

ASSESSMENT OF THE RISK OF RESISTANCE TO
BENZAMIDE FUNGICIDES IN Phytophthora infestans

by

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ABSTRACT

The in vitro sensitivity of 43 isolates of Phytophthora megasperma, which had never been exposed to benzamide or acylalanine fungicides under field or glasshouse conditions, ranged from ED₅₀-values of 0.159µg-9.614µg R157001/ml, and 0.005µg-37.749µg metalaxyl/ml.

Forty of 101 isolates of Phytophthora infestans, collected worldwide, were resistant in vivo to metalaxyl, but no isolates were resistant to R157001. An additional 35 isolates, examined in vitro only, were also relatively sensitive to R157001.

Isolates of Phytophthora citricola were generally more resistant to the benzamides R157001 and R157846, and metalaxyl, than isolates of P. megasperma. The latter were more resistant to R157846 than isolates of P. infestans.

P. megasperma f.sp. medicaginis adapted readily to a high concentration (100µg/ml) of metalaxyl, but with difficulty to a low concentration (5µg/ml) of R157001. Adapted strains demonstrated reduced virulence to host plants, and metalaxyl-adapted strains grew slowly on unamended agar.

Despite many attempts, no benzamide-resistant mutants were obtained following ultra-violet irradiation and N-methyl-N'-nitro-N-nitrosoguanidine mutagenesis of sporangia of P. infestans. Under similar conditions, P. infestans grew readily at 100µg metalaxyl/ml.

Variants of P. megasperma f.sp. medicaginis resistant to high concentrations (20µg/ml or more) of metalaxyl, and which grew vigorously on unamended medium, were obtained following NTG-mutagenesis. No benzamide-resistant variants were isolated under similar conditions.

The rate of degradation of R157001 and R157846 in agar medium was more rapid than that of metalaxyl, and was increased, probably by a change to alkaline conditions, following the growth of P. megasperma.

Metalaxyl was fungistatic, and R157846 lethal, to the growth of P. infestans. Sporangia and zoospores of P. infestans and/or zoospores of P. megasperma f.sp. medicaginis germinated in at least 100µg metalaxyl/ml and 50µg R157001/ml, although subsequent hyphal extension was inhibited. 10µg mancozeb/ml prevented the germination of sporangia of P. infestans.

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INTRODUCTION

Fungicides play a major role in the control of crop diseases, particularly in the developed countries. In the last 20 years several new classes of fungicides have been introduced, most of which are site-specific in their mechanism of action. Unfortunately, many of these no longer provide the disease control originally observed because the pathogens have developed resistance* to the chemicals.

One of the most rapid and devastating examples of resistance followed the introduction of the acylalanine fungicide, metalaxyl, used to control diseases caused by members of the fungal order Peronosporales. Metalaxyl-resistant strains of target fungi arose within 2 years of commercial use of the fungicide.

Preliminary studies undertaken by the manufacturer of metalaxyl suggested that fungicide resistance was unlikely to pose a threat to the compound. Later studies established that there was a risk, but unfortunately were published when the fungicide was already in widespread commercial use.

In spite of the difficulties, it is widely recognised that agrochemical companies are probably in the best position to assess the likelihood of resistance arising to their own novel fungicides, mainly because such studies can be undertaken before a new compound is introduced into widespread use.

* The Food and Agriculture Organisation Panel of Experts on Pest Resistance to Pesticides (1979) decided that the term "resistance" should be applied to genetically-based heritable resistance in fungi or bacteria. It should not be used to describe the low susceptibility either of certain species or individuals that were temporarily influenced by the environment. The Panel also advised that the word "tolerance" should not be used since it is ambiguous. These recommendations were followed in the preparation of this thesis, to the extent that "resistance" replaced terms such as "tolerance" when these were cited in source references.

Since 1980, ICI has expended considerable effort towards the development of a new class of fungicides, specifically active against members of the Peronosporales, known as the benzamides. The problems associated with previous cases of fungicide resistance, and acylalanine resistance in particular, prompted the company to instigate a resistance-risk assessment with the new chemicals at an early stage in their development. The present study was the outcome of that decision.

1 LITERATURE REVIEW

1.1 Chemical Control of Oomycetes

The importance of pests in reducing crop yields is well recognised, and although estimates are understandably lacking in detail, losses worldwide have been calculated at 25-45% of production (Dekker, 1982a). Pesticides play a major role in plant protection in the developed countries, where growers are more readily able to finance their use than growers in developing countries. It has been argued that chemicals are the most effective means at present for the control of fungal plant diseases (Schwinn, 1982).

Table 1 was ICI's (1984) estimate of the 1980 world fungicide market, by major disease groups. This reveals that chemicals were generally used on high value crops and against a relatively small proportion of plant pathogens. As might be expected, fungicides are used especially on crops produced in environments conducive to the pathogen (Fry, 1977). Chemical control of Oomycete plant pathogens represents the largest sector of the fungicides market (Table 2). Within this category, vine downy mildew caused by P.viticola and potato late blight caused by P.infestans account for 57% and 20% respectively of sales to users. The value of chemical control of these diseases will be demonstrated by reference to their economic importance, biology and epidemiology.

