH, and then viewed using polarised or transmitted light (Kable & Ballantyne 1963) respectively. Under conditions of low light intensity (Zitter et al. 1996), 95-97% relative humidity (Zaracovitis 1965), temperatures ranging from 22-31°C, and the absence of free water (Alexopoulos & Mims 1979), conidial germination commences within 2 h on a suitable host (Sitterly 1978). Conidia are self-sufficient in water and nutrients and can remain viable for 7-8 days (Zitter et al. 1996). If kept in contact with water on the plant surface they can be rendered nonviable, or may germinate abnormally and exhaust their food reserves without forming appressoria. The first or primary germ tube which emerges is short, and forms a convoluted appressorium which attaches to the epidermal cell 2-6 h post-inoculation. The appressorial germ tube matures at 8-12 h after inoculation and attempts penetration (Carver & Zeyen 1993).

Erysiphe cichoracearum was considered to be the primary causal organism of powdery mildew throughout most of the world before 1958 (Zitter et al. 1996). Today *S. fuliginea* is more commonly reported in glasshouses worldwide. It is thought that a shift in the predominance of these two fungi may have occurred, or that *S. fuliginea* was in the past misidentified on cucurbit hosts as *E. cichoracearum*, since criteria for identifying these

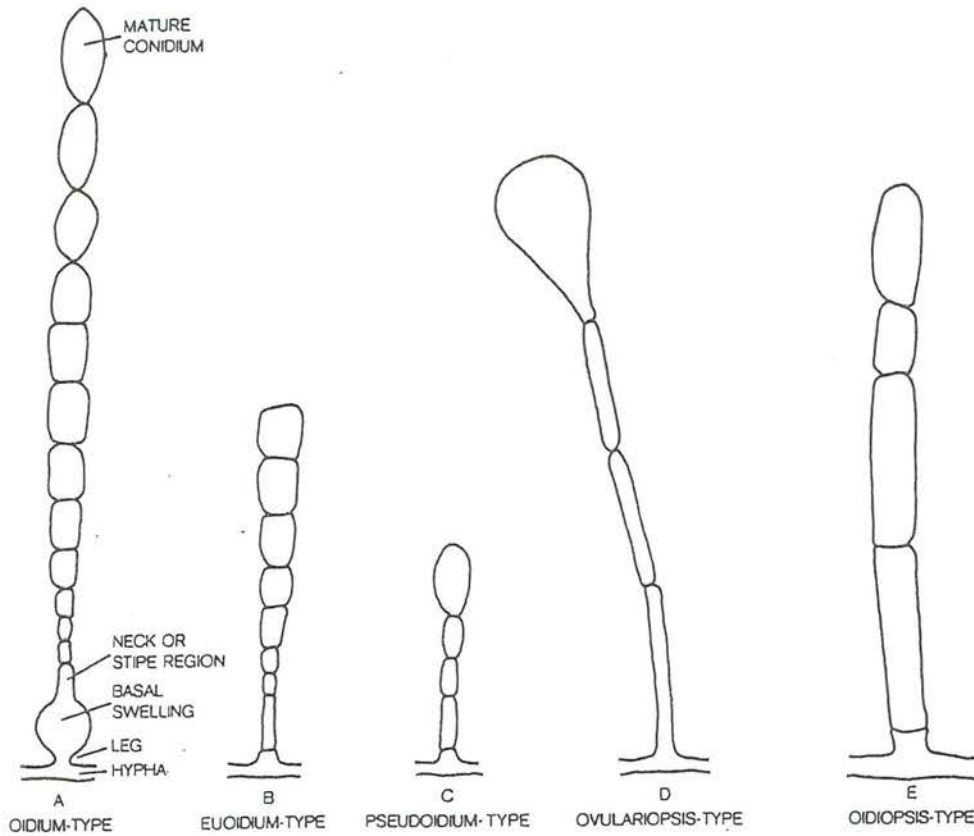


Figure 1.3 Morphology of Erysiphaceae (powdery mildew) conidial chains. Examples of each are A-*Erysiphe graminis* f.sp. *tritici*; B- Cucurbit powdery mildew (*Sphaerotheca fuliginea*); C-Grape powdery mildew (*Uncinula necator*); D-*Ovulariopsis* sp. (on *Vitex lucens*); E-*Oidium* sp. (on *Magnolia*) (Adapted from Hammett 1977)

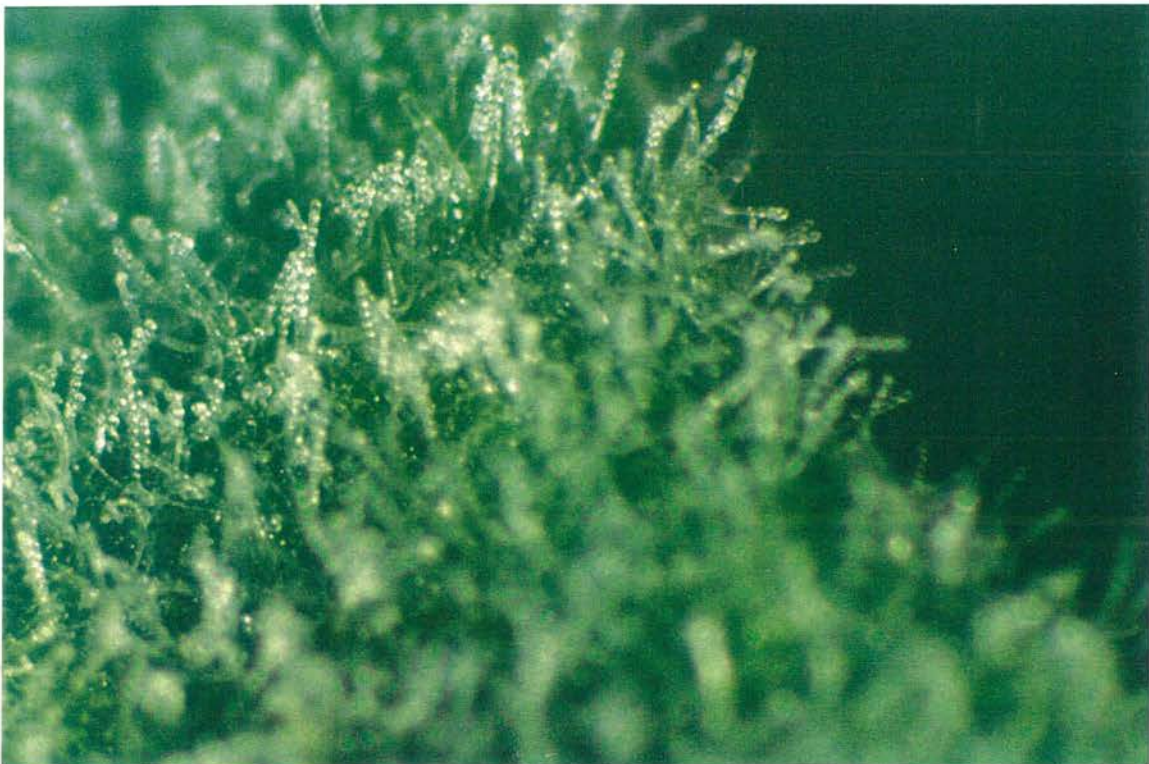


Figure 1.4 Conidial chains of *S. fuliginea* arising from a leaf of cucumber 'Lebanese'

fungi from the conidial stage were not developed and published until the 1960's. Modern classification systems by Yarwood (1973) and Hammett (1977) distinguish the species on the basis of:

- i. differences in climatic requirements:-*S. fuliginea* is more prevalent during the warmer months.
- ii. morphological features (Blumer 1967):-edge lines produced by immature conidial chains are sinuate in *E. cichoracearum* and crenate in *S. fuliginea*
 - fibrosin bodies are found only in *S. fuliginea*
 - The germ tubes of *S. fuliginea* are short, thick and occasionally *forked* (Zaracovitis 1965; Hammett 1977), ending in convoluted appressoria (Sitterly 1978), whereas those of *Erysiphe* spp. are simpler in form (ie. straight and unlobed (Zitter et al. 1996)).

An infection peg forms beneath the appressorium, and penetrates through the host cuticle and epidermal cell wall, growing into the centre of the cell lumen. The plasma membrane invaginates, enlarges, and a specialised globular or club-shaped structure called an haustorium is formed in the cell lumen (Kapoor 1967; Clarkson 1992). This is separated from the neck by a septum and consists of a central body with many lobes embedded in an extrahaustorial matrix surrounded by an extrahaustorial membrane (Heintz & Blaich 1990). Due to the manner in which *S. fuliginea* obtains nutrition, the fungus is referred to as a biotrophic or obligate parasite, since growth and reproduction are entirely dependant on the fungus obtaining nutrition from a living host whose metabolism is still functional. Unlike necrotrophs which derive their nutrition from dead or dying cells (Clarkson 1992), powdery mildew fungi cannot utilise the breakdown products of host degradation. They rely on the assimilates produced by photosynthesis of the host plant, at least at the beginning of the infection cycle (Heisterüber et al. 1994).

It is through haustoria that mineral nutrients, water, amino acids, and carbohydrates (such as hexoses), are absorbed from the protoplast, in a complex interactive process

between the cell cytoplasm and specialised tissue surrounding the body of the haustorium (Bushnell & Gay 1978). While the first haustorium is establishing, additional germ tubes are formed from other points on the same conidium, and hyphae grow out from the primary appressorium along the leaf surface (Sitterly 1978). Hyphal cells are thin-walled, vacuolate, and contain a single, large nucleus. Along the hypha are lateral swellings or lobed appressoria, one on each alternate hyphal cell (Sitterly 1978) which infect epidermal cells as before. This growing network of haustoria securely anchors the fungus to the host surface (Alexopoulos & Mims 1979). This non-ramification of fungal mycelium deeper than the epidermal cells results in ectoparasitism.

Physiological studies of infected cucumber leaves have shown increases in the rate of respiration, and impaired rates of photosynthesis (Abo-Foul et al. 1996). A temporary stimulation of the rate of photosynthesis, is often followed by sudden decline in both net and gross photosynthesis (Abo-Foul et al. 1996). Evidence provided by electron micrographs, associates this decline with marked deteriorations in the morphological organisation of chloroplast membranes, including damage to thylakoids, and loss of the stroma (and Calvin cycle enzymes through tears in the chloroplast envelope), with consequent increases in levels of uncoupled chlorophyll that can be utilised by haustoria. This reduction in light-harvesting capacity of the plant's primary photosynthetic organs, ultimately results in poor crop production.

The time between infection and appearance of the pathogen is usually short and 3-7 days after infection, many conidiophores have formed (Sitterly 1978; Zitter et al. 1996). A generative cell at the apex of each conidiophore produces conidia which are not abstricted, but cling together in chains. The actual method of abstriction for dispersal is unknown, but it is thought to follow a diurnal cycle (Yarwood 1977), and dispersal is almost exclusively by air currents in glasshouses (Blancard et al. 1994). One life cycle is estimated to be completed in 5-6 days (Yarwood 1957).

When environmental or nutritive conditions of the host become unfavourable (Sitterly 1978), and two sexually compatible mating types come into contact, the fungus may produce one or several asci inside a closed ascocarp, the cleistothecium (Sitterly 1978; Alexopoulos & Mims 1979; Agrios 1988). These overwintering fruiting bodies are 66-98 μm in diameter, globose, with a variable number of basal, mycelioid (resembling mycelium

in being flaccid and indefinite (Alexopoulos & Mims 1979)) taxonomically characteristic appendages that are interwoven with the mycelium (Kapoor 1967). When ascospores are mature they are forcibly discharged as the cleistothecium ruptures under the strain of swelling asci. Increased genetic diversity resulting from sexual reproduction poses a threat to disease control for it could include new combinations of virulence genes and fungicide resistance (McGrath et al. 1996a).

Although the conidial (anamorphic) state of *S. fuliginea* is extremely common, cleistothecia (teleomorph) have rarely been reported on cucurbitaceous plants in fields or glasshouses around the world. McGrath et al. (1996b) recently charted their global location, and shows that to date, cleistothecia have only been observed in New Zealand in 1956 (Boeswinkel 1976) and in 1958 (Hammett 1977).

Three genetically distinct races (ie. mating groups within the species *S. fuliginea*) have been identified on cucurbits (Cohen et al. 1996; Zitter et al. 1996). They show pathological specialisation, attacking specific host genotypes within a species and also differ in their tolerance to fungicides (O'Brien & Weinert 1994). Race 1 and 2 are the most cosmopolitan, with race 3 being restricted at the moment to the United States (Floris & Alvarez 1996).

1.2 HOST BIOLOGY

1.2.1 Taxonomy And Growth Habit

The cucumber (*Cucumis sativus* L) is classified within the family of flowering plants known as the Cucurbitaceae Juss. It is indigenous to India, where it has been cultivated for over 3000 years (Sitterly 1978). A chromosome count of 14 and morphological features including angular stems, distinguish the cucumber from other members of the genus *Cucumis*, which have 24 chromosomes. Cucumbers are annuals, frost-sensitive, and have adopted a mesophytic requirement for water and humidity (Watson & Dallwitz 1996). Most grow as vines, but some modern cultivars produce compact, bushlike plants (Zitter et al. 1996).

1.2.2 Floral And Fruit Morphology

Cucurbit flowers are solitary or aggregated in axillary inflorescences. They range in colour from green, white, yellow to orange, and are variable in size. Species have a gynoeocious flowering habit ie. produce mainly female flowers and few male on the same plant. Fruits are usually fleshy and indehiscent (ie. do not split open to release seeds contained within) (Watson & Dallwitz 1996). Mature fruit are consumed fresh or cooked, and are valued for flavour and flesh texture qualities that make them ideal meat accompaniments, dessert and salad ingredients. Fruit possess a high water content and are relatively low in nutritional value, the most notable constituents being vitamins (A and C), and minerals (Zitter et al. 1996).

Seeds of several cultivars are important sources of oil and protein in parts of Africa, Asia and Latin America, and the skins have been utilised as material for baskets, insulation, oil filters, and as alternatives to pottery utensils (Sitterly 1978). The two major types of cucumbers are processing (pickling) and fresh (slicing). Varieties of the former are grown almost exclusively outdoors, while slicing varieties are grown outdoors and in glasshouses.

In New Zealand, cucumbers are grown solely for the local market. Estimates of crop production and the impact of powdery mildew disease with respect to this are sketchy due to a lack of complete survey data (M. Nicholls pers. comm.). Recent estimates place the total yield at 10 ha (c. 250 tons/ha) per growing season (M. Nicholls pers. comm.), with growers harvesting between 2-3 crops a season. Table 1.1 indicates the market value of cucumbers that make it an important speciality crop.

Table 1.1 Average prices (\$) per container for New Zealand-grown cucumbers^a

Average Prices Per Container							Average Prices Per Container						
	Difference between May 1995 and May 1996							Difference between May 1995 and May 1996					
	Jan	Feb	Mar	Apr	May	%		Jan	Feb	Mar	Apr	May	%
Apples	22.13	24.80	15.61	14.95	13.14	-22.98%	Cucumber short	15.04	19.74	16.07	14.94	25.76	28.10%
Avocado	21.13	24.70	36.98	32.84	29.05	-39.54%	Cucumber field	15.47	20.69	21.92	21.28	36.46	17.20%
Broccoli	14.59	15.25	15.94	13.56	14.79	-52.18%	Kumara	40.4	44.50	28.81	31.44	30.55	15.37%
Cabbage	4.23	6.27	7.36	6.42	4.48	-58.86%	Lettuce	9.51	10.30	6.28	5.52	17.07	-18.13%
Capsicum	15.90	9.20	10.76	13.26	20.90	34.66%	Lettuce Fancy	10.27	12.95	9.17	7.35	13.07	-21.92%
Carrots	11.56	11.50	6.46	6.42	6.60	-23.79%	Mushrooms	13.53	16.92	16.18	16.37	16.78	-10.69%
Cauliflower	8.38	7.00	17.12	9.46	9.00	-50.63%	Onions (table)	10.22	6.29	3.99	4.26	3.24	-74.61%
Celery	16.49	10.93	10.54	9.07	8.40	-39.91%	Potatoes	4.76	5.54	5.41	5.34	4.81	-46.56%
Chinese Cabbage	6.03	7.64	6.01	6.47	8.74	1.04%	Strawberries	18.48	30.18	33.68	36.58	43.99	38.03%
Courgettes	15.90	14.08	14.85	15.14	31.45	63.53%							

The above figures should be taken as a guide only. They encompass more than one size of container in some products, so will be misleading if used as finite references for values.

^a Source of figures: Turners & Growers Wholesale Fruit And Veg Report, Horticulture News, July 1996.

1.3 PLANT DEFENCE MECHANISMS

To survive the selection pressure imposed by herbivores, pests and pathogens during coevolution, plants have acquired effective structural features and biochemical mechanisms for disease resistance to defend themselves against attack (Keen 1990; Simms 1996). The mechanisms determining the resistance phenotype are unknown, but it is generally accepted that plants utilise a large arsenal of constitutive and inducible (active) defence mechanisms to prevent colonisation by pathogens (Kombrink et al. 1993). The same defence mechanisms are often utilised by a diversity of plant species against different types of pathogen: fungal, bacterial and viral, in both host and non-host resistance. Depending on the nature of invasion, products of the "same" biosynthetic pathway might accumulate, thus limiting the spread of a pathogen (El Ghaouth 1994). Examples of common defence reactions are given in Figure 1.5. These defences can be grouped into preformed and post-formed barriers to infection which may be structural (physical) or biochemical in nature.

The literature on plant defence mechanisms is extensive. The scope of this work does not allow for more than a brief overview of these. Several excellent reviews are available (Blanchette & Biggs 1992; Isaac 1992; Bennett & Wallsgrave 1994) and should be referred to for fuller comment. A thorough account of protein synthetic responses to

environmental stresses, including pathogen challenge can be found in Artlip & Funkhouser (1995).

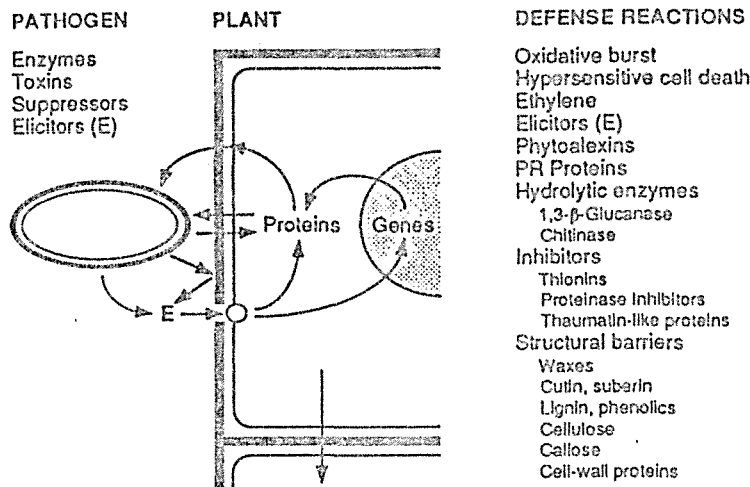


Figure 1.5 Schematic representation of plant-pathogen interactions and a list of typical plant defence responses (adapted from Kombrink et al. 1993).

1.3.1 Physical Barriers To Disease

Physical or mechanical impedance of hyphal growth is thought to be the first line of defence (Taylor 1987) against fungal pathogens. The first barriers to invasion are the morphological characteristics of the host's surface which influence the arrival and retention of inoculum (Royle 1976). Morphological features may operate in resistance in two ways:

- i. passively- as preinfectious barriers characteristic of the unchallenged, healthy host, such as the thickness of the cuticle, trichome density, structure and composition of cell walls, timing of stomata opening.
- ii. actively post-infection- to restrict pathogens and toxins from entering healthy tissue. Cork production, tylose formation in lumen of xylem vessels, extra lignification of cell walls and papillae, are examples of structures formed in response to the pathogen's

invasion (Royle 1976). The significance of papilla formation in host cells in response to fungal attack were discussed in depth by Aist (1976).

1.3.2 Biochemical Barriers To Disease

In most cases, infection by plant pathogenic microorganisms stimulates an active defence response in the metabolism of their target plants. Responses of plants vary, but depend ultimately on the interaction of genetic factors in both the host and pathogen (El Ghaouth 1994). These generally begin with molecular events:

- i. alterations in protein synthesis overall and,
- ii. shifts in levels of specific proteins (Artlip & Funkhouser 1995). Responses further depend on the nature, duration, and severity of the stress; and are frequently expressed as a combination of structural and biochemical changes (Matern & Kneusel 1988) which can be detected by modern microscopy, assaying and molecular techniques. Preinfectious biochemical barriers have included fungitoxic compounds such as phenolics inherent in the waxy cuticle (Adaskaveg 1992), cuticular exudates and leachates. Several phenolics have been observed in healthy cucumber leaves (Callebaut 1984; Daayf et al 1995) but their identity is unknown. Cyanogenic glycosides have also been detected in Cucurbitaceae, though most plants are noncyanogenic (Watson & Dallwitz 1996). There is still some debate as to their involvement in plant resistance to fungal pathogens.

The most frequently observed and best characterised biochemically active defence reactions are:

- i. Rapid, localised cell death in and around the infected area (hypersensitive response: **HR**)- The hypersensitive reaction of plants to obligate parasites results in increased oxidative enzyme activity and a rapid enhancement of oxygen uptake. This is a sequence of events that occurs in the latter stages of a compatible host-pathogen interaction. The pattern is similar in both compatible (susceptible) and incompatible (resistant) host-pathogen interactions, however, when incompatible, the events proceed with greater rapidity. It is thought that the rapid development of the HR confers resistance upon the host plant (Artlip & Funkhouser 1995).

- ii. Synthesis and deposition of phenolic compounds and proteins in the cell wall- phenolics are usually defined as substances that possess an aromatic ring bearing a hydroxyl group or its functional derivative (Mettraux & Raskin 1994).
- iii. Increases/decreases in an existing pool of proteins known as **PRs** (pathogenesis-related proteins) reported to have a role in defence against pathogen development (Punja & Raharjo 1996). This pool includes the oxidative enzymes peroxidase and polyphenoloxidase, the hydrolytic enzymes β -1,3-glucanase, hydroxyproline-rich glycoproteins and the chitinases. Two general groups of chitinase are known to occur in plants, acidic and basic. Acidic (extracellular) chitinases have been postulated to lyse hyphae of invading fungal pathogens during the early stages of pathogenesis and to release fungal cell wall fragments, which in turn may activate other defence-related mechanisms (Punja & Raharjo 1996). Several acidic chitinase isoforms have been induced in cucumber leaves susceptible to *S. fuliginea* in response to treatment with chitosan, that were constitutive in resistant cucumbers (Zhang & Punja 1994). The basic (vacuolar) forms may affect hyphae following plant cell collapse, when the vacuolar contents are released into the extracellular space.
- iv. The *de novo* appearance and accumulation of protein(s)- this includes low molecular weight nonproteinaceous antimicrobial compounds, phytoalexins (Matern & Kneusel 1988), and structural materials like suberin and lignin after challenge by microorganisms and exposure to stresses. The presence of phytoalexins in cucumber has been claimed (Strange & Alavi 1981; Hammerschmidt & Kuc 1982) but there are also reports on the failure to detect them (Bailey & Mansfield 1982). Lignin reinforces cell walls at the site of attempted penetration. It is also an important component of papillae, structures deposited by the host cytoplasm, especially in response to biotrophic fungi, between the plasma membrane and cell wall, to likewise prevent penetration. Changes in the morphology of the host cell wall and plasmalemma, and also differences in the extent and rate of lignification, speed and quality (ie molecular composition) of papillae formation, are considered critical to the success of the expression of resistance against ectoparasites.

1.4 CURRENT METHODS OF CONTROLLING GLASSHOUSE DISEASES

1.4.1 Chemical Control

Glasshouse production forms the basis of high-value crop industries around the world (Elad et al. 1996). In general, commercial glasshouse operations aim at supplying high quality, high value products to specific markets (Menzies & Bélanger 1996). To achieve adequate economic returns, these crops are grown with intensive labour inputs and a high degree of advanced technology (Jarvis 1992). Disease management of glasshouse crops offers a particularly difficult challenge to growers because environmental conditions maintained at an optimum for plant growth are often conducive to disease development (ie. benefit the development and spread of pathogens) (Menzies & Bélanger 1996). Striking a balance between the two using whatever means necessary has invariably been the goal.

To avoid a loss of crop quality and reductions in yield (McGrath 1996), control strategies against *S. fuliginea* have relied heavily on two groups of active ingredients, azoles (dimethylation inhibitors: DMIs) and morpholines/morpholine-like compounds (Felsenstein et al. 1994). Both inhibit ergosterol biosynthesis of the fungus, but act on different steps in this pathway (Felsenstein et al 1994). Ergosterol plays an important role in the structure and function of fungal membranes. These synthetic materials also differ in their fungicidal modes of action, acting in either of two ways, as:

- i. Protectant fungicides- Are most active when applied before inoculum arrival, persisting as a fungitoxic barrier on the plant surface to infection and re-infection. These are phytotoxic if they enter the plant in any appreciable quantities (Manners 1993). They usually affect several fungal metabolic processes (O'Brien et al. 1988), so are often referred to as 'broad spectrum' or 'non-specific' in activity. Application reduces sporulation and ultimately the amount of inoculum that is available in glasshouses to infect crops, and also the rate of increase of the pathogen ('r'- see below).
- ii. Systemic fungicides- Have post-infection activity. These are translocated through the host to the site of infection where they act on specific pathways of fungal energy metabolism and nuclear processes to reduce sporulation and eradicate established

infections. They are often referred to as ‘site-specific inhibitors’, and are applied less frequently than protectants, as they are not degraded by environmental forces such as wind and rain. New growth is protected but the strength of chemical activity becomes diluted as time passes due to loss of plant organs.

Inoculum pressure and the high crop value demand that frequent applications of the most effective fungicides be applied before the most significant part of an epidemic, the “logarithmic phase”, to minimise the infection rate (Elad et al. 1996). Van der Plank (1968) suggested that the rate of disease development could be mathematically described at any time by the following equation:

$$X = X_0 e^{rt}$$

where: X = Pathogen population (level of infection) at time ‘t’

X_0 = Pathogen population at time ‘0’ (or the initial amount of inoculum)

r = rate of increase of pathogen

t = time period being considered

e = mathematical constant: 2.718

Any factor that reduces ‘ X_0 ’, ‘r’ or ‘t’ will reduce ‘X’, the amount of disease. A reduction in ‘t’ is often more difficult to achieve in practice, especially with respect to glasshouse crops, so more emphasis is placed on reducing ‘ X_0 ’. Where ‘t’ can be influenced, typical strategies employed could include a manipulation of planting or harvest dates, or fungicide usage to delay infection.

Of the few fungicides registered for the control of powdery mildew on glasshouse crops, several are rapidly losing their efficacy because of the emergence of resistant pathogen strains (Menzies & Bélanger 1996). Repeated usage of at least three popular systemic fungicides (benomyl, pyrazophos and triadimefon) over several growing seasons, has led to a partial or complete loss of sensitivity amongst races of *S. fuliginea*. This has occurred in production areas around the world, including the United States (as early as 1969) (Elad et al. 1989; Ziv & Zitter 1992), Europe, the Middle East and New Zealand. Their use in cucurbit production is now discouraged (Anon. 1996).

The success of resistant strains is in their ability to evolve new metabolic pathways to replace those targeted by site-specific fungicides, thus allowing them to function pathologically as normal (McGrath et al. 1996). Once they appear, they can survive for several years, so with continued use/long-term exposure of pathogens to previously effective fungicides, there is a high risk of promoting resistant populations of increased fitness in a short time (Reuveni et al. 1996).

This situation has resulted in renewed efforts to develop biologically-based control methods against powdery mildew. Various management strategies have been proposed for grower adoption to provide a balance between the effective control provided by systemic fungicides and the resistance problems that are associated with their over-use (McGrath 1996). Most are based on the alteration or mixture of site-specific with multi-site inhibitors, restricting their application to a specific use, or limiting the number of applications in glasshouses per year (O'Brien et al. 1988).

Reducing fungicide inputs necessary for profitable agriculture is also desirable, where feasible, because their application constitutes a considerable input cost (Warrington 1996), and local and international societies have expressed concern (Menzie & Bélanger 1996) about adverse effects of pesticides on:

- i. The environment and ultimately the sustainability of the ecosystem- from air, water and soil contamination (Tzeng et al. 1996);
- ii. Human (and animal) health- fungicides should not constitute a hazard to workers who apply agricultural chemicals, or to consumers of fresh produce as a result of residues detected on/in the final product (Anon. 1996). Consumers have become fearful of pesticide residues and their possible side-effects if ingested. Some have opted for organically grown produce but there are limits to the number of sulphur and copper sprays that can be applied in organic orchards without having detrimental effects on soil nutrients.
- iii. Trade in primary produce- Fungicides are used extensively to eradicate diseases on horticultural produce in order to satisfy the quarantine requirements of importing countries (Anon 1996). Many countries now enforce strict regulations on levels and types of chemical residues permitted on domestic and imported produce destined for human consumption (Manners 1993). One example of this concerns produce treated with

Benlate™ (Du Pont) (McGrath 1996). The use of agricultural compounds should not result in breaches of domestic and overseas food standards.

- iv. Non-target organisms, beneficial insects and natural enemies- especially those in glasshouses (the crop ecosystem) that biologically control insect pests.

1.4.2 Resistant Varieties

Genetic engineering of disease resistance is one of the main components of integrated plant protection systems (Simms 1996). Disease-tolerant hybrids of the long and short-type cucumbers grown in New Zealand glasshouses for example, 'Suprami F.1' and 'Maestro F.1' (Watkins New Zealand Ltd.), that display desirable characteristics and which are only moderately susceptible to powdery mildew, are available to growers to reduce 'X_t' (see above).

When developing resistant cultivars, attention must be given to the type of resistance bred for. In many cases, resistance is controlled by single dominant resistance genes in the host cultivars that match cognate dominant avirulence genes in the pathogen race (Kmecl et al. 1995). Although this type of resistance (also known as vertical or major gene resistance) can be very effective, problems arise concerning its stability or longevity and durability (Floris & Alvarez 1996). Durability is determined by:

- i. the mutation rate of the pathogen,
- ii. selection pressure placed upon mutant strains. Race-specific resistance is often short-lived because of the relatively frequent evolution of new mutant pathogen races that overcome this resistance (Kmecl et al. 1995). There are many instances where major gene resistance has been defeated by populations of *S. fuliginea* (McGrath et al. 1996a,b).

A more stable type of resistance is quantitative field resistance, which describes the degree of pathogen-resistance observed in the field. It may be the best approach to avoid the easy breakdown of resistance by the pathogen, and to reduce the rate of pathogen multiplication, if not entirely preventing infection. Briefly, new cultivars are bred with several different genes for resistance, with different mechanisms of action. The level of resistance observed may actually be the composite result of different resistances such as environmentally determined resistance, acquired resistance which can be triggered by biological and chemical inducers, resulting in an increase in nonspecific resistance to many different pathogens; and is usually

inherited polygenically (Kmecl et al. 1995). However, at present it is not anticipated that genetic control will eliminate entirely the need for fungicides in glasshouses (McGrath et al. 1996). A thorough discussion of the genetics of plant (host) resistance and pathogen virulence can be found in an article by Simms (1996).

1.5 ALTERNATIVES TO CONVENTIONAL FUNGICIDES

Alternatives to existing synthetic compounds or at least methods for reducing the dependence on chemicals through integration with existing programs of pest and disease management together with resistant cultivars must be considered, thereby reducing injudicious pesticide use and the risk of resistance to all fungicide groups in the programme.

1.5.1 Biological Control

Research has demonstrated the potential of biological approaches for plant disease control, but there are only a few commercialised systems for the biocontrol of foliar diseases. This lack arises for several reasons (Elad et al. 1996):

- i. availability of cheap and effective fungicides which can be easily applied;
- ii. biotic and abiotic factors in glasshouses, including the microclimate and surface conditions of plant parts, do not favour survival and activity of introduced microorganisms;
- iii. some systems perform less effectively than potent chemicals, and their effectiveness is not always consistent;
- iv. the formulation and distribution of biocontrol preparations is regarded as far more difficult than for synthetic chemicals.

The literature on biological approaches to the suppression of powdery mildew diseases is extensive, and excellent overviews are provided by Elad et al. (1996) and Menzies & Bélanger (1996), which may be consulted for a thorough treatment of the subject. It appears that powdery mildew fungi are vulnerable to antagonistic microorganisms, due to the

superficial development of all powdery mildew fungal parts except haustoria (Elad et al. 1996). Three natural antagonists have been tested beyond the laboratory and show promise for commercialisation (Menzies & Bélanger 1996):

- i. The hyperparasitic coelomycete *Ampelomyces quisqualis* Ces. Schlecht. (Abo-Foul 1996) which inhibits conidial production and cleistothecial formation. Problems with high humidity requirements hampered the efficacy of early formulations of this antagonist (Menzies & Bélanger 1996). Recently however, the Ecogen company developed a formulation based on an isolate (AQ10) selected for its tolerance of lower humidities. AQ10 is currently being evaluated under glasshouse conditions for the control of *S. fuliginea* (Hofstein 1994; Menzies & Bélanger 1996).
- ii. Several species belonging to the ballistospore-forming yeast genus *Tilletiopsis* (Hoch & Providenti 1979; Urquhart et al. 1994) have reduced the density of *S. fuliginea* conidia on glasshouse-grown cucumbers through the act of hyperparasitism
- iii. Several species belonging to the yeast-like epiphytic genus *Sporothrix* (Bélanger et al. 1994) have also restricted the development of *S. fuliginea* on glasshouse-grown cucumbers through a different mode of action, antibiosis.

1.5.2 Cultural Control

Heated glasshouses, and restricted ventilation coupled with wet conditions, especially on the lower, shaded plant parts, often results in the glasshouse atmosphere remaining saturated for long periods of the day (Elad et al. 1996), and thus makes the environment very conducive to disease development. The temperature requirements of *S. fuliginea* for growth and reproduction overlap with conditions prevailing in glasshouses, up to a maximum of c. 30-35°C (Elad et al. 1996). Therefore modifications of the glasshouse environment based on climate control alone have so far had little effect against *S. fuliginea*. Sanitation which generally involves removal or destruction of all disease material is also practiced.

1.5.3 Biocompatible Fungicides

A Coating polymers-Antitranspirants, mineral oils (derived from highly refined petroleum), plant oils (eg. canola, safflower, soybean), and surfactants have been used in multiple-application programs as artificial barriers to infection on abaxial leaf surfaces. Research indicates that they have a broad spectrum of action against foliar pathogens on various host plants, including powdery mildew on cucurbits (Ziv & Zitter 1992), rose, hops, lilac, euonymous, apple, hydrangea; and powdery and downy mildews on grapevines (Northover & Schneider 1996). Extra benefits of these polymers include:

- a lack of phytotoxicity
- their biodegradability
- permeability to gases
- resistance to weathering in the short-term.

Surfactants are integral constituents of many pesticides because they favour absorption and cuticular penetration of the active ingredient (Mayr et al. 1994). There is some evidence that surfactants effect changes in photosynthetic and transpirational behaviour, and phenolic biosynthesis. Mayr et al. (1994) observed that Triton-X-100 induced phenol synthesis when applied to leaves and fruit of apple trees, and further, phenol accumulation after treatment was more pronounced in young than in mature leaves.

B Photoactivated pesticides-Control of powdery mildew on cucurbits and other crops, in recent field and glasshouse trials, has been achieved to levels equivalent or superior to conventional chemicals by weekly applications of a formulation developed from a methionine-riboflavin mixture (Tzeng et al. 1996). The observed morphological destructive effects on mildew colonies have suggested that the biocidal activity is one of eradication, through the action of various highly reactive oxygen radicals or active oxygen derivatives in this complex, which are generated by light. Particularly vulnerable are microbial cells hyaline in colour, such as the thalli of ectoparasitic powdery mildew fungi. The formulation is unique because it contains mainly food constituents and biodegradable ingredients in quantities considered too small to pose any danger to the environment.

☞ Simple inorganic salts eg. silicates, bicarbonates and phosphates in conjunction with surfactants- Chemicals with low mammalian and environmental toxicities (Horst et al. 1992) such as oxalate, bicarbonate, potassium and di- and tri-phosphate salts, alone and in combination with surfactants, have been investigated worldwide for their potential to control plant diseases. Sodium bicarbonate (NaHCO_3), commonly used by humans as a medicine, a toothpaste, an ingredient in baking powder and as a food additive for swelling bread and biscuit dough (Long et al. 1994), in quantities well above those which would be consumed from spray residues on fleshy plant organs and vegetative material, combined with mineral oil or liquid detergents has also shown considerable potential as a non-toxic disease control material.

Sodium bicarbonate is produced from the treatment of a strong NaCl solution with ammonia and CO_2 (Youngson 1994). The fungicidal properties of sodium bicarbonate against powdery mildew pathogens were first recognised by Currey (1924) as an effective way to control rose powdery mildew (*Sphaerotheca pannosa* var. *rosae* (Wallr. ex Fr.) Lev. In the late 1970's, research in Japan by Homma et al. showed that sodium bicarbonate inhibited powdery mildew on cucumber and in addition, the fungicidal effect was greatly enhanced when it was combined with surfactants (Homma et al. 1981a; Homma et al. 1981b; Homma et al. 1981c). Surfactants (surface-active agents) are materials usually chemically fairly inert, which are often added to agrichemicals to improve their adhesion and distribution on leaf surfaces, or their penetrating properties (Manners 1993) if a systemic. Many surfactant oils and emulsifiers are fungicidal in their own right (Amer et al 1993). Several researchers found that mixtures of fatty alcohols, methyl esters of fatty acids (Burchill et al 1979) and nitrogen-containing surfactants (Clifford et al 1981) eradicated apple powdery mildew (*Podosphaera leucotricha*) from infected buds. Hunter et al (1982) considered that the ability of a surfactant to control powdery mildew was independent of the electrical charge which its particles carried (Manners 1993): non-ionic, cationic, anionic or amphoteric, a property reputed to affect the efficacy of the material. Researchers in Israel recently found that soapy water in the form of a new detergent Zohar LQ-215, acted directly on developmental stages of white fly and on transmission of two viruses, ZYMV and CULTIVARYV in melons (Cohen et al. 1996).

Recent studies by researchers at Massey University showed that sodium bicarbonate/surfactant mixtures provided good control of powdery mildew on glasshouse cucumbers and roses (Weeds 1992), and that application to grapevines during field trials proved cost-effective compared with a standard fungicide spray programme (Wood et al. 1994). Sodium bicarbonate is a relatively inexpensive compound and research is continuing to determine effective doses for application on various plant hosts. Very little is known about the mode of action of these materials, though some degree of synergism is suggested. Synergism is defined as the joint action of two or more agrichemicals that is greater than the sum of their activity when used alone (Anon. 1996).

An alternative hypothesis is that application of chemical salts causes plants to generate “immunity” signals (Xiang et al. 1995), which in turn contributes to disease resistance and the level of control observed. This may be useful for plant protection in the short-term. These chemical inducers (CI) are considered to have the following advantages (adapted from Xiang et al. 1995):

- i. Eliminate the potential problems associated with the introduction of plant pathogens as inducers.
- ii. More readily produced, distributed, and stored than pathogens.
- iii. Can be applied with the standard spraying equipment, thus eliminating the need for extra labour and expense.
- iv. Induced resistance is generally nonspecific, being effective against a wide spectrum of fungal, bacterial, and viral diseases unlike systemic fungicides.
- v. Main advantage over standard fungicides is that application of one inducer would protect plant against many diseases.
- vi. Because CI appear to work through the activation of plant defence mechanisms, this type of protection should be durable, and since the mechanisms activated appear to be similar to those reported for resistant plants developed by breeders, induced resistance should be as safe as resistant plants in the environment.
- vii. Since the mechanisms activated for defence are similar to those reported for resistant plants developed by breeders, induction with alternative chemicals could be as safe as resistant plants.

Two issues have still to be thoroughly addressed before CI can find routine application in agriculture: phytotoxicity and variability in efficacy:

Whether the fungitoxic activity of sodium bicarbonate/surfactant mixtures is associated with pH (Long et al. 1994): toxic ion concentrations that antagonise pathogen development, the triggering or enhancement of some aspect of host resistance, or some other way, their adoption should lead to a cheaper, more environmentally friendly material in the arsenal of disease control materials available to growers of a wide range of crops in New Zealand.

D Composts and plant extracts-Aqueous extracts of composts (especially animal manures) incorporated into the soil which contain a mixture of microorganisms, have controlled *S. fuliginea* on cucumbers and zucchini by up to 60% in recent studies (Tränkner 1991; Weltzein 1992; Elad et al. 1996). The mechanisms suggested for the underlying activity of these materials include effects on host resistance, and direct inhibition of the pathogens (Weltzein 1992). Similar levels of disease control have been achieved against *E. cichoracearum* by the high pressure spray application of a 5% emulsion of garlic extract (Qvarnstrom 1992).

Under semi-commercial conditions, weekly applications of Milsana (BASF, Germany), a 2% (v/v) commercial formulation prepared from aqueous plant extracts of the perennial giant knotweed (*Reynoutria sachalinensis* F. Schmidt (Nakai.) have significantly reduced disease incidence on Long English cucumber, and increased crop yield by 50% despite severe inoculum pressure (Herger et al. 1988). On treated plants, *S. fuliginea* colonies showed a lower hyphal density, and produced fewer conidiophores. Milsana reduced by more than half, the number of fungicide applications necessary for disease control (Dik & Van der Straay 1995).

The mode of action remains unclear, although it is known that it does not have any direct fungicidal properties (Menzies & Bélanger 1996). Biochemical studies of the treated plants revealed increased chlorophyll values, stimulation in the activities of several defence enzymes, such as peroxidase, polyphenyloxidase, phenylalanine ammonia-lyase (PAL) (Kowalewski & Herger 1992), β -1,3-glucanase, chitinase, and increased ethylene

production (Herger & Klingauf 1990) *in planta*. A rapid accumulation of antifungal phenolic compounds, especially in infected cucumber leaves treated with Milsana, additional to those produced naturally in host tissues in response to pathogen attack, has also been observed using the technique of thin-layer chromatography (Daayf et al. 1995). To date, it appears that the extracts are only effective against diseases caused by obligate parasites (Menzie & Bélanger 1996), they offer only protective not curative activity, and the current price is a major deterrent as small-scale production keeps the cost of processing Milsana high. As such, high value crops for home gardens and glasshouses are the only markets that may be able to justify use of this product (Menzie & Bélanger 1996).

1.6 MODES OF ACTION OF PLANT DISEASE CONTROL

MATERIALS

Very little is known about the mode of action of sodium bicarbonate/additive mixtures. There are three ways in which plant disease control materials act, and good basic information is required to decide whether the mode of action of sodium bicarbonate/additive mixtures is one of the following (adapted from Guest (1995)):

- i. Indirect- the material acts as an elicitor which stimulates host defence mechanisms to produce antifungal compounds that are responsible for disease control.
- ii. Direct- the fungicide acts on the pathogen alone. Control occurs through a number of physical and physiological processes such as interruption of fungal metabolism or membrane disruption. This mode is common to protectant and systemic fungicides.
- iii. Complex Interaction- pathogen control occurs via a combination of indirect and direct effects.

A knowledge of the mode of action of a potential disease control material defines when the material can be used to greatest advantage in the disease cycle (in relation to the time of inoculation, infection, lesion appearance and sporulation). Consideration of additional effects on host resistance and residual activity of the material allows the development of disease prevention programs with application intervals defined accordingly.

1.7 RESEARCH OBJECTIVES

Based on a knowledge of the host, pathogen, fungicidal modes of action and plant defence mechanisms the primary aim of this work was to study the host-parasite-complex of *Cucumis sativus*-*Sphaerotheca fuliginea* to determine whether stimulation of host defences is a mode of action of bicarbonate/additive mixtures. Subsequent chapters deal with the four approaches taken to assess the role in defence of cucumbers against powdery mildew of;

1. Phenolic compounds
2. The enzyme-Peroxidase
3. The enzyme-Phenylalanine ammonia-lyase
4. Induced structural barriers

2. GENERAL MATERIALS AND METHODS

2.1 THE PATHOGEN

S. fuliginea, was obtained from diseased plants purchased from a local garden centre, and was maintained during the course of this work on 'Leb' plants. Its identity was confirmed by two simple methods, on the basis of conidial attributes.

i. Mode of conidial germination (adapted from Zaracovitis 1965)

Clean, dry, glass microscope slides were touched momentarily to diseased leaves attached to plants which 24 h previously had been shaken vigorously by hand to remove old and perhaps nonviable conidia, a greater incidence of which occur on indoor grown plants (Zaracovitis 1965). In glasshouses, ventilation is restricted and plants rarely brush together. Therefore, conidia are less likely to be distributed as frequently from older colonies on the host as if plants were situated outdoors where normal wind activity distributes spores almost as soon as they are delimited on the conidiophore. Slides were placed over bent glass rods on the bottom of a humid chamber (a plastic container lined with moistened tissue paper). The lid was closed and the entire unit incubated in the dark at $20 \pm 1^\circ\text{C}$ for 24 h. On removal, conidia were examined for production of the forked germ-tubes which are characteristic of this pathogen a light microscope (Model Nr 340 172; Reichert, Austria).

Results and Discussion

Conidia germinating on glass were identifiable after 24 h incubation as those of *S. fuliginea* by the shape of their primary germ tubes. A number were forked and shorter than the long, straight germ tubes formed by *Erysiphe cichoracearum* described by Zaracovitis (1965). Figure 2.1 shows conidia as hyaline and elliptical. Germinated conidia appeared to contain fewer vacuoles than ungerminated conidia: one or two in the former compared with at least 6 in the latter. The rate of germination from surveys of 200 conidia ($n=6$) was relatively low, 40% (ie. 80 ± 11). This was lower than Zaracovitis (1965). Factors such as natural variation in the fungal population and suboptimal environmental conditions in the

humid chamber may have restricted greater germination in the present study (Dhingra & Sinclair 1985; Paxton 1991).

ii. Presence of fibrosin bodies as recognised by Clare (1958) (adapted from Kable & Ballantyne 1963)

Fresh conidia were dusted onto clean glass slides, several drops of 3% aqueous KOH added, and a coverslip overlayed. Slides were viewed using transmitted light and conidia examined for the presence of cell inclusions called fibrosin bodies. These appeared as dark-outlined, oblong to arc-shaped bodies in the cytoplasm (Figure 2.2).

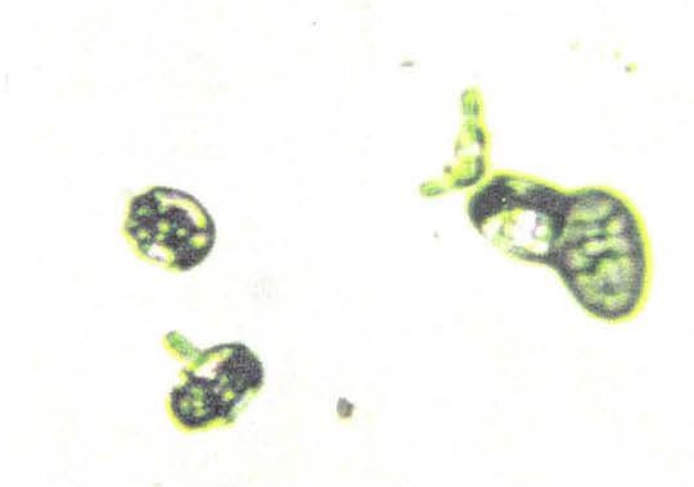


Figure 2.1 Germinated conidia of *Sphaerotheca fuliginea* incubated at 21°C for 24 h produced forked germ tubes. Conidia were viewed with transmitted light, magnification x160.

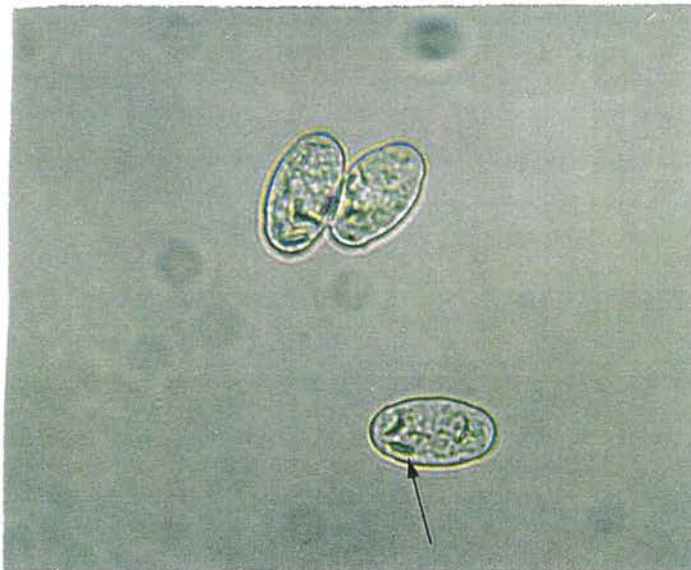


Figure 2.2 Conidia of *Sphaerotheca fuliginea* mounted in 3% KOH and viewed with transmitted light. Cellular inclusions- fibrosin bodies (arrowed) are clearly visible. Magnification x160.

2.2 PLANT CULTURE

Three cultivars of cucumber (*Cucumis sativus* L.) were selected on the basis of their resistance (Table 2.1) to give one cultivar for each of the resistances defined in Table 2.2. Seeds were sown in plastic pots (5 cm diam), containing a moist seed-raising mix (Appendix 1).

Table 2.1 Description of cucumber (*Cucumis sativus* L.) cultivars studied

Cultivar	Seed distributor	Description
'Lebanese' ('Leb')	Yates NZ Ltd	An open pollinated line sourced from USA, probably over 20 years old (J. Phillips pers. comm.). Smooth-skinned. Distinguished from other cucumbers in being sweeter and almost acid-free (burpless). High yielding, large fruit. Germination: 6-10 days. Maturity: 7 weeks. Susceptible to powdery mildew (<i>S.fuliginea</i>)
'Slicemaster' ('SM')	Yates NZ Ltd	A hybrid purchased from Petoseed USA. Produces dark green uniform fruit with a slight taper at each end, and fleshy white interiors (J. Phillips pers. comm.). High yielding. Germination: 5-10 days. Maturity: 9 weeks. Disease resistance / tolerance claimed by breeders against: Cucumber mosaic virus, anthracnose (<i>Colletotrichum lagenarium</i>), angular leaf spot (<i>Pseudomonas lachrymans</i>), powdery mildew and scab (<i>Cladosporium cucumerinum</i>)
'Slice King' F.1 Hybrid ('SK')	Watkins Seeds	Crisp tender cucumber with smooth fruit up to 20 cm long. High yielding. Very resistant to diseases caused by: <i>S. fuliginea</i> , <i>C. lagenarium</i> and <i>C. cucumerinum</i> . Germination: 10-21 days. Maturity: 12 weeks.

Table 2.2 Definitions of resistance genotypes (adapted from Anon. 1966 and Stewart 1995)

Resistant-	Plants which possess qualities that hinder the development of a given pathogen. Resistance involves some interaction between specific host plants and specific pathogens.
Disease tolerant-	Plants that are able to endure colonisation by a particular pathogen without showing severe symptoms of disease, or are able to compensate for the effects of disease.
Susceptible-	Plants that are not immune to infection by a certain disease, they have low resistance

They were placed on benches overlaid with capillary matting, and enclosed in a plastic cloche in a glasshouse containing no diseased plants at the Plant Growth Unit (PGU), Massey University. All three cultivars were germinated at $30 \pm 5^\circ\text{C}$ during the day and night and seedlings were watered by overhead mist sprays. Transplanting was performed with great care as it was found that cucumber plants were very sensitive to any disturbance of their root systems

2.3 CHEMICAL TREATMENTS

Chemical treatments (listed in Table 2.3) were prepared in sterile distilled water and applied and sprayed to the point of runoff. Plants each received 25 ml of test solution applied to the adaxial leaf surface, from a 700 ml plastic spray bottle which was held approximately 15-20 cm away from the subject plant. The water-treated control represented the effect of the inoculum load during the investigation, thus allowing a basis for judging comparative efficacy of the various treatments (Dooley 1978). All pots were removed from the experimental site for spraying to reduce the chance of cross-contamination from spray drift.

Table 2.3 Chemical treatments tested in this investigation

	Material	Recommended dilution rate
1	Tap water	n/a
2	Milsana fluessig (BASF, Germany) ^a	2%
3	Sodium bicarbonate (general purpose reagent grade)	2 g/litre ^b
4	Shellspray mineral oil (a highly refined petroleum distillate; Shell Chemicals New Zealand Limited) ^c	5 ml/litre
5	Sodium bicarbonate-plus-Shellspray oil	2 g/litre + 5 ml/litre
6	Sunlight hand dishwashing liquid (a blend of anionic surfactants; Lever Rexona Petone, New Zealand) ^c	1 ml/litre
7	Sodium bicarbonate-plus-Sunlight dishwashing liquid	2 g/litre + 1 ml/litre

^a Manufacturers recommended dosage of concentrated extracts from the giant knotweed (*Reynoutria sachalinensis*)

^b Rate recommended by Weeds (unpublished) and Wood et al. (1994)

^c Product description and their active ingredients can be found in Appendix 1.2

3. THE ROLE OF PHENOLIC COMPOUNDS IN DEFENCE OF CUCUMBER AGAINST POWDERY MILDEW

3.1 INTRODUCTION

In studies to determine whether disease control materials have an indirect mode of action, researchers have examined the production of antifungal phenolic materials in plant tissues. Daayf et al. (1995) employed the technique of thin-layer chromatography to visualise whether treatment with the plant extract Milsana stimulated production of these materials in healthy and infected cucumber leaves in addition to those produced naturally in response to pathogen attack. They found an increase in glycosidically-bound antifungal phenolics (aglycones) following treatment with Milsana, infection by *S. fuliginea* and by treatment of infected plants. Since concentrated salt solutions are also known to stimulate systemic induced resistance (Mucharromah & Kuc 1991) it is possible that sodium bicarbonate could have a similar effect.

The objectives of this work were to:

- i. Confirm the effect of Milsana on host resistance
- ii. Determine whether sodium bicarbonate and/or additives stimulate production of antifungal phenolic materials.
- iii. Compare the disease control performance of sodium bicarbonate/additive mixtures with that of Milsana.

3.2 MATERIALS AND METHODS

The estimation of antifungal materials produced in cucumber in response to treatment with sodium bicarbonate/additive mixtures in the presence and absence of *S. fuliginea*, was carried out by the procedures of Daayf et al. (1995) and comparisons were made between phenolic patterns from extracts of cucumber cultivars that differ in disease susceptibility.

3.2.1 Experimental Design

Seeds of cucumber cultivars ‘Lebanese’, ‘Slicemaster’ and ‘Slice King’ were sown in a moist seed-raising mix as before and germinated on benches in a temperature-controlled glasshouse ($30 \pm 5^\circ\text{C}$) located at PGU. Ten days after sowing, ie. when both cotyledons were fully expanded, 48 plants per cultivar were transplanted into a short-term soil mixture in plastic pots (20 x 15 cm diam), and moved to a larger glasshouse. Pots were positioned on glasshouse benches according to a randomised block design:

BLOCK 1			BLOCK 2			BLOCK 3								
A	B	C	A	B	C	B	A	C	B	A	C	B	A	
h	h	h	+	+	+	h	h	h	+	+	+	h	h	h
h	h	h	+	+	+	h	h	h	+	+	+	h	h	h
h	h	h	+	+	+	h	h	h	+	+	+	h	h	h
h	h	h	+	+	+	h	h	h	+	+	+	h	h	h
h	h	h	+	+	+	h	h	h	+	+	+	h	h	h
h	h	h	+	+	+	h	h	h	+	+	+	h	h	h
h	h	h	+	+	+	h	h	h	+	+	+	h	h	h
h	h	h	+	+	+	h	h	h	+	+	+	h	h	h

KEY: A=‘Lebanese’; B=‘Slicemaster’; C=‘Slice King’; h= healthy plants; += diseased plants

Figure 3.0 Position of pots in the greenhouse which contained three cucumber cultivars in three replicate blocks.

A. Inoculation

Plants were grown to the third-true leaf stage, and those from inoculated treatments (+) (Figure 3.1) were removed from the glasshouse and inoculated with fresh *S. fuliginea* conidia by artificially inoculated by dusting over the test plants with well-sporulating diseased ‘Leb’ leaves with a mildew rating of 3 or 4 (Reuveni & Bothma 1985). Although the inoculation procedure used was not quantitative, relatively uniform infections developed on plants of the same cultivar. Plants were removed for inoculation to reduce inoculum pressure in the glasshouse, thus reducing the chance of healthy control plants becoming

infected. After inoculation, plants were covered with moistened polythene bags, returned to their original bench position, and the bags removed after 24 h. Control (healthy) plants were similarly covered with polythene bags for 24 h.

Once inoculated plants developed approximately 20% infection (determined by Reuveni & Bothma (1985) as scale 2 infection; with individual leaves (2 and 3) bearing 51 to 100 infection sites), treatments listed in Table 2.3, were applied to runoff in a random order. Plants were returned to the experimental site when dry. Pots were watered from the bottom daily for the first 2 weeks, then twice a day to allow for growth. During this experiment no pesticides were used, though prior to transplantation the main glasshouse had been thoroughly disinfected.

Leaf material was harvested 24 h later and pooled to give a total of 20 g fresh material (FM) per cultivar, per treatment, where leaf samples from healthy and from diseased plants were considered separate treatments thus giving a total of 42 samples.

3.2.2 Detection Of Fungitoxic Free State And Glycosidic-Linked Phenolics In Fresh Leaf Material

A. Extraction from fresh leaf material

Cucumber leaves (20 g) were placed in pre-chilled 250 ml conical flasks, together with 80% methanol (acidified to pH 2.0 with 4-5 drops of concentrated HCl per 100 ml), at a rate of 10 ml/g fresh weight leaf tissue to allow liberation of chlorophyll, waxes and other phospho- and glycolipids so that they could be removed more completely by later partitioning. Tissues were homogenised mechanically using a high speed blender (Polytron, Kinematica GmbH) while the flasks were in an ice-water mixture to cool the plant material.

Plant cells contain many different enzymes which are able to attack phenolic compounds, and the enzymic activity remains active in leaves even after their excision from the parent plant (Draper 1979). Particularly active are polyphenol oxidases and glycosidases. The latter degrade complex glycosides into simpler ones, or even to aglycones

(Ribereau-Gayon 1971). Two precautionary measures were taken to prevent enzymic oxidation and chemical hydrolysis: i. the extraction temperature was maintained at 4°C throughout; ii. flasks were flushed with nitrogen (c. 1.5 min) following homogenisation. In preliminary trials to examine the effects of high speed blending versus grinding in a mortar and pestle and of two types of antioxidant (nitrogen flushing versus addition of ascorbic acid, 0.352 g/20 g FM), high speed blending combined with nitrogen flushing gave the most consistent results.

Following homogenisation, flasks were covered with aluminium foil and placed on a Certomat M rotary shaker (175 rpm) at 4°C, in the dark for 48 h.

B. Filtration

Extracts were clarified under suction through moistened Whatman No. 1 filter paper (11 cm diameter) placed in a Buchner funnel. A volume of 20 ml 80% acidified methanol was used to wash the residue.

C. Fractionation of crude extracts

Fractionation to separate the main classes of constituent from each other is necessary to remove components other than those of primary interest (Draper 1979) prior to chromatographic analysis (Harborne 1984). The technique employed by Daayf et al. (1995) and used here, is based on differential solubility of materials in aqueous and non-aqueous solvents to separate and concentrate them.

Samples were partitioned against half-volumes (125 ml) of a non-polar organic solvent (Seikel 1964; Ribereau-Gayon 1971), light petroleum ether (30-40°C b.pt.; density g/ml 0.62; Ajax Chemicals UNIVAR), in separating funnels to remove non-phenolic, non-polar substances such as chloroplast pigments, lipids, carotenoids and waxy matter (Figures 3.1 and 3.2). Both extract and ether were shaken vigorously together and the ether-soluble and methanol-soluble fractions allowed to settle out for 60-90 s. The ether fraction (Fraction I) containing chlorophyll etc. was discarded while the methanolic fraction was retained. Partitioning was repeated twice more.

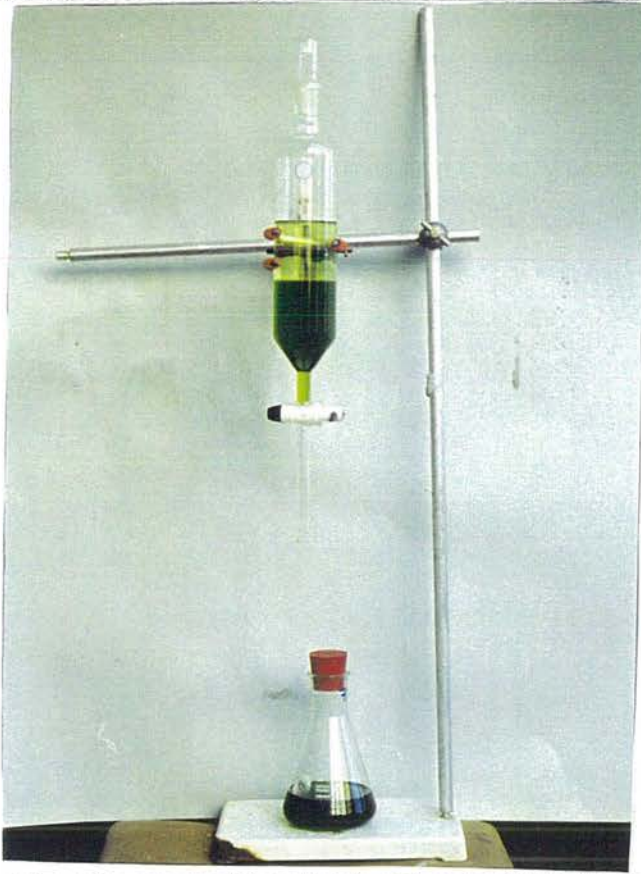


Figure 3.1 Separating funnels were used to separate and concentrate the main classes of constituent in cucumber leaf extracts in order to obtain phenolic compounds

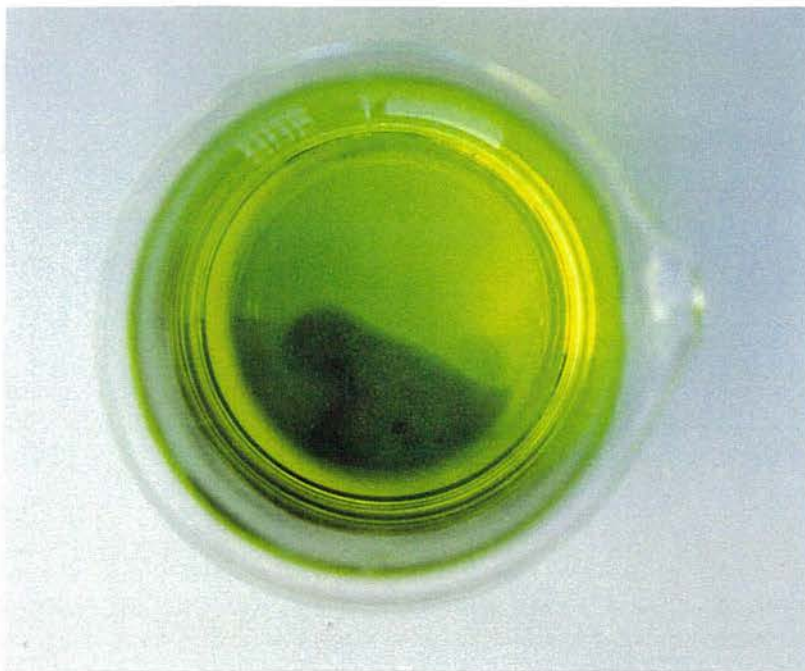


Figure 3.2 Non-phenolic substances removed from cucumber leaf extracts after the first partitioning process included chloroplast pigments, lipids, carotenoids and waxy matter

The methanolic residue from the partitioning of Fraction I containing the phenolic constituents, was rotary-evaporated (Büchi Rotavapor-R, Watson Victor Ltd, New Zealand) under pressure, at 38°C, to remove most of the solvent and leave 50 ml of extract. The aqueous residue was partitioned three times with 30 ml of a non-miscible solvent (diethyl ether anhydrous), to give Fraction II- "*free phenolics*" contained in the ether fraction. Phenolics occurring in the free state ie. are not glycosidically- or ester-bound, include simple phenols, the degradation products of more complex polyphenols such as flavonoids, and phenolic acids (Harborne & Baxter 1993). The aqueous fraction still contained hydrophilic compounds, namely phenolic esters and glycosides.

D. Acid hydrolysis

Acid hydrolysis cleaves glycosidic and acyl substituents -C-O-C- binding phenolics, with the release of free aglycones (Harborne 1989). These compounds are generally more toxic than the parent phenolic and often result from hydrolysis by the plant in response to pathogen challenge. The aqueous fraction was diluted with equal volumes of 4N HCl. Conical flasks used to contain the extracts were weighted down with lead bands and placed in a water bath (Model No. 5540-2; Grant Instruments (Cambridge) Ltd. England) at 100°C for 1.5 h. Flasks of this shape provided a larger surface area for hydrolysis. In most cases there was an obvious colour change in the extracts during acid hydrolysis from orange/yellow to:

- i. shades of darker orange for extracts from noninoculated treated and control plants,
- ii. red-brown for extracts from inoculated treated and control plants. Red-brown colourations expressed during heating were possibly the conversion or polymerisation products of leucoanthocyanidins, anthocyanidins (anthocyanin aglycones) or phlobaphenes respectively, which are often seen when tissues containing few anthocyanins are heated in acid solutions (Ribereau-Gayon 1971).

It was necessary to filter some extracts after hydrolysis because of the presence of a material likened to black "grit", possibly sugar residues from the breakage of glycosidic bonds or membrane-bound protein residues. Extracts were cooled to room temperature and

partitioned three times against 30 ml diethyl ether anhydrous as before, in order to retrieve the “*aglycones of glycosidically-bound phenolics*” -Fraction III. Phenolics occurring as glycosides are thought to have high phytotoxicity in the free state, hence their conjugation. They are commonly sequestered in plant vacuoles in this harmless form (because conjugation deactivates the phenolic nucleus), where they cannot interfere with important enzyme processes of plant metabolism (Harborne 1978).

E. Concentration of extracts *in vacuo*

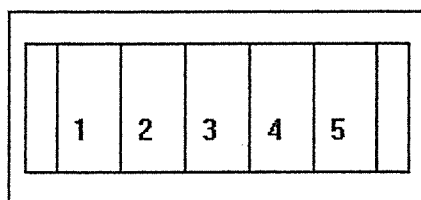
Fractions II and III were reduced to a concentration equivalent to 20 g leaf material per ml by two methods; rotary-evaporation which was first utilised to reduce the volume to 5-10 ml, and fume cupboard evaporation which took it down to the desired 1 ml. Concentrated extracts were stored at 4°C in 5 x 1.5 cm stoppered soda glass specimen tubes (SACO).

F. Thin layer chromatography for separation of compounds in plant extracts

i. Preparation of silica gel plates (chromatograms) for chromatography-

Setting up the 'Movable-Spreader' type apparatus

1. Five glass plates of equal width (20 x 20 cm) (Stahl 1969), and 2 smaller plates (8 x 20 cm) were laid together in a row on an aligning tray;



2. The end lever attached to two parallel clamping brackets, was lowered to hold the plates firmly in place.
3. All were wiped clean with cold 100% isopropyl (=isopropanol) to remove grease (lipid residues), using lint-free tissue paper.

4. The entire apparatus was levelled with a spirit level, thus ensuring plates were spread evenly.

The thickness of the silica layer (adsorbent) spread over each plate was predetermined using a rectangular trough and key template, such that a depth of 0.25 mm was maintained across the row of plates.

Spreading procedure for applying adsorbent to plates.

1. 61.0 g of silica gel (GF₂₅₄ Sigma Chemical Company) containing the fluorescent indicator- fluorescein was weighed into a 250 ml screw-top glass jar.
2. 130 ml water (determined as the optimum volume to achieve the consistency of a quality slurry) was added and the mixture shaken vigorously by hand for c. 1.5 min.
3. This aqueous slurry was poured into the trough (spreader) which was passed in one movement over the row of plates, leaving a thin even layer of silica gel, 0.25 mm thick.
4. Plates were left to dry for 2 h at room temperature (20-22°C), at which time the transparency of the layers had disappeared. Manilla folders placed above shielded wet plates from dust and other particles settling and causing disruption of their surfaces.
5. Semi-dry plates were stacked on a light alloy rack, and the drying process completed in an oven heated to 105-110°C, for 1 h. This reduction in water content ensured that the activity of the adsorbent was maximised.
6. Activated plates were stored until required in dry air-tight polystyrene containers lined with indicating silica beads (Scientific Supplies Ltd.). These beads served as a desiccating agent, which ensured that atmospheric moisture was not absorbed by the plates, leading to a subsequent loss of adsorbent activity.
7. Those plates showing a uniformity of the adsorbent layer in transmitted light (Stahl 1969) were selected for chromatographic separation of extracts.

ii. Spot application of extracts for one-dimensional separation by ascension-

1. A sharp, hard pencil was used to mark each chromatogram with the point of origin (ie. where spotting began) c. 2 cm. above the lower edge of the plate; the line of the solvent

front (10 cm above the origin); and the reference number of the substances applied. Two extracts were applied to each plate in duplicate, and a duplication made of each plate.

2. Fractions II and III were spotted equidistant apart on chromatograms at a loading of 20 μl^* using a fine 25 μl micropipette (Clay Adams). To stop material diffusion, small volumes were applied successively and the diethyl ether solvent removed between additions with a current of cool air produced from a small hair-drier. The final diameter of the spot applied was 0.75-1.0 cm. Chromatograms were left to air dry for 1 h before developing. Also spotted were (1) replicates of the solvent, to determine whether it was responsible for any subsequent inhibition; (2) replicates of a fungicide, Benlate™ (Du Pont; active ingredient: benomyl) at 0.05 g/100 ml, recommended by the “New Zealand Agrichemical Manual 1996” for control of the tomato leaf mould pathogen *Fulvia fulvum* (previously classified as *Cladosporium fulvum* Cooke), a fungus closely related to *Cladosporium herbarum* (Pers.) Link, the Deuteromycete fungus used in subsequent bioassays; (3) replicates of treatments listed above in raw material form, to determine whether they had any inherent antifungal activity. Replicates served two purposes; 1) TLC chromatograms were liable to vary slightly in the thickness of silica-gel coated on each. This can affect the rate at which the solvent front travels up the chromatogram and the degree of separation of the extracts. 2) Some bands may be clearer on different chromatograms.
3. The success of substance separation on a chromatogram can depend on the degree of saturation of the atmosphere inside a chamber with the solvent phase (Stahl 1969; Randerath 1970; Harborne 1984). Thus it was necessary to prepare an equilibrated chamber for chromatogram development several hours before use (Stahl 1969). This involved lining the inner walls and base of a flat-bottomed, rectangular glass tank with smooth sheets of blotting paper (15 x 40 cm; Ford’s Gold Medal). 200 ml (a volume sitting 1 cm deep in the tank which fell below the point of origin when a plate was inserted) of a solvent mixture sensitive to phenolic development- dichloromethane:n-hexane:methanol (6:4:1 v/v) (Daayf et al. 1995) was poured into the chamber, and the tank tilted to soak the paper. After being thus moistened, the paper was pressed against

*1 Reasons for extract application at this volume are given in Results 3.1.2

the sides. Silicon grease (MOLYKOTE 111, Dow Corning Australia Pty. Ltd.) was pasted over the top edges of the tank and corresponding surfaces on the glass lid, to ensure that the tank was air-tight when the lid was replaced. Sealing the chamber prevented differential evaporation of the different components of the solvent mixture which may result in uneven development (edge effects).

4. Separation was carried out at room temperature (19-21°C) in a fume cupboard, in diffuse daylight (Stahl 1969). Two plates were lowered vertically into one chamber, remaining in this position until the solvent had ascended to the front marking, c. 25 ± 5 min later, at which time they were removed and placed on a drying rack for 1.5-2 h. The solvent system was re-used only once before refreshing.



Figure 3.3 Glass tank-blotter paper system used for separation of substances in cucumber leaf extracts spotted on thin-layer chromatograms

G. Bioassay for detection of antifungal compounds

Chromatograms were air-dried for 1-2 h, laid on a bench at a slight incline (25°) and sprayed with a concentrated conidial suspension of *Cladosporium herbarum* mixed (1:1, v/v) in a solution of potato-dextrose agar (20 g/litre; GIBCO BRL Life Technologies Ltd. U.K: PDA), using an Arnold DeVilbiss hand sprayer attached via plastic tubing to compressed air. Spore suspensions were prepared by pouring 5 ml sterilised 0.02% Tween 20 (SERVA FEINBIOCHEMICA GmbH & Co. Heidelberg) onto a PDA culture of *C. herbarum*, scraping off the spores with a sterile glass slide and passing them through a nylon Falcon cell strainer (pore diameter 40µm; Becton Dickinson & Company, New Jersey) into a McCartney bottle. The density of the suspension was enumerated with a Spencer Bright-Line Phase haemocytometer (Becton Dickinson & Company, New Jersey) and adjusted to contain 1×10^7 conidia per ml. PDA, cooled to 55-60°C, could be mixed with conidia without affecting viability or viscosity of the PDA.

***Cladosporium herbarum* (Pers.) Link (Figure 3.4)**

The original culture was obtained as a contaminant and was maintained by periodic transfer of small pieces of the thallus to petri plates of 2% PDA. Plates were sealed with Parafilm to minimise contamination, then were incubated in the dark at 25°C until sporulating colonies were observed. They were then stored in a refrigerator at 12°C.

Chromatograms were laid flat on a support consisting of bent glass rods located within a humidity chamber fashioned from a 50 cm plastic container lined with water-saturated paper towels. This assembly was inserted into a clear plastic bag, the bag sealed with sticky-tape, and incubated for 48-72 h ($21 \pm 1^\circ\text{C}$), under a low light intensity illumination regime of 12 h light (2 x 40W Gro-Lux white fluorescent tubes) : 12 h darkness. Light intensity as measured with a quantum meter (LI-COR #Li-185; Lambda Inst. Co. Inc) was 55-60 µE/m²/s.

i. Assessment of bioassay

Assays of this type essentially screen for the presence of antifungal materials and with the large number of extracts prepared it was only possible to qualitatively assess results as visual estimates of zones of fungal inhibition seen as white areas devoid of dark mycelial growth. The diameter and R_f value of fungitoxic areas were recorded. The R_f or “relative front” value, is a measure of the distance travelled by the compound from the point of origin in a given solvent system and is defined as:

$$R_f = \frac{\text{distance moved by compound}}{\text{distance moved by solvent front}}$$

Spot diameter (mm) was rated to represent the concentration of antifungal materials (A/f) in the extract at any particular R_f value. This was defined as: 0 cm=zero, 0.1-1 cm in diameter=low, 1.01-2 cm=moderate, 2.01-3 cm=high, 3.01 cm-4⁺=very high concentration of A/f.



Figure 3.4 Test organism for detecting antifungal compounds in cucumber extracts, separated on thin-layer chromatograms: *Cladosporium herbarum* (Pers.) Link, seen cultured on 2% PDA

H. Visualisation of phenolic compounds with ultraviolet light

i. Introduction

Phenolic compounds may be located after chromatogram development by examination under visible light which has a wavelength (λ) from 430-790 nm, and short- and long-wavelength ultraviolet light (UV) (Stahl 1969; Van Sumere 1989) with emission maxima or peaks at 254 nm and 365 nm respectively. Either they appear coloured and fluoresce vividly, or they absorb light. Anthocyanins, quinones and the deeply coloured aurone and chalcone pigments can easily be distinguished in daylight without any further treatment (Seikel 1962). UV fluorescence reveals many more flavonoid compounds such as polyphenols, and differentiates between many pigments which are colourless or appear merely pale yellow in visible light (Seikel 1962).

Phenols which absorb light in the region of 254 nm will stand out as dark zones on a green fluorescing background (Bailey & Burdon 1973) on plates coated with silica gel containing a fluorescent indicator (Harborne 1984) such as fluorescein, when exposed to short wave UV light (UVC). The exception are flavanones, isoflavones, catechins and leucoanthocyanins (Seikel 1962) which remain colourless and require exposure to a specific chromogenic reagent alone or in combination with UV light, to be visible.

One set of developed plates was examined under visible, short- and long-wavelength UV concurrent to the bioassay. Photographs of plates exposed to UV light were taken on Kodak Gold III print film (Kodak (Australasia) Pty. Ltd.; ISO 200) in a darkroom with a Minolta x-700 MPS (Japan) camera fitted with a 55 mm macro lens and yellow filter. The camera was mounted on a tripod angled over the chromatogram, which was illuminated by a clampstand suspended, six-watt UV lamp (Model UVGL-58 Mineralight Lamp; UVP, Inc., Upland, California) operating both shortwave and longwave UV radiation. The optimal exposure times of 11 and 20 s respectively for short- and longwave photography were determined from a series of photographs taken at different exposures.

The usefulness of UV light of different radiations for detecting fungitoxic compounds prior to bioassaying was explored by comparing R_f values for UV-absorbing

bands at 254 nm, and fluorescent bands at 365 nm were compared with antifungal zones in bioassays.

I. Colour reactions for confirmation and partial characterisation of antifungal phenolic compounds

i. Introduction

The methods of detection, isolation, identification and analysis of phenolic compounds as they occur in biological materials are based on the polarity of the hydroxyl group (Thomson 1964). Fuming or spraying with suitable reagents (Stahl 1969), activates the benzene ring which reacts to give distinctive colours and it may also show absorption in the UV region of the spectrum (Thomson 1964). For instance, flavonoid compounds may be converted into more deeply coloured or fluorescent derivatives which can be observed in visible and/or UV light (Seikel 1962). Spray reagents can be particularly useful for characterisation of pale substances (Seikel 1962; Ribereau-Gayon 1971). Phenols on account of their weakly acidic character, have different colours in the presence of acid and alkali (Ribereau-Gayon 1971).

Spraying was done with a DeVilbiss handheld atomiser fitted with a No. 15 head connected to an aquarium pump (Maxima, air output 2800 cc/min² ; Rolf C. Hagen (UK) Ltd.) via plastic tubing. This was carried out in a fume cupboard as both spray reagents were extremely noxious. The reagents were applied as a fine spray according to the scheme of Waldi (Stahl 1969) illustrated in Figure 3.5. This ensured that the spray was distributed uniformly over the whole plate. The spray jet was swept several times over the plate with the sprayer held away at a distance of 30-40 cm.

Table 3.1 Phenolic test reagents used in this work

Phenolic Test (Chromogenic) Reagent	Spot colour response on silica gel if phenolics present	Specificity	Reference source(s)
Prussian blue reaction: Colour test based on the ease of oxidation of the aromatic ring by the mild inorganic oxidising agent -the ferricyanide ion. Reagent consisted of equal volumes of freshly prepared 1% aq. $K_2Fe(CN)_6$ and 1% $FeCl_3 \cdot 6H_2O$. Colours were intensified by subsequent over spraying of 2 N HCl.	deep blue as ferric ferrocyanide is produced	locates phenolic compounds of all types	Harborne (1964; 1989) Randerath (1970)
Acid vanillin test: 1 g vanillin ($CH_3O \cdot C_6H_3(OH) \cdot CHO$ - Mr=152.15) was dissolved in 10 ml conc. HCl)	pink-red ^a	most phenols, especially resorcinol and phloroglucinol derivatives, flavans and leucoanthocyanins	Ribereau-Gayon (1971) Harborne (1984, 1989), Van Sumere (1989)

^a Catechins and leucoanthocyanins (monomeric forms of flavans) turn blue to grey or brown by acid vanillin reagent. These substances do not occur in plant tissues in the form of glycosides, and are changed by heating in acid solution, therefore they may be present in fraction II because this was not subjected to acid hydrolysis (Ribereau-Gayon (1971))

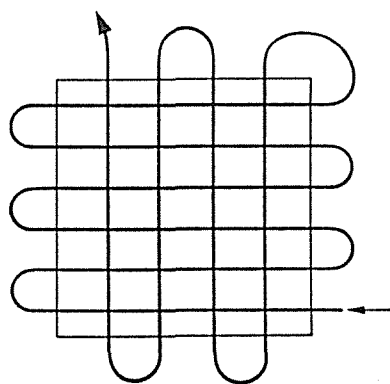


Figure 3.5 Waldi's scheme for applying chromogenic reagents as a fine spray. The jet of solution is swept over the layer surface in the direction indicated by the arrows (adapted from Stahl 1969)

3.2.3 Glasshouse Trials for Disease Severity

A. Experimental design

Ten days after sowing, ie. when both cotyledons were fully expanded, 56 plants per cultivar were transplanted into 10 litre plastic buckets containing a short-term soil mixture (Appendix 1). A bamboo cane was inserted into each pot at this stage for support as the cucumbers grew. Buckets were placed on the floor of a large glasshouse and treatments were applied in a randomised complete block design (Figure 3.6) consisting of 4 replicate blocks per cultivar, each containing 7 treatments randomly assigned to plants within each block. This design was chosen because the experimental units were essentially homogenous (Steel & Torrie 1980), being of the same cultivar, and differing little in their appearance or age. Temperatures in the glasshouse were set at $27 \pm 2^\circ\text{C}$ day and night.

-	I	I	I	II	II	II	@	@	III	III	III	IV	IV	IV	-
<i>g</i>	<i>g</i>	<i>g</i>	<i>g</i>	<i>g</i>	<i>g</i>	<i>g</i>	<i>g</i>	<i>g</i>	<i>g</i>	<i>g</i>	<i>g</i>	<i>g</i>	<i>g</i>	<i>g</i>	<i>g</i>
<i>g</i>	A	B	C	B	A	C	<i>g</i>	<i>g</i>	C	B	A	C	B	A	<i>g</i>
<i>g</i>	A	B	C	B	A	C	<i>g</i>	<i>g</i>	C	B	A	C	B	A	<i>g</i>
<i>g</i>	A	B	C	B	A	C	<i>g</i>	<i>g</i>	C	B	A	C	B	A	<i>g</i>
<i>g</i>	A	B	C	B	A	C	<i>g</i>	<i>g</i>	C	B	A	C	B	A	<i>g</i>
<i>g</i>	A	B	C	B	A	C	<i>g</i>	<i>g</i>	C	B	A	C	B	A	<i>g</i>
<i>g</i>	A	B	C	B	A	C	<i>g</i>	<i>g</i>	C	B	A	C	B	A	<i>g</i>
<i>g</i>	<i>g</i>	<i>g</i>	<i>g</i>	<i>g</i>	<i>g</i>	<i>g</i>	<i>g</i>	<i>g</i>	<i>g</i>	<i>g</i>	<i>g</i>	<i>g</i>	<i>g</i>	<i>g</i>	<i>g</i>
-	I	I	I	II	II	II	@	@	III	III	III	IV	IV	IV	-

KEY: A='Lebanese'; B='Slicemaster'; C='Slice King'; *g*=Guard row plants; @=Aisleway; I-IV=replicate blocks

Figure 3.6: Glasshouse layout for trials comparing the efficacy of sodium bicarbonate/additive mixtures and Milsana plant extract against cucumber powdery mildew.

B. Inoculation

When 3 true leaves were fully expanded (4 weeks), cucumber plants were inoculated by the method described in Chapter 3.1.2. Relatively uniform infections developed on plants of the same cultivar. Visible mildew lesions generally appeared 1 week after inoculation. The plants were then sprayed to the point of runoff with the test materials (Table 2.3.1). Treatments were applied thereafter at weekly intervals for the minimum of four applications needed to discover any phytotoxicity from repeated applications (Dooley 1978). Plants were returned to the experimental site when dry. Pots were watered from the bottom daily for the first 2 weeks, then twice a day to compensate for growth. During this experiment no pesticides were used, though prior to transplantation the main glasshouse had been thoroughly disinfected.

C. Assessment of disease severity

The fungicidal activity of each treatment was determined 5 weeks after treatment commenced (ie. one week after the final spray) by visually assessing the severity of disease. This period was chosen for assessment because 'Leb' matures earlier than the other two varieties: 7 weeks compared with 9 ('SM') and 12 ('SK'). Plants had reached the stage of growth at which 6-10 leaves were present along the primary stem and the percentage area visibly affected by disease was assessed on the adaxial surfaces of five fully-expanded leaves along the main stem (excluding laterals) beginning from the third node upwards. A transparent plastic grid-square was placed over each surface and the total number of dots falling within the leaf area were counted. A second measurement was made of the total number of dots falling over mildew lesions, including areas of chlorotic tissue associated with these. The percentage leaf area infected was calculated by the formula:

$$\frac{\text{diseased leaf area}}{\text{total leaf area}} \times 100$$

An average was taken over the leaves in each replicate block and these measurements averaged to give an overall mean and its standard error for treatment efficacy.

D. Analysis of data

The effect of test materials on disease control was analysed by an analysis of variance (ANOVA) using the Quattro Pro v6.0 spreadsheet/statistical package. Individual treatment means were compared for differences after necessary transformation with the appropriate controls using Fisher's protected LSDs, since the standard errors of differences between pairs of treatments were very similar.