1.2 Vine Downy Mildew and Potato Late Blight

1.2.1 Vine Downy Mildew (Plasmopora viticola)

Grape vines are grown throughout the world, primarily in Europe (7.5 million ha), followed by Asia (1.5 million ha), the

Table 1 World fungicide market in relation to major diseases, 1980^a

Disease group	Sales to users (£ million)
Oomycete diseases (see also Table 2)	269.8
Cereal diseases (includes rusts, mildew, foot diseases, <u>Rhynchosporium</u> , <u>Septoria</u>).	193.2
Diseases caused by <u>Botrytis</u> / <u>Sclerotinia</u> / <u>Alternaria</u> (of vines, tomatoes, potatoes, top fruit, vegetables, turf and ornamentals).	159.2
Non-cereal powdery mildew (of vines, top fruit, vegetables, tobacco, hops, sugar beet, ornamentals).	120.4
Rice diseases (rice blast and various seed-borne pathogens).	130.6
Non-cereal rusts and Basidiomycetes (coffee rust, rice sheath blight, apple and pear rust, tea blister blight, and diseases of vegetables, turf, ornamentals).	55.0
Diseases caused by <u>Cercospora</u> (coffee, sugar beet and peanut leaf spots, banana sigatoka).	50.1
Bacterial diseases (rice leaf blight, citrus canker, pear fire blight, diseases of vegetables).	8.7
Miscellaneous diseases (apple/pear scab, soybean complex, citrus post-harvest/ melanose/scab/greasy spot, and diseases of vines, rubber, tobacco, etc).	182.9
TOTAL	1,169.9

^a Source : ICI Plant Protection Division (1984)

Table 2 World fungicide market for Oomycete diseases, 1980^a

Disease	Pathogen	Sales to users (£ million)
Vine downy mildew	<u>Plasmopora viticola</u> (Berk. & Curt.) Berlese & de Toni	154.0
Potato late blight	<u>Phytophthora infestans</u> (Mont.) de Bary	53.7
Tomato Late Blight	<u>P. infestans</u>	19.0
Vegetables	Various including <u>Bremia lactucae</u> Regel and <u>Peronospora parasitica</u> (Pers. ex Fr.) Fr.	17.0
Turf/ornamentals/ domestic	Various including <u>Phytophthora cinnamomi</u> Rands and <u>Pythium ultimum</u> Trow	10.0
Tobacco blue mould	<u>Peronospora tabacina</u> Adam	7.0
Cocoa black pod	<u>Phytophthora palmivora</u> (Butler) Butler	5.0
Hop downy mildew	<u>Pseudoperonospora humuli</u> (Miy. & Tak.) Wilson	4.1
TOTAL		269.8

^a Source : ICI Plant Protection Division (1984)

Americas (0.9 million ha), Africa (0.5 million ha) and small areas of Australia (Weltzien, 1981). Vine downy mildew caused by P. viticola occurs in all these areas, attacking mainly the varieties of Vitis vinifera L. which constitute the majority of vineyards under cultivation (Lafon & Bulit, 1981). The history and economic importance of the disease was described by Viennot-Bourgin (1981). Since the introduction of the disease into Europe a century ago, France has suffered numerous 'mildew years' when prolonged periods of rain encouraged development of the disease. In 1930 and 1932 severe attacks of downy mildew in certain vineyards in the south of France reduced the harvest by one-third, and in 1957 the attacks were described as 'disastrous'.

In 1977, and despite the efficacy of the fungicides used, 12-15 treatments were required to reduce attacks, again in the south of France.

A description of vine downy mildew, its biology and epidemiology, was provided by Lafon & Bulit (1981), and is summarised here.

All the green parts of the vine, the leaves and tendrils, shoots, inflorescences and bunches, may be attacked by P. viticola. Invasion of the tissues results in yellowing and browning of affected organs, malformations and necroses. Under wet conditions white sporangiophores cover those infected parts bearing stomata. Sporangial liberation occurs in moist air, following the dissolution of a cross-wall of callus which connects the sporangia with the sporangiophores. Subsequent transport is ensured by the wind and sporangia remain viable in dry air for up to 5 days. Each sporangium gives rise to between one and six motile zoospores which are released exclusively in water. After 20 - 30 min, the zoospores lose their flagella and encyst. Under conditions of

persistent moisture a germ tube is produced which penetrates the host almost exclusively via the stomata. The fungus grows intercellularly through the tissue and visual lesions become apparent over a 5 - 18 day period.

P. viticola usually overwinters in the form of oospores in fallen leaves. In spring, as soon as the temperature reaches 11°C, the oospores germinate in water. Each produces a single terminal sporangium from which zoospores are released. These zoospores are rain-splashed onto nearby vine leaves and the infection cycle is repeated.

Vine downy mildew is favoured by those factors which increase the moisture content of soil, air and plant. Rain is a major contributing factor to epidemics. It stimulates the production of young, susceptible shoots, and provides the wet conditions under which infection can then occur. Temperature plays a less important role, by regulating the speed of development of the disease.