3.3 RESULTS

3.3.1 Bioassay Results

In the absence of inhibitory compounds, *C. herbarum* grew extensively on the silica plates in the humid chamber, and dark olive-green hyphae and spores were clearly visible. Inhibitory zones were white areas devoid of mycelium.

i. Chromatogram loadings

Preliminary experiments to examine the effects of applying different volumes (5,10,15,20 and 25 μ l) of extracted material on chromatograms, showed that all volumes of extracts containing antifungal compounds resulted in inhibition zones. Increasing the loading decreased the number of spots that could be applied, since there was a considerable increase in diameter of the zone of antifungal activity at higher loadings. A maximum of four spots could be applied to plates at a volume of 15-25 μ l which is why a loading of 20 μ l was used in all experiments described here.

ii. Bioassays for detection of antifungal compounds

One inhibition zone (zone 1) was detected on chromatogram bioassays for free-state phenolics (F2), while three (zones 2, 3, and 4) occurred on chromatograms bioassayed for aglycone fractions (F3).

All four inhibition zones were found in at least one of the six cultivar/inoculation combinations for each of the seven treatment results given in Tables 3.2-3.8. There was little consistency in the patterns- four times for 'Leb', three times for 'SM' and twice for

‘SK’, and five times for inoculated plants compared with four for healthy ones. However, with the exception of inoculated ‘SM’ treated with shellspray oil or sodium bicarbonate-plus-sunlight liquid (where all inhibition zones were similar) zone II was always the largest zone. There was considerable variation in the results and no consistent enhancement of any antifungal zones by any treatment or by infection.

Table 3.2 Chromatogram bioassay of free-state phenolics (F2) and aglycone fractions (F3) from leaf extracts of healthy and of *Sphaerotheca fuliginea* inoculated cucumber plants. Treatment: Water

Cultivar / Host Status ^a	Zone I	Zone II	Zone III	Zone IV
	F2 (Rf=0.70-0.80)	F3 (Rf=0.00-0.10)	F3 (Rf=0.50-0.60)	F3 (Rf=0.70-0.80)
‘Lebanese’ H	0	17 (±0.5)	10 (±0.5)	16 (±1.0)
‘Lebanese’ I	10 (±1.0)	41.3 (±1.0)	8 (±0.6)	10 (±0.3)
‘Slicemaster’ H	0	17 (±2.0)	0	0
‘Slicemaster’ I	0	0	6 (±0.0)	11 (±0.5)
‘Slice King’ H	9 (±0.25)	26 (±0.8)	10 (±0.3)	15 (±0.5)
‘Slice King’ I	0	0	10 (±1.0)	12 (±0.3)

^aH=healthy, I=inoculated

Table 3.3 Chromatogram bioassay of free-state phenolics (F2) and aglycone fractions (F3) from leaf extracts of healthy and of *Sphaerotheca fuliginea* inoculated cucumber plants. Treatment: Milsana

Cultivar / Host Status ^a	Zone I	Zone II	Zone III	Zone IV
	F2 (Rf=0.70-0.80)	F3 (Rf=0.00-0.10)	F3 (Rf=0.50-0.60)	F3 (Rf=0.70-0.80)
‘Lebanese’ H	10 (±0.3)	37 (±0.8)	7 (±0.0)	21 (±0.8)
‘Lebanese’ I	0	12 (±1.0)	10 (±0.3)	14 (±0.3)
‘Slicemaster’ H	0	31 (±0.5)	6 (±0.3)	22 (±0.8)
‘Slicemaster’ I	0	0	7.5 (±0.2)	11 (±0.3)
‘Slice King’ H	0	44 (±0.3)	14 (±0.5)	21 (±1.0)
‘Slice King’ I	17 (±0.5)	31 (±0.8)	0	14 (±0.3)

^aH=healthy, I=inoculated

Table 3.4 Chromatogram bioassay of free-state phenolics (F2) and aglycone fractions (F3) from leaf extracts of healthy and of *Sphaerotheca fuliginea* inoculated cucumber plants. Treatment: Sodium bicarbonate.

Cultivar / Host Status ^a		Zone I F2 (Rf=0.70-0.80)	Zone II F3 (Rf=0.00-0.10)	Zone III F3 (Rf=0.50-0.60)	Zone IV F3 (Rf=0.70-0.80)
'Lebanese'	H	0 ^b	28 (±0.3)	0	14 (±0.5)
'Lebanese'	I	0	0	0	0
'Slicemaster'	H	0	23 (±0.3)	4 (±0.0)	22 (±0.3)
'Slicemaster'	I	10 (±1.0)	22 (±0.8)	9 (±0.5)	16 (±0.2)
'Slice King'	H	0	17 (±0.3)	7 (±0.3)	11 (±1.0)
'Slice King'	I	0	0	0	10 (±0.5)

^aH=healthy, I=inoculated

Table 3.5 Chromatogram bioassay of free-state phenolics (F2) and aglycone fractions (F3) from leaf extracts of healthy and of *Sphaerotheca fuliginea* inoculated cucumber plants. Treatment: Shellspray oil

Cultivar / Host Status ^a		Zone I F2 (Rf=0.70-0.80)	Zone II F3 (Rf=0.00-0.10)	Zone III F3 (Rf=0.50-0.60)	Zone IV F3 (Rf=0.70-0.80)
'Lebanese'	H	13.5 (±1.3)	34 (±0.3)	0	16 (±0.5)
'Lebanese'	I	0	34 (±0.8)	4 (±0.3)	12 (±0.3)
'Slicemaster'	H	0	-	-	-
'Slicemaster'	I	10 (±0.5)	9 (±0.2)	6 (±0.0)	10 (±0.3)
'Slice King'	H	0	46 (±0.8)	10 (±0.4)	20 (±0.7)
'Slice King'	I	0	0	14 (±0.6)	13 (±0.5)

^aH=healthy, I=inoculated

Table 3.6 Chromatogram bioassay of free-state phenolics (F2) and aglycone fractions (F3) from leaf extracts of healthy and of *Sphaerotheca fuliginea* inoculated cucumber plants. Treatment: Sodium bicarbonate-plus-shellspray oil

Cultivar / Host Status ^a		Zone I F2 (Rf=0.70-0.80)	Zone II F3 (Rf=0.00-0.10)	Zone III F3 (Rf=0.50-0.60)	Zone IV F3 (Rf=0.70-0.80)
'Lebanese'	H	13.5 (±0.3)	34 (±0.2)	17 (±0.8)	25 (±0.5)
'Lebanese'	I	0	4 (±0.0)	4.5 (±0.3)	4.5 (±0.0)
'Slicemaster'	H	0	31 (±1.2)	11 (±0.5)	28 (±0.2)
'Slicemaster'	I	10 (±0.8)	10 (±0.3)	9 (±0.3)	11 (±0.0)
'Slice King'	H	0	21 (±1.0)	0	10 (±0.3)
'Slice King'	I	0	0	0	23 (±0.5)

^aH=healthy, I=inoculated

Table 3.7 Chromatogram bioassay of free-state phenolics (F2) and aglycone fractions (F3) from leaf extracts of healthy and of *Sphaerotheca fuliginea* inoculated cucumber plants. Treatment: Sunlight liquid

Cultivar / Host Status ^a		Zone I F2 (Rf=0.70-0.80)	Zone II F3 (Rf=0.00-0.10)	Zone III F3 (Rf=0.50-0.60)	Zone IV F3 (Rf=0.70-0.80)
'Lebanese'	H	13.5 (±0.3)	20 (±0.0)	4 (±0.2)	7.5 (±0.3)
'Lebanese'	I	0	8.5 (±0.5)	18 (±0.3)	13 (±0.8)
'Slicemaster'	H	0	10 (±0.2)	0	9 (±0.4)
'Slicemaster'	I	10 (±0.8)	23 (±1.0)	11 (±0.5)	21 (±0.3)
'Slice King'	H	0	40 (±1.2)	10 (±0.2)	22 (±0.8)
'Slice King'	I	0	14 (±0.5)	0	4 (±0.0)

^aH=healthy, I=inoculated

Table 3.8 Chromatogram bioassay of free-state phenolics (F2) and aglycone fractions (F3) from leaf extracts of healthy and of *Sphaerotheca fuliginea* inoculated cucumber plants. Treatment: Sodium bicarbonate-plus-sunlight liquid

Cultivar / Host Status ^a		Zone I F2 (Rf=0.70-0.80)	Zone II F3 (Rf=0.00-0.10)	Zone III F3 (Rf=0.50-0.60)	Zone IV F3 (Rf=0.70-0.80)
'Lebanese'	H	13.5 (±0.3)	43 (±1.0)	10 (±0.2)	14 (±0.3)
'Lebanese'	I	0	0	18 (±0.5)	10 (±0.3)
'Slicemaster'	H	0	22 (±0.5)	4 (±0.2)	11 (±0.8)
'Slicemaster'	I	10 (±0.8)	14 (±0.6)	10 (±0.8)	14 (±0.5)
'Slice King'	H	0	27 (±0.8)	0	17.5 (±0.3)
'Slice King'	I	0	8.5 (±0.3)	34.5 (±1.2)	31 (±1.0)

^aH=healthy, I=inoculated

3.3.2. Characterisation of Antifungal Substances

The location of inhibitory areas was compared with areas of colour, fluorescence, and absorption on duplicate plates photographed under three light sources: visible, longwave and shortwave ultraviolet radiation (UV) respectively (Table 3.9).

Table 3.9: Appearance of antifungal compounds I-IV under three light sources

Light Source (wavelength: nm)	Zone I F2 (Rf=0.70-0.80)	Zone II F3 (Rf=0.00-0.10)	Zone III F3 (Rf=0.50-0.60)	Zone IV F3 (Rf=0.70-0.80)
visible (430-790 nm)	none	orange/brown	none	none
longwave (365 nm) ^a	vivid red	none	faint green	faint green
shortwave (254 nm) ^b	darkly absorbing	darkly absorbing ^c	darkly absorbing	darkly absorbing

^a Appearance against a dark background

^b Appearance against a bright-green background

^c Most intense absorbance

i. Visible light

Examination of duplicate plates to those bioassayed revealed several differences between areas of antifungal activity in the free phenolic (F2) and aglycone fraction (F3) that appeared common to all cultivars and treatments. Several areas in F2 extracts only faintly coloured with visible light, inhibited fungal growth. Compounds that are known to be colourless or pale included isoflavones, flavones and flavonols. In most cases inhibition was demonstrated at an Rf value of 0.70-0.80. The width and location of these inhibition areas could not have been predicted from plates observed with visible light. Chlorophyll a and b pigments which absorb visible light principally from the red, blue and violet regions of the spectrum to appear green, may have escaped the partitioning process as residues when F2 was partitioned off first and could be responsible for the bluish/green and green bands observed. F2 bands located at Rf 0.30-0.50 contained yellow, green, bluish/green and yellow/green pigments of various shades. Aurones and chalcones are examples of natural flavonoid compounds that could appear yellow (Seikel 1962).

Pigments seen in F3 extracts appeared in definite orange/brown bands at the origin (Rf 0.10-0.20). These areas were revealed to be strongly inhibitory, while other areas caused inhibition beyond the origin (Rf 0.70-0.80) were not visible with daylight. Pink pigments were visible in several bands at Rf 0.40-0.55. Both orange and pink bands could represent anthocyanins (Seikel 1962).

ii. Longwave ultraviolet light

At 365 nm, prominent bands of colour appeared bright against a dark background. All areas that fluoresced red in F2 extracts were strongly inhibitory. Lighter yellow/green or orange areas were inhibitory in some cases but weaker in activity. The most distinct zones of inhibition in F3 extracts were invisible under longwave UV or appeared faintly fluorescing green, this included the origin. While, areas of faint inhibition appeared as bright-green bands.

For F2 extracts, two contrasting coloured areas were observed. All green and bluish/green areas seen with visible light (and even some that were not observed) fluoresced a vivid red (chlorophyll), while all orange/brown areas fluoresced yellow to yellow/orange. Several classes of flavonoid may have contributed to this colour including, anthocyanins (3,5-diglycosides) (Seikel 1962), confirming the colourings observed with visible light.

Many bands were distinguished in F3 extracts including several that were invisible or very pale coloured in daylight. All bands fluoresced bright yellow/green to green. Pink bands seen with daylight were a more intense green and orange/brown bands a more subtle green.

iii. Shortwave ultraviolet light

Compounds absorbing shortwave UV light appeared dark against a bright green background. Shortwave photographs were almost complimentary to bioassays, because the most absorbing (darkest) areas were the most antifungal, for most F2 and F3 extracts. Several less absorbing areas were weakly fungitoxic.

All orange/brown or green F2 zones seen in visible light, absorbed UV. Absorption was greatest at the origin (0.00-0.15) and in areas of orange colour. F3 absorption was also greatest at the origin. At higher Rf values, several bands of absorption invisible in daylight could be discerned.

Some areas that fluoresced red under longwave UV in F2 extracts absorbed light at shortwave UV at a weaker intensity than others, and weaker than all areas that fluoresced orange, which may indicate the presence of different compounds. Actual bands of materials were more readily distinguished by shortwave than longwave UV. Longwave UV suggested

close relationships between compounds in extracts, and did not distinguish differences as distinct bands. Bands of materials were more distinguishable in F3 than F2 extracts.

Bands distinguished with longwave UV as areas of bright fluorescence, absorbed less intensely under shortwave UV than other bands faintly visible and vice versa (Figure 3.7). Shortwave UV did distinguish as many types of compounds as longwave UV, but it also defined other bands.

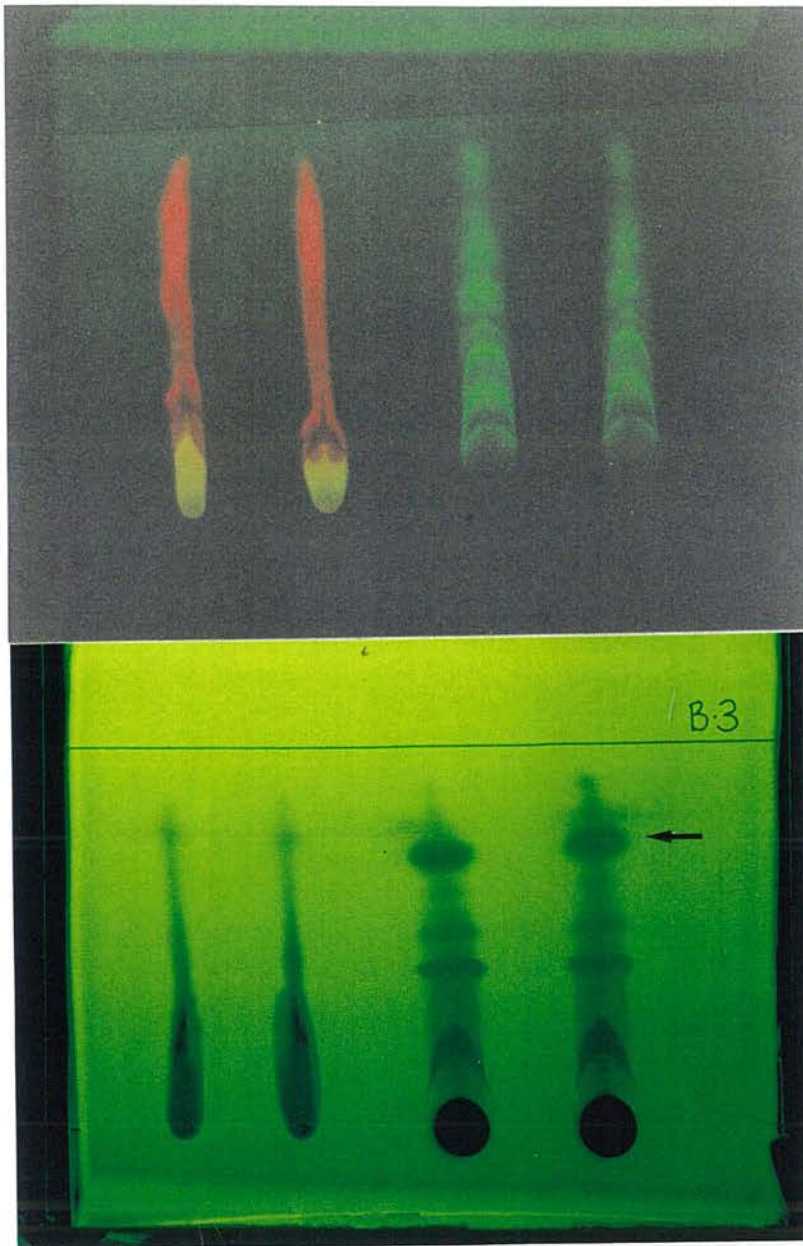


Figure 3.7 Bands of antifungal substances in chromatogram bioassays (arrowed) were not distinguished with longwave UV (top photo). These substances absorbed strongly under shortwave UV (bottom photo). Extract shown: Noninoculated 'Slicemaster' cucumber treated with sodium bicarbonate.

Based on these results, shortwave UV appeared to be the most reliable/likely indicator of possible areas of antifungal activity in these extracts. Banding patterns were mostly complimentary. The series of photographs (Figures 3.8-3.19) which follow illustrate leaf extracts from three cucumber cultivars as they appeared under shortwave UV, and inhibitory zones revealed by bioassay. The scale alongside each plate refers to the Rf value (ie. distance from the spot origin to the solvent front).

Key to figures 3.8-3.19:

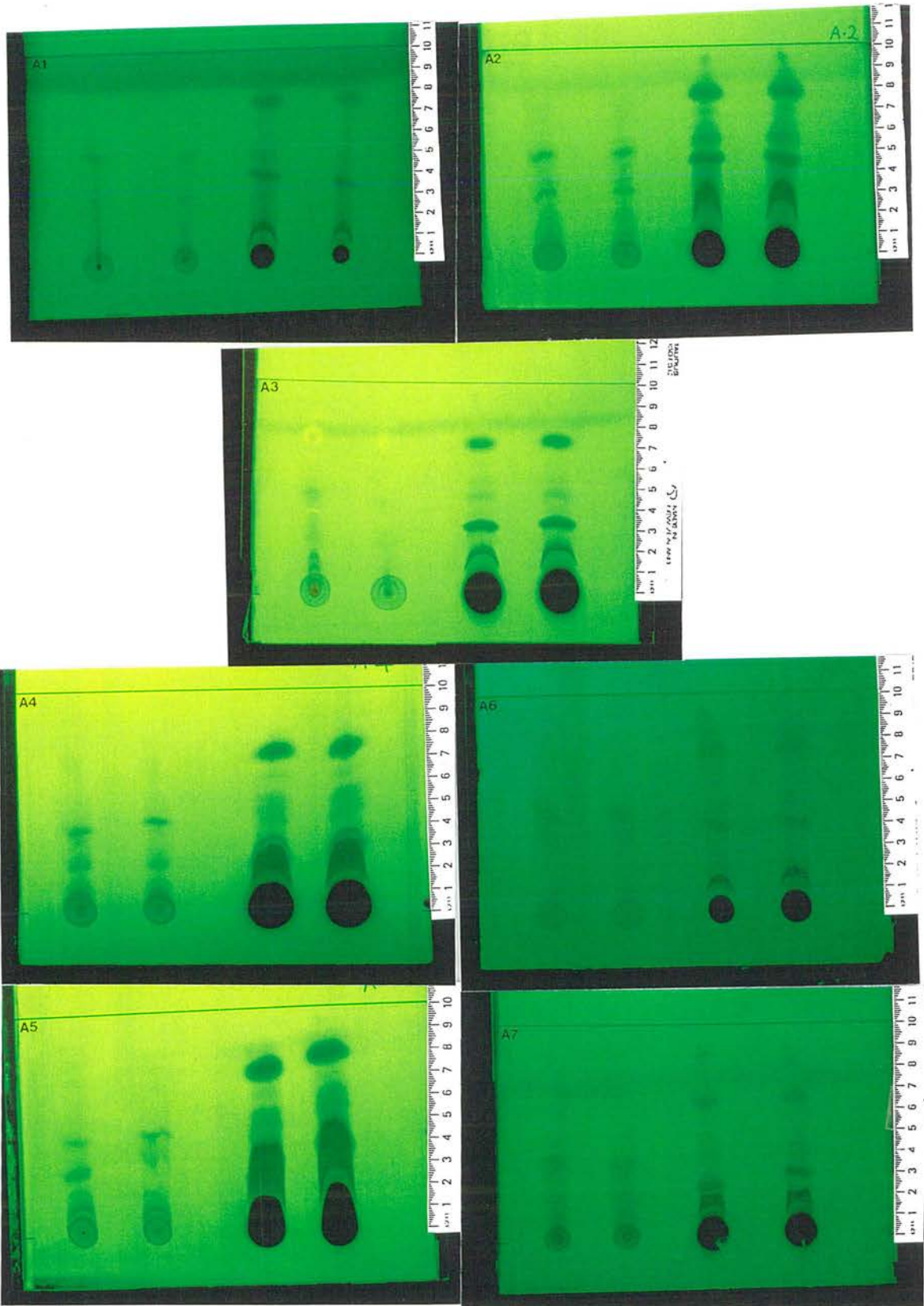
Letter assigned to Cultivar each cultivar

A	'Lebanese' extracts
B	'Slicemaster' extracts
C	'Slice King' extracts

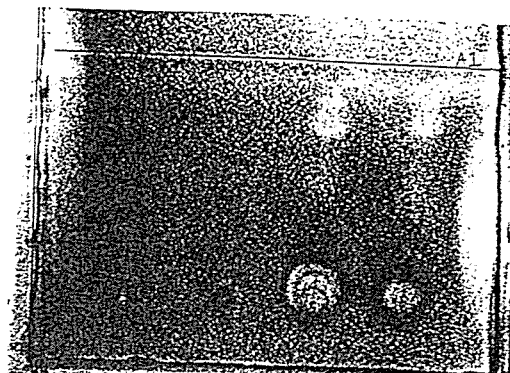
Letter assigned to each treatment

Treatment

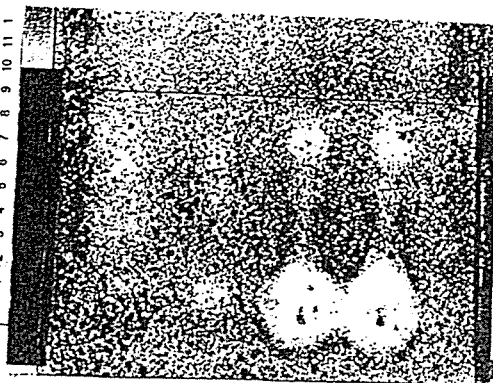
1	Noninoculated	Water-treated
2	~	Milsana
3	~	Sodium bicarbonate
4	~	Shellspray oil
5	~	Sodium bicarbonate-plus-shellspray oil
6	~	Sunlight liquid
7	~	Sodium bicarbonate-plus-sunlight liquid
8	Inoculated	Water-treated
9	~	Milsana
10	~	Sodium bicarbonate
11	~	Shellspray oil
12	~	Sodium bicarbonate-plus-shellspray oil
13	~	Sunlight liquid
14	~	Sodium bicarbonate-plus-sunlight liquid



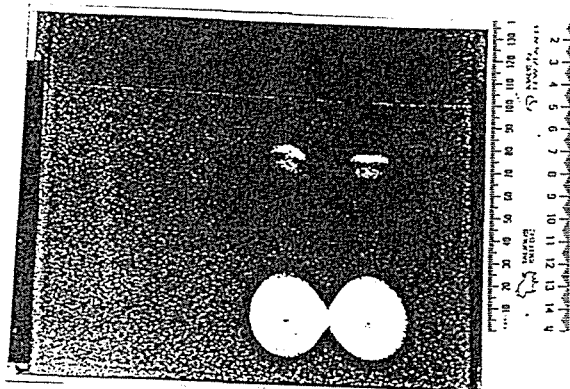
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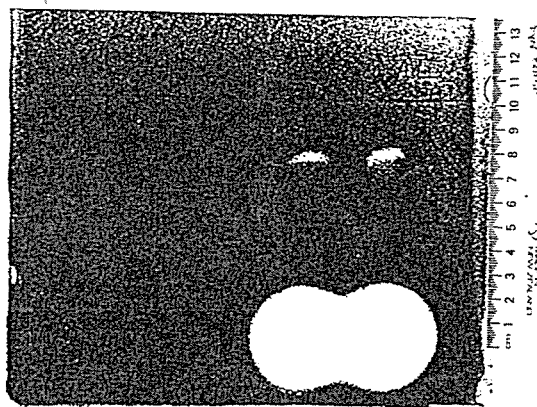
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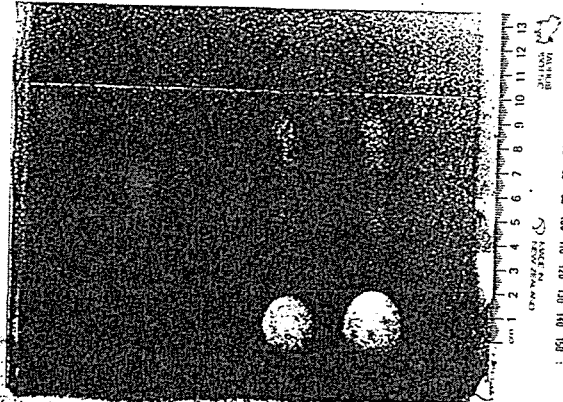
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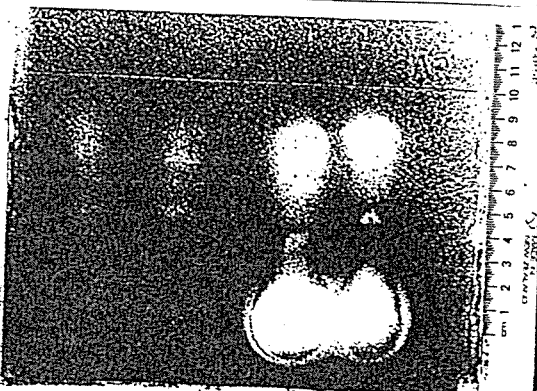
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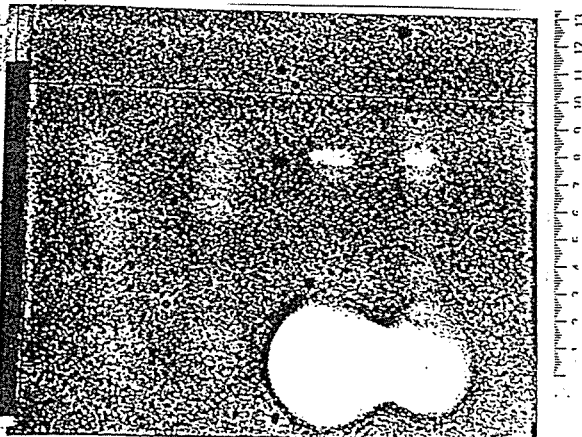
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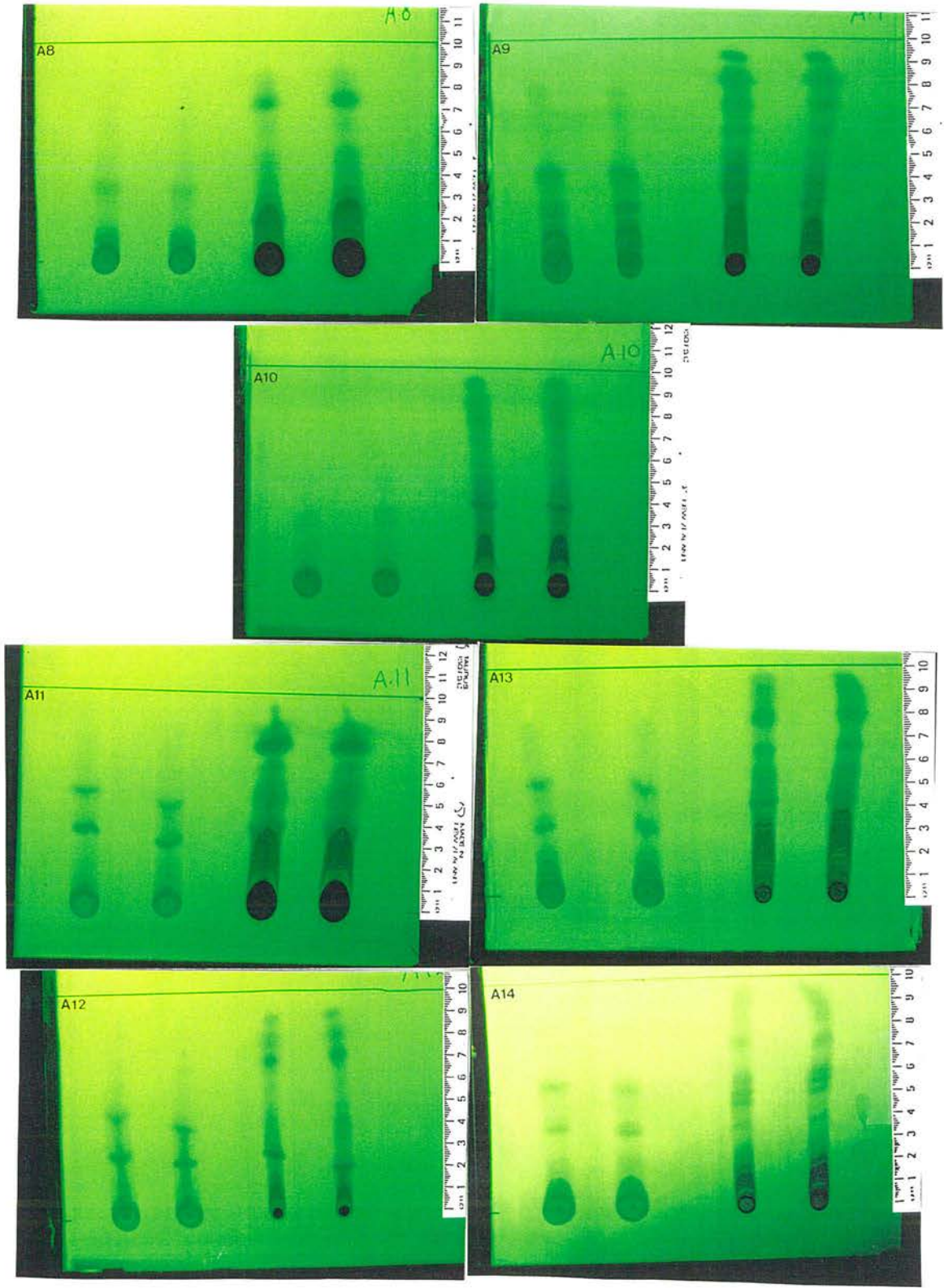


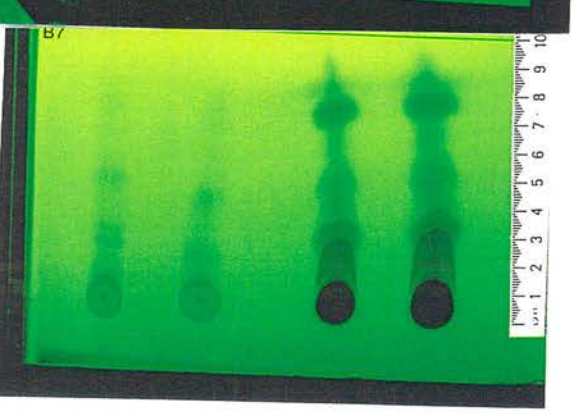
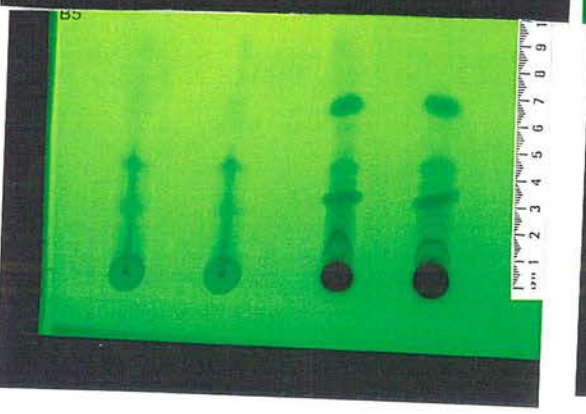
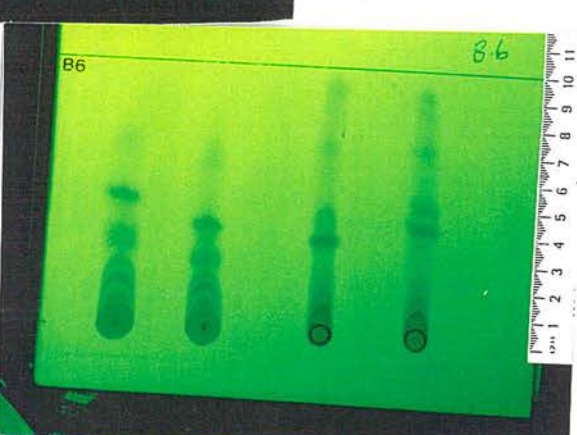
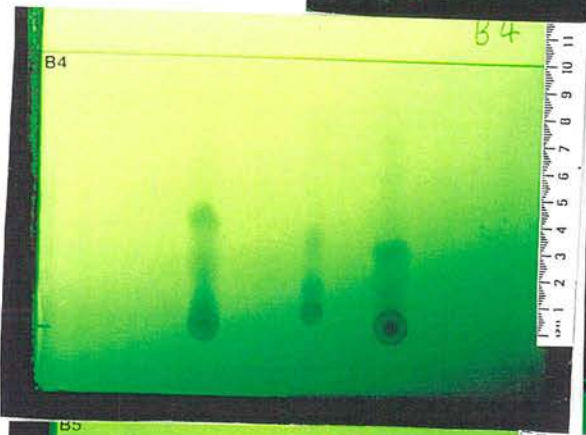
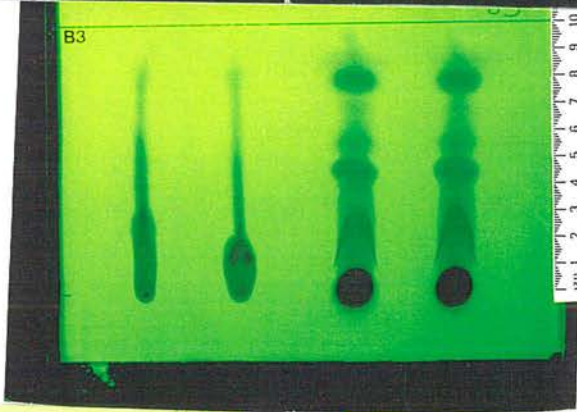
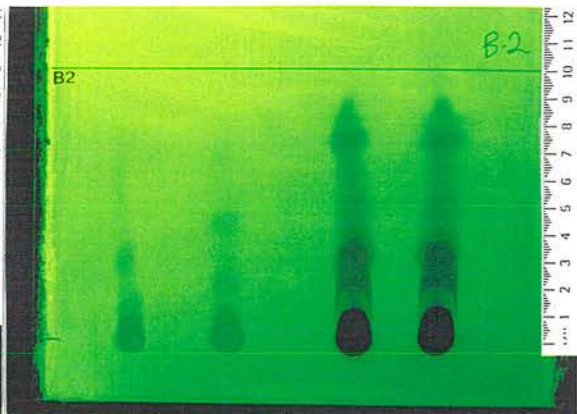
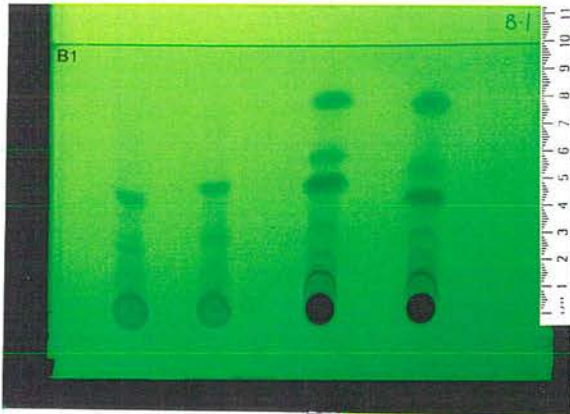
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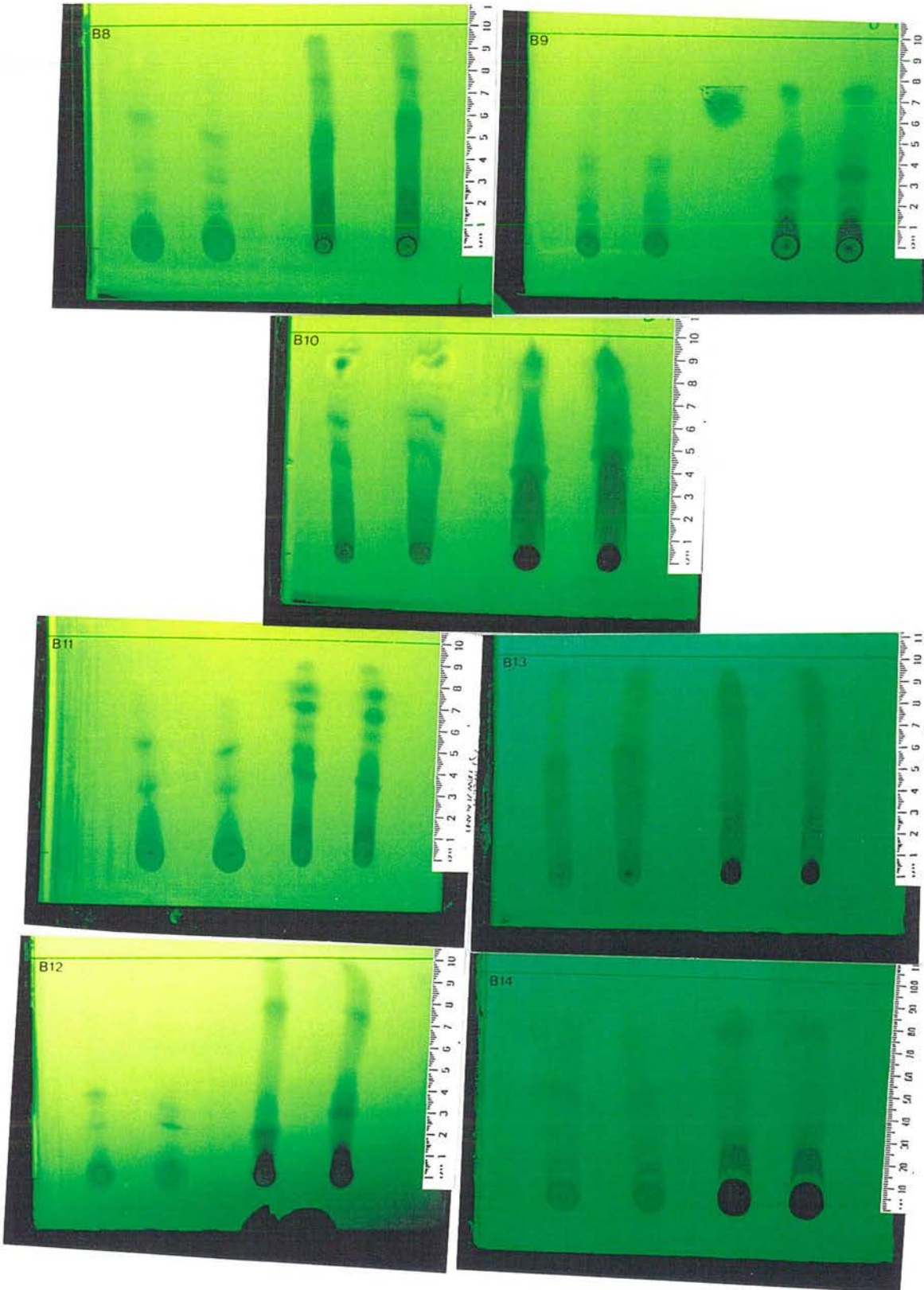


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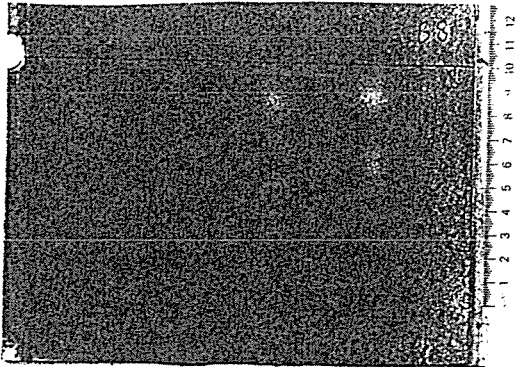




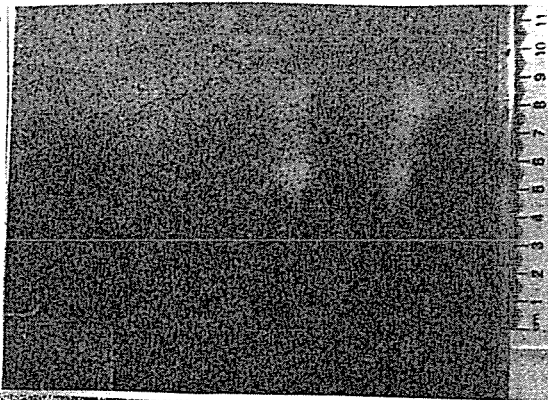




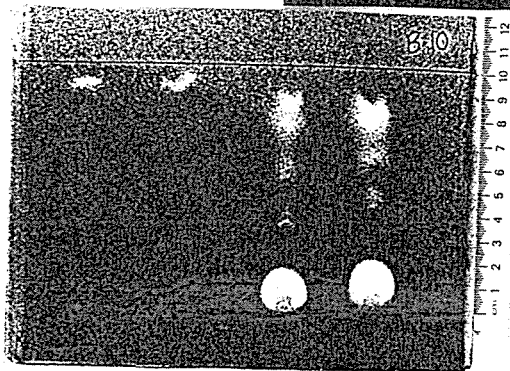
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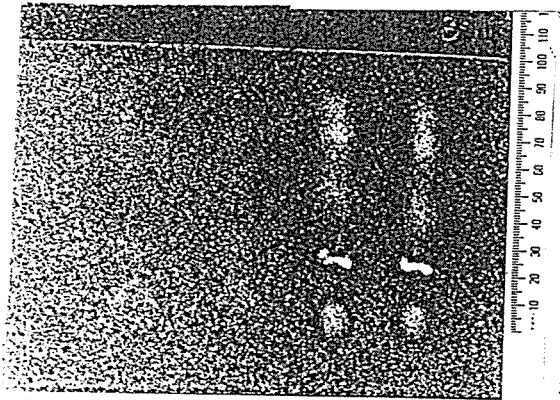
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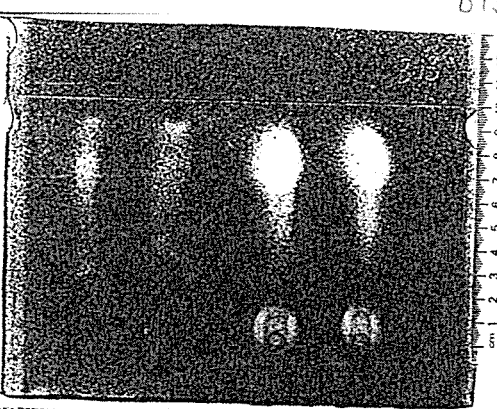
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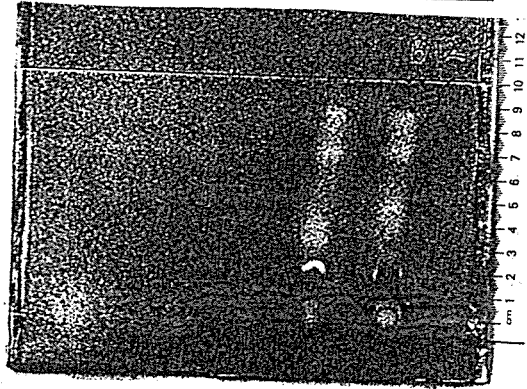
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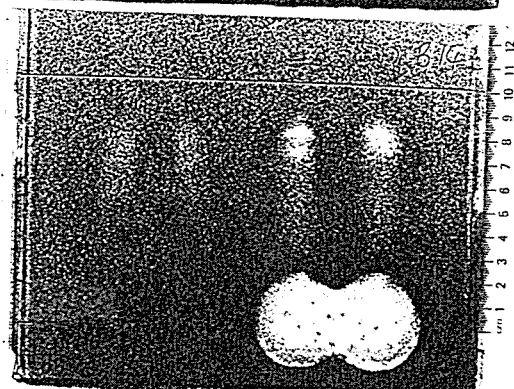
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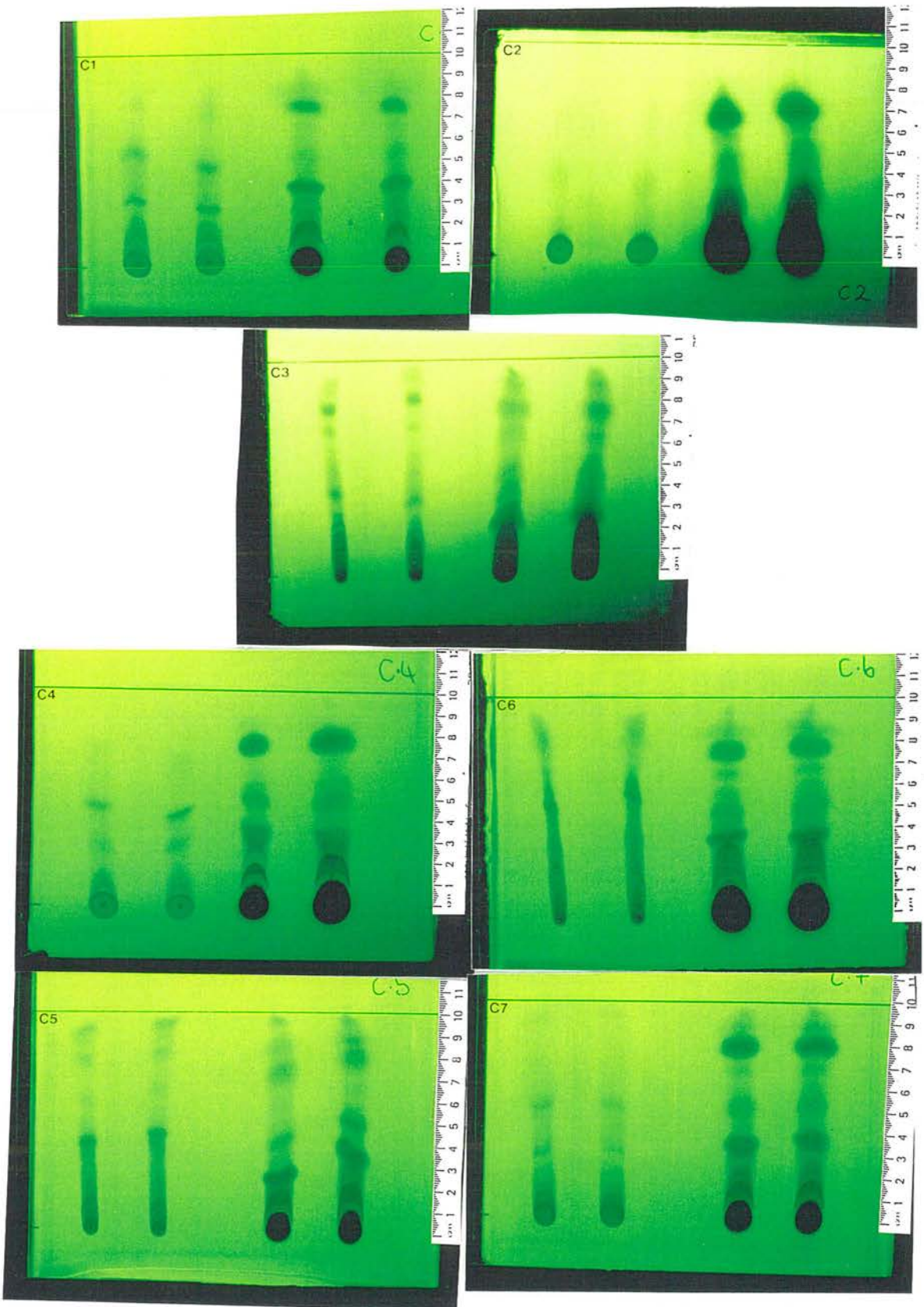


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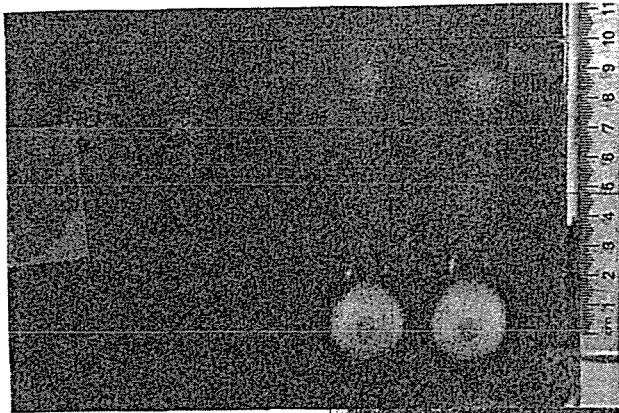


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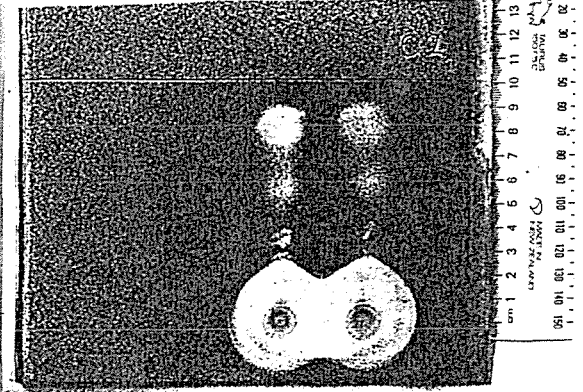




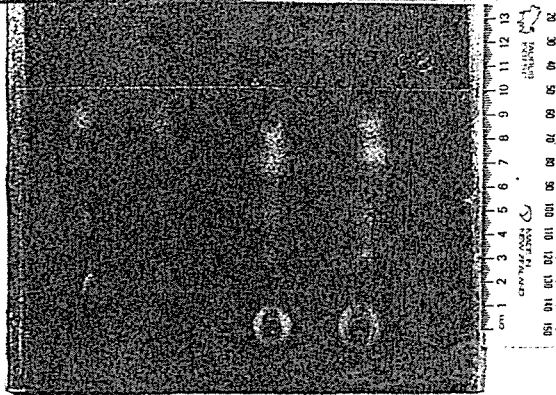
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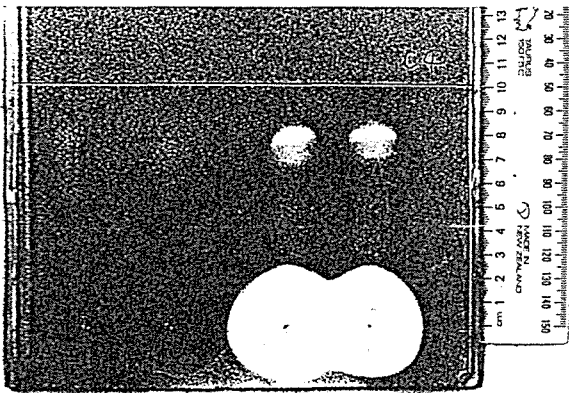
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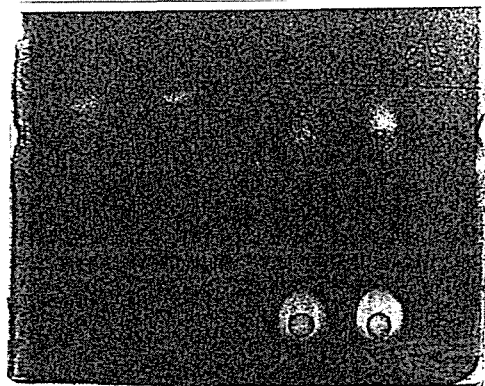
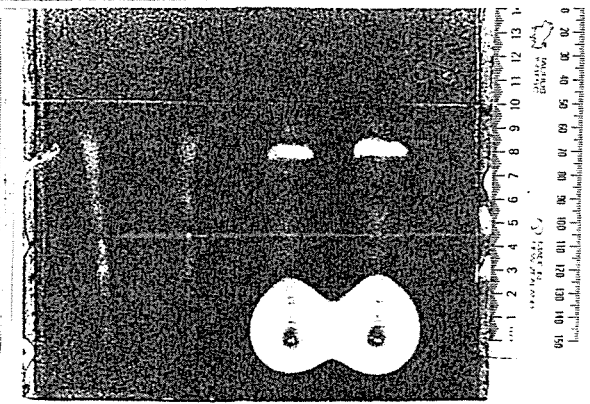
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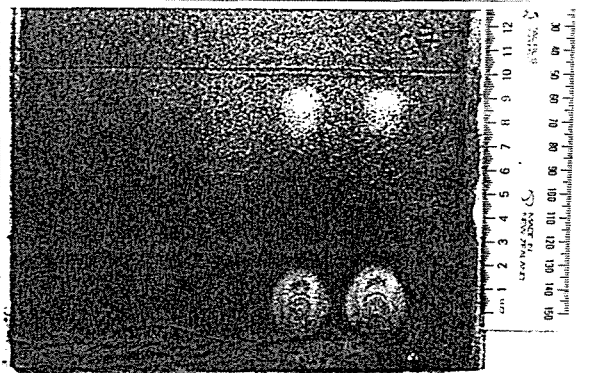
C4



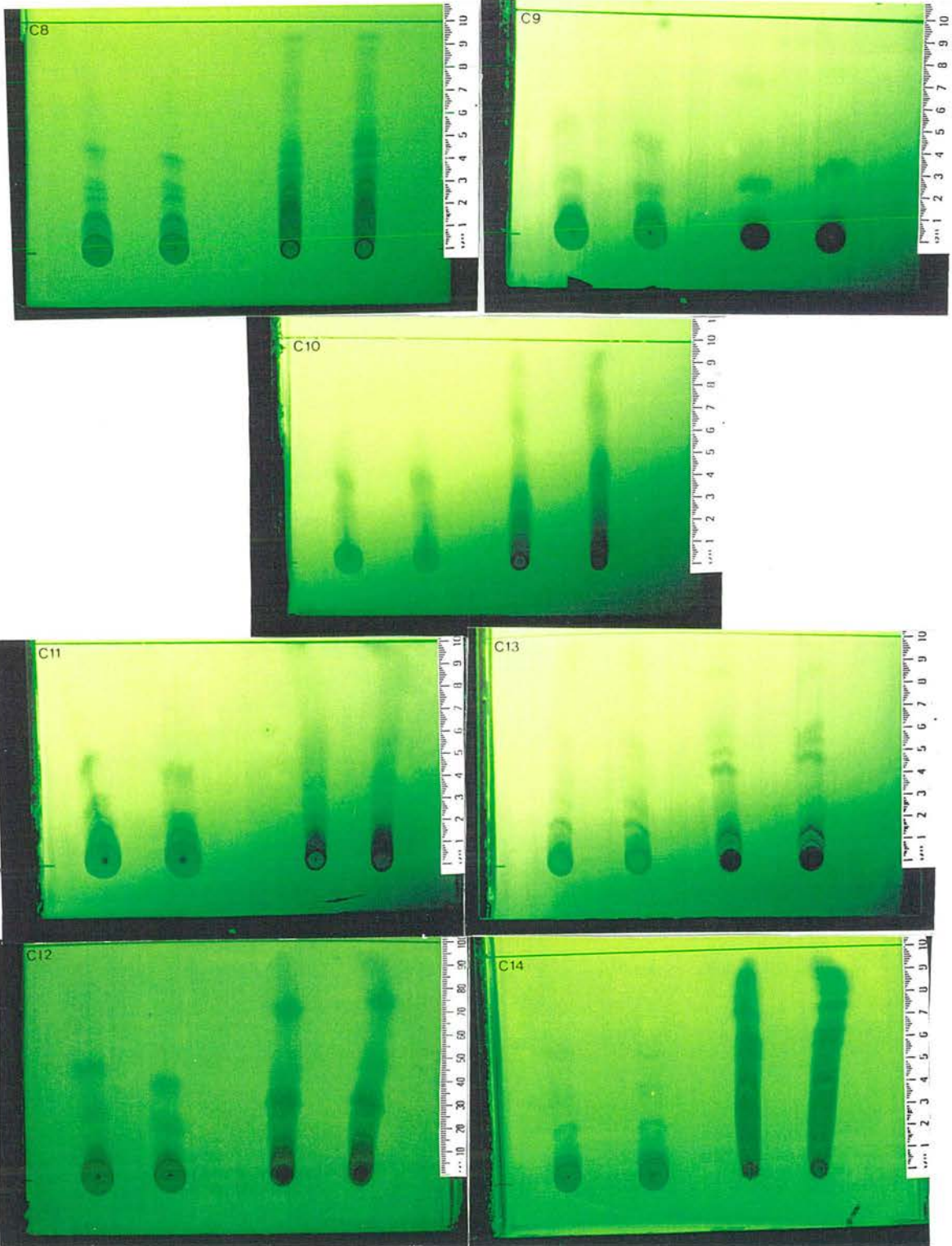
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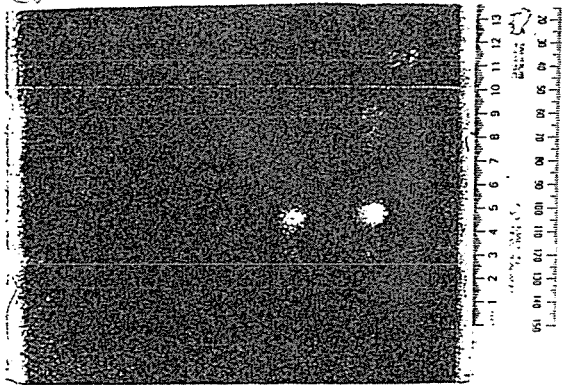
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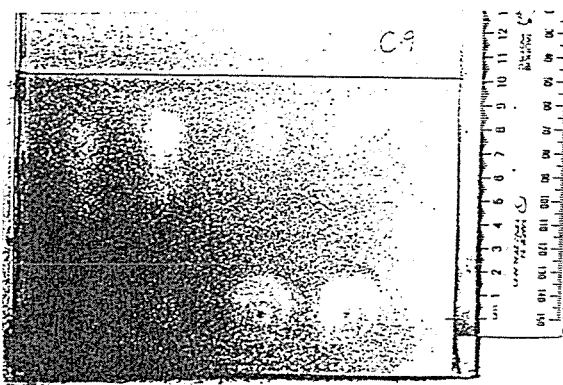
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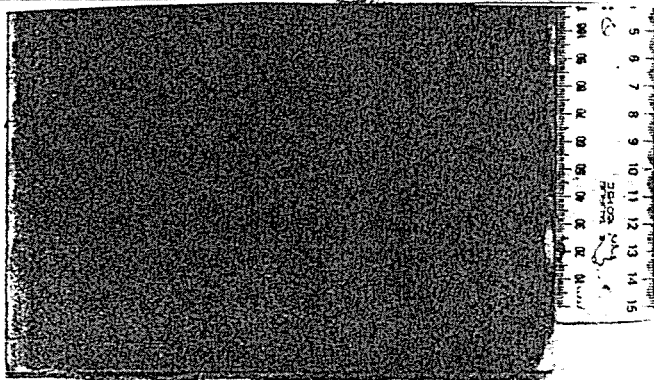
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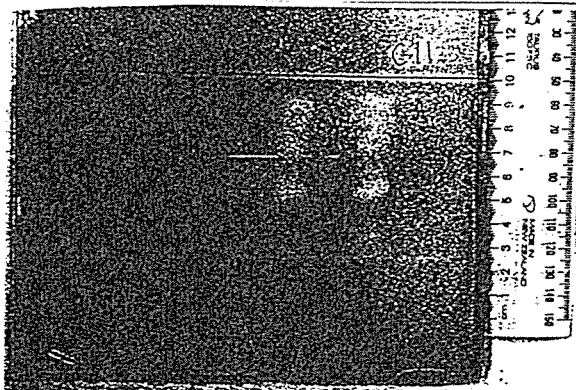
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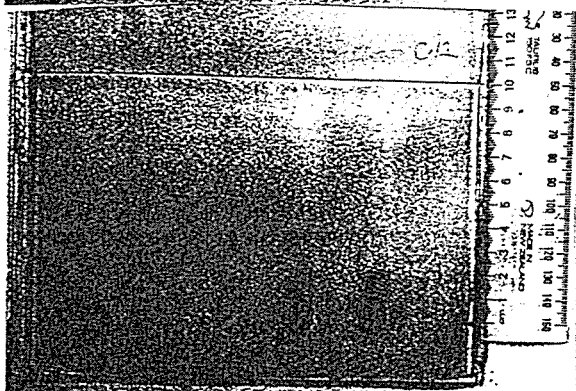
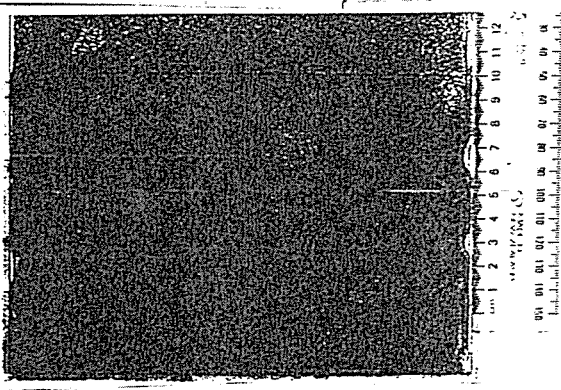
C10



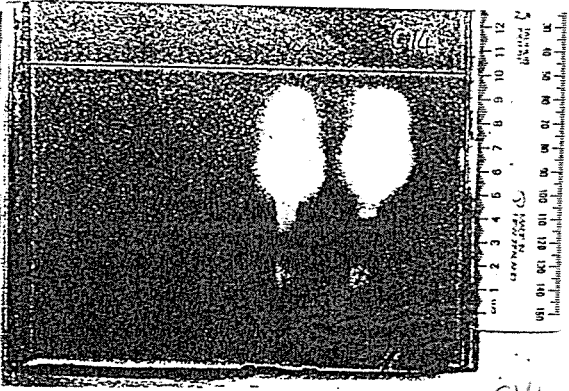
C11



C13



C12



C14

iv. Colour reactions for confirmation and partial characterisation of antifungal phenolic compounds.

Many coloured bands seen in visible light corresponded to inhibitory zones on replicate plates and to UV-absorbing areas (254 nm). Figure 3.20 is an example of the response of materials in F2 and F3 fractions to vanillin-HCl. Table 3.10 summarises the responses of antifungal compounds in zones I-IV to the two chromogenic reagents utilised, and suggests their possible identity. Other bands were detected at different R_f values with these reagents, however bioassays revealed that they had no antifungal activity.

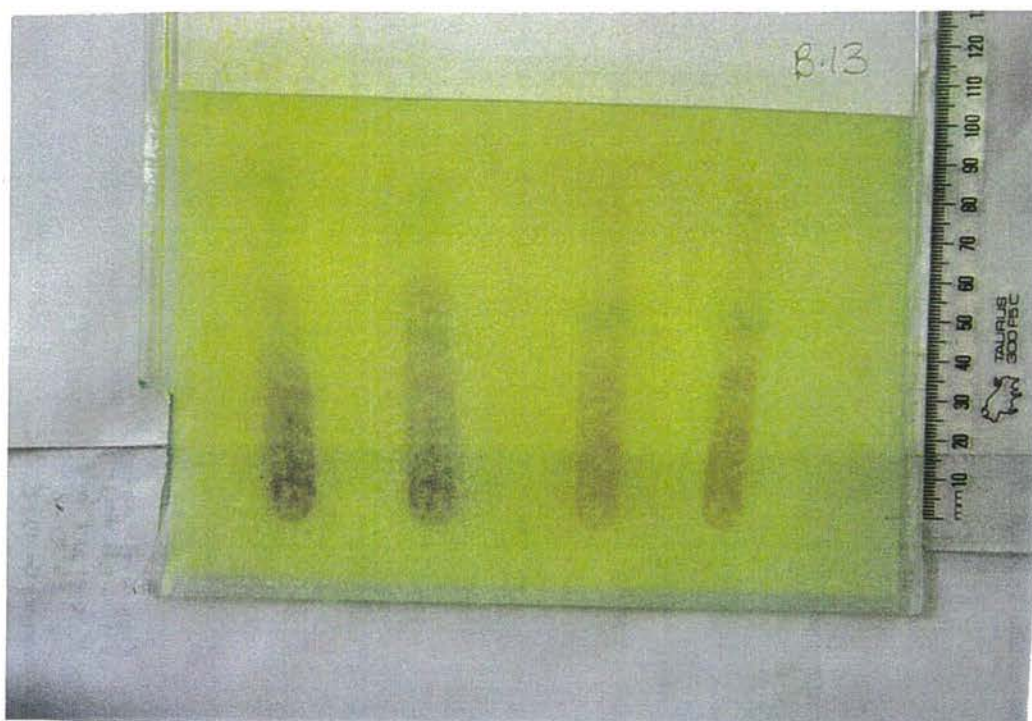


Figure 3.20 The response of materials in F2 and F3 fractions extracted to vanillin-HCl. Extract shown: Inoculated 'Slicemaster' cucumber treated with sunlight liquid.

Table 3.10: Colour reactions of the antifungal compounds in zones I-IV in cucumber leaf extracts, as they appeared in visible light after treatment for the Prussian blue reaction with $K_2Fe(CN)_6 \cdot FeCl_3 \cdot 6H_2O$, and with acid vanillin.

Chromogenic spray	Zone I F2 (Rf=0.70-0.80)	Zone II F3 (Rf=0.00-0.10)	Zone III F3 (Rf=0.50-0.60)	Zone IV F3 (Rf=0.70-0.80)
$K_2Fe(CN)_6 \cdot FeCl_3 \cdot 6H_2O^a$	blue	blue	blue	blue
Acid Vanillin ^b	grey/brown	orange/brown ^c interspersed with pink	pink	pink
Tentative identity	1. phenolics 2. catechins or leucoanthocya- nins	1. phenolics 2. catechins or leucoanthocya- nins possibly, resorcinol or phloroglucinol derivatives, flavans	1. phenolics 2. resorcinol or phloroglucinol derivatives, flavans	1. phenolics 2. resorcinol or phloroglucinol derivatives, flavans

^aThis reagent is specific for phenolic compounds, turning them blue on oxidation of the aromatic ring by the ferricyanide ion.

^b1 g vanillin ($CH_3O \cdot C_6H_3(OH) \cdot CHO$ -Mr=152.15) was dissolved in 10 ml conc. HCl. This reagent is specific for resorcinol and phloroglucinol derivatives, flavans turning them pink-red. In the presence of this reagent catechins and leucoanthocyanins turn blue to grey or brown.

^cAs they appeared in visible light

3.3.3 Glasshouse Trials of Disease Severity

A. Effects of treatments on disease severity

A standard analysis of variance (ANOVA) performed on raw data collected for disease severity indicated that there were highly significant differences ($P < 0.01$) between the seven treatments in their ability to control the development of powdery mildew disease on leaves of all cultivars. As this percentage data violated at least one assumption or prerequisite of the ANOVA, that of variance homogeneity or equality, a transformation of some nature was required. The most appropriate transformation found using the procedure of Fernandez (1992) was a power transformation ($\lambda = 0.7$). Because a common transformation scale was used for all observations in the data, treatment ranks were not altered, and the mean comparisons remained valid (Fernandez 1992). Table 3.11 shows the results of the ANOVA calculated for this data. The difference in powdery mildew severity

between cultivars of different resistances varied for the 7 treatments applied. As a result of the significant ($p < 0.05$) treatment x cultivar interaction the simple effects were examined by lsd comparisons. Figure 3.21 shows the responses of each cultivar to disease.

Table 3.11 Analysis of variance of the severity of powdery mildew on cucumber as affected by host resistance and seven treatments.

Source of variation	df	Sum of squares	Mean square	F-value
A (cultivar resistance)	2	99.9	49.9	39.4**
B (material applied)	6	695.5	115.9	92.6**
AB interaction	12	32.2	2.7	2.1*
Error	147	186.1	1.3	
Total	167	1013.7		

** Indicates differences between treatment means are highly significant at $p < 0.01$. *Indicates differences between treatment means are highly significant at $p < 0.05$.

Comparison of treatment means according to Fisher's protected lsd test indicated that the severity of powdery mildew disease was similar on water-treated 'SM' and 'SK'. Both had significantly ($P < 0.05$) less disease than 'Leb'. Milsana repressed disease to a significantly lower level on all three cultivars, especially 'SK' compared with the water control. This control was not significantly different between 'Leb' and 'SM', and 'SM' and 'SK'. Protection against disease was not significant for cultivars treated with sodium bicarbonate alone, though 'Leb' and 'SK' differed significantly. Sunlight liquid was equally effective on all three cultivars but control was significant on 'Leb' and 'SM' only. Shellspray oil emulsions equally and significantly controlled disease on all three cultivars, and disease severity was similar to that observed with Milsana treatment. Significant disease control was achieved with sodium bicarbonate-plus-sunlight liquid mixtures. Disease severity was similar to that obtained with sunlight liquid. Sodium bicarbonate-plus-shellspray oil was the most effective treatment, controlling disease to levels below quarter of those observed on plants treated with water and sodium bicarbonate.

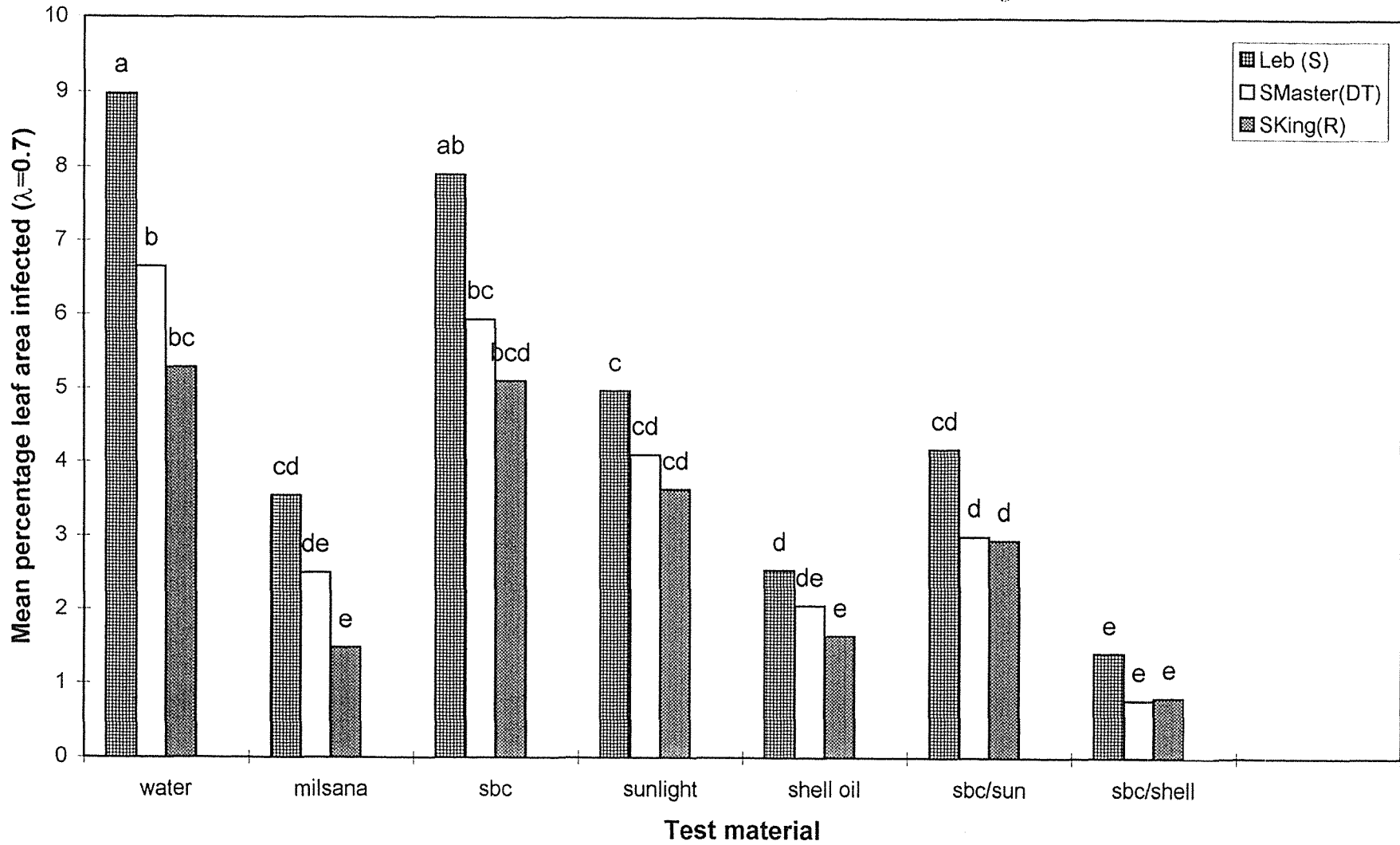


Figure 3.21: Severity of powdery mildew on cucumber cultivars that differed in disease resistance, as affected by seven test materials

3.4 DISCUSSION

Qualitative assessments of chromatogram inhibition bioassays revealed much variability in free-state and aglycone phenolic patterns between cultivar resistance/treatment combinations, in the presence and absence of disease. Production of antifungal free-state phenolics (Fraction 2) was exhibited as a prominent inhibition zone at Rf 0.70-0.80. Their location was similar to compounds detected by Daayf et al. (1995) in extracts from healthy and infected control and Milsana-treated leaves of a disease-tolerant cultivar 'Mustang' (R.R. Bélanger pers. comm.), separated in an identical solvent system. However, from the outset, results differed from Daayf et al. (1995) because this zone of fungitoxicity was not uniform in intensity among the different samples. This indicated differential activity among the plant material tested, and suggested that data did not support the concept of stimulation of host materials by either mildew infection or the treatments applied. The disappearance of phenolics in infected leaves (particularly for 'Leb') which were detected in healthy ones treated with other materials, suggested that the antifungal compounds found previously at this Rf had been inactivated by *S. fuliginea*, or were degraded into non-fungitoxic substances during the infection process. Detoxification of phenolics was noted by Lizzi et al. (1995) who observed that the aglycones luteolin and apigenin were less toxic against *Phytophthora parasitica* than their glycosides. It was suggested that β -glucosidase-activity of this fungus played a prominent role in the detoxification.

Colour reactions with acid vanillin and a positive Prussian blue reaction tentatively suggested that free phenolics were catechins (Chakraborty & Saha 1994). Catechins are flavan-3-ols with 2 hydroxyl groups in the side ring (Figure 3.22). Their presence has not been reported in cucumber, but they are biologically highly active and are known to have a widespread occurrence in nature, especially in woody plants (Harborne & Baxter 1993). Catechins from tea leaves extracted and bioassayed in a similar manner have displayed activity against *Streptococcus mutans* (Kawamura & Takeo 1989) and the fungus *Bipolaris carbonum* (Chakraborty & Saha 1994). Before we can be certain of the identity of F2 phenolics in these crude extracts, further purification is necessary. This would probably involve removal of antifungal compounds from silica gel, chromatography in a different

solvent system to separate out compounds, repeat bioassays, and co-chromatography and ultraviolet spectral comparisons with authentic catechin substances. Several excellent treatises on the isolation and identification of phenolic compounds in biological material are available and would be useful in future investigations (Seikel 1962; Harborne 1989; van Sumere 1989).

The Cucurbitaceae are the major source of tetracyclic triterpenoids collectively known as cucurbitacins (Lavie & Glotter 1971), which occur both free and in glycosidic combination (Harborne & Baxter 1993). These substances were first characterised as the bitter principles of cucumber, marrow, and squash (Harborne & Baxter 1993), which provide effective defence against many herbivores. It has been speculated that these substances play a role in host defence as antifungal compounds resident in plant tissues before infection, or as phytoalexins synthesised after pathogen challenge (Bar-Nun & Mayer 1990). It is possible that cucurbitacins were present in phenolic fractions bioassayed in the present study and contributed to fungitoxicity observed. The effect of cucurbitacins on fungal pathogens was investigated in two studies, which showed that application of partially purified cucumber extracts or authentic cucurbitacin I (Figure 3.23) to cucumber plants and fruit inhibited the growth of *Phytophthora cactorum* (Nes & Patterson 1981) and *Botrytis cinerea* (Bar-Nun & Mayer 1990). In the latter study, the protective ability of cucurbitacin I was associated with an inhibition of induction of extracellular laccase formation by *B. cinerea*. Laccase, a polyphenoloxidase, is capable of oxidising and hence inactivating phenolics such as *ortho*- and *para*-dihydroxyphenols and the free phenol hydroquinone in plants (Mahadevan 1982). Co-chromatography and UV spectral comparisons (Harborne 1986) of antifungal compounds purified from extracts in the present study with authentic cucurbitacins, might unravel their identity or even provide further evidence for cucurbitacins having a role in defence. This would be of particular interest if sodium bicarbonate/additive mixtures enhanced production of cucurbitacins.

Glycosidically-bound phenolics extracted from cucumber leaves, hydrolysed into their free form (aglycones-Fraction 3), displayed prominent antifungal activity at three R_f values on chromatograms. The majority of these zones were darkly absorbing at 254nm and gave a positive Prussian blue reaction, confirming that phenolics were present. Longwave

ultraviolet light suggested that all compounds fluorescing the same colour were the same, or if not identical, then very closely related; this remains to be seen. Bands of materials were more distinguishable in F3 extracts than the F2. Perhaps because F3 passed through several more extraction procedures, the final extract contained less erroneous material than F2.

For most extracts the greatest inhibition was encountered at the origin (Rf 0.00-0.10) which suggested that the highest concentration of aglycones and/or the most active components were located there. Fungitoxicity of compounds appeared less intense at Rf 0.50-0.60 and Rf 0.70-0.80. As the majority of extracts from healthy leaves treated with the various materials contained aglycones at these three Rf values, and because of the large variability in spot diameters, it was difficult to say whether stimulation of aglycone production had occurred in infected leaves, and as a result of any specific treatment. Thus the presence of these highly fungitoxic substances could not account for differences in disease severity on cultivars in the present study. Results differed from bioassays by Daayf et al. (1995), for they did not observe aglycones at the origin in healthy, infected, and Milsana-treated controls of the single cultivar studied. Daayf et al. (1995) also reported a rapid and distinct accumulation of antifungal phenolic compounds in leaves treated with Milsana, especially in infected leaves. Similar increases were not observed for Milsana treatment, however antifungal phenolics were detected at similar Rf values.

As there were no obvious increases in aglycones for any treatment/cultivar combination, one way to distinguish differences could be on the basis of the identity of antifungal compounds in cucumber extracts. It is not sufficient to simply measure "total phenolics in crude extracts" because this could mask variation in the concentration of a minor component critical to resistance (Pollock & Drysdale 1976). Once isolated and their structure determined by mass spectroscopy, changes in the levels of specific substances in healthy and infected cucumber leaves, treated with the various materials, could be quantified by high-performance liquid chromatography (HPLC) as a time series. Chakraborty & Saha (1994) followed their initial screening work for antifungal materials in tea leaves on TLC-plates with such a method. This led them to discover that pyrocatechol levels were always less in susceptible than in resistant tea leaves. The composition of phenolic acids can be very different between susceptible and resistant cultivars (Trajkovski

1974). For example, while some kinds and amounts of flavonol glycosides were found in black current (*Ribes nigrum* L.) cultivars both resistant and susceptible to powdery mildew (*Sphaerotheca mors-uvae* (Schw.) Berk)), phenolic acids common to more resistant cultivars were absent from susceptible ones (Trajkovski 1974).

Substances in the present study at all Rf values partially or strongly reacted with acid vanillin, indicating that they were possibly phenols derived from resorcinol, or contained phloroglucinol units in their structure, flavans, catechins or leucoanthocyanins (Harborne 1986). Of these, it is known that catechin is oxidatively cleaved to simpler phenols and phenolic acids, such as pyrocatechol, phloroglucinol and protocatechuic acid (Sambanam et al. 1982). Protocatechuic acid is a water-soluble phenolic acid which may occur in glycosidic combination, and is thought universal in angiosperm plants (Harborne & Baxter 1993). The antifungal activity of protocatechuic acid has been demonstrated in at least two host-parasite interactions:

- i. Levels of protocatechuic acid and catechol are high in resistant onion bulb scales (*Allium cepa*). Owing to their water solubility, these substances can diffuse into inoculum-containing droplets and inhibit germination and penetration of spores from *Colletotrichum circinans* (onion smut disease) (Isaac 1992);
- ii. protocatechuic acid inhibited the growth of a fungus causing canker disease in eucalypt (Harborne & Baxter 1993).

Purification and analysis of these antifungal compounds should reveal their true identity. The use of the spray reagent Folin-Ciocalteu which reacts with phenols with catechol nuclei colouring them blue, would confirm whether such phytochemicals were present. Phenolic substances may be partly terpenoid-derived (Harborne & Baxter 1993), and hence antifungal aglycones may be related to cucurbitacins. Future work might reveal such a relationship.

Chromatogram inhibition bioassays proved successful as a screening method for antifungal compounds in these cucumber extracts. The assay was simple, sensitive and results achieved in a relatively short time. In conclusion:

1. Data do not support the concept of stimulation of host materials by either mildew infection or the treatments applied. It appears they had no effect on host physiology, and that experimental errors may have been made in sample collection, during the extraction process, or during separation of substances in the solvent system which contributed to

these results. Development of chromatograms in a different solvent system such as butanol-acetic acid-water (Harborne 1986) may separate substances more completely.

2. High levels of disease control provided by sodium bicarbonate/additive mixtures were therefore probably the result of fungitoxic effects directly on *S. fuliginea*.
3. It was difficult to determine whether variation in phenolic patterns was correlated with differential resistance to powdery mildew.

4. THE ROLE OF PEROXIDASE IN DEFENCE OF CUCUMBER AGAINST POWDERY MILDEW

4.1 INTRODUCTION

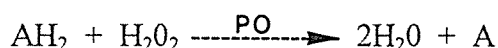
4.1.1 Peroxidase

Peroxidase (E.C. 1.11.1.7; PO) is an oxidative enzyme ubiquitous in higher plants, and thought to be involved in many molecular, physiological, and morphological events that occur during normal plant development (Goodman et al 1986; Reuveni & Reuveni 1995). PO and other oxidative enzymes, particularly polyphenoloxidases, have increasingly been implicated in the resistance responses of plants towards pathogens. Recent physiological and biochemical studies (Reuveni & Bothma 1985; Reuveni et al 1991, 1992) have suggested that the 'level' of PO in plant tissues is indicative of a plant's potential to respond advantageously to environmental stresses such as fungal invasion, though environmental and physiological factors can affect its relative activity (Artlip & Funkhouser 1995). Further, several researchers have proposed that the level of inherent PO activity in plants before infection takes place, to be of value as a biochemical marker for disease resistance in several host-pathogen systems (Reuveni & Bothma 1985; Reuveni et al 1991, 1992). This could be used in breeding programmes to select individuals with the best potential for resistance.

PO exists in soluble form in the cytoplasm, or linked by ionic or covalent bonds to other components in cell walls (Reuveni & Reuveni 1995). Wall-bound anionic peroxidases are readily detected in lignifying tissues (Goodman et al 1986). The importance of the PO enzyme appears to lie in its role in the final steps of lignin biosynthesis. PO is classified as a polymerising enzyme which scavenges and polymerises phenoxy-free radicals, precursors of lignin and suberin (Kolattukudy et

al. 1980; Poole et al 1993). These free radicals are generated from the oxidation of the phenolic hydroxyl groups of coniferyl and hydroxycinnamyl alcohol monomers (Poole et al 1993), compounds which frequently accumulate in response to infection.

The general reactions catalysed by peroxidase involve H_2O_2 as the source of the oxidising power, and are described by the equation:



AH_2 may represent a phenolic compound such as chlorogenic or caffeic acid (the donor). Hydrogen atoms derived from these phenol substrates are combined with the oxygen of the peroxide itself (Goodman et al 1968). A represents the free-radical (oxidised donor).

PO then proceeds to aid in the deposition of these lignin and suberin polymers in cell walls for the purpose of wall reinforcement perhaps, and in papillae, which may physically block penetration at the sites of infection (Asiegbu et al 1995). In this way, PO is considered to interfere with the further growth and development of the challenging pathogen (Kolattukudy et al. 1980), though there is continuing debate as to whether PO is involved directly or indirectly in defence. A further consequence of phenolic compound oxidation is the production of quinones, which in a hypersensitive response (HR) then polymerise to give brown coloured compounds, often more toxic to microorganisms than the original phenols. Several other functions are suggested for PO:

1. PO may function as a scavenger of cytotoxic oxidants produced by pathogens, that would otherwise lead to 'hypersensitive' cellular collapse
2. Alternatively, PO may enhance defence by the production of toxic radicals (Asiegbu et al 1995)
3. PO may be involved in defence by cross-linking phenolic compounds into papillae (Cadena-Gomez & Nicholson 1987), and in the control of cell elongation

processes by altering the phenolic cross-links of cutin in the epidermal cells in which phenolic compounds have been detected.

4.1.2 Peroxidase Activity and Compatible-Incompatible Host Reactions

PO activity has been studied in diseased plants of many host-pathogen associations, *C. sativus*-*S. fuliginea* among them, in connection with their susceptibility (compatibility) or resistance (incompatibility). Elevated PO activity has often been associated with the post-infection responses of both resistant and susceptible plants to pathogens (Reuveni et al 1991). Positive correlations have been drawn between this and resistance phenomena such as lignification and increases in the rate of polymerisation of phenoxy-free radicals into lignin-like substances, changes in PAL activity, and phenol accumulation. In the absence of disease control materials, the activity of PO has been observed at higher levels in the infected tissues of resistant varieties than in infected susceptible ones and the non-infected healthy plants (Agrios 1988) of either resistance.

Frequently associated with these shifts in activity during infection is the formation of new forms of the enzyme (isozymes) in infected tissues (Svalheim & Robertsen 1990). Anionic forms of these isozymes are considered to be particularly significant in deposition (Kolattukudy et al. 1980). There are several lines of evidence to suggest that the induction of PO participates in the mechanisms of plant disease resistance:

- i. PO activity has been seen to increase locally- regularly increasing in the cells surrounding lesions where wounding or localisation of a pathogen has occurred (Ahl-Goy et al. 1992).
- ii. PO activity has been seen to increase systemically- Slow-migrating isozymes were found in muskmelon cultivars resistant to *S. fuliginea*, but absent from susceptible varieties (Reuveni & Bothma 1985). It was suggested that possibly the enzyme protein was active but present in undetectable amounts, or that it was present but inactive.

iii. The resistance of a host to disease has been negated by oxidative enzyme inhibitors.

4.1.3 Peroxidase Activity and Chemical Treatments

Peroxidases have been studied extensively in many host-parasite interactions. However, little work has been published on the effects of modern fungicides on peroxidase activity. It is only in recent times, when attention has turned towards finding alternative disease control materials, or ways of reducing or better utilising existing fungicides, that research focussing on host responses to their application has been conducted.

Treatment with inorganic phosphate (K_2HPO_4) salts (Irving & Kuc 1990) and silica preparations (Schneider & Ullrich 1994) has elevated levels of β -1,3-glucanase, chitinase, and peroxidase activity in cucumber plants. Phosphate treatment also stimulated a 3-fold increase in soluble peroxidase activity in non-infected grape berries. Even more unusual was an 8-fold increase in soluble peroxidase activity in phosphate-treated berries infected with powdery mildew (*Uncinula necator*), and a 2-fold increase in ionically-bound peroxidase (Reuveni & Reuveni 1995).

Prophylactic Milsana treatment (a formulation prepared from extracts of the giant knotweed-*Reynoutria sachalinensis*) has also enhanced the activities of several potentially defence-related enzymes in cucumber and tobacco (*Nicotiana tabacum* L.) (Schneider & Ullrich 1994). Correlations were drawn between the increased activities and induction of resistance to *S. fuliginea* and bacterial pathogens of tobacco, however no clear relationships could be found between resistance and the increases in specific enzyme activities. This suggested to the researchers that resistance was not due to any particular enzyme, but more likely the result of a series of molecular, cellular and biochemical interactions and recognition processes between the host and pathogen.

The objectives of this enzymatical study were to:

- i. Investigate whether shifts in peroxidase-activity were stimulated in leaves from cucumber cultivars that differed in disease susceptibility, in the presence and absence of the pathogen, when treated prophylactically with sodium bicarbonate/additive mixtures.
- ii. Determine whether peroxidase was useful as a biochemical marker of host resistance for cultivars under testing.

4.2 MATERIALS AND METHODS

4.2.1 Plant Culture, Chemical Treatment and Inoculation

Plant material pertaining to three cucumber cultivars, 'Leb', 'SM' and 'SK' was raised and maintained under the same environmental regime and soil conditions as described in Chapter 2.1.1. Once seedlings had reached 3 weeks of age, those uniform in height and having the second-true leaf unfolded were selected out and arranged on a greenhouse bench in eight blocks containing rows of pots 7 x 6 in length.

Once the third-true leaf had fully expanded, the seven materials listed in Table 2.3 were applied to run-off as before, with a. When plants were dry, they were returned to the experimental site and arranged with the treatments randomised in eight replicate blocks. This provided the samples for peroxidase assays which were harvested from plants at six time intervals, 0 (ie. 1 h after inoculation), 1, 3, 7, 10, 14 days) following inoculation with *S. fuliginea*, which occurred 2 h after treatment as described in Chapter 3.2.1. Four of the eight blocks were treated in a similar way but were not inoculated.

4.2.2 Assessment of Powdery Mildew Disease

14 days after treatment inoculation, leaf 2 was examined and the severity of disease rated according to a 0-4 scale devised by Reuveni & Bothma (1985):

Rank	Rank defined
0	no powdery mildew lesions observed on leaves
1	1-50 infection sites
2	51-100 infection sites
3	101-200 infection sites
4	>200 infection sites (sporulation)

4.2.3 Determination of Peroxidase Activity

A. Preparation of crude enzyme extract

The procedure undertaken to extract and determine peroxidase activity was based on one published by Reuveni et al. (1992) with few modifications.

1. Leaf 2 was harvested from 4 plants. Samples were then washed, blotted dry, placed in labelled re-sealable bags, quick frozen in liquid air, and stored at -75°C until analysed for soluble peroxidase activity.
2. Two leaf discs, 10 mm in diameter, were removed from both sides of each sample leaf from the same approximate position. Leaf discs were ground with a mortar and pestle at 4°C , in 1 ml ice-cold 0.015 M sodium phosphate buffer (pH 6.0) which was prepared according to Gomori (1955). At room temperature, a volume totalling 6.15 ml 0.2 M- Na_2HPO_4 was added to 43.85 ml 0.2M NaH_2PO_4 and diluted with deionised water to 100 ml. The pH was checked with a meter.
3. The homogenates were decanted into 1 ml eppendorf tubes and clarified by centrifugation at 10,000 g for 10 min at 4°C . The resulting supernatants were used for peroxidase assays.

B. Determination of peroxidase activity in supernatant

1. Fifty microlitres of the supernatant was added to 3 ml of reaction mixture or “synthetic substrate” (containing 1 x 1 mg tablet of 3,3',5,5'-tetramethylbenzidine (TMB; Sigma Chemical Company) dissolved in 100 μl dimethyl sulphoxide ($(\text{CH}_3)_2\text{SO}$; Mr=78.13; Ajax Chemical Pty), 5 ml 0.2 M sodium acetate, 195 μl 0.2

M citric acid, 5 μ l 30% H₂O₂, and 5 ml deionised water) (Dewey 1996) in test tubes, to initiate the reaction.

2. Contents were vortexed, then emptied into clean 3 ml plastic disposable curvettes.
3. Peroxidase reacted quantitatively with the reaction mixture to give a coloured product (Williams & Wilson 1981). The colour exhibited was complimentary to that which was absorbed by the chromophore TMB, so that in visible light, samples appeared greenish-blue because they absorbed light in the orange region of the spectrum; a wavelength of 595-650 nm. Before commencing with the assay proper, trial runs determined that the most appropriate wavelength at which to measure the rate of increasing absorbance in the samples (ie. increase in the concentration of the reaction product per unit of time- which is a direct measure of the catalytic activity in the sample assayed (Davies & Littlewood 1979); or rate of appearance of the chromophore), was 620 nm. The reaction (colour development) was followed at room temperature ($21 \pm 1^\circ\text{C}$) for 10 mins., then stopped with 3 ml of 3 M H₂SO₄, and a final reading taken at 470 nm. Addition of H₂SO₄ produced a colour change in samples from blue to yellow, hence, estimates of absorbance were made in the blue region of the spectrum. Each supernatant was assayed in duplicate, and two controls were included in each set of assays; boiled leaf material (*boiling for 10 min at 90-100°C was sufficient to destroy enzyme activity but retained a comparable quantity of chlorophyll protein), and substrate lacking enzyme. No increase in absorption occurred when the enzyme extract was assessed.

4.2.4 Peroxidase as a Biochemical Indicator of Resistance in Cucumber

In 1990, a rapid, non-destructive assay was devised by Reuveni et al. that demonstrated a high correlation between peroxidase activity in muskmelon (*Cucumis melo*) leaves and the known levels of resistance or susceptibility of whole plants to downy mildew, *Pseudoperonospora cubensis*. Their procedure was repeated to see if a similar relationship could be found between the levels of resistance and

susceptibility that the three cucumber cultivars under consideration were regarded by their seed distributors to have.

Leaf discs, 5 mm in diameter, were removed from the third leaf of non-inoculated plants. One disc was placed in separate wells in a 96-well ELISA cassette (Nunc. Immuno Plate MaxiSorp™) each containing 0.5 ml of the above mentioned reaction mixture. After 5 min, leaf discs were removed and the reaction stopped with 0.25 ml of 3 M H₂SO₄. An ELISA plate reader (DYNATECH MR 5000) was used to quantitate colour development in wells at 450 nm.

4.3 RESULTS

4.3.1 Disease Severity

Symptoms were observed first on leaves of untreated control plants and individuals treated with sunlight liquid and sodium bicarbonate, approximately five days after inoculation. Associated with bicarbonate treatment were circular areas or speckles, yellow-brown in colour and approximately 2-5 mm in diameter, on the adaxial surface of leaves of 'Leb' plants. These appeared within three days after treatment.

A standard analysis of variance performed on the data collected for disease severity indicated that there were highly significant differences ($P < 0.01$) between the seven treatments in their ability to inhibit powdery mildew disease on leaves of all cultivars. When the responses of each cultivar to prophylactic treatments were compared, it was seen that the more resistant cultivar 'SK' had significantly ($P < 0.05$) less foliar infection than 'Leb' and 'SM'. Milsana reduced disease on all three cultivars but the reduction was significant on 'Leb' and 'SM' only. There was no significant reduction among cultivars with sodium bicarbonate treatment. Application of shellspray oil alone significantly reduced disease severity on all three cultivars. Sunlight liquid significantly reduced disease on 'SM' only. Protection against powdery mildew was complete on all three cultivars with sodium bicarbonate-plus-

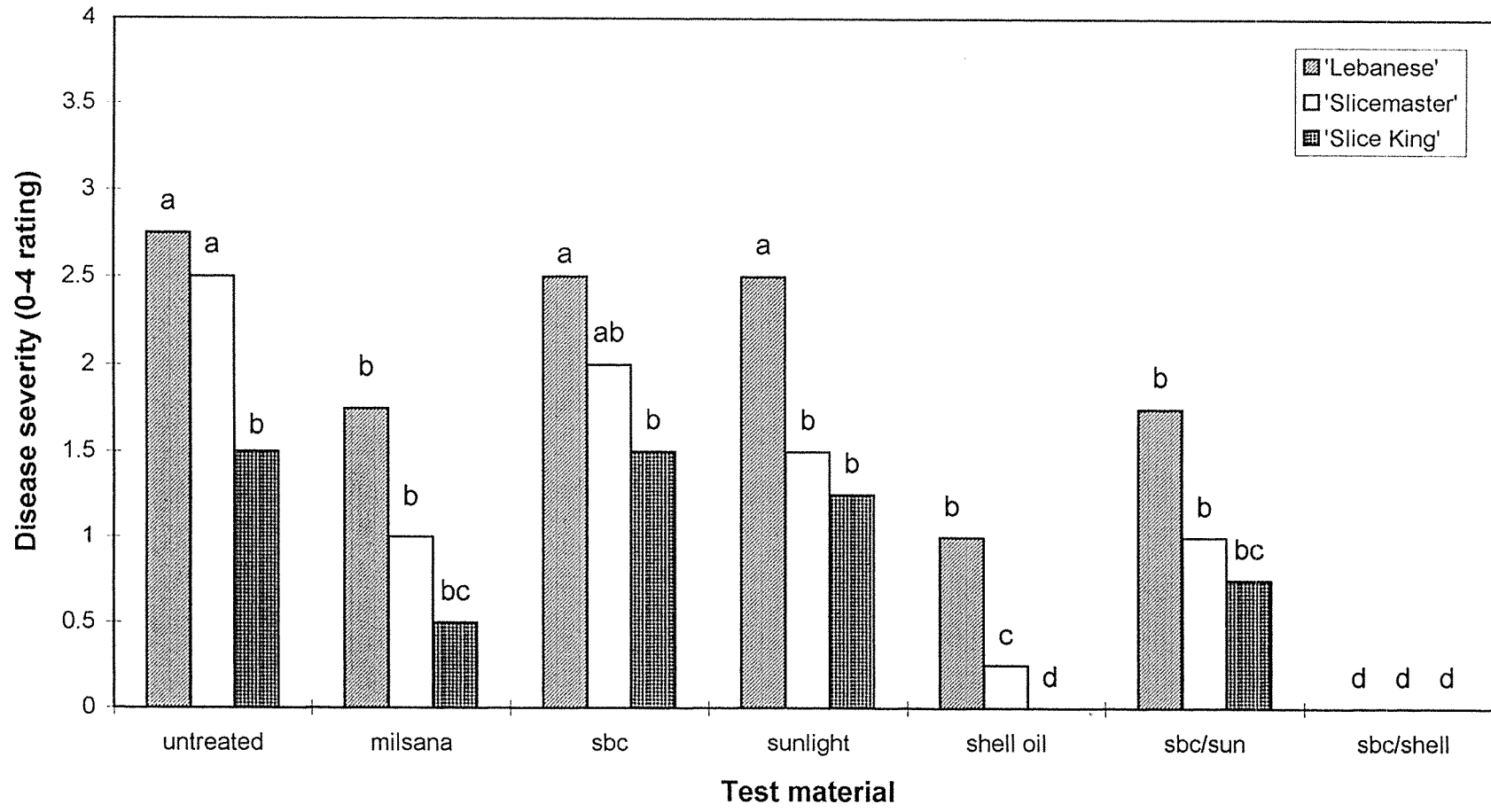


Figure 4.0 Protection of cucumber foliage against powdery mildew disease by foliar sprays of Milsana or sodium bicarbonate/surfactant mixtures

shellspray oil treatment. Disease severity on plants treated with sodium bicarbonate-plus-sunlight liquid was similar to that obtained with Milsana and was also significant on 'Leb' and 'SM' only.

Individual treatment means were compared after transformation with the appropriate controls using Fisher's protected LSDs, since the standard errors of differences between pairs of treatments were very similar. Text-bars with different letters are significantly different ($P < 0.05$) according to Duncan's Multiple Range test (Figure 4.0).

4.3.2 Effects of Treatments on Peroxidase Activity

i. Non-inoculated 'Lebanese' (Figure 4.1)

Compared to the control, peroxidase (PO) activity 1 h post-treatment was not significantly ($p < 0.01$) different. The following day, activity was significantly greater in plants treated with sodium bicarbonate ($p < 0.01$) alone. Three days later PO activity had increased slightly for all treatments, but was only greater in plants treated with bicarbonate. By 7 days, PO activity was only significantly greater in Milsana and bicarbonate treated plants. However in contrast with this, only shellspray oil did not differ significantly 10 and 14 days after inoculation. From Figure 4.1 an apparent trend for the majority of treatments was a gradual increase in activity over time from c. 0.23-0.26 total absorbance (abs.) units (day 0) to an almost plateau of c. 0.60-1.09 abs. units (day 14).

ii. Inoculated 'Lebanese' (Figure 4.2)

PO activity was significantly higher ($p < 0.05$) in bicarbonate treated leaves only, 1 h post-inoculation (p.i). The next day, activity had significantly increased in leaves collected from Milsana and bicarbonate only. Three days after inoculation only Milsana and sodium bicarbonate treated leaves measured significantly higher activity. Activity in plants treated with all materials had significantly increased 7 days p.i. except shellspray oil and bicarbonate-oil mixtures which were significantly lower in treated leaves. By 10 days p.i., PO activity had risen substantially in water treated controls. This level was significantly greater ($p < 0.01$) than for all other treatments

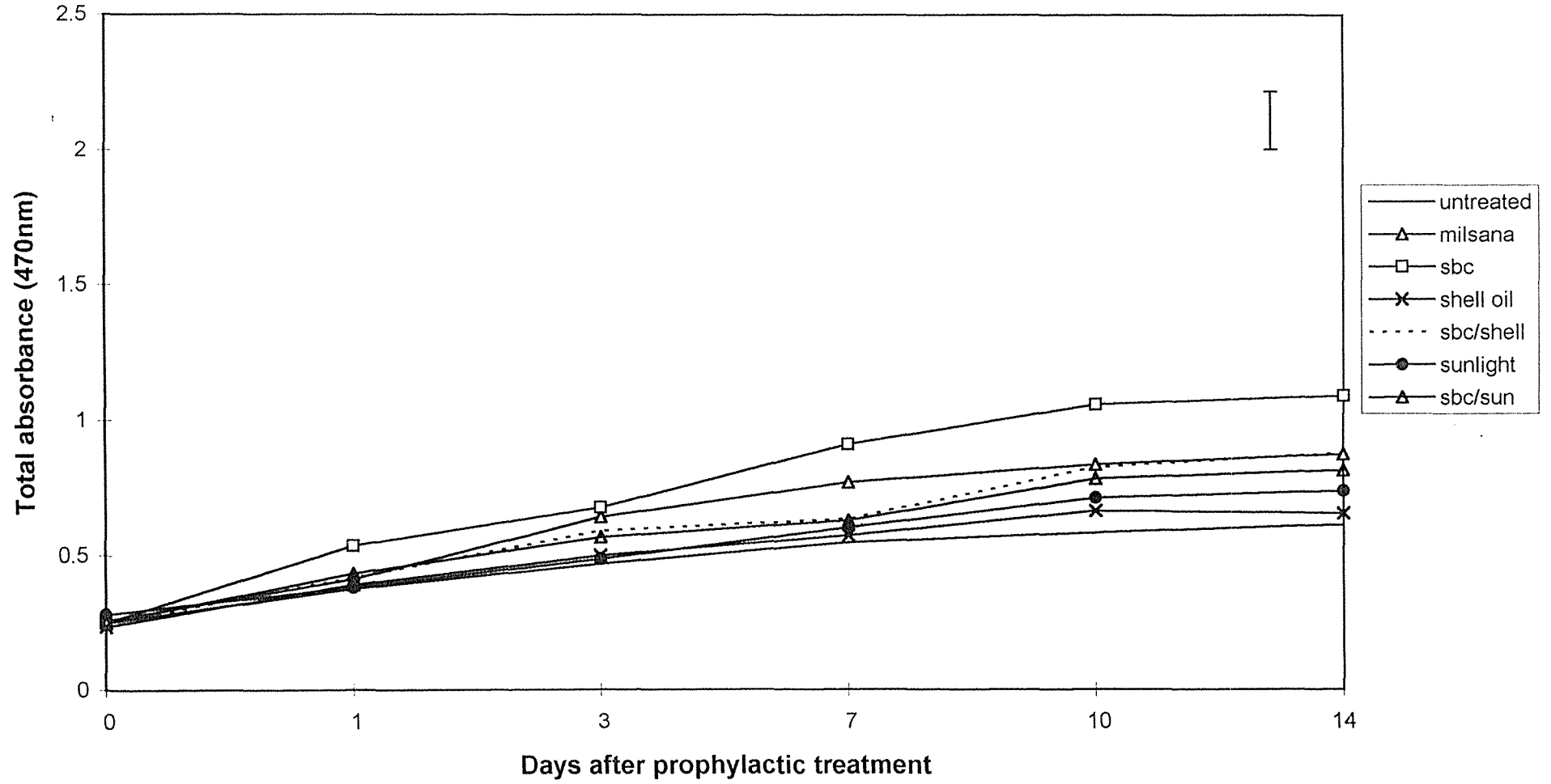


Figure 4.1 Daily comparison of peroxidase activities in leaf 2 sampled from noninoculated 'Lebanese'

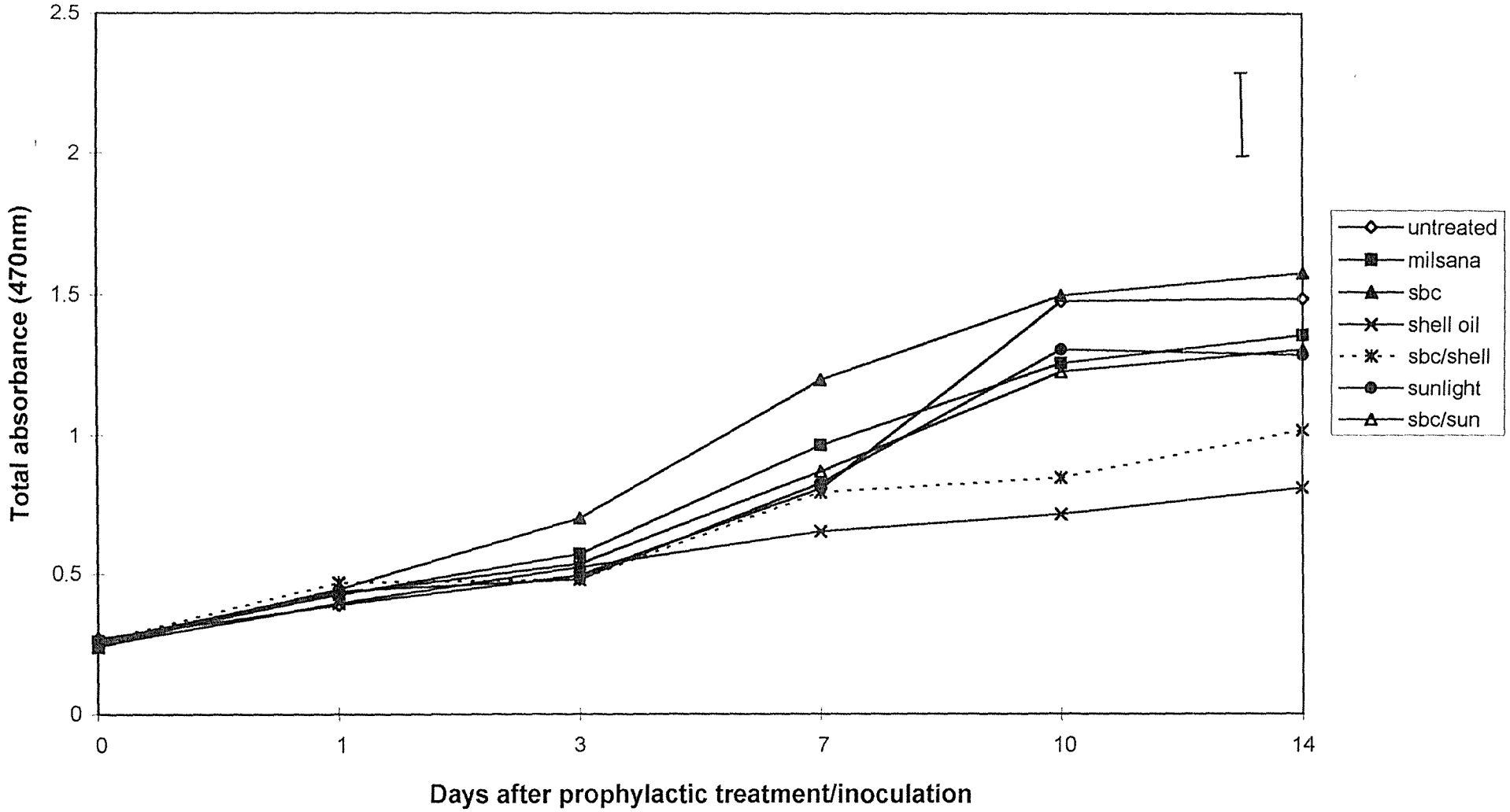


Figure 4.2 Daily comparison of peroxidase activities in leaf 2 sampled from inoculated 'Lebanese'

except sodium bicarbonate and sunlight liquid. This pattern was repeated on day 14 ie. significantly greater for all except Milsana and bicarbonate that activity was not significant from that measured in Milsana, and it had increased significantly ($p < 0.01$). Activity in sunlight liquid-treated leaves was now significantly lower. The rate of increase in activity (steepness of the slope) appeared to be the greatest over the 7-10 day p.i. period.

iii. Noninoculated 'Slicemaster' (Figure 4.3)

PO activity did not differ significantly between treatments in leaves collected from plants 1 h p.i. (average c. 0.50-0.60 total abs. units). One day later PO activity had increased slightly in leaves from all treatments, but had significantly in sodium bicarbonate-treated leaves only. After 3 days, PO activity was significantly higher for two materials applied ie. Milsana and sodium bicarbonate. This pattern was repeated on day 7-10. In addition, activity in bicarbonate-plus-sunlight liquid-treated leaves was now significantly higher ($p < 0.01$). Fourteen days p.i. only activity in shellspray oil treated leaves did not differ significantly, though for both shellspray oil-plus-sodium bicarbonate and sunlight alone the difference was only significant at the 5% level. The final readings for total absorbance lay between c. 0.95-1.15 units which meant that PO activity had virtually doubled over the 14 day period.

iv. Inoculated 'Slicemaster' (Figure 4.4)

As for non-inoculated plants, there was no significant ($p < 0.01$) difference in activity which ranged from c. 0.50-0.60 abs units on average, between treatments applied when the first samples were collected. This changed on day 1 where both sodium bicarbonate alone and combined with sunlight liquid increased significantly. PO levels continued to be higher in leaves treated with these two materials on day 3. In addition significantly higher levels were measured in plants treated with Milsana. By day 7, PO was significantly higher at the 5% not 1% level of significance in all plants, except those treated with shellspray oil alone and combined with bicarbonate. Activity measured 10 days p.i. did not differ between water, Milsana, bicarbonate,

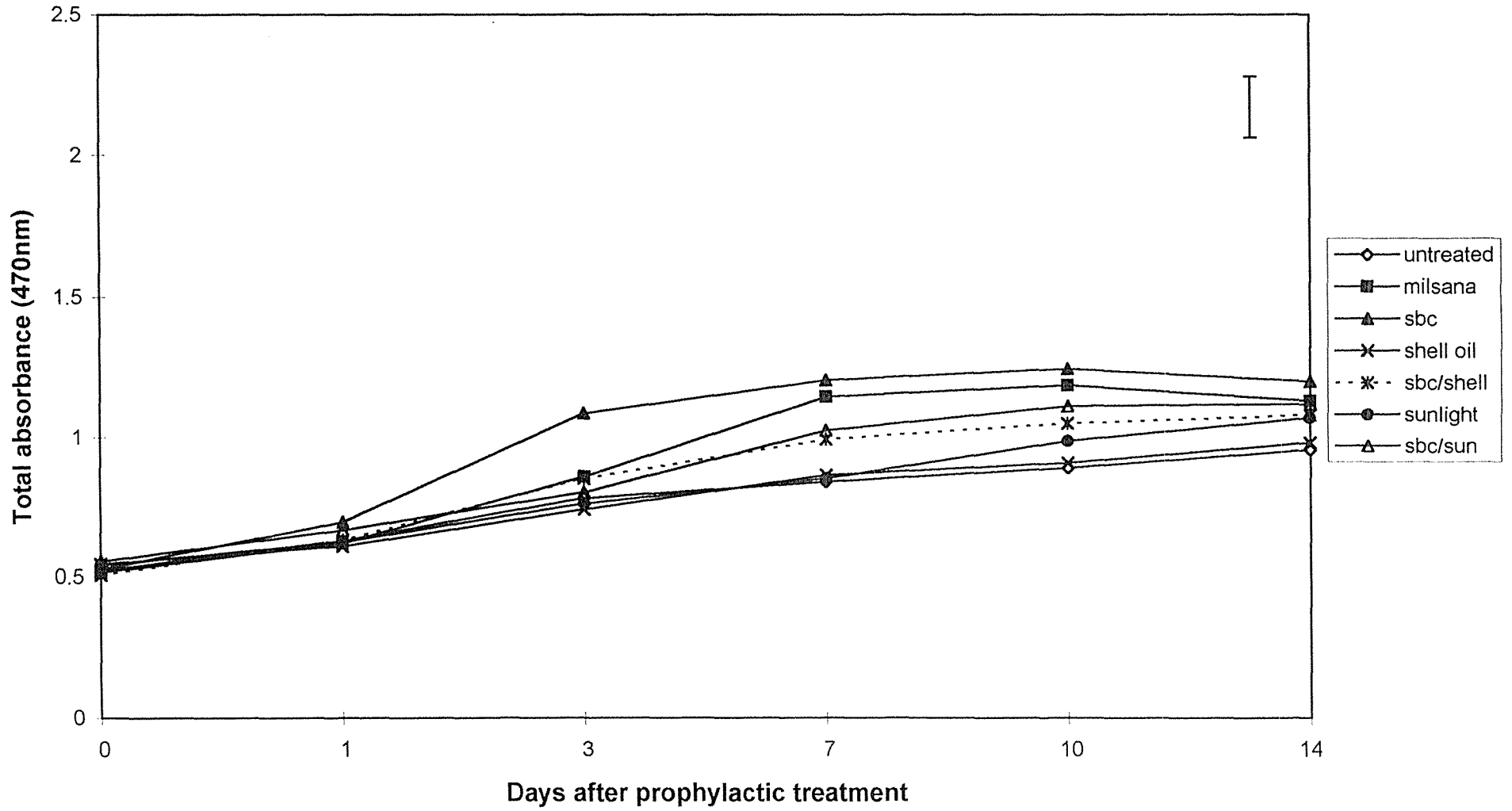


Figure 4.3 Daily comparison of peroxidase activities in leaf 2 sampled from noninoculated 'Slicemaster'

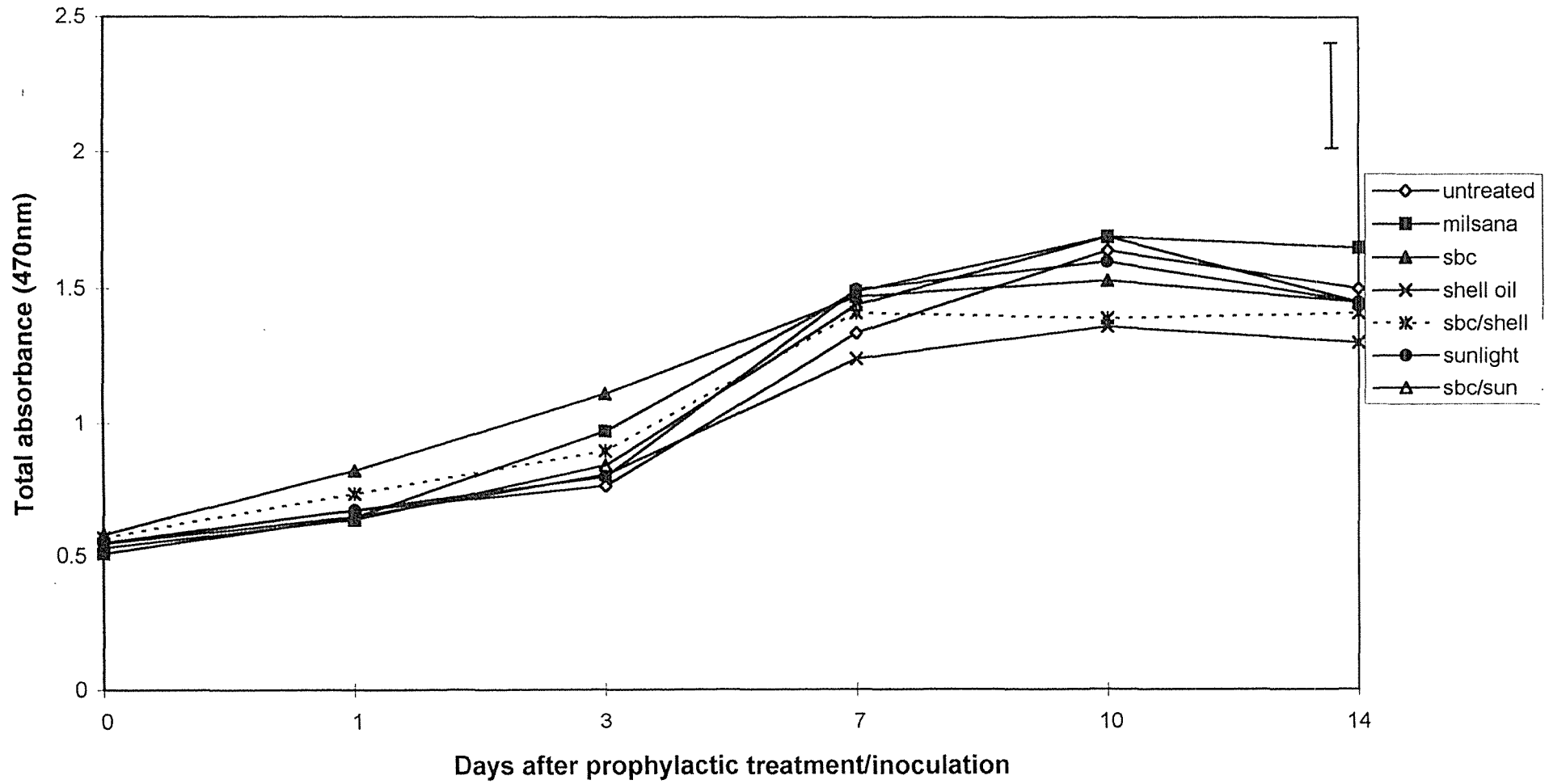


Figure 4.4 Daily comparison of peroxidase activities in leaf 2 sampled from inoculated 'Slicemaster'

sunlight alone and combined with bicarbonate. Activity in leaves treated with shellspray oil alone and combined with sodium bicarbonate was significantly less ($p < 0.01$). By 14 days p.i. PO activity was significantly higher in Milsana ($p < 0.05$) and significantly less in shellspray oil only. Looking at Figure 4, the increase in activity was greatest (of the steepest slope) over days 3-7 p.i. for the water treated control.

v. Noninoculated 'Slice King' (Figure 4.5)

Leaves collected 1 h and 1 day p.i. did not differ significantly ($p < 0.01$) in activity whichever treatment was applied 3 and 4 days earlier. Average levels of activity were measured at c. 0.85-0.95 absorbance units. By day 3 leaves treated with Milsana and sodium bicarbonate had significantly higher activity compared to the water treated controls. This pattern was continued on days 7 to 14 except that on day 10 plants treated with sunlight liquid had significantly ($p < 0.01$) less PO activity than the control, and on day 14 there was no significant difference between levels in leaves treated with this material and the water control.

vi. Inoculated 'Slice King' (Figure 4.6)

No significant differences in activity were measured on days 0-3 p.i. Average levels of activity began at c. 0.95-1.2. 7 days p.i. PO activity in shellspray oil and sodium bicarbonate/shell oil treated leaves was significantly less ($p < 0.01$). On day 10, PO activity remained the highest in water, Milsana and sodium bicarbonate treated plants. Significantly less activity was measured in plants treated with all other materials. By day 14 PO activity was still increasing (Figure 6) for all treatments except sodium bicarbonate/sun. There was no significant difference between levels in leaves treated with water, Milsana and sunlight. Plants treated with all other materials had significantly less activity.

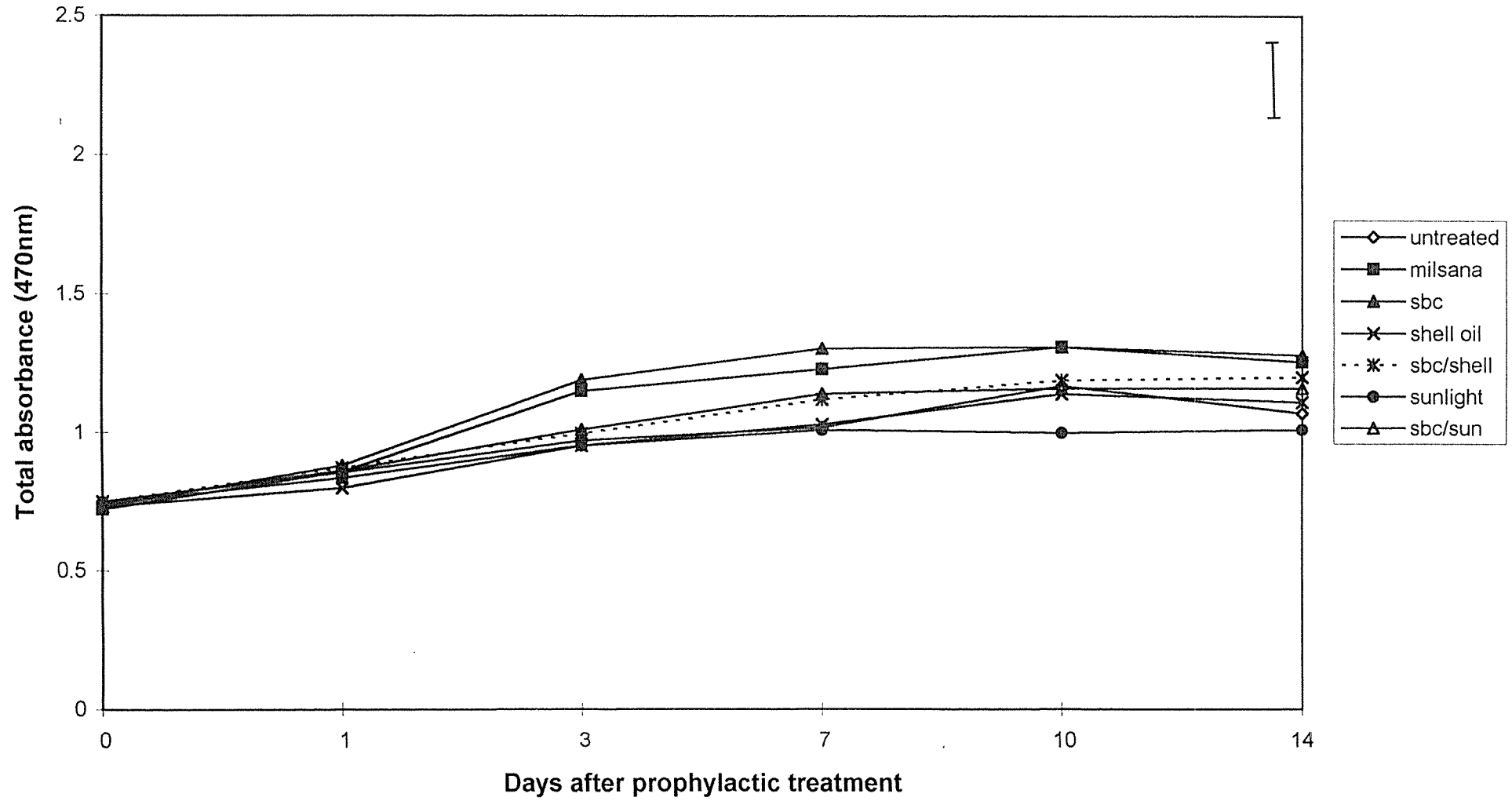


Figure 4.5 Daily comparison of peroxidase activities in leaf 2 sampled from noninoculated 'Slice King'

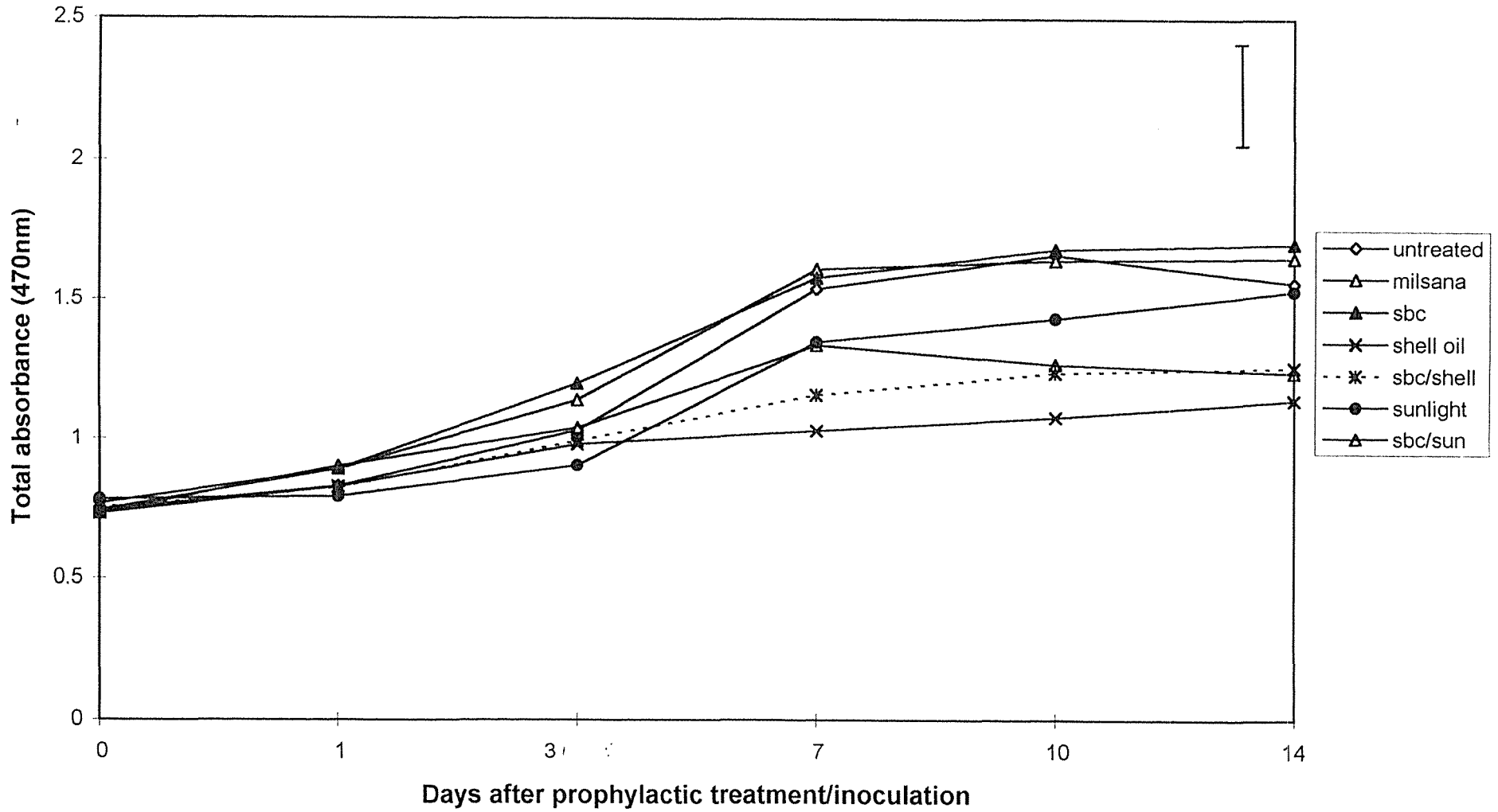


Figure 4.6 Daily comparison of peroxidase activities in leaf 2 sampled from inoculated 'Slice King'

4.3.3 Peroxidase as a Biochemical Indicator of Resistance in Cucumber

Table 4.1: Peroxidase activity in ELISA plate wells containing leaf discs of cucumber with various levels of resistance to *Sphaerotheca fuliginea*

Cultivar	Predicted resistance ^a	Inherent peroxidase activity (expressed as total absorbance at 450nm)	Severity of disease compared to the most susceptible cultivar 'Lebanese'
'Lebanese'	least resistant	0.813 (\pm 0.14) ^b	a ^c
'Slicemaster'	disease tolerant	1.18 (\pm 0.11)	b**
'Slice King'	highly resistant	1.27 (\pm 0.17)	b**

^a As regarded by the seed distributors

^b Data represent the mean values for peroxidase activity measured 5 minutes after leaf discs were placed in the reaction mixture and the reaction stopped with 3M H₂SO₄. Standard errors are in brackets. There were 10 plants per assay.

^c Values followed by the same letter are not significantly different. **=significantly less disease than 'Leb'

It can be seen from this assay that the inherent peroxidase levels are significantly lower in 'Leb', while they are higher and more similar in cultivar of greater resistance. Based on comparisons with studies by Reuveni et al. (1992), a positive correlation could be drawn between PO levels and resistance of the above cultivars to *S. fuliginea*.

4.4 DISCUSSION

This study began by asking the question, are shifts in PO-activity stimulated by treatment of cucumbers of different resistances to *S. fuliginea* with sodium bicarbonate/surfactant mixtures, in the absence of the pathogen?.

Inherent levels of PO-activity measured in the first samples collected from the water controls (the best standard for comparison) were found to be the lowest in 'Leb', significantly higher in 'SM', and the highest in 'SK'. This division in activity closely paralleled the levels of known resistance possessed by these cultivars to *S. fuliginea*. A review of the literature on PO-activity indicated that these results

supported the work of several independent groups who found a significant correlation between higher constitutive levels of peroxidase activity in non-infected plants of a variety of unrelated species and their genotypic susceptibilities and resistances to pathogens. Some examples include: tomato and resistance to *Verticillium dahliae* (Reuveni & Ferrira 1985), muskmelon (*Cucumis melo* L.) cultivars and breeding lines and resistance to *S. fuliginea* (Reuveni & Bothma 1985), wheat lines and resistance to powdery mildew (Patykowski et al. 1988), and maize resistance to *Bipolaris maydis* (Akhtar & Garraway 1987). Similarly associated was the activity in lettuce cultivars with the highest level of field-resistance to downy mildew (*Bremia lactucae*) (Reuveni et al. 1991). In this study also, lettuce cultivars with moderate-low resistance exhibited intermediate levels of PO-activity, and highly susceptible cultivars had the lowest PO-activity of all.

Within 1 day after treatment, increases in PO-activity were detected in 'Leb' leaves treated with sodium bicarbonate but not in the more resistant cultivars 'SM' and 'SK', or for any other materials applied to all 3 cultivars. These increases in activity may be related to the appearance of circular yellow-brown areas on 'Leb' leaves treated with sodium bicarbonate within 3 days after treatment. Similar lesions were described occurring on healthy leaves (Weeds 1992) and inoculated leaves (Ziv & Zitter 1992) within 1 week after treatment, when sodium bicarbonate was applied to susceptible cultivars at concentrations of 2.0-5.0% without surfactants. Ziv & Zitter (1992) considered this to be a sign of phytotoxicity of the material, which they attributed to solution concentration, and in part to leaf damage caused by *S. fuliginea* which may have facilitated the entry of bicarbonate salts into epidermal cells. This is a quality most often associated with protectant fungicides which are toxic if they enter plants in any appreciable quantities (Manners 1993). As the same tissue reaction was obtained when sodium bicarbonate was applied to punctured cucumber cotyledons, this suggested to them that some means of entry was required for phytotoxic damage to occur.

Given that lesions appeared on 'Leb' leaves only, and on noninoculated plants, this also suggested that 'Leb' was more sensitive to bicarbonate salt than the two

other cultivars, and/or that they may be the result of a phenomenon known as the 'lens effect'. Wherein, droplets of water that are retained by dense trichomes (hairs) on the leaf surface may act as a type of convex lens which increases the convergence of parallel light rays to bring them together to a focus at a point on the other side (Youngson 1994) of the droplet, similar to how a magnifying glass functions. Intense heat is a net result of this convergence, and as easily as a magnifying glass can burn holes in paper and rubber, burns to leaves seem entirely possible. Presumably these droplets contained bicarbonate salt as sodium bicarbonate readily dissolved in tap water. The presence of salt may reduce the convergence, but at the same time if a burn was created in leaf tissue this could have provided an entry for bicarbonate salt, much like deliberate wounding or damage by *S. fuliginea* was thought to contribute to phytotoxicity observed by Ziv & Zitter (1992).

Alternatively, the tissue might have been sufficiently cauterised that sodium bicarbonate could not pass into adjacent cells and hence damage observed was not related to sodium bicarbonate at all. Elevated PO-activity may be a response to wounding of this type, because in cucumber leaves levels increased locally in cells surrounding lesions where wounding or localisation of a pathogen had occurred (Ahl-Goy et al. 1992), and in the phenomenon of induced systemic resistance. Inducer leaves sprayed with oxalate-containing extracts from spinach or rhubarb leaves developed chlorotic and necrotic lesions and were more resistant to anthracnose (*Colletotrichum lagenarium* (Pass.) Ell. and Hallst.). It was suggested that as a result of low-level persistent stress, the inducer leaf exported an alarm signal which conditioned plants so that resistance mechanisms such as enhanced PO-activity and lignification were quickly activated in response to subsequent challenge (Doubrava et al. 1988). The nature of the receptor of this signal was discussed by Hotter (1996). Oil emulsions and detergents as surfactants may have helped to distribute the salt uniformly over the leaf surface as a thin film and therefore beads of water did not occur. This may explain the absence of injury when sodium bicarbonate was combined with these additives, and when Milsana which is formulated with surfactants was applied.

Three to 14 days after treatment slight increases in foliar PO were detected in leaves from all 3 cultivars, for all treatments. Particularly significant was the activity in plants treated with sodium bicarbonate and Milsana. The time-course of PO-activity in plants following Milsana treatment was similar to one described for susceptible cultivars (Herger et al. 1988; Schneider & Ullrich 1994), although the extent of the increase was not as substantial in the present study. In addition, levels of PO varied between cultivars for they increased 3-fold in 'Leb', doubled in 'SM' and had risen by little more than half in 'SK' leaves. Concurrent studies of tobacco leaf tissue (Schneider & Ullrich 1994) may provide some explanation for this variability. Results indicated that the extent and time-course of the increase varied not only according to the inducer material but also according to the type of host plant. Regardless of all plants in this study being cucumbers, and phenotypically and genetically similar, it should not be assumed that they should respond similarly. To begin with, 'Leb' was derived from an old breeding line sourced from America (J. Phillips pers. comm.), while 'SM' and 'SK' were both modern hybrids bred for resistance to a number of diseases. Heredity may be one factor that has influenced the response of cultivars to materials applied.

Gradual increases in PO-activity in noninfected plants over time have been described in at least 2 plant species, muskmelon (Reuveni & Bothma 1985) and tobacco (Wyatt et al. 1991). In the study concerned with muskmelon, samples were collected from several cultivars with differing resistances to *S. fuliginea* (Reuveni & Bothma 1985) and *Pseudoperonospora cubensis* (Reuveni et al. 1990), that were of a comparable age to plants used in this study. For tobacco, correlations were drawn between the activities of several pathogenesis-related enzymes including PO, chitinase, and β -1,3-glucanase, and the observation that plants become naturally resistant to blue mould (*Peronospora tabacina*) with age. There are no reports of cucumber becoming similarly resistant with age, perhaps because the plant is an annual and reaches maturity in a relatively short time.

The differences in PO-activity on successive days between leaves treated with materials other than sodium bicarbonate and Milsana suggested that:

i. PO was present, but relatively inactive. PO is considered to play a role in normal plant developmental processes such as leaf senescence (Wyatt et al. 1991), respiration, and the regulation of plant growth hormones like indoleacetic acid (Macko et al. 1968). Non-inoculated plants were grown in the greenhouse in an environment conducive to plant growth. They did not lack water, soil nutrients, light, or even suffer damage by grazing insects such as white-fly, important factors which can disturb the normal physiological functioning of plants (Mayr et al. 1994; Schneider & Ullrich 1994). Such biological stresses often generate molecular responses in the host similar to those induced for defence against pathogens, which can influence the relative activity of PO in plant tissues (Artlip & Funkhouser 1995). For example, moisture stress altered metabolic reactions in marigold (*Tagetes erecta* L.), inducing a marked increase in specific PO-activity and enhancing biosynthesis of stress phenols, which resulted in lignin formation. Lignin reduced cell shrinkage and loss of water, thus protected plants from further stress (Kurup et al. 1994). The protein synthetic responses of plants to environmental stresses were reviewed by Artlip & Funkhouser (1995).

If normal cucumber plant growth proceeded in the absence of external provocation, then it might be reasonable to expect that levels of PO-activity should remain virtually constant aside from slight changes in a young actively growing plant. Evidence on the constancy of PO-activity was reported in a study by Johnson & Lee (1978) where the PO-activity in healthy leaves of wheat varieties susceptible and resistant to leaf rust showed almost no change during 7-15 days of plant development, and there was no significant difference between varieties. Looking at the PO-activity in water treated controls a similar description could be applied, except that there were significant differences between cucumber varieties to begin with.

ii. It could take longer than 3 days for activation of the enzyme protein by surfactants alone and in combination with sodium bicarbonate to be expressed by the host in any appreciable amounts. Work on the development of 'induced resistance' in noninoculated cucumber and tobacco plants treated with several materials

including Milsana found that enzyme activities did increase, but only after a lag period of 2-3 days following treatment (Schneider & Ullrich 1994). Changes in activity are likely to be governed by the reception and sending of a signal from the site of application to the host genome. If an appropriate signal is received, recognised and translated, a molecular response in the host which includes the synthesis of proteins related to defence may be generated. However, if there was insufficient provocation at the site of application and hence, no signal was sent to begin with, then the expression of heightened activity would not be expected to occur. So in this sense, surfactants applied alone and in combination with sodium bicarbonate may not be stimulating signal relay.

Information on the effects of surfactants on host physiology is lacking though much is known about their action directly on pathogens (see Calpouzos 1966; Northover & Schneider 1996 for reviews). Early work by Calpouzos et al. (1959) showed that petroleum oils decreased photosynthesis and transpiration but increased respiration. This led Calpouzos (1966) to speculate that the therapeutic effect of oil on leaves infected by *Mycosphaerella musicola*, the causal agent of sigatoka disease of banana, was not due to a direct effect on the pathogen, but possibly due to an indirect effect by altering the physiology of the host. Results obtained in a more recent study by Northover & Schneider (1996) where the regrowth and reappearance of powdery mildew lesions after treatment with oils showed that oils had a fungistatic rather than a fungicidal effect, also compelled them to consider that possibly there was a temporary effect on host physiology.

The second question asked of this study was whether shifts in PO-activity were stimulated by treatment of cucumbers of different resistances to *S. fuliginea* with sodium bicarbonate/surfactant mixtures, in the presence of the pathogen?. As was found for noninoculated 'Leb', PO was significantly more active in Milsana and sodium bicarbonate treated leaves on the second day of tissue collection and on subsequent days. There were also few significant differences between activity in leaves from inoculated controls and plants treated with other materials until 3 days

after treatment for all cultivars. This provided further support to two ideas, that the physiology of 'Leb' was more sensitive to sodium bicarbonate treatment, and that a lag period is observed before any measurable increase in PO-activity in 'SM' and 'SK' cultivars. The actual values of activity at this time were considerably higher in inoculated than noninoculated 'Leb' and 'SM' plants, and of a comparable level in 'SK'. This result was also observed by Reuveni & Bothma (1985), where the ratio of activity in inoculated to that in noninoculated leaves changed little in resistant cultivars.

Seven days after inoculation, levels were substantially higher in control plants, and in those treated with milsana, sodium bicarbonate and sunlight liquid applied by itself and in combination with sodium bicarbonate, for all cultivars. In contrast, levels attained in leaves treated with shellspray oil alone and sodium bicarbonate-oil mixtures were significantly lower. These patterns of activity were repeated on successive days but the rate of increase had slowed considerably. Comparing these results with the severity of disease on cucumbers at the end of the 14 day period it was found that disease was greatest on 'Leb' and considerably less on 'SM' and 'SK' water treated controls. This actually reflected the order in which symptoms were observed first within 5 days of inoculation. In addition, shellspray oil alone and sodium bicarbonate-oil mixtures proved superior in efficacy than all other materials applied to 'Leb' and 'SM'. The same observations were made for disease development on 'SK' except that Milsana provided comparable control. These results suggested that:

- i. The materials which controlled disease development with the greatest efficacy inhibited the pathogen directly, rather than by stimulating a physiological host response involving PO. This might also apply to sunlight liquid formulations with and without sodium bicarbonate for they provided some obstacle to disease development and did not appear to stimulate increases in PO-activity significantly different from the controls.
- ii. The extent of the increase in PO-activity in all cultivars following sodium bicarbonate and Milsana treatment was not related to the level of protection

afforded by these materials except for the more resistant cultivar 'SK' where PO levels were relatively high to begin with. This suggested that disease control may be the result of a complex interaction between Milsana and the natural activation of resistance that occurs in 'SK' when challenged by *S. fuliginea*.

- i. Sodium bicarbonate had activity against *S. fuliginea* but this was not realised until the salt was combined with a surfactant such as shellspray oil or sunlight liquid. This enhancement of activity was earlier suggested in glasshouse studies (see Chapter 4.1) examining the efficacy of sodium bicarbonate/surfactant mixtures over a longer period of time.

The inherent levels of PO-activity in cucumber cultivars and the rate of increase following inoculation may influence the success of a material applied. PO values in 'Leb' were half to three times lower than those measured in 'SM' and 'SK'. In addition, the rate of increase in PO was steepest over the 7-10 day period in 'Leb' tissues for all materials except oil and bicarbonate formulations, while PO-activity increased most over the 3-7 day period in 'SM' and 'SK'. Several researchers (Reuveni & Bothma 1985; Reuveni et al. 1991; Shimoni et al. 1991) consider that susceptible plants activate their peroxidases after infection, but that this occurs too late to prevent or restrict disease development. This may apply to 'Leb' for PO was seen to increase in the control but not with such rapidity as took place in the more resistant cultivars.

For the cucumber cultivars studied, the lowest levels of inherent PO were detected in leaves of 'Leb' and the highest levels in 'SM' and 'SK' respectively, although there was no significant difference between the two latter. On the basis of these results a weak positive correlation could be drawn between the severity of powdery mildew disease observed on whole plants and PO levels in leaves. This supports in part the relationship demonstrated by Reuveni et al. (1990). The PO test devised by them appears useful as a tool for rapidly selecting out moderately resistant plants for breeding purposes, assuming that PO is an enzyme indicative of a host's

potential for resistance to a (specific) disease. However, this was only a small trial with few cucumber individuals compared. Screening of a larger range of cultivars would highlight whether real benefits could be achieved from this test, and also provide a scale against which the activities of individuals and successive generations could be compared and rated for resistance, though it cannot be said specifically against which diseases. For PO has been shown to be induced against a range of pathogens in cucumber in addition to powdery mildew (Menke & Walker 1963), which indicates that it is non-specific in activity. It should be remembered that the activities of several enzymes are likely to be influenced upon pathogen challenge, and in response to different pathogens.

5. THE ROLE OF PHENYLALANINE AMMONIA-LYASE IN DEFENCE OF CUCUMBERS AGAINST POWDERY MILDEW

5.1 INTRODUCTION

Pathogen attack on plants appears to induce alterations in enzyme synthesis in plants that can lead to the development of various degrees of resistance around infection sites. An enzyme present during normal plant development, that generally exhibits increased activity in diseased tissues is phenylalanine ammonia-lyase (E.C. 4.3.1.5: PAL). PAL catalyses the elimination of ammonia (deamination) from phenylalanine to give *trans*-cinnamic acid (Koukol & Conn 1961). This reaction is the first step of the phenylpropanoid pathway, a branch of the shikimic acid pathway. *Trans*-cinnamic acid provides phenylpropane skeletons which serve as building blocks for lignin, or are utilised in the synthesis of flavonoids and other phenolic derivatives (Tena & Valbuena 1982; Goodman et al. 1986) (Figure z). PAL-activity is correlated with the synthesis of various phenolic compounds (Hahlbrock & Grisebach 1979). Consequently, PAL has been proposed to play a regulatory role in phenolic biosynthesis (Creasy & Zucker 1974).

In recent years, considerable attention has been devoted to the response of PAL to various stimuli (chemical, stress conditions, and environmental factors) applied to plant tissues (Tena & Valbuena 1982; Artlip & Funkhouser 1995). Apart from the expression of hydrolytic enzymes, changes in PAL-activity have also been observed in relation to plant diseases, particularly in localised incompatible host-parasite interactions rather than in systemic interactions. This has been shown to occur at the level of both transcription and translation. Several workers have indicated that the activation of PAL and subsequent increase in phenolic contents, are general responses associated with plant disease resistance (Henderson & Friend 1979; Brueske 1980). However, this general involvement of PAL activation in resistant reactions is still under debate.

The objective of this enzymatical study was to:

- i. Investigate whether shifts in PAL-activity were stimulated in leaves from cucumber cultivars that differed in disease susceptibility, in the presence and absence of the pathogen, when treated prophylactically with sodium bicarbonate/additive mixtures.

5.2 MATERIALS AND METHODS

5.2.1 Plant Culture, Chemical Treatments And Inoculation

Plant material pertaining to three cucumber cultivars, 'Leb', 'SM' and 'SK' was raised and maintained under the same environmental regime and soil conditions as described in 2.1.1, except that pots were shallower and smaller in diameter (20 x 15 cm). Once seedlings had reached 3 weeks of age, those uniform in height and having the second-true leaf unfolded were selected out and arranged on a glasshouse bench in eight blocks containing rows of pots 7 x 6 in length.

Once the third-true leaf had fully expanded, the seven materials listed in Table 2.3 were applied to runoff as before from a 700 ml plastic spray bottle. When plants were dry, they were returned to the experimental site and arranged with the treatments randomised in eight replicate blocks. This provided the samples for PAL assays which were harvested from plants at six time intervals, 0 (ie. 1 h after inoculation), 1, 3, 7, 10, 14 days following inoculation with *S. fuliginea* 3 days after treatment. Four of the eight blocks were treated in a similar way but were not inoculated.

5.2.2 Determination Of PAL-Activity By Spectrophotometry

A. Preparation of crude enzyme extract

Extraction and spectrophotometry of PAL-activity was based on to the method described by Zucker (1965).

1. Leaf 2 was harvested from 4 plants. Samples were then washed, blotted dry, placed in labelled re-sealable bags, quick frozen in liquid air, and stored at -75°C until analysed for PAL-activity.
2. Two leaf discs (0.75 g), 10 mm in diameter, were removed from both sides of each sample leaf used in assays for peroxidase activity, from the same approximate position. Leaf discs were ground in a mortar at 4°C with ice-cold 0.025M sodium tetraborate buffer (pH 8.8: STB) (Bates & Bower 1956) 12.5 ml/g tissue, containing 0.01 μl /2 g of tissue 0.02M 2-mercaptoethanol ($\text{HS}\cdot\text{CH}_2\cdot\text{CH}_2\text{OH}$; BDH Ltd.) (added to the medium just before extraction to protect PAL from inactivation).
3. The homogenates were decanted into 1 ml eppendorf tubes and clarified by centrifugation at 15,000 g for 10 min at 4°C to remove cell debris. The resulting supernatants were assayed for PAL-activity. The total protein content in these crude extracts was measured by the method of Bradford (1976) which utilised the principle of protein-dye binding. Pure bovine serum albumin (BSA) used as the standard.

B. Determination of protein concentration (after Bradford (1976)).

i. Preparation of protein standard

0.08 g pure bovine serum albumin (2x crystallised: BSA) was prepared in 0.15M NaCl, giving a total of 400 μg /100 μl .

ii. Preparation of protein reagent dye concentrate

0.100 g Coomassie Brilliant Blue G-250 ($\text{C}_{47}\text{H}_{49}\text{N}_3\text{O}_7\text{S}_2\cdot\text{Na}$; Mallinckrodt Inc. Kentucky, USA) was dissolved in 50 ml 95% ethanol and 100 ml 85% (w/v) phosphoric acid (orthophosphoric acid) added to this. The resulting solution was diluted with distilled water to a volume of 200 ml. This new solution, the concentrate, was further diluted 5-fold with distilled water.

iii. Protein assay (standard method)

- a) Standard curve: BSA solution containing 10, 15, 20, 25, 30, 40 μg of protein was pipetted into test tubes (12 x 100 mm in length). 5 ml of diluted protein reagent dye was added and the contents vortexed for 10 s, then left to stand for not less than 2 min. Samples were emptied into plastic curvettes and the protein content of BAS measured at a wavelength of 595nm against a reagent blank containing 100 μl STB buffer and 5 ml of diluted protein reagent, using a Hitachi U-2000 Spectrophotometer.
- b) Sample assay: 0.10 ml of enzyme extract was added to 5 ml protein reagent and assayed as above.

C. Determination of PAL-activity in supernatant

1. In test-tubes 0.5 ml of extract (determined by Bradford's (1976) method to contain 25-30 μg protein) was added to a reaction-mixture consisting of 0.65 ml 0.025M of STB and 0.65 ml 60 μM of L-Phenylalanine (Sigma Chemical Co: L-Phe).
2. Contents were vortexed for 5 s, incubated at 37°C in an incubator for 5 min, and then emptied into clean 3 ml plastic disposable curvettes.
3. The activity was determined spectrophotometrically by measuring the increase in absorbance (due to the enzyme-catalysed deamination of L-Phe to *trans*-cinnamic acid), at 290nm against a reaction-mixture blank lacking L-Phe. Readings were taken at 10 min intervals using a Hitachi U-2000 Spectrophotometer, and were expressed in absorbance units (AU)/min/ μg protein. The reaction-mixture was returned to the incubator between measurements.
4. Each supernatant was assayed in duplicate, and two controls were included in each set of assays; boiled leaf material (*boiling for 10 min at 90-100°C was sufficient to destroy enzyme activity), and substrate lacking enzyme. No increase in absorption occurred when the enzyme extract was assessed.

5.2.3 Assessment Of Powdery Mildew On Cucumber Leaves

14 days after inoculation, leaf 2 was examined and the severity of disease rated according to a 0-4 scale devised by Reuveni & Bothma (1985) described in Chapter 4.2.2.

5.3 RESULTS

5.3.1 Disease Severity

Symptoms observed and the severity of disease with respect to different host resistances and the seven treatments applied, were described in Chapter 4.3.1.

5.3.2 Effects of Treatments on PAL-Activity (Figures 5.1-5.6)

i. Noninfected plants

Compared to controls, PAL-activity 1 h post-treatment did not increase significantly ($p < 0.01$) for any cultivars. Within 1 day, activity was significantly greater in 'Leb' plants only, treated with sodium bicarbonate ($p < 0.01$) alone (Figure 5.1). Three to 14 days later PAL activity had increased slightly for all treatments applied, but was only significantly greater in bicarbonate and Milsana treated plants (Figures 5.1-5.3). By 7 days, these increases observed with the latter treatments had returned to similar levels as for other treatments.

ii. Inoculated plants

PAL-activity was significantly higher ($p < 0.05$) in Milsana and bicarbonate-treated 'Leb' leaves 24-72 h post-inoculation/treatment (Figure 5.4). PAL-activity had also significantly increased in leaves of 'SM' and 'SK' treated with these materials (Figures 5.5 and 5.6 respectively) and also sunlight liquid. These increases just as suddenly decreased after 72 h, followed by slight increases that were not significantly different from controls ($p < 0.05$). Activity in plants treated with shellspray oil and

bicarbonate-oil mixtures had only risen slightly, which was significantly less than control activity levels.

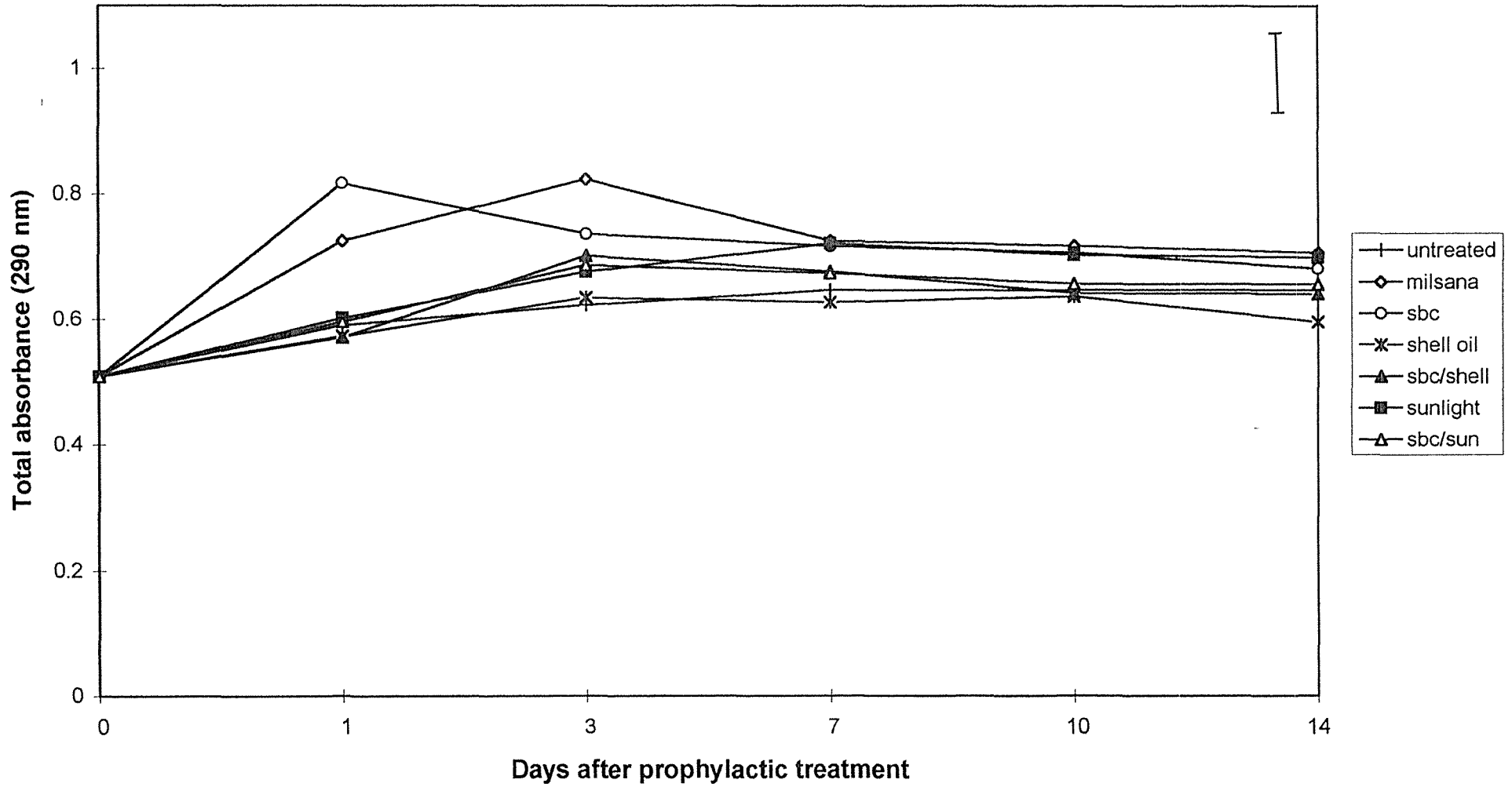


Figure 5.1 Daily comparison of PAL activities in leaf 2 sampled from noninoculated 'Lebanese'

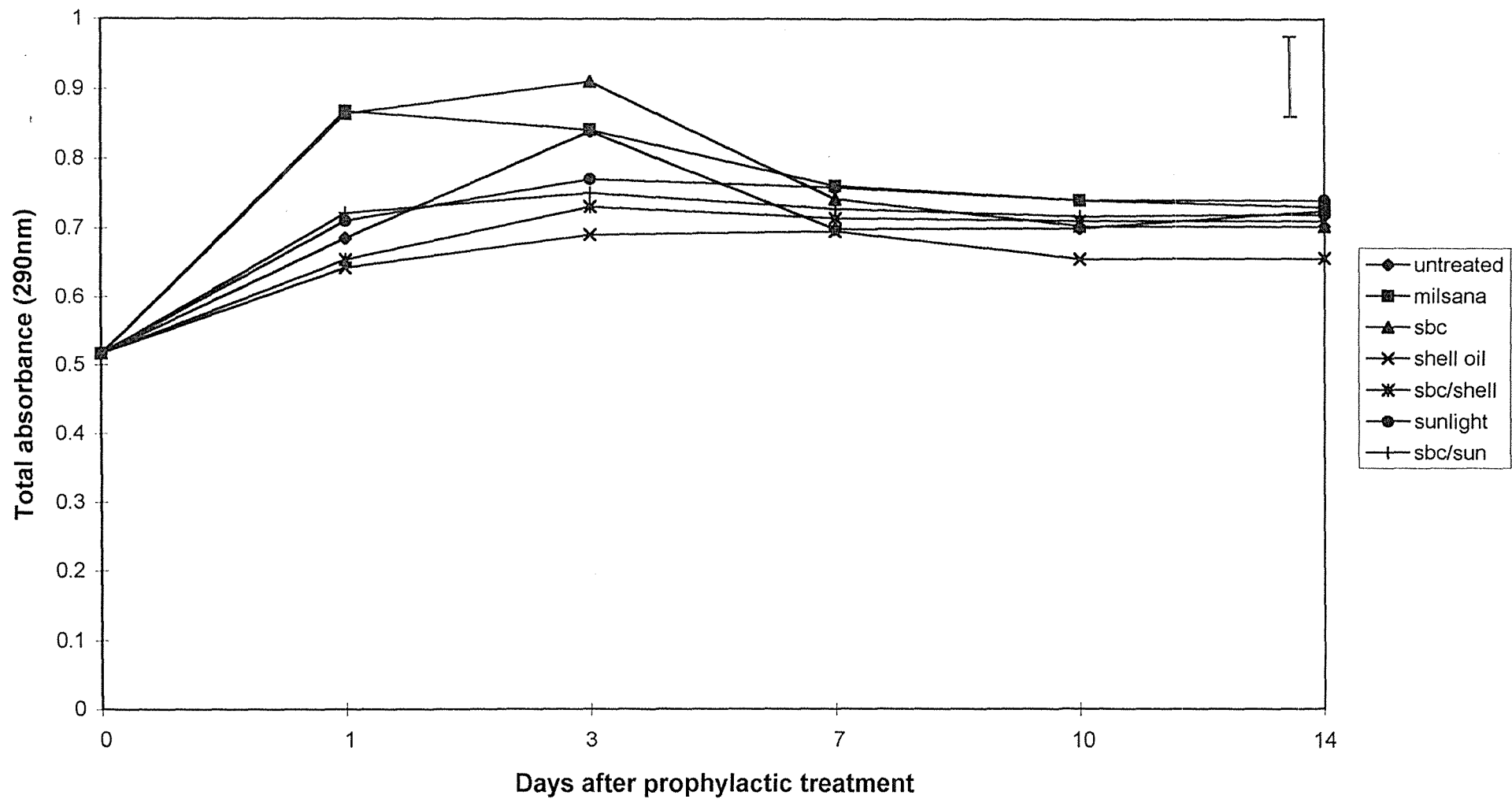


Figure 5.2 Daily comparison of PAL activities in leaf 2 sampled from inoculated 'Lebanese'

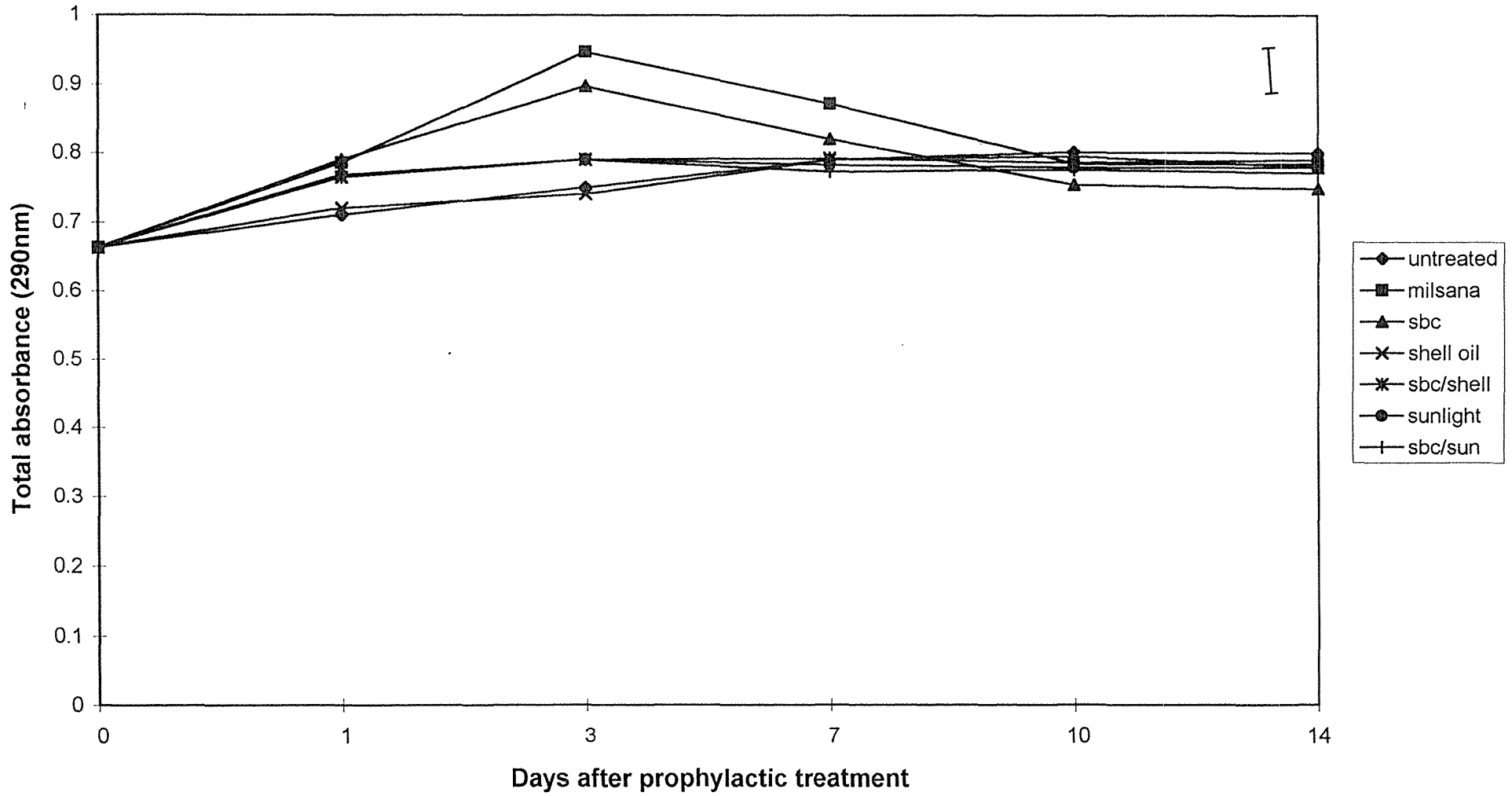


Figure 5.3 Daily comparison of PAL activities in leaf 2 sampled from noninoculated 'Slicemaster'

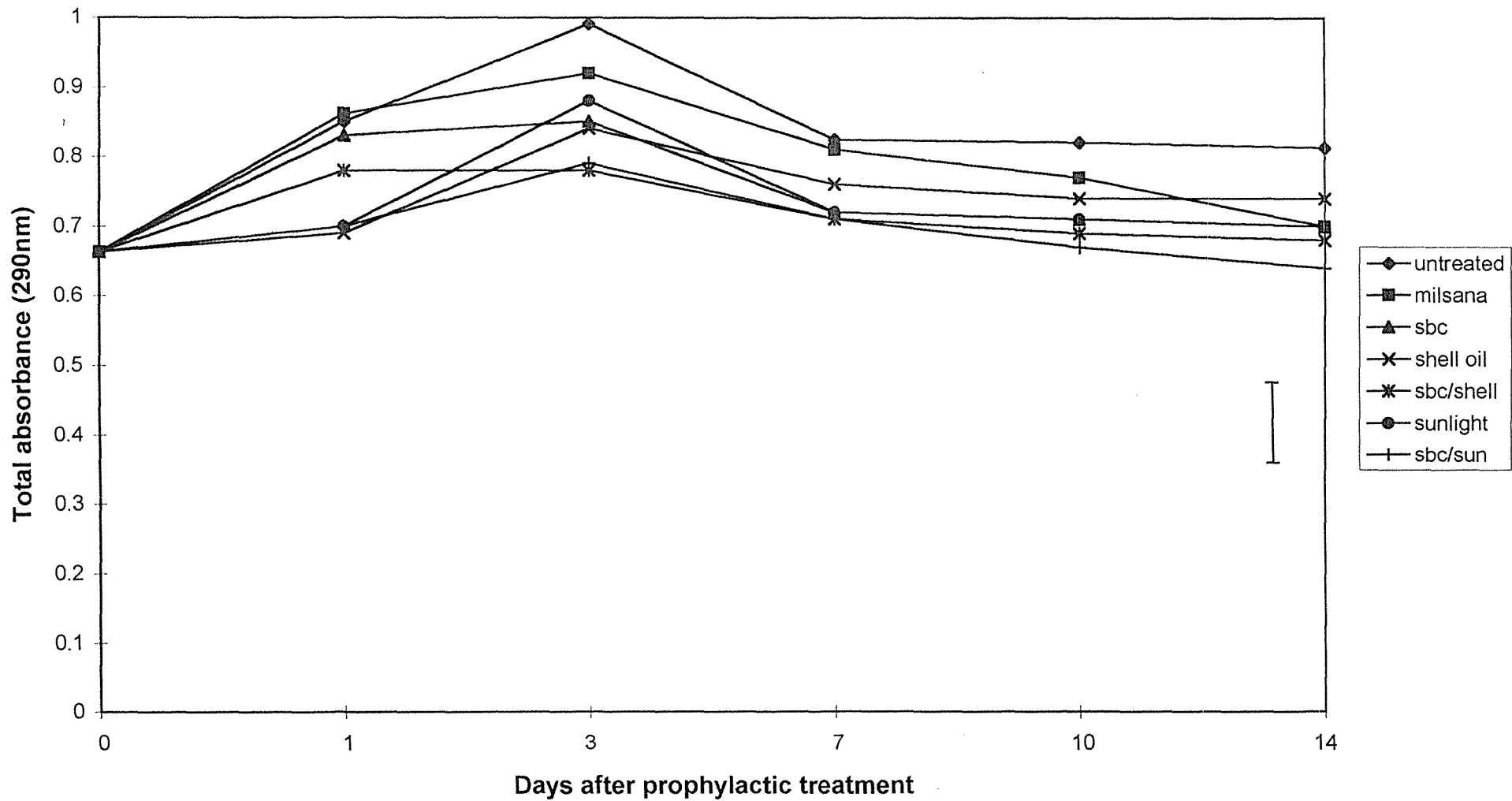


Figure 5.4 Daily comparison of PAL activities in leaf 2 sampled from inoculated 'Slicemaster'

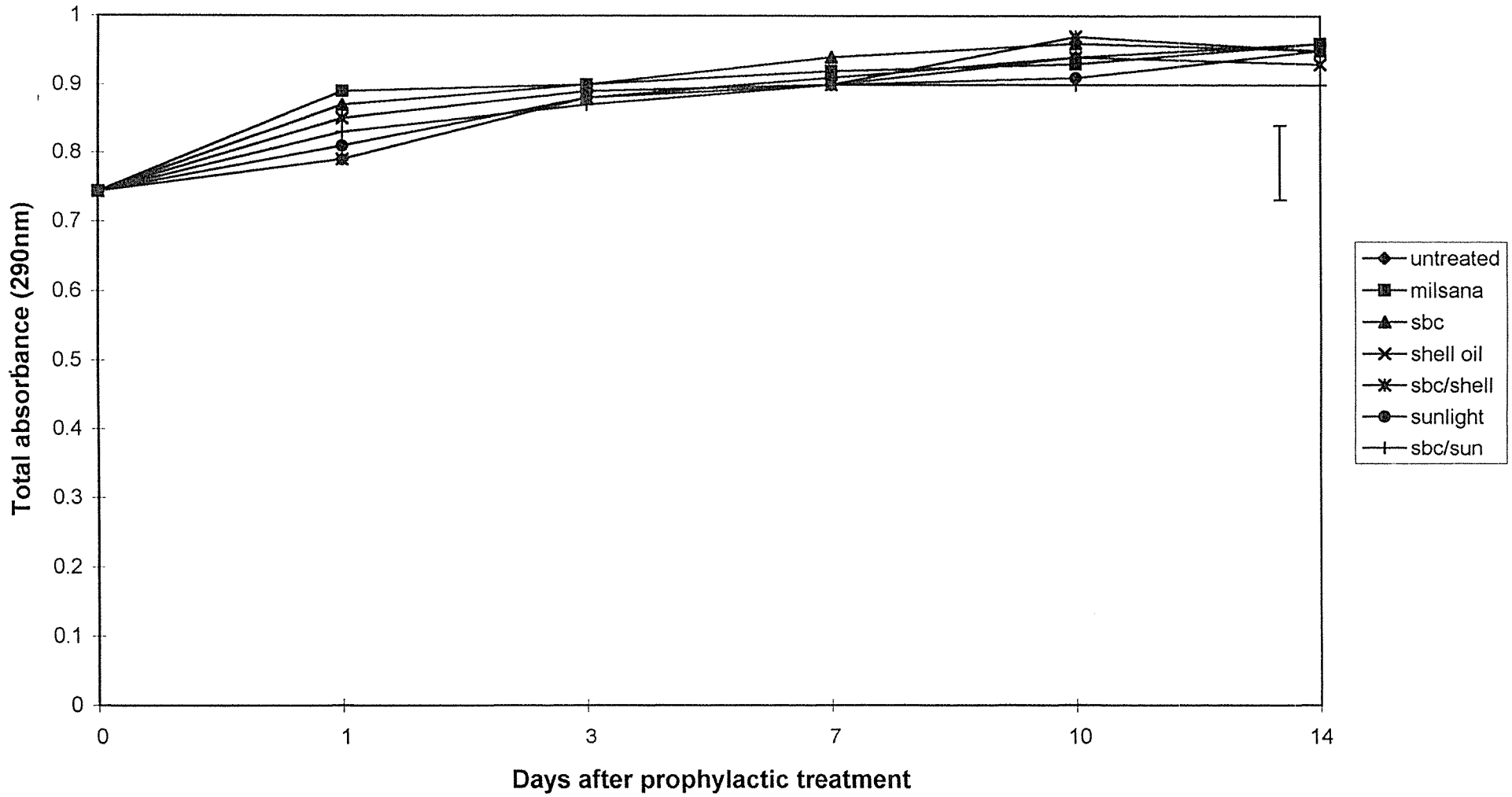


Figure 5.5 Daily comparison of PAL activities in leaf 2 sampled from noninoculated 'Slice King'

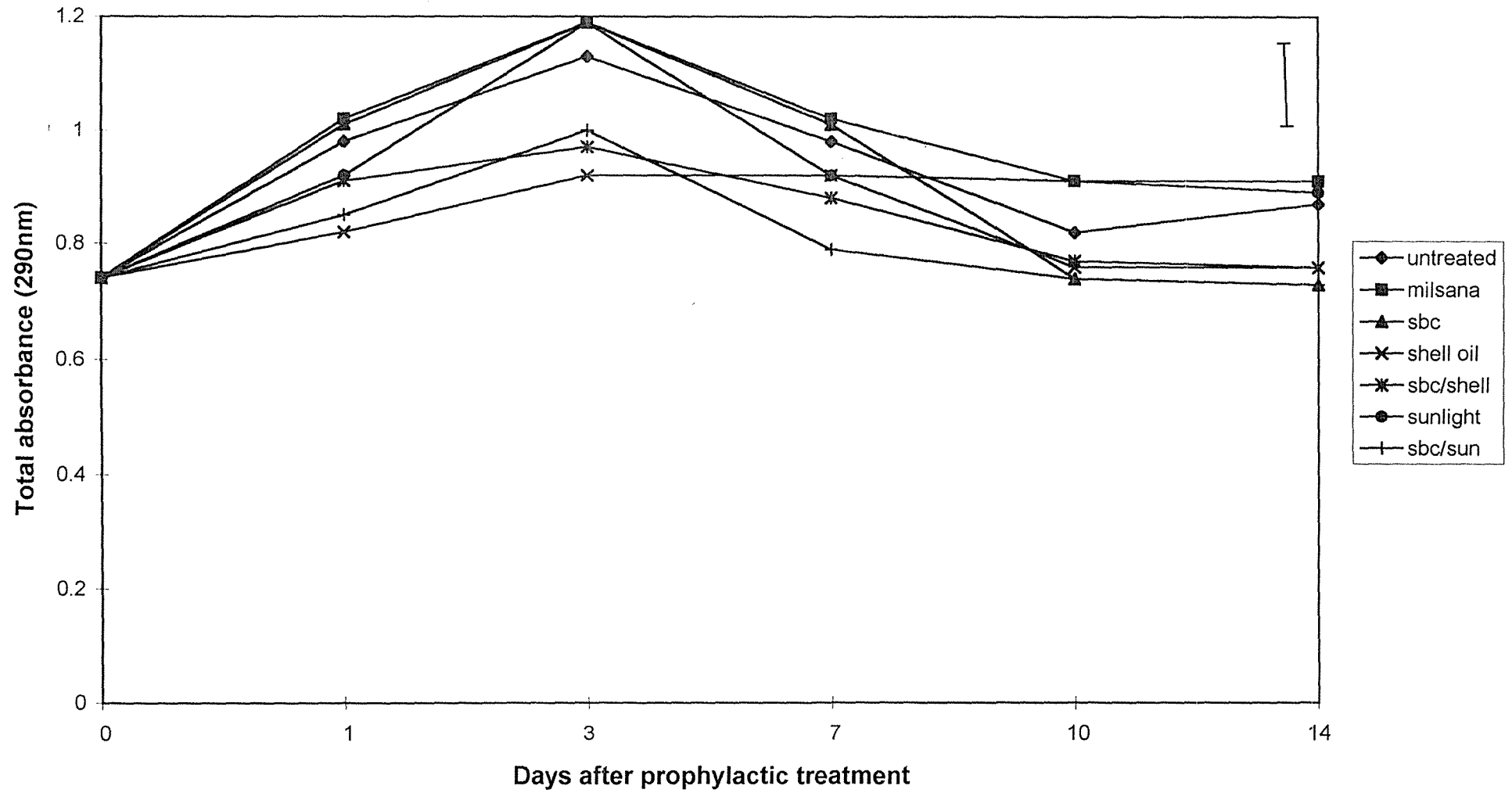


Figure 5.6 Daily comparison of PAL activities in leaf 2 sampled from inoculated 'Slice King'

5.4 DISCUSSION

The first part of this investigation was to determine whether shifts in PAL-activity were stimulated by treatment of cucumbers of different resistances to *S. fuliginea* with sodium bicarbonate/surfactant mixtures, in the absence of the pathogen. Inherent levels of PAL-activity measured in the first samples collected from the water-treated controls were found to be lowest in 'Leb', significantly higher in 'SM', and the highest in 'SK'. This division in activity closely paralleled the results of physiological studies of sunflower seedlings resistant and susceptible to downy mildew (*Plasmopara halstedii*) (Tena & Valbuena 1982).

Within 1 day after treatment, slight increases in PAL-activity were detected in all 3 cultivars, for all materials applied. Particularly significant increases were seen in enzymic activity in 'Leb' leaves only, treated with sodium bicarbonate, which did not occur in the more resistant cultivars 'SM' and 'SK', or for any other materials applied to the 3 cultivars. These increases may be related to the appearance of necrotic lesions on 'Leb' leaves 3 days after treatment with sodium bicarbonate. These lesions were discussed in detail in Chapter 4.4 in reference to changes in foliar peroxidase-activity and the sensitivity of this cultivar to the bicarbonate salt. The same principles may apply to PAL-activity, for stimulated enzyme activities are characteristic not only of infections but also of injury and accelerated aging (Goodman et al. 1986). These responses are therefore not specific for infection-based injury. This is illustrated by the following examples:

-The most pronounced effects of UV-B irradiation of sensitive cucumber seedlings were a 27-28% increase in foliar PAL-activity (Krizek et al. 1993), and severe leaf chlorosis.

-The appearance of necrotic lesions on needles of Scots pine (*Pinus sylvestris* L.) seedlings 24 h after treatment with high concentrations of ozone (O_3^2 : a major phytochemical pollutant), was accompanied by high levels of PAL-activity (Rosemann et al. 1991).

-Pressure treatment of apple leaf discs significantly enhanced PAL-activity, which peaked relatively soon after treatment onset (Fünfgelder et al. 1994).

Three to 14 days after treatment, increases in PAL were detected in leaves from all 3 cultivars, for all treatments. However, sodium bicarbonate/additive mixtures applied as individual components or together, did not stimulate a physiological response. Particularly significant was the stimulation of activity in plants treated with sodium bicarbonate and Milsana. This reached a maximum 3 days after treatment, and then rapidly returned to levels similar for other treatments at the same point in time. The time-course of PAL-activity in plants following Milsana treatment was similar to one described by Schneider & Ullrich (1994) for susceptible cultivars.

The similarities in PAL-activity on successive days in leaves treated with all materials suggested that:

- i. PAL was present and functioned in normal plant developmental processes such as aging and leaf senescence, and respiration (Goodman et al. 1986). If normal plant growth proceeded in the absence of biotic and abiotic stress (described in Chapter 3.4), then it might be expected that levels of PAL-activity should remain constant.
- ii. It might take longer than 24 h for activation of the enzyme protein by surfactants alone and in combination with sodium bicarbonate to be expressed by the host in any appreciable amounts. The occurrence of a possible lag period before increased activity and the reception of a correct signal for stimulation were discussed in work on peroxidase (Chapter 3.4).

The second part of this investigation was to determine whether shifts in PAL-activity were stimulated by treatment of cucumbers of different resistances to *S. fuliginea* with sodium bicarbonate/surfactant mixtures, in the presence of the pathogen. As was found for noninoculated 'Leb', PAL was significantly more active in Milsana and sodium bicarbonate treated leaves 24-72 h after inoculation. Similar increases in PAL have been induced in cucumber leaves susceptible to *Erysiphe cichoracearum* in response to treatment with tomato leaf extracts and mycelial sonicates of *Monilinia fructigena* and *Fusarium oxysporum*. These increases were

associated with a reduction in disease development by c. 71-78% (Tyuterev et al. 1994). PAL-activity in Milsana and sodium bicarbonate treated leaves of 'SM' and 'SK' was seen to have increased significantly 24-72 h after inoculation, more so than in 'Leb'. Which suggested that a physiological response had been induced in the host.

PAL-activity was higher in control leaves from inoculated more resistant cultivars than in inoculated 'Leb'. These absolute values were actually higher than in noninoculated 'Leb' and 'SM' leaves, and comparable to levels for noninoculated 'SK'. When a fungal pathogen enters a resistant host cell, a general acceleration of the metabolism of the host is caused (Goodman et al 1986). This might explain why differences between cultivars were observed. The general division in activity closely paralleled the results of physiological studies of the resistance of cucumber cotyledons and primary leaves to *S. fuliginea* by Kabsch (1988). PAL-activity in the resistant cultivar only, exceeded control values within 12 h after inoculation, and fungal infection resulted in higher activities of several other enzymes during the early stages of infection, which catalyse reactions in the shikimic acid pathway.

The lack of stimulation by shellspray oil and sodium bicarbonate-oil mixtures suggested that application of these materials did not induce a physiological response in the host, or that they prevented penetration so that a response was not generated as a result of pathogen challenge. Hence increases in PAL were not significantly different from activities in noninoculated leaves. Seven-14 days after inoculation, levels were similar in all cultivars for all treatments. This levelling off of activity has been reported for several host-pathogen interactions (Creasy & Zucker 1974; Tena & Valbuena 1982).

Comparing these results with the severity of disease on plants at the end of the 14 day period, it was found that disease was greatest on 'Leb' and considerably less on 'SM' and 'SK' water treated controls. This actually reflected the order in which symptoms were observed first within 5 days of inoculation. In addition, shellspray oil alone and sodium bicarbonate-oil mixtures proved superior in efficacy than all other materials applied to 'Leb' and 'SM'. The same observations were made for disease

development on 'SK' except that Milsana provided comparable control. These results suggested that:

- i. The materials which controlled disease development with the greatest efficacy inhibited the pathogen directly, rather than by stimulating a physiological host response involving PAL. This might also apply to sunlight liquid formulations with and without sodium bicarbonate for they provided some obstacle to disease development and did not appear to stimulate increases in PAL-activity significantly different from the controls.
- ii. The extent of the increase in PAL-activity in all cultivars following sodium bicarbonate and Milsana treatment was not related to the level of protection afforded by these materials except for the more resistant cultivar 'SK', where PAL levels were relatively high to begin with. This suggested that disease control may be the result of a complex interaction between Milsana and the natural activation of resistance that occurs in 'SK' when challenged by *S. fuliginea*.
- iii. Sodium bicarbonate had activity against *S. fuliginea* but this was not realised until the salt was combined with a surfactant such as shellspray oil or sunlight liquid. This enhancement of activity was earlier suggested in greenhouse studies (see Chapter 4.1) examining the efficacy of sodium bicarbonate/surfactant mixtures over a longer period of time.
- iv. Genetic variation governing the speed and extent of enzyme induction may account, at least in part for the differences between resistant and susceptible cultivars (Bennett & Wallsgrove 1994) with respect to disease severity observed after 14 days.

6. THE ROLE OF INDUCED STRUCTURAL BARRIERS IN DEFENCE OF CUCUMBERS AGAINST POWDERY MILDEW

6.1 INTRODUCTION

Papillae or cell wall appositions are structural barriers produced in leaf tissue of several plant species (Sherwood & Vance 1982), including cucurbits (Taylor 1987; Kovats et al. 1991). Some workers consider this to be a cellular response initiated by the host, in the attempt to prevent the entry of fungal pathogens into epidermal cells. Even if a papilla fails to prevent actual penetration, any impedance may essentially gain the host time to activate other defenses mechanisms (Matern & Kneusel 1988).

A papilla is described as a body of heterogeneous materials deposited often rapidly (within 2-3 h after inoculation), by the host cytoplasm between the plasma membrane and cell wall at the site of fungal attack (Aist 1983). They are often localised beneath appressoria. Histochemical staining of papillae produced in cucumber leaves in response to powdery mildew invasion has shown that callose is the main structural component (Kovats et al. 1991). The importance of callose in papilla formation and in host resistance was confirmed by an experiment using 2-deoxy-D-glucose as a callose-formation inhibitor (Bayles et al. 1990). Callose is embedded in a matrix containing pectic materials, suberin, cellulose, gums, calcium, silicon (Carver & Ingerson 1987), some phenolics, and proteins such as peroxidase and other hydrolytic enzymes which are suggested to have a role in degrading components of penetration pegs (Inoue et al. 1994b).

Work by Schneider & Ullrich (1994) has shown that cucumber leaves can be stimulated to produce papillae in greater numbers after treatment with certain biotic and abiotic materials, including Milsana. Correlations were drawn between this increase and superior disease control, though it was acknowledged that papillae were unlikely to be the only mechanism responsible for control.

The objectives of this work were to:

- i. Determine whether sodium bicarbonate/additive mixtures stimulated an increase in papillae per unit area in infected leaves.
- ii. Determine whether any enhancement differed with the host genotype for resistance.
- iii. Investigate the effects of treatment and cultivar genotype on the early stages of infection in order to quantify the host-pathogen response to treatment.

6.2 MATERIALS AND METHODS

Quantification of the effects of treatment and cultivar genotype on host-pathogen responses in the early stages of infection were based on whole plant bioassays.

6.2.1 Plant Material And Sample Collection

1. Cucumber plants ('Lebanese', 'Slicemaster', and 'Slice King') were grown from seed and transplanted according to the protocol described in Chapter 2.2.
2. At four weeks, when 3 true leaves were fully expanded, plants were sprayed to the point of runoff with the seven test materials listed in Chapter 2.3.
3. Three days after treatment, half were inoculated on the second leaf with *S. fuliginea* conidia by gently pressing the infected surface of a donor leaf onto the adaxial surface.
4. On three separate occasions, 1, 3 and 5 days post-inoculation, two discs 12 mm in diameter, were removed from the same approximate position on opposing halves of the inoculated leaf using a cork borer. The results of Cohen et al. (1990) suggested that observation over this time interval would be appropriate to detect resistant responses. Concurrently, leaves were collected from noninoculated, treated plants. For each cultivar, treatment and time interval, four leaves were harvested.
5. Disease severity was assessed 12 days after inoculation of using a 1-4 rating scale devised by Descalzo et al. (1990) for investigating the effects of phosphate salts on the susceptibility of cucumbers to infection. Each fully opened second leaf was

rated according to the number of powdery mildew colonies present: 1=0, 2=1-25, 3=26-50, 4=51-128, 5=>128 colonies per leaf.

6.2.2 Histochemistry

Preliminary trials using the staining techniques outlined below indicated that important details of the host-pathogen interaction could be discerned from leaf discs prepared as cleared whole mounts. The visual contrast and resolution between fungal structures and host tissues was very good. Leaf discs were studied instead of epidermal strips as the latter were too difficult to peel from soft cucumber tissue in any appreciable size and with any uniformity of shape. Both discs were prepared for histochemical staining as described below.

1. Discs were cleared according to the method of Hood & Shew (1996). Clearing tissues for microscopical examination is a common procedure used to soften tissue, release light absorbing pigments such as chlorophyll and light-scattering interfaces in cytoplasm, mitochondria, vacuoles and chloroplasts (O'Brien & McCully 1981), which can affect the resolution of the image. In this research it also made tissues more transparent for viewing with transmitted light (they were more than one cell thick), and more receptive to stain absorption. Discs were autoclaved for 15 min at 121°C, in 150 ml glass beakers with 50 ml of 1 M KOH (Mr 56.11; pelletised) which cleared and softened tissue by partial hydrolysis (Fox 1991).
2. When cool, specimens were floated onto holders constructed from metal mesh, to keep them flat and eliminate handling, during removal from the clearing solution. They were blotted dry of excess KOH on paper towels and transferred to 10 cm deep glass dishes containing distilled water for rinsing. Tissues were washed to remove the alkali before any subsequent staining (Fox 1991). The washing process was repeated three times with 5 min per rinse and tissues were blotted between rinses.
3. One disc per leaf was transferred via mesh holders to freshly prepared aniline blue. Aniline blue fluorescence has been widely used in botanical histochemistry, to stain

callose (β -1,3-glucans) plugs in phloem (Hood & Shew 1996), and in phytopathology to stain the callose component in papillae. The affinity of aniline blue for callose is thought to be attributable to loose packing of these polymers, providing greater access to them by the fluorochrome (Smith & McCully 1978).

Stain solution prepared according to the procedure of McNicol et al. (1985) several days previous as 0.1% aniline blue water-soluble dye (CI 42755; Gurr BDH Chemicals) in 0.1 M K_2PO_4 (pH 9), was stored subsequently at room temperature ($21 \pm 1^\circ C$) in a brown-glass bottle. Leaf disc samples were refrigerated at $4^\circ C$ and stained for a minimum of 40 min. Longer immersion in aniline blue stain (ie. several days) was not detrimental to imagery, provided that the samples were refrigerated and the stain was not allowed to evaporate.

4. Samples were removed, excess stain blotted from the metal holders, and the specimens rinsed once in distilled water. Blotting was repeated and the specimens transferred into a fluorescent-brightener Calcofluor White M2R (CI 40622), 0.1% in distilled water) for several minutes, before floating onto glass microscope slides with the aid of a paintbrush. A drop of DIFCO FA mounting fluid was added to further preserve fluorescence, followed by a coverslip, and the slide blotted gently dry between two sheets of lint-free paper towel.
5. Material was examined with an Olympus BH-2 RFC reflected light fluorescence microscope. The microscope was equipped for epifluorescence microscopy with an HBO 100 W/2 mercury vapour lamp, G365 nm exciter and LP 420 nm barrier filters. Photographs were taken with a Nikon HFX-IIA camera. Three aspects of the infection process and host response were quantified:
 - i. Conidial germination (per 200 spores surveyed) after 24 h
 - ii. Germinated conidia producing successive germ tubes after 72 h
 - iii. Number of papillae per colony in epidermal cells 120 h after inoculation (mean of 20 colonies).
6. The second cleared disc was transferred to trypan blue (CI 23850; Gurr microscopy materials) in alcoholic lactophenol for 2 h, a stain prepared according to Kmecl et al. (1995) to examine the development of wheat powdery mildew in cleared leaves.

After blotting dry as described above, samples were mounted in Shear's mounting fluid. The coverslip was ringed in clear nail varnish to reduce evaporation (Fox 1991) of the lactophenol complex, thus providing a semi-permanent mount until such time as when the slide could be assessed. Quantitative observations were attempted under bright field microscopy. The number of haustoria produced by the fungus in epidermal cells associated with development of 20 colonies derived from single conidia were counted for each treated host cultivar.

7. Toluidine blue O stain prepared according to Smith & McCully (1978) was utilised to determine qualitatively whether papillae contained lignin.

6.2.3 Analysis Of Results

Pathogen development, the production of papillae, and the effect of test materials on disease control were analysed by analysis of variance using the Quattro Pro v6.0 spreadsheet/statistical package. Individual treatment means were compared for differences after necessary transformation ($\sqrt{y+0.5}$) with the appropriate controls using Fisher's protected lsd test, since the standard errors of differences between pairs of treatments were very similar.

6.3 RESULTS

6.3.1 Fungal Growth On Susceptible And Resistant Cultivars

i. Fluorescence microscopy

Conidia were visible on the adaxial leaf surface, fluorescing mauve to blue-white against a dark background under UV-epifluorescence microscopy. By 24 h after inoculation conidia had produced their first or primary germ tube which appeared unbranched, some were forked. Analysis of variance on data adjusted with a square root transformation (Table 6.1) indicated that the difference in the number of conidia germinated on cultivars of different resistances treated with the same materials was not significant ($p \leq 0.01$). However, there was a significant difference ($p \leq 0.01$) between the effects of the seven treatments applied. Comparison of treatment means showed that for all cultivars treated with shellspray oil and sodium bicarbonate-plus-shellspray oil, significantly ($p \leq 0.01$) fewer conidia produced their first-germ tube. In contrast, the mean number germinated for other treatments was not significantly different from the water-treated control (Table 6.2).

Table 6.1 Analysis of variance for *Sphaerotheca fuliginea* conidial germination on leaf discs taken from different genotypes of cucumber 24 h after inoculation

Source of variation	df	Sum of squares	Mean square	F-value
A (cultivar resistance)	2	2.54	1.27	0.76
B (material applied)	6	420.50	70.09	41.70**
AB interaction	12	12.10	1.01	0.60
Error	63	105.70	105.70	
Total	83	540.89		

** Indicates differences between treatment means are highly significant at $p < 0.01$. *Indicates differences between treatment means are highly significant at $p < 0.05$.

Table 6.2 A comparison of *Sphaerotheca fuliginea* conidial germination on leaf discs taken from different genotypes of cucumber 24 h after inoculation

	'Lebanese' (s) ^a	'Slicemaster' (dt)	'Slice King' (r)
Tap water	104.8 ^b	121.5	106.3
Milsana fluessig	100.0	103.8	112.5
Sodium bicarbonate	107.0	95.3	119.5
Sunlight liquid	114.0	128.3	126.8
Shellspray mineral oil	37.5**	58.5**	36.0**
Sbc/sunlight liquid	104.0	100.3	120.3
Sbc/shellspray oil	20.3**	19.5**	24.9**

^aGenotype for resistance to powdery mildew: s=susceptible, dt=disease tolerant, r=resistant

^bUntransformed means

* =values are significant at $p \leq 0.05$ **=values are significant at $p \leq 0.01$

Three days after inoculation, four to five germ tubes had been produced from conidia on water-treated controls. Analysis of variance (Table 6.3) showed that there were significant differences in numbers of conidia forming multiple germ tubes on cultivars with different resistances, for the 7 treatments applied. Comparison of means revealed that multiple germ tube production decreased with increasing resistance of the cultivar in the order of 'Leb', 'SM' and 'SK'. The most effective treatments for reducing production were shellspray oil alone and combined with sodium bicarbonate (Table 6.4). Sodium bicarbonate-plus-sunlight liquid also caused a major, significant ($p < 0.01$) reduction in multiple germ tube production. Sodium bicarbonate and sunlight liquid individually and Milsana were less effective and did not significantly reduce multiple germ tube production on 'SK', or in the sodium bicarbonate, sunlight liquid, or sodium bicarbonate 'Leb' combinations.

Table 6.3 Analysis of variance for the mean number of *Sphaerotheca fuliginea* conidia which produced four or five germ tubes on leaf discs taken from different genotypes of cucumber 72 h after inoculation

Source of variation	df	Sum of squares	Mean square	F-value
A (cultivar resistance)	2	60.66	30.33	82.00**
B (material applied)	6	839.34	139.90	378.10**
AB interaction	12	30.40	2.53	6.84**
Error	63	23.30	0.37	
Total	83	953.70		

** Indicates differences between treatment means are highly significant at $p < 0.01$. *Indicates differences between treatment means are highly significant at $p < 0.05$.

Table 6.4 A comparison of the mean number of *Sphaerotheca fuliginea* conidia which produced four or five germ tubes on leaf discs taken from different genotypes of cucumber 72 h after inoculation

	'Lebanese' (s) ^a		'Slicemaster' (dt)		'Slice King' (r)	
Tap water	146.8 ^b	a ^c	90.8	a	65.5	a
Milsana fluessig	94.0	b	61.5	b	45.0	a
Sodium bicarbonate	130.3	a	73.8	b	71.8	a
Sunlight liquid	97.5	b	80.5	ab	56.5	a
Shellspray oil	8.4	d	7.0	c	6.8	b
Sbc/sunlight liquid	60.7	c	65.3	bc	50.8	a
Sbc/shellspray oil	7.3	d	3.3	c	2.4	b

^aGenotype for resistance to powdery mildew

^b Remaining conidia of the 200 surveyed per replicate, produced 1-3 germ tubes on average

^c Values in the same column followed by the same letter do not differ significantly at $p \leq 0.05$ according to Fisher's protected lsd test

After 5 days incubation, sporulating structures of *S. fuliginea* were clearly visible (Figures 6.1 and 6.2), appearing morphologically similar to specimens previously described (Cunningham 1956; Hammett 1977). Fungal development was particularly extensive on water-treated 'Leb'. Unicellular, barrel-shaped conidia arose in chains of 3-4 on short euoidium-type conidiophores above a network of mycelium on the leaf surface. Both conidiophores and conidia fluoresced blue-white. Septation between conidia and in mycelium was clearly visible. Host trichomes (epidermal hairs) were readily discernible from fungal tissue by their morphology, and fluorescence (brilliant green).



Figure 6.1 (above) and 6.2 (below) Callose deposition in epidermal cells on the adaxial surface of a cucumber leaf from cultivar 'Lebanese', 120 h after inoculation. Callose fluoresced an intense yellow, host cells and fungal structures (conidia, germ tubes, hyphae) blue-white, and trichomes a brilliant yellow-green (white in the photographs). Note white areas in the host cell cytoplasm appear circular with a small perforation midway (arrow), especially those located beneath a mycelial strand. x 200 magnification.



6.3.2 Fungal Penetration And Host Responses

i. Fluorescence microscopy

Areas where callose-like material had accumulated or been deposited in epidermal cells of the adaxial leaf surface were located 120 h after inoculation by an intense yellow fluorescence. This was in sharp contrast to the fluorescence of host cells and fungal structures (conidia, germ tubes, hyphae) described above. Yellow areas in the host cell cytoplasm appeared circular and many possessed a small perforation in their centre, especially when located beneath a mycelial strand (Figures 6.1 and 6.2). When non-inoculated cucumber tissues of all cultivars were compared, these discrete formations were absent from epidermal cells. This evidence suggested that they were papillae, surrounding infection pegs. The perforation was likely to be the point at which an infection peg produced from beneath an appressoria had penetrated the cell. Besides the fluorescence of papillae, callose was found in lateral walls of penetrated cells and adjacent non-penetrated cells, especially in the cytoplasm around haustoria (Figure 6.3). This phenomenon was common to all cultivars regardless of treatment, though qualitatively, the reaction of cell walls in 'Leb' tissues containing haustoria seemed less intense than those from 'SM' and 'SK'.

Analysis of variance (Table 6.5) showed that there were significant differences in numbers of papillae formed in leaf tissues from cultivars with different resistances to disease, for the 7 treatments applied.

Table 6.5 Analysis of variance for the mean number of papillae counted in leaf discs taken from different genotypes of cucumber 120 h after inoculation

Source of variation	df	Sum of squares	Mean square	F-value
A (cultivar resistance)	2	40.71	20.34	84.70**
B (material applied)	6	120.10	20.00	83.30**
AB interaction	12	13.13	1.09	4.54**
Error	63	15.13	0.24	
Total	83	189.07		

** Indicates differences between treatment means are highly significant at $p < 0.01$. *Indicates differences between treatment means are highly significant at $p < 0.05$.

Water-treated leaves from the most susceptible genotype ‘Leb’ contained significantly fewer papillae in their epidermal cells than those from similarly treated ‘SM’ and ‘SK’ (Table 6.6). The number of papillae associated with each germinated spore was greater in Milsana-treated leaves for all cultivars, but this was significant for ‘SM’ and ‘SK’ only. Sodium bicarbonate pre-treatment also increased numbers of papillae although the increase was significant in ‘Leb’ only. No significant increases followed shellspray oil pre-treatment when it was used alone or in combination with sodium bicarbonate on any of the three cultivars. Papillae occurred in similar numbers in leaves treated with sunlight liquid and water. Combined with sodium bicarbonate, sunlight liquid increased papillae numbers in all cultivars but the increase was significant in ‘SK’ only.

Table 6.6 A comparison of the mean number of papillae counted in leaf discs taken from different genotypes of cucumber 120 h after inoculation

	‘Lebanese’ (s) ^a		‘Slicemaster’ (dt)		‘Slice King’ (r)	
Tap water	4.5 ^b	c ^c	12.8	cd	16.5	c
Milsana fluessig	12.6	bc	27.0	ab	36.0	a
Sodium bicarbonate	13.6	ab	24.0	bc	19.7	bc
Sunlight liquid	2.7	c	15.0	cd	20.1	bc
Shellspray oil	2.2	c	4.4	d	4.7	d
Sbc/sunlight liquid	11.1	bc	17.9	c	37.3	a
Sbc/shellspray oil	1.7	cd	3.8	d	4.4	a

^a Data show the average number per colony derived from a single conidium (n=4)

^b Genotype for resistance to powdery mildew

^c Untransformed means

^d Values in each column followed by the same letter do not differ significantly at $p \leq 0.01$

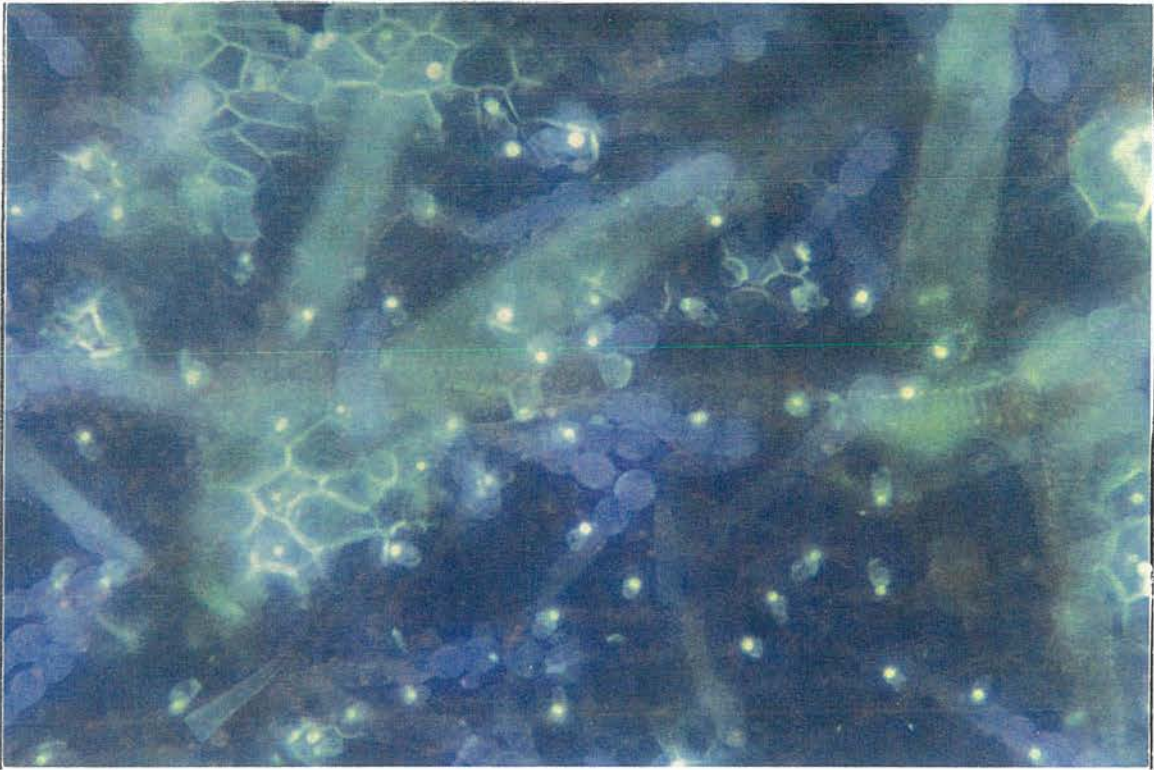


Figure 6.3 Callose fluorescence in papillae, lateral walls of penetrated cells, non-penetrated cells adjacent to these, and in the cytoplasm around haustoria in a cucumber leaf from cultivar 'Lebanese'. x 62.5 magnification.

ii. Bright field microscopy

First penetration of the host occurred from the primary germ tube 24 h after inoculation and was seen in detail under bright field microscopy. Figures 6.4 and 6.5 show this event occurring in a water-treated 'SK' leaf. At x 500 magnification viewed with oil immersion (Figure 6.4), this conidium was clearly seen to have produced a germ tube, 2 cells long (delimited by 1 septum), ending in an appressorium. At this magnification, two features stood out. The lateral walls of the host cell adjacent to the conidium, and several globular bodies contained in the cytoplasm (consisting of over half the cell area), were heavily stained. This may have been a sign of a hypersensitive response in process as this reaction was not observed in cells where conidia were absent. Secondly, the epidermal cell directly beneath the appressorium was darkly stained in the centre region. Higher magnification x 625 (Figure 6.5), revealed an infection peg produced from beneath the appressorial germ tube which had penetrated into the cell and was surrounded at the point of penetration by a dark halo (later revealed by aniline blue fluorescence as callose-containing).

By 72 h, in addition to epidermal cells containing globular bodies, 1-3 cells on average per colony derived from a single conidium were stained distinctly darker in tissues of the more resistant varieties 'SM' and 'SK' (Figure 6.6). Figure 6.7 shows that the cytoplasm had become granular at this time there was no such response from 'Leb'. In several instances, an epidermal cell apparently in the process of collapse, was surrounded by haustoria in adjoining living cells (Figure 6.8). Haustoria stained a light blue with the trypan blue-lactophenol stain and appeared almost circular from the outset (Figure 6.9). Focussing down through the epidermal cells at higher magnification defined the shape of haustoria as almost pear-shaped (Figure 6.10).

Analysis of variance (Table 6.7) showed significant differences in numbers of haustoria formed by *S. fuliginea* in leaf tissues from cultivars with different resistances to disease, for the 7 treatments applied. However, the treatment x cultivar interaction was not significant ($p < 0.01$), indicating that these two factors were independent.

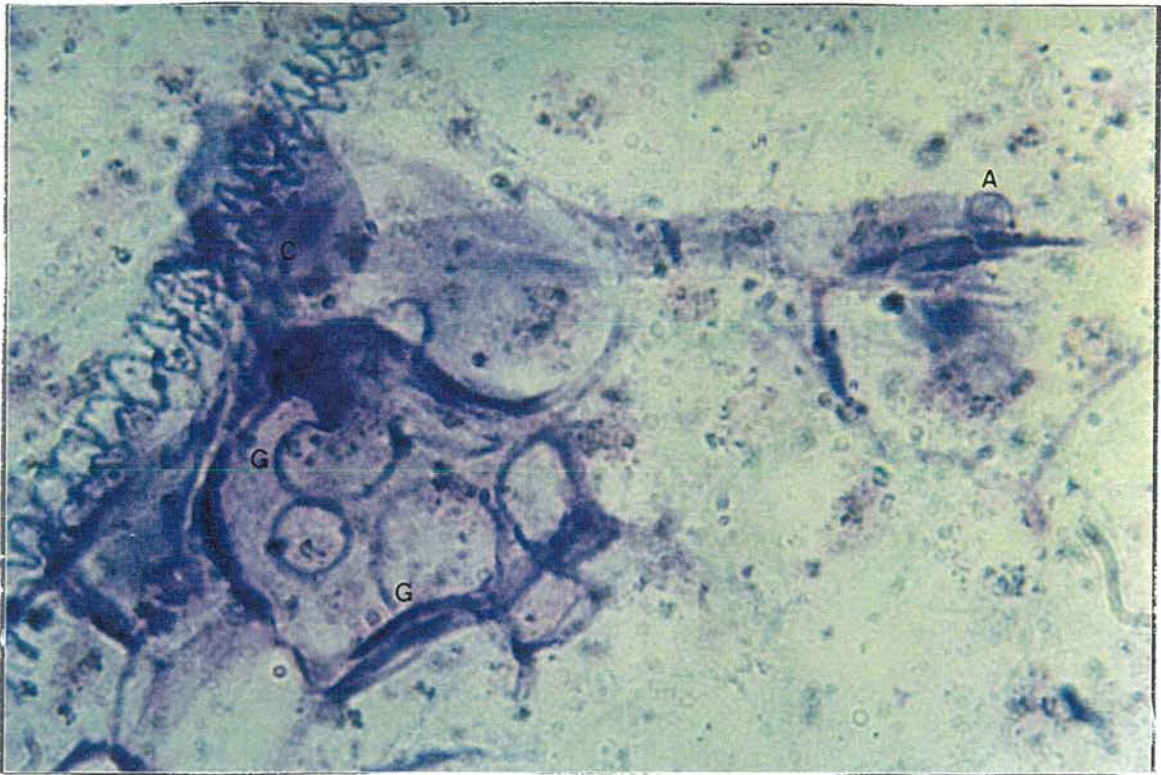


Figure 6.4 (above) First penetration of the host by the primary germ tube 24 h after inoculation of a water-treated 'Slice King' leaf. Lateral walls of the host cell adjacent to the conidia (C), several globular bodies (G) in the cytoplasm, and the epidermal cell directly beneath the appressoria (A) were heavily stained. x 500 magnification viewed with oil immersion.

Figure 6.5 (below) An infection peg (arrow) produced from beneath the appressorial germ tube which had penetrated into the cell was surrounded at the point of penetration by a dark halo (H). x 625 magnification.

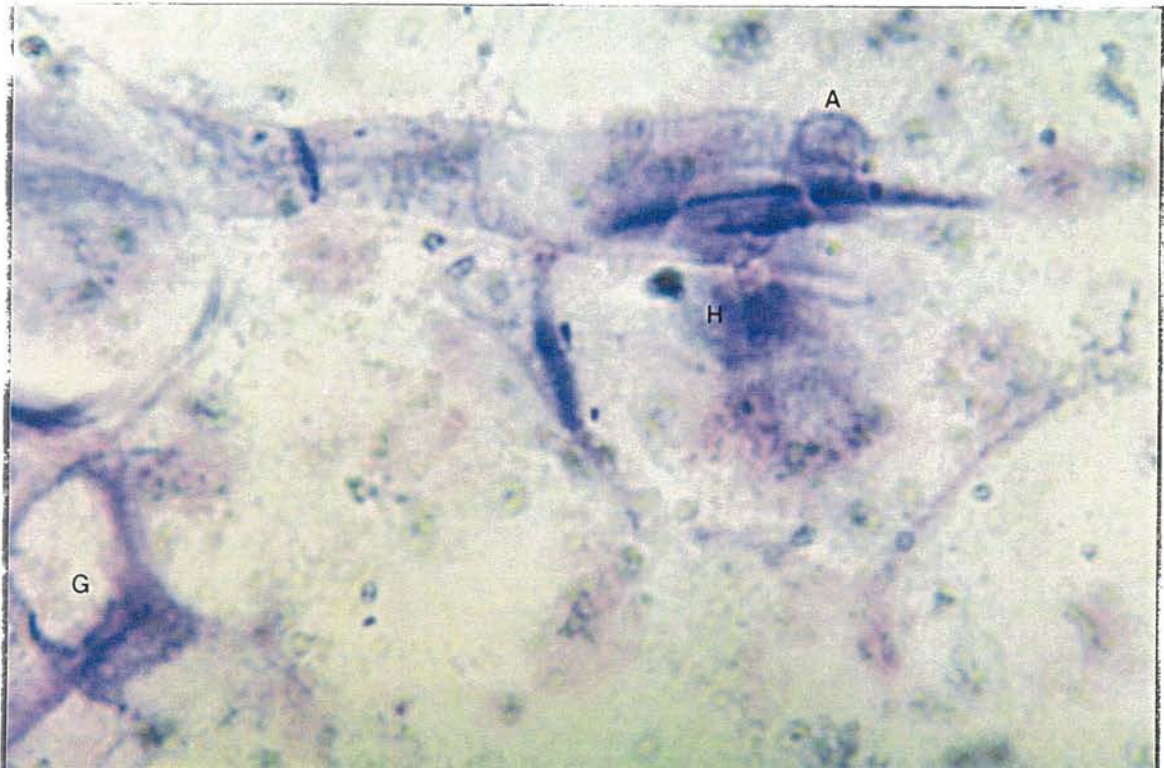
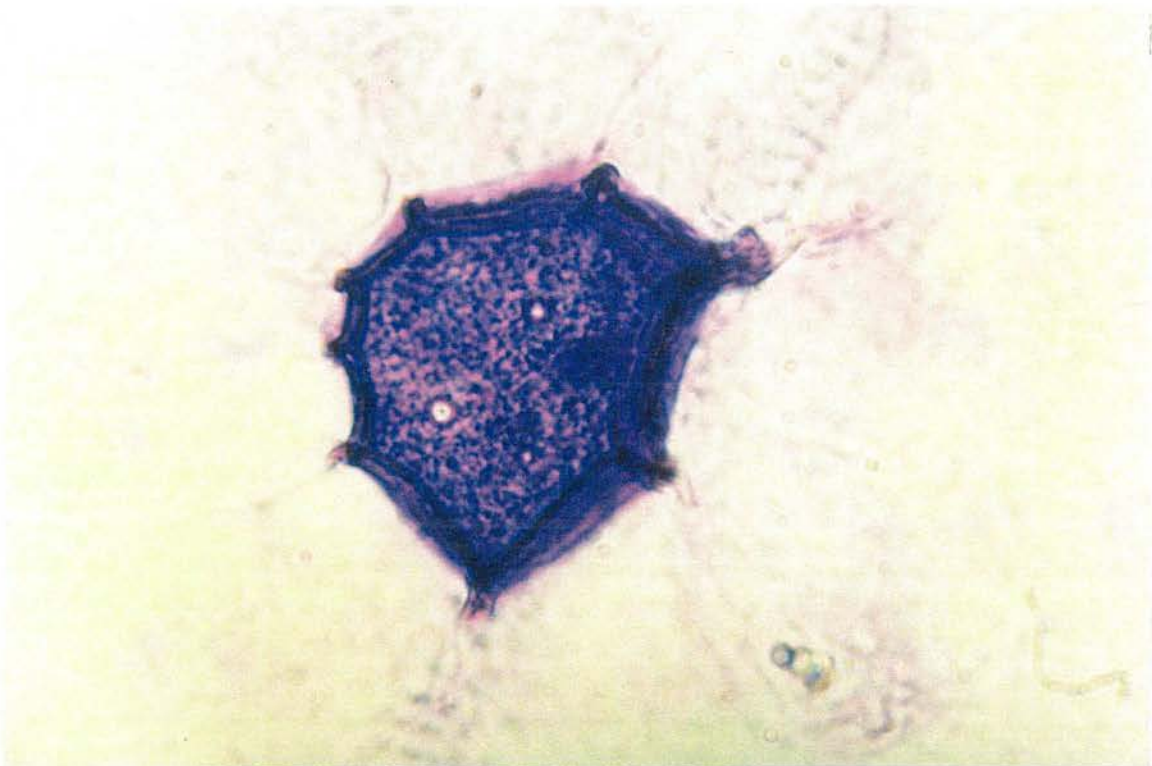




Figure 6.6 Darkly staining epidermal cells in tissues of the more resistant cucumber cultivar 'Slice King', 72 h post inoculation. x 125 magnification.

Figure 6.7 The cytoplasm of these cells (above) had become granular as if their contents were disintegrating. x 500 magnification.



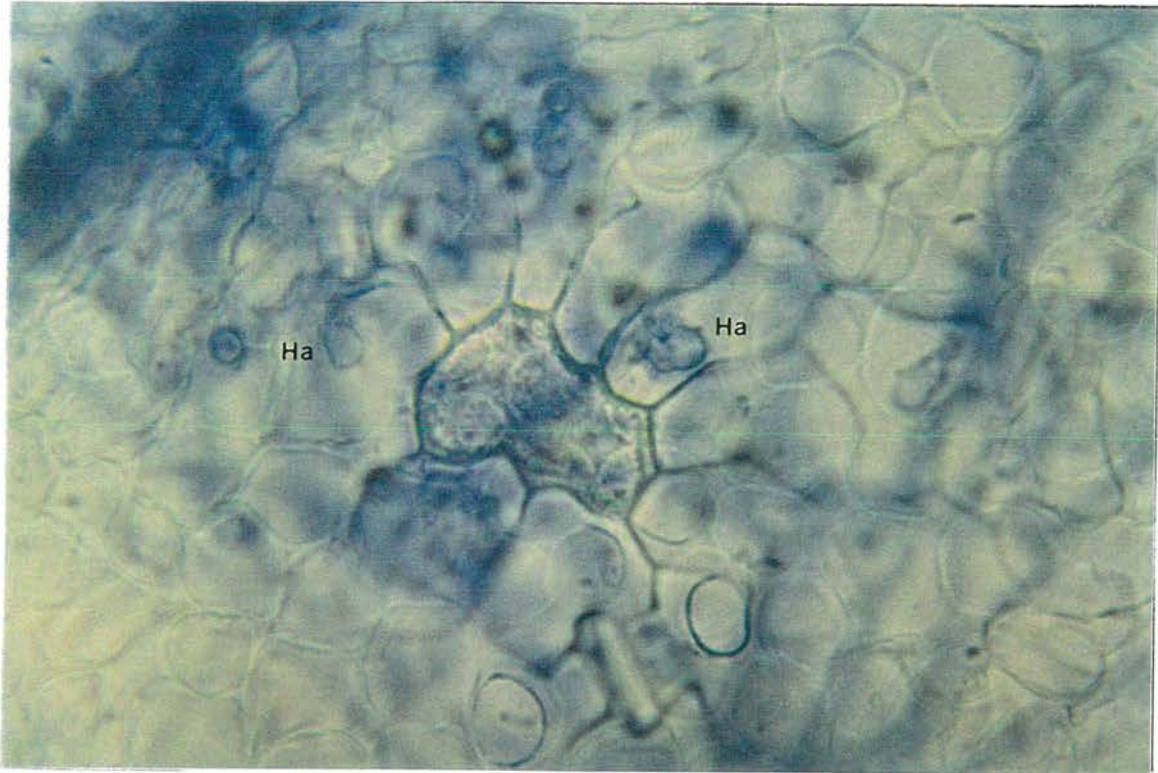


Figure 6.8 An epidermal cell possibly in the process of collapse, surrounded by haustoria (Ha) formed in adjoining living cells. x 200 magnification.



Figure 6.9 An haustorium (Ha) of *Sphaerotheca fuliginea* in epidermal cells of cucumber cultivar 'Lebanese' stained a light blue with trypan blue-lactophenol. x 62.5 magnification.

Figure 6.10 Close up of an haustorium (Ha) of *Sphaerotheca fuliginea* with appendages (a), 72 h after inoculation. x 500 magnification.

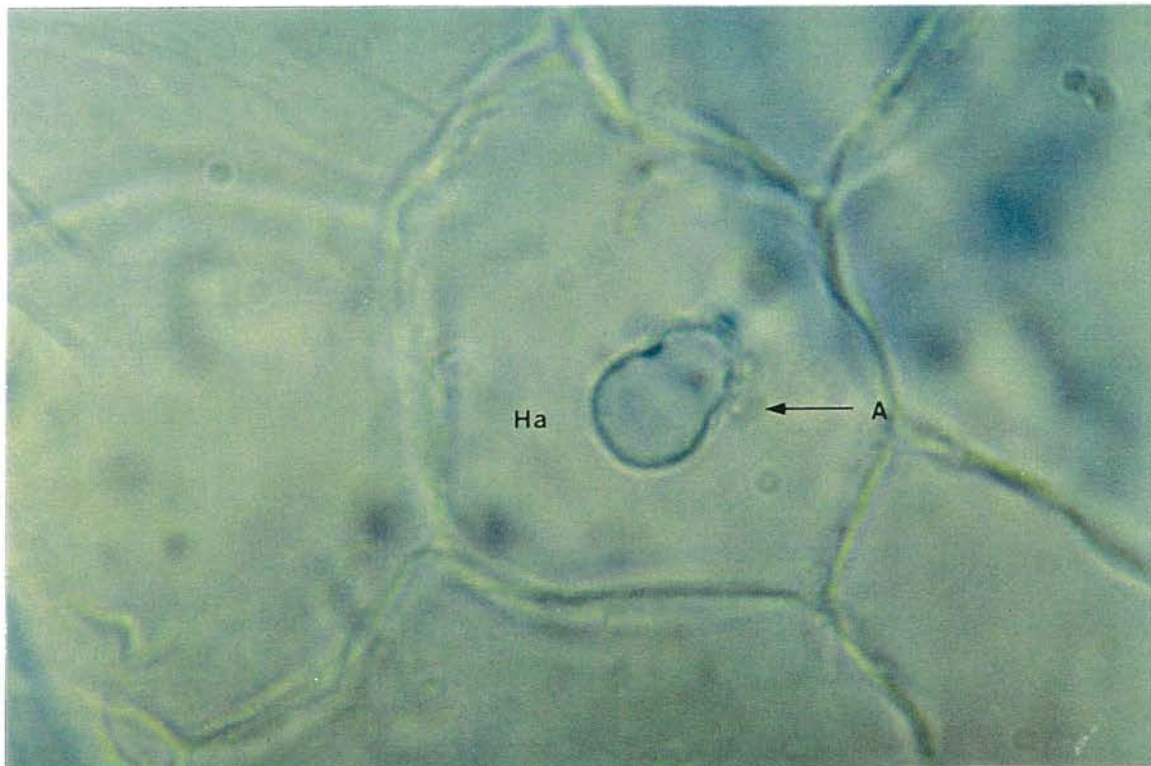


Table 6.7 Analysis of variance for the mean number of haustoria counted in leaf discs taken from different genotypes of cucumber 120 h after inoculation

Source of variation	df	Sum of squares	Mean square	F-value
A (cultivar resistance)	2	23.07	11.54	44.10**
B (material applied)	6	70.30	11.72	44.78**
AB interaction	12	3.23	0.27	1.03
Error	63	16.49	0.26	
Total	83	113.09		

** Indicates differences between treatment means are highly significant at $p < 0.01$. * Indicates differences between treatment means are highly significant at $p < 0.05$.

Comparisons of haustoria per colony on water-treated leaves found that numbers were significantly higher for 'Leb' than for 'SK' (Table 6.8) which did not differ significantly from 'SM'. Milsana treatment reduced haustorial numbers overall, but did not significantly restrict haustorial development in leaves of any one host genotype. Sodium bicarbonate and sunlight liquid by themselves also failed to suppress haustorial development across cultivars. Significantly fewer haustoria developed in leaves, irrespective of host genotype, treated with shellspray oil alone or in combination with

Table 6.8 A comparison of the mean number of haustoria counted in leaf discs taken from different genotypes of cucumber 120 h after inoculation

	'Lebanese' (s) ^a		'Slicemaster' (dt)		'Slice King' (r)	
Tap water	29.7 ^b	a ^c	21.5	ab	10.8	ab
Milsana fluessig	19.4	ab	16.2	bc	8.3	b
Sodium bicarbonate	27.0	a	22.5	ab	12.7	ab
Sunlight liquid	20.1	ab	18.2	b	10.9	b
Shellspray oil	7.8	c	5.3	cd	3.0	c
Sbc/sunlight liquid	11.8	bc	10.3	bc	7.9	bc
Sbc/shellspray oil	7.2	cd	4.7	d	2.5	c

^a Data show the average number per colony derived from a single conidium (n=4)

^b Genotype for resistance to powdery mildew

^c Untransformed means

^d Values in each column followed by the same letter do not differ significantly at $p \leq 0.01$

sodium bicarbonate. Sunlight liquid-plus-sodium bicarbonate restricted haustoria numbers on all cultivars but was significant for 'Leb' only.

Papillae appeared as intensely stained blue-green halos (Figure 4.11) in inoculated tissues from all cultivars when stained with toluidine blue O, a stain specific for lignin. A similar colour reaction for xylem elements, confirmed that papillae were lignified.



Figure 6.11 Papillae (P) in inoculated tissues from all cultivars stained as blue-green halos with toluidine blue O, indicating that they contain lignin. Note the similar colour reaction for xylem elements (X). x 62.5 magnification.

6.3.3 Disease Severity

The difference in disease severity between cultivars of different resistances to powdery mildew varied significantly ($p < 0.01$) with both genotype for resistance and treatment applied (Table 6.9). The results of treatment application are listed in Table 6.10. Twelve days after inoculation, water-treated 'SK' had significantly less infection than 'Leb' and 'SM' plants. Milsana controlled disease significantly and equally well on all cultivars. Sodium bicarbonate applied alone provided no significant disease control. Significantly lower levels of disease were assessed on plants of all three cultivars treated with a single application of shellspray oil. Control of powdery mildew was similar across all cultivars with sodium bicarbonate-plus-shellspray oil treatment. Sunlight liquid significantly controlled disease on 'Leb' and 'SM' only but when combined with sodium bicarbonate, it gave significant control on all 3 cultivars.

Table 6.9 Analysis of variance for the difference in powdery mildew severity between cultivars of different resistances varied for the 7 treatments applied.

Source of variation	df	Sum of squares	Mean square	F-value
A (cultivar resistance)	2	1.13	0.57	11.40**
B (material applied)	6	7.97	1.33	2.66**
AB interaction	12	0.50	0.04	0.80
Error	63	3.45	0.05	
Total	83	13.05		

** Indicates differences between treatment means are highly significant at $p < 0.01$. *Indicates differences between treatment means are highly significant at $p < 0.05$.

Table 6.10 Mean disease development on the second leaf of different genotypes of cucumber 12 days after inoculation according to the rating scale of Descalzo et al. (1990)^a.

	'Leb' (s) ^b		'SM' (dt)		'SK' (R)	
Tap water	4.75	a ^c	4.00	a	2.75	a
Milsana fluessig	3.50	b	3.00	b	1.75	b
Sodium bicarbonate	4.75	a	4.50	a	3.25	a
Sunlight liquid	3.50	b	3.25	b	2.50	ab
Shellspray mineral oil	1.50	cd	1.25	d	1.25	c
Sbc/sunlight	2.25	c	2.00	c	1.50	bc
Sbc/shell oil	1.25	d	1.25	d	1.25	c

^aRating used to quantify powdery mildew disease development was as follows: 1=0, 2=1-25, 3=26-50, 4=51-128, 5=>128 colonies per second leaf

^bGenotype for resistance to powdery mildew

^cValues followed by the same letter do not differ significantly at $p \leq 0.05$

6.4 DISCUSSION

The severity of powdery mildew on the water-treated controls decreased with increasing resistance of the cultivars. This result confirmed resistances predicted from information provided by the seed distributor. Shellspray oil and sodium bicarbonate-plus-shellspray oil mixtures were the most effective treatments, followed by sodium bicarbonate-plus-sunlight liquid, and then Milsana and sodium bicarbonate. This agreed with the findings of Chapter 2 (see this Chapter for a fuller explanation and review of the relevant literature).

Fluorescence microscopy permitted observations on the development of *S. fuliginea* on cucumber as influenced by different host genotypes for resistance, and prophylactic treatment of the host with sodium bicarbonate/additive mixtures and the plant extract Milsana. During the early stages of infection, surveys of conidial germination on cucumber leaves 24 h after inoculation, reported a maximum of 65% for any one cultivar. A similar percentage of germination on water-treated controls was found by Homma et al. (1981), but they offered no explanation for why this number was not higher. Factors such as natural variation in the fungal population and suboptimal environmental conditions in the glasshouse may have restricted greater germination in the present study. Paxton (1991) considered humidity important in

producing susceptible growth and promoting satisfactory disease development, when screening materials for their fungicidal ability. Alternatively, the quality of inoculum may have been overestimated although measures were taken to generate fresh conidia for inoculation of recipient plants. Laboratory tests to determine the viability of conidia can prove futile for a high germination reading does not necessarily indicate a high infective potential (Dhingra & Sinclair 1985).

A germination rate of 48-65% was universal across cultivars, as was a low rate of germination for plants treated with oil and bicarbonate-oil mixtures. The similarities in development on susceptible and more resistant cultivars at this early stage in the infection process agreed with several studies of *S. fuliginea* development on cucurbits (Cohen et al. 1988; Cohen et al. 1990; Menzies et al. 1991; Aalbersberg & Stolk 1995; Floris & Alvarez 1996). Similar observations have also been made on the interaction between resistant cucumbers and the pathogen *Colletotrichum lagenarium*, the causal agent of cucumber anthracnose (Kovats et al. 1991; Stein et al. 1993). Based on the 4 physical modes of action of petroleum oils and reference fungicides on grapevine powdery mildew described by Northover & Schneider (1996), the poor rate of germination in the presence of shellspray oil and sodium bicarbonate-plus-shellspray oil suggested that these materials had a direct effect on the fungus at the host-pathogen interface which caused the inhibition of germination, an attribute commonly associated with true protectant fungicides.

The formation of additional germ tubes from conidia 72 h after inoculation decreased with increasing resistance of the cultivar, indicating that some mechanism for resistance may have been activated in the host which retarded pathogen development. Hijwegen & Verhaar (1996) reported similar differences in the development of secondary germ tubes and hyphae in a comparable study which examined the effect of host genotype on the induction of resistance to *S. fuliginea*, by 2,6-dichloroisonicotinic acid. Germ tube production was also retarded for conidia observed on all cultivars treated with shellspray oil alone and in combination with sodium bicarbonate. This supported the notion suggested after only 24 h, that these materials had either a fungistatic or fungicidal mode of action, or both. Materials that

these terms are applied to differ in their severity on a mycological pathogen. A fungistat has the ability to prevent the growth or development of fungal spores without killing them, whereas a fungicide kills spores or mycelium outright (adapted from Anon 1968).

Papillae, purported physical barriers to the mechanical forces of fungal penetration, were correctly identified by an intense yellow fluorescence under ultraviolet epi-illumination (Sherwood & Vance 1976; Cohen et al. 1990; Kovats et al. 1991), the result of a histochemical reaction between their callose component and basic aniline blue stain. The number of papillae formed beneath infection pegs in epidermal cells of water-treated leaves at 120 h after inoculation increased with increasing resistance of the cultivar. A similar association was found in wheat resistant to powdery mildew (Kmecl et al. 1995), in lines of pearl millet resistant and susceptible to downy mildew (*Sclerospora graminicola*) (Sharada et al. 1995), and was suggested by Cohen et al. (1990) for cucumber cultivars more resistant to *S. fuliginea*, although the numbers of papillae deposited were not quantified. Cucumbers in which systemic resistance had been induced also displayed enhanced formation of papillae, which correlated with reduced development of infection hyphae of *C. lagenarium* (Kovats et al. 1991).

Milsana stimulated the greatest increase in papillae deposition in 'SM' and 'SK' tissues, while sodium bicarbonate appeared to have promoted numbers in 'Leb'. It was not clear why a significant increase following bicarbonate treatment occurred in leaves of this cultivar. Whatever the reason, the increase did not appear to be related to the level of protection afforded by this material over time. Schneider & Ullrich (1994) also found an increase in cucumber leaves following Milsana treatment within 10 days post-infection, but they did not specify the resistance status of the cultivar tested. From the work it appeared that their cultivar was reasonably susceptible to disease. Little is known about the effect of chemicals and plant extracts on the production of physical barriers in hosts as a response to pathogen challenge. Results of this present study suggested that Milsana pre-treatment enhanced the resistance response of more disease

tolerant cultivars which resulted in the increased production of papillae and better control of disease, although not the best control.

The existence of compounds in plant extracts which might induce papilla formation against powdery mildew fungi has been suggested (Inoue et al. 1994). This might explain the increase observed in leaves treated with Milsana in this research, and in the work of others (Schneider & Ullrich 1994). Yokoyama et al. (1991) found that treatment of barley coleoptiles with an extract from healthy barley leaves known as papilla-regulating extract (PRE), increased the frequency and rate of appearance of papillae, and decreased the penetration efficiency of *Erysiphe graminis* f.sp. *hordei*. The active component in this extract was recently identified as potassium phosphate, and it was suggested that the presence of phosphates in plant extracts that enhance the resistance response may have been overlooked (Inoue et al. 1994). Investigations by Kowalewski & Herger (1992) concerning the chemical nature of the active constituents in Milsana preparations excluded proteins, terpenoids, phenolics, and regular sugars as active ingredients. They concluded that the resistance-inducing factor was most likely a carbohydrate with a hydrophobic part, but complete characterisation of inducing and induced molecules are still being investigated (Daayf et al. 1995). Phosphate and potassium salts formulated with Tween 20 have induced local and systemic resistance against powdery mildew in glasshouse-grown cucumber (Reuveni et al. 1994, 1996) where they gave comparable or superior control to sodium bicarbonate. If one or other of these salts were present in Milsana this may explain why disease control obtained was superior to control afforded by bicarbonate. As yet, the mode of action of foliar-applied salts in controlling powdery mildew on cucumbers has not been clearly determined (Reuveni et al. 1996).

Significantly fewer papillae were seen in plants treated with shellspray oil applied singly or in combination with sodium bicarbonate. Sunlight liquid alone did not stimulate an increase above the control in leaves of any cultivar, except when combined with bicarbonate, which was particularly significant for 'SK'. These results suggested that additive mixtures which gave superior disease control did not stimulate host defences as part of their mode of action against *S. fuliginea*.

Twenty-four to 72 h post inoculation, epidermal cells of water-treated 'SM' and 'SK' associated with conidia and later haustoria, were observed by light microscopy to be heavily stained, some contained globular bodies in the cytoplasm, and others appeared to be in a state of collapse. Cohen et al. (1990) observed similar pre- and post-haustorial reactions in muskmelon leaves resistant to *S. fuliginea*, which they interpreted as a hypersensitive response. With powdery mildew fungi, a pre-haustorial resistance reaction is thought characteristic of non-host and partial resistances, while race-specific resistance (mostly associated with hypersensitivity) is often post-haustorial (Lebeda & Reinink 1994).

Haustoria appeared morphologically similar to ones photographed by Menzies et al. (1991). The number produced in epidermal cells significantly decreased with increasing resistance of the cultivars to *S. fuliginea*, and appeared to be related to the levels of disease severity observed. Less frequent formation of haustoria has been correlated with resistance of muskmelon to *S. fuliginea*, pearl millet to *S. graminicola* (Sharada et al. 1995), soybean to *Peronospora manshurica* (downy mildew) infections (Riggle 1977), and in other biotrophic infections (Clark & Spencer-Phillips 1994). For cucumber plants a reduction in haustoria production was also achieved following treatment with silicon (implicated in resistance to *S. fuliginea* and a component of papillae) (Menzies et al. 1991). The most effective treatments which reduced haustoria production were shellspray oil alone and in combination with sodium bicarbonate, followed by sodium bicarbonate-plus-sunlight liquid. This was universal across cultivars. That fewer haustoria developed in the presence of these materials after 120 h, and that the severity of disease was least in their presence, suggested that they were active against the fungus early in the infection process. By themselves, sodium bicarbonate and sunlight liquid were less effective across cultivars which suggested that there was some degree of synergism between these materials.

In summary,

1. Conidia germinated similarly on all cultivars 24 h after inoculation, except for those treated with shellspray oil and sodium bicarbonate-plus-shellspray oil where fewer germinated.
2. With increasing host resistance- the number of multiple germ tubes produced 72 h post-infection was less, the number of papillae deposited in epidermal cells had increased, and the number of haustoria was less 120 h post-infection.
3. Shellspray oil and sodium bicarbonate-plus-shellspray oil gave- the most effective disease control, the most significant reduction in production of multiple germ tubes and haustoria, and the least apparent stimulation of papillae production.

7. GENERAL DISCUSSION

The host-parasite-complex of cucumber (*Cucumis sativus* L.) and *Sphaerotheca fuliginea* (Schlechtendal ex Fr.) Pollacci, the causal agent of cucumber powdery mildew, was studied to determine whether stimulation of host defences was a mode of action of sodium bicarbonate/additive mixtures. Additives included shellspray mineral oil and sunlight dishwashing liquid. The roles in cucumber defence of phenolic compounds, enzymes: peroxidase (PO) and phenylalanine ammonia-lyase (PAL), and induced structural barriers, papillae, were investigated. Comparisons were made between the effects of bicarbonate/additive mixtures and the plant extract Milsana on these host defences, as related to three levels (cultivars) of host resistance to disease: susceptible, disease-tolerant and resistant.

Long and short duration glasshouse studies employing sodium bicarbonate/additive mixtures found that applied on its own at 2 g/litre, sodium bicarbonate failed as a protectant on all cultivars. This generally agreed with studies of control of cucurbit (Weeds 1992) and wheat (Bell 1996) powdery mildews, and also bean white mould (Ludy Powelson 1996). It is possible that the waxy leaf cuticle and dense cover of trichomes influenced adhesion of material which resulted in poor disease control. Several papers have implicated the plant cuticle as the primary barrier to pesticide penetration (Tan & Crabtree 1994). Bicarbonate was applied in a polar liquid, distilled water, while the cuticle is composed of insoluble, non-polar lipid polymers. General chemistry textbooks would relate that polar and non-polar compounds are immiscible due to their opposite charges. Therefore it would seem feasible that this simple salt spray would be repelled by the leaf surface, leaving little active ingredient to have a prophylactic effect.

When combined with sunlight liquid or shellspray oil, the fungicidal activity of bicarbonate was enhanced. This enhancement supported the findings of several studies on standard fungicides and bicarbonate salts (listed previously) combined with surfactants when tested for control of powdery mildew diseases. There is still some discrepancy between the low efficacy of bicarbonate/additives against other fungal

pathogens of a range of plant species. Information regarding the mechanism of surfactant-enhancement in general is lacking, but it may be the result of a complex interaction between disease control materials, surfactants, plant surfaces and environmental conditions (Shafer & Bukovac 1987). Research by Tan & Crabtree (1994) suggested that surfactants interact with both cutins and waxes of the cuticle, which results in their being adsorbed by the cuticle for up to 2 months.

Milsana was less efficacious against powdery mildew than bicarbonate/additives, and additives applied as emulsions in water, except when used to protect the most resistant cultivar 'SK'. This suggested that Milsana enhanced 'SK's' high level of natural resistance, which interacted with any possible fungitoxicity of this material to reduce the incidence of disease. From these glasshouse studies, it was not clear why Milsana was ineffective on less resistant cultivars. These results conflicted with at least two studies of the disease controlling abilities of Milsana (Herger & Klingauf 1988; Daayf et al. 1995).

On the basis of chromatogram bioassays for antifungal phenolics there was much variability in free-state and aglycone phenolic patterns between cultivar resistance/treatment combinations, in the presence and absence of disease. This suggested that data did not support the concept of stimulation of host materials by either mildew infection or the treatments applied, proposed by Daayf et al. (1995) for Milsana.

Glycosidically-bound phenolics hydrolysed into their free form, aglycones, were more abundant in leaf extracts than free phenolics, with 3 groups of antifungal substances detected. Most extracts from healthy leaves contained aglycones at 3 Rf values, and because of the large variability in spot diameters, it was difficult to say whether stimulation of aglycone production had occurred in infected leaves as a result of any specific treatment or level of host resistance. Thus the presence of these highly fungitoxic substances could not account for differences in disease severity on cultivars in the present study. Results differed from bioassays by Daayf et al. (1995) in terms of the presence and location of antifungal materials on chromatograms. They did not

observe aglycones at the origin in healthy, infected, and Milsana-treated controls of the single cultivar studied, and also reported a rapid and distinct accumulation of antifungal phenolic compounds in leaves treated with Milsana, especially in infected leaves. Similar increases were not observed for Milsana treatment, however antifungal phenolics were detected at similar Rf values.

There were obviously several strongly fungitoxic compounds in leaf extracts, although these could not be associated with any particular genotype for resistance. Colour reactions suggested that free-state phenolics, and substances concentrated in the aglycone fractions may be catechins, while other aglycones possibly resorcinol or phloroglucinol derivatives. Forms of these substances are reported to have antifungal activity, although catechins have not been detected in cucumber. Several studies have implicated cucurbitacins, occurring particularly in the Cucurbitaceae, as being antifungal substances. It was possible that cucurbitacins contributed to antifungal activity detected in bioassays. If their production could be stimulated in healthy plants by materials other than bicarbonate/additive mixtures (eg. phosphate salts (Reuveni et al. 1991), to prime the host for pathogen challenge, then disease may be controlled effectively with frequent applications. Repeated exposure might provide the right stimulus to maintain substance levels. Before the identity of phenolics in these crude extracts is certain, further purification is necessary. This would probably involve removal of antifungal compounds from silica gel, chromatography in a different solvent system to separate out compounds, repeat bioassays, and co-chromatography and ultraviolet spectral comparisons with authentic catechin substances. Several excellent treatises on the isolation and identification of phenolic compounds in biological material would be useful in future investigations (Seikel 1962; Harborne 1989; van Sumere 1989).

Peroxidase (PO)- and phenylalanine ammonia-lyase (PAL)- activity in healthy leaves resembled the increasing levels of host resistance to disease. That is, activity was least in 'Leb', and highest in 'SK'. This supported work by several groups (listed in Chapter 4.4) who found a significant correlation between higher constitutive levels

of these enzymes in noninfected plants of a variety of unrelated species, and their genotypic susceptibilities and resistances to pathogens.

Rapid stimulation of enzymic-activity in healthy and inoculated 'Leb' leaves treated with bicarbonate, may have symptomised a response to lesions which appeared on leaves within 3 days after treatment. These were possibly the result of phytotoxicity (Weeds 1992; Ziv & Zitter 1992) or burns due to interactions between light rays and water droplets on leaves. Several studies have associated stimulated activities of both enzymes as characteristic not only of infections, but also of injury (Goodman et al. 1986). Oil emulsions and detergents may have distributed the salt uniformly over the leaf surface as a thin film and therefore beads of salt-containing water did not occur. This may explain why lesions and increased activities were not observed when sodium bicarbonate was combined with these additives, or when Milsana (formulated with surfactants) was applied.

During the 14 days after treatment significant increases in PO were detected in leaves treated with sodium bicarbonate and Milsana, while gradual increases occurred in noninoculated cucumber leaves treated with other materials. Increases in PAL were detected in leaves from all cultivars, for most treatments, except bicarbonate/additive mixtures applied as individual components or together where increases were gradual. Particularly significant was the stimulation of PAL-activity in plants treated with bicarbonate and Milsana. This reached a maximum 3 days after treatment, and then rapidly returned to levels similar for other treatments at the same point in time. The time-course of PO- and PAL-activities in plants following Milsana treatment was similar to one described (Herger et al. 1988; Schneider & Ullrich 1994).

Similar gradual increases in PO- and PAL-activity on successive days between leaves treated with materials other than bicarbonate and Milsana suggested:

- i. PO- and PAL-activity detected was due to their involvement in normal plant developmental processes (Macko et al. 1968; Goodman et al. 1986; Wyatt et al. 1991).
- ii. It might take longer than 24 h for PAL-activation, and 3 days for PO-activation, by surfactants alone and in combination with sodium bicarbonate, to be expressed by

the host in any appreciable amounts. The occurrence of a possible lag period before increased activity in noninoculated leaves, and the reception and relaying of a correct signal for stimulation were discussed in Chapter 3.4. Briefly, changes in activity are likely to be governed by reception and relaying of a signal from the site of application to the host genome. If an appropriate signal is received, recognised and translated, a molecular response in the host which includes the synthesis of proteins related to defence may be generated (Hotter 1996). However, if there was insufficient provocation at the site of application and hence no signal sent, then the expression of heightened activity would not be expected to occur. In this sense, surfactants applied alone and in combination with bicarbonate may not be stimulating signal relay, and superior disease control may have resulted from direct effects on the pathogen rather than effects on host physiology.

Increases in PO-activity was stimulated in inoculated 'Leb' by Milsana and sodium bicarbonate. PAL also was significantly more active in leaves treated with these materials, especially 24-72 h after inoculation, and in 'SM' and 'SK' rather than in 'Leb'. PO-levels activity in leaves from inoculated controls and plants treated with other materials were not significantly different until 3 days after treatment for all cultivars. This suggested that 'Leb' physiology was more sensitive to bicarbonate treatment, and that a lag period was observed before any measurable increase in PO-activity in 'SM' and 'SK' cultivars. Activity at this time was considerably higher in inoculated than noninoculated 'Leb' and 'SM' plants, and of a comparable level in 'SK'. In the initial phase of plant diseases the activity of pathways normally operating also in the healthy plants has been reputed to be enhanced (Goodman et al. 1986).

On successive days, high activity in controls and plants for all cultivars treated with materials other than shellspray oil alone and bicarbonate-oil mixtures, suggested that infection and these materials had stimulated host physiology. Disease incidence at the end of the test period was greatest on 'Leb', and considerably less on 'SM' and 'SK' water-treated controls. Shellspray oil and bicarbonate-oil mixtures proved

superior in efficacy than all other materials applied to all cultivars, except that Milsana provided comparable control on 'SK'. These results suggested:

- i. Materials which controlled disease development with the greatest efficacy inhibited the pathogen directly, rather than stimulating a physiological host response involving PO and PAL. They may also have prevented penetration so that a response was not generated as a result of pathogen challenge. Hence, increases in PAL especially, were not significantly different from activities in noninoculated leaves. This might have applied to sunlight liquid formulated with and without bicarbonate for they impeded disease development, and did not appear to stimulate increases in PO- and PAL-activity.
- ii. The extent of increase in PO- and PAL-activity in all cultivars following bicarbonate and Milsana treatment was not related to the level of protection afforded by these materials, except for the more resistant cultivar 'SK', where PO and PAL levels were initially high. Thus, disease control may be the result of a complex interaction between Milsana and natural activation of resistance that may occur in 'SK' when challenged by *S. fuliginea*.
- iii. Bicarbonate had direct activity against *S. fuliginea* but this was not realised until combined with oil or detergents. This enhancement of activity was earlier suggested in glasshouse studies examining the efficacy of bicarbonate/additive mixtures over a longer period of time.
- iv. Genetic variation governing the speed and extent of enzyme induction may account at least in part, for the differences between resistant and susceptible cultivars (Bennett & Wallsgrove 1994) with respect to disease severity observed after 14 days.

In the context of this research, the PO test devised by Reuveni et al. (1990) was useful for rapidly selecting moderately resistant plants for breeding purposes, assuming that PO is an enzyme indicative of a host's potential for resistance to a (specific) disease. It should be remembered that the activities of several enzymes are likely to be influenced upon pathogen challenge, and in response to different or the same pathogens, as PAL was. Other biochemical processes might yield better markers

of resistance. Screening of a larger range of cultivars would indicate whether real benefits could be achieved from this test.

Fluorescence microscopy revealed similar numbers of conidia germinated 24 h after inoculation, irrespective of treatment and cultivar, although, significantly fewer conidia germinated on leaves treated with oil and bicarbonate-oil mixtures. The poor rate of germination in the presence of these materials, suggested they acted directly on the fungus at the host-pathogen interface which caused the inhibition of germination (Northover & Schneider 1996), an attribute commonly associated with true protectant fungicides. With increasing host resistance the number of multiple germ tubes produced 72 h post-infection was less, the number of papillae deposited in epidermal cells had increased, and the number of haustoria produced by the fungus was less 120 h post-infection, indicating that some mechanism for resistance may have been activated in the host which retarded pathogen development. Shellspray oil and bicarbonate-oil mixtures provided the most effective disease control, the most significant reduction in production of multiple germ tubes and haustoria, and the least apparent stimulation of papillae production. Sunlight liquid alone did not stimulate an increase above the control in leaves of any cultivar, except when combined with bicarbonate, which was particularly significant for 'SK'. These results suggested that additive mixtures which gave superior disease control, did not stimulate host defences as part of their mode of action against *S. fuliginea*, rather that they were either fungistatic or fungicidal, or both. Milsana stimulated the greatest increase in papillae deposition in 'SM' and 'SK' tissues, while bicarbonate promoted numbers in 'Leb', although this latter increase did not appear related to the level of protection afforded by this material.

Little is known about the effect of chemicals and plant extracts on the production of physical barriers in hosts as a response to pathogen challenge. Results of this present study suggested that Milsana pre-treatment enhanced the resistance response of more disease tolerant cultivars which resulted in increased production of papillae and improved control of disease. Twenty-four to 72 h post inoculation,

epidermal cells of water-treated 'SM' and 'SK' associated with conidia and later haustoria, were observed by light microscopy to be heavily stained, some contained globular bodies in the cytoplasm, and others appeared to be in a state of collapse. Such hypersensitive resistance reactions have been associated with partial and race-specific resistances (Lebeda & Reinink 1994).

The number of haustoria produced in epidermal cells significantly decreased with increasing resistance of the cultivars to *S. fuliginea*, and appeared related to disease severity observed. Less frequent formation of haustoria has been correlated with resistance of several plant species to biotrophic infections (Riggle 1977; Menzies et al. 1991; Clark & Spencer-Phillips 1994; Sharada et al. 1995). The most effective treatments which reduced haustoria production were shellspray oil alone and in combination with sodium bicarbonate, followed by bicarbonate-plus-sunlight liquid. This was universal across cultivars. That fewer haustoria developed in the presence of these materials after 120 h, and that the severity of disease was least in their presence, suggested that they were active against the fungus early in the infection process. By themselves, sodium bicarbonate and sunlight liquid were less effective across cultivars which suggested that there was some degree of synergism between these materials.

Although bicarbonate/additive mixtures did not stimulate any apparent production of phenolics, or host defences, the disease controlling performance of these materials in glasshouses lends further support to sodium bicarbonate/additive mixtures being worthy of inclusion into integrated disease management programmes.

Future work on these materials could include:

1. Testing of sodium bicarbonate/surfactant mixtures as eradicants of cucumber powdery mildew.
2. Testing the eradicant activity of standard fungicides in combination with these additives.

It is important that modern disease control incorporates a range of materials in order to reduce the frequency of, and thereby use of, applications of conventional fungicides.

3. Testing of sodium bicarbonate/surfactant mixtures as protectants and eradicants of other important cucurbit diseases such as anthracnose (*Colletotrichum lagenarium*) and downy mildew (*Pseudoperonospora cubensis* Rostov.), and diseases of other crops.
4. Testing of sodium bicarbonate/surfactant mixtures in full-scale field plot trials under commercial conditions. It is likely that the concentrations of materials used in this investigation would need to be increased in trials such as these, to account for weather dilution of activity, and the larger volume of plant material to be covered.

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APPENDIX 1

1.1 COMPOSITION OF BARK-BASED GROWING MEDIA

1.1.1 Seed-Raising Mix

Fertiliser components	Rate (g/100 litre)
Dolomite	300 g
Agricultural lime	300 g
PGU (multi-nutrient)	60 g
Bark	100 litre

1.1.1 Media For Transplanted Seedlings

Fertiliser components	Rate (g/100 litre)
Dolomite	300 g
Agricultural lime	300 g
Osmocote 3-4months (short-term mix)	400 g
Lime sulphate	50 g
Bark	100 litre

SUNLIGHT LIQUID (Levers Rexona, before)

Sunlight Liquid is a hand dishwashing liquid.

COMPOSITION

Sunlight contains a blend of anionic surfactants which wet and emulsify greasy soils in order to clean cutlery, glasses, crockery etc. and provide a lather through the washing process.

Alcohol and urea are used to stabilise the product, keep the detergents in solution and adjust the viscosity of the neat product.

A perfume and a colourant are included.

PRESENTATION

Sunlight Liquid is a thick yellow-green liquid packed in 1 litre and 500ml cylindrical plastic squeeze bottles. Volumes are declared on all packs.

USES

The product is designed to be used for hand dishwashing. It can also be used for any task where a high foaming liquid detergent is suitable.

CARE IN USE

For best results, dishwashing liquid should be rinsed off with clean water before crockery, etc is dried or left to drain. Accidental ingestion should be treated by drinking plenty of milk or water and entry into the eye by bathing with water. After treatment, medical attention should be sought.

HISTORY

Sunlight Liquid was introduced in 1972 and continuously developed over the years.

New Product

SHELLSPRAY All Purpose Spraying Oil

PRODUCT BRIEF

A highly refined narrow distillation range spray oil. Enhances crop safety by the removal of undesirable oil fractions. To control scale, mites, aphids, mealy bug and thrips. For use on citrus, grapes, pipfruit and stonefruit.

Chemical group: Mineral oil.

Active ingredient: Shellspray contains not less than 970 ml/litre of mineral oil in the form of an emulsifiable concentrate and has a minimum unsulfonated residue content of 92%. Contains emulsifiers to facilitate mixing with water.

Maximum transport quantity: No limit.

DGD category: Not applicable.

Mode of action: Exhibits a physical action on mites, scale and aphids by coating their exterior with oil and thereby interfering with their metabolic processes.

Toxicity: Low oral and dermal toxicity.

PRECAUTIONS

User: Avoid prolonged skin exposure.

Contact re-entry: When fully dry.

Non-contact re-entry: When fully dry.

Crops: Do not use Shellspray in temperatures above 32°C or when high winds, showery weather or drought conditions prevail. Do not apply to russet prone apple varieties eg Gala, after tight cluster. Do not apply to citrus trees in hot, dry weather when trees are likely to be suffering from a lack of soil moisture. Lemons in particular may under certain conditions result in leaf and fruit drop — ensure conditions are suitable before applying. Also see compatibility below.

RECOMMENDED USES

Apples, Pears (including nashi) Stonefruit: *San Jose scale, European red mite and aphids*

Green tip — tight cluster: Use 2 litres/100 litres of water and apply to ensure complete coverage. For severe insect populations, add a suitable organo-phosphate insecticide.

Summer application: Use 1 litre/100 litres of water and apply to ensure complete coverage. Do not apply on russet prone apple varieties eg Gala, from tight cluster to third cover. Where insect populations assume severe proportions add a suitable organo-phosphate insecticide.

Citrus: *Scale, aphids, citrus red mite, mealy bug and thrips*

Use 1 litre/100 litres of water and apply to achieve complete coverage. Where insect populations are high or where mealy bug is a problem add a suitable organo-phosphate insecticide.

Grapes: *Mealy bug and mites*

Use 1 litre/100 litres of water. Where mites or mealy bug populations are high add a suitable organo-phosphate insecticide.

Wetting agent: Shellspray is itself an effective wetter/spreader. Additional surfactant is not necessary. Shellspray may be used as an adjuvant at 0.5-1% with insecticides and fungicides on vegetable and potato crops, but do not apply if plants are suffering from moisture stress.

Rainfall: Do not apply if rain is likely to fall within 3 hours after treatment.

Compatibility: May be tank mixed with many currently used insecticides. Do not use within 7-10 days either side of a sulphur application, nor mix with any sulphur spray, and do not use within 14 days either side of a captan application. Oil sprays are not compatible with captan, Phaltan, Karathane, carbaryl or dicloran. Oils are compatible with hard and soft water and in tank combination with Bordeaux mixture.

Application: Apply as a high volume spray to achieve complete coverage of all leaf and stem surfaces.

Pack sizes: 5, 20 and 205 litres.

Distributed by: Shell Chemicals New Zealand Limited.

SHIRLAN® Fungicide

PRODUCT BRIEF

A protectant fungicide for the control of botrytis and downy mildew in grapes. Recommended for use over the flowering period only.

Chemical group: Pyridinamine.

Active ingredient: Shirlan contains 500 g/litre fluzinam in the form of a suspension concentrate.

Maximum transport quantity: 200 litres.

DGD category: 9 3082 2X III.

Mode of action: A protectant fungicide which prevents disease establishment.

Toxicity: Low toxicity. Acute oral LD50 > 5000 mg/kg rats. Acute dermal LD50 > 2000 mg/kg rats.

Warning: May cause skin reaction in sensitive individuals.

PRECAUTIONS

User: Avoid contact with skin or inhalation of spray mist.

Contact re-entry: When fully dry.

Non-contact re-entry: When fully dry.

RECOMMENDED USES

Grapes: *Botrytis, downy mildew*

Use 100 ml/100 litres (or not less than 1 litre/ha). Apply at early and late blossom and again just prior to bunch closure. Do not apply after bunch closure. Use alternative fungicides for the rest of the season. Add 'Contact' surfactant at a rate of 25 ml/100 litres. The rates expressed are for high volume spraying to run off. For concentrate spraying adjust dilution rate accordingly.

Withholding period: 56 days.

Rainfall: Do not apply if rain is likely to fall within 6 hours following treatment. If heavy rain falls soon after treatment and disease pressure remains high, re-treatment will be necessary.

Compatibility: May be tank mixed with commonly used fungicides and insecticides. Do not mix with alkaline materials such as Bordeaux mixture or lime sulphur.

Application: Use conventional spray equipment ensuring thorough spray coverage of the crop. Dilution rates given are for high volume spraying to run-off and should be adjusted accordingly for concentrate spraying.

Pack size: 1 litre.

Disposal: Refer to the local regional authority. Triple rinse the empty container and add residue to spray tank, then burn container if circumstances, especially wind direction, permit. Otherwise bury in landfill.

Distributed by: ICI Crop Care.

Shirlan® is a Trademark of Imperial Chemical Industries PLC. England.

SIMAZINE Herbicide

Trade Names: CHEMAGRO SIMAZINE 500 FL, FLOWABLE SIMAZINE, GESATOP® 90 WG, GESATOP® 500 FW, SIMATOX™ 900 WG

PRODUCT BRIEF

A selective pre-emergence herbicide for weed control in lucerne, orchards, vineyards, forestry and certain horticultural crops. It is very effective in preventing the emergence of a wide range of annual and perennial grass and broadleaf weeds. The soil residual life ranges