1.2.2 Potato Late Blight (Phytophthora infestans)

The potato is the most important dicotyledenous source of human food (Hooker, 1981). It ranks as the fifth major food crop of the world, exceeded only by the cereals (wheat, rice, maize and barley). The percentage of arable land in potato production ranges from less than 1% in Canada and the United States, to 18% or more in the Netherlands and Poland (van der Zaag, 1976).

Late blight caused by P. infestans is probably the single most important disease of potatoes worldwide (Thurston & Schultz, 1981). It is destructive wherever potatoes are grown, except in hot, dry, irrigated areas. Many of the potato late blight epidemics throughout the

world were described by Cox & Large (1960). The best known example of the damage wrought by the disease was the Irish potato famine of the 1840's, a graphic account of which is given by Large (1940). Potato late blight first appeared in Ireland in 1845, and destroyed the potato crop in this and subsequent years, causing much human suffering. Between 1845 and 1860 one million people died and one and a half million emigrated, out of a total population of eight million, as a direct consequence of the famine.

A useful description of potato late blight and the biology and epidemiology of P. infestans was provided by Thurston & Schultz (1981). This, and an account of the disease under field conditions (Anonymous, 1980), are summarised here.

Late blight is first apparent on potato foliage as dark green spots which under favourable conditions may rapidly enlarge to necrotic lesions. Once the infection is widespread, and there are approximately ten blight spots per plant, the disease may progress rapidly, and kill off most of the foliage within a fortnight.

Under moist conditions sporangiophores appear at the edge of lesions. Sporangia are dispersed by wind or rain-splash and germinate directly by means of a germ tube, or, more commonly, they release zoospores. These encyst, germinate in water, and usually enter the host by penetration of the cuticle. Mycelia ramify intercellularly throughout the affected tissue and haustoria are produced which develop intracellularly.

Potato tubers can become infected by spores washed into the soil, or at lifting. These may subsequently rot in storage, but blight does not spread between tubers. More importantly, infected seed tubers may be a source of inoculum the following season.

Until recently, sexual reproduction was only known in Mexico where both mating types (A1 and A2) occurred with equal frequency

(Gallegly & Galindo, 1958). Isolates from elsewhere were of the A1 mating type (Smoot et al., 1958). However, the A2 mating type has now been recorded from Switzerland (Hohl & Iselin, 1984) and Great Britain (Malcolmson, 1985; Shaw et al., 1985). Sexual reproduction involves the formation of oospores by the union of oogonia and antheridia (Thurston & Schultz, 1981). Oospores germinate to produce a germ tube with a terminal sporangium. Either zoospores are liberated from the sporangium, or it forms another germ tube. Leaves touching the soil are often infected first, indicating that oospores play a role in the survival of P. infestans under adverse conditions.

Like vine downy mildew, potato late blight is very dependent on climatic conditions. In a dry season it causes little damage, but under wet conditions premature haulm destruction can cause losses of as much as half of the potential crop (Anonymous, 1980).

1.3 Disease Control

Cultural practices, the use of resistant varieties and the application of chemicals, each play a role in the control of vine downy mildew (Palti & Rotem, 1981) and potato late blight (Shea & Broadbent, 1983). Successful biological control of the causal organisms of both diseases has not been demonstrated.

1.3.1 Cultural Practices

Cultural control of crop diseases involves sanitation practices and manipulation of the environment to the advantage of the host and the detriment of the pathogen. For vine downy mildew, Palti & Rotem (1981) recommended ploughing to bury as deep as possible, vine leaves carrying oospores of P. viticola. Dumps of unwanted potatoes are an important source of P. infestans, and can be destroyed by levelling,

covering with a shallow layer of soil and applying a suitable herbicide treatment (Anonymous, 1980). Additionally, delaying potato lifting until the foliage is mechanically or chemically killed reduces the incidence of tuber infection by late blight (Thurstun & Schultz, 1981).

When periods of plant wetness are limited, moisture management, especially the use of irrigation, probably affects the development of vine downy mildew more than any other cultural practice (Lafon & Bulit, 1981). The same workers recommended that the choice of irrigation technique and optimal timing of water application should ensure an adequate supply of water to the crop, without unduly favouring the pathogen.

Shea & Broadbent (1983) remarked that timing of the sowing date of potatoes enables farmers to take advantage of the seasonal variation in inoculum levels of P. infestans, as well as variations in host susceptibility, throughout the season. Furthermore, it is advised (Anonymous, 1980) that potatoes should not be grown for successive seasons in the same ground. This avoids the potential carry-over of late blight from year to year in infected tubers which remain in the soil.

1.3.2 Varietal Resistance

A comprehensive review of breeding for resistance to potato late blight, by Umaerus et al. (1983) is summarised here.

The serious epidemics of potato late blight in Europe between 1845-6 soon initiated programmes of breeding for resistance to P. infestans. First attempts sought what is now recognised as general resistance, which is polygenically inherited. The Mexican species Solanum demissum L., used as early as 1849, has been the most important source of resistance of all the wild species of Solanum tested.

In crosses between S. demissum and Solanum tuberosum L. it

was found that the former could confer resistance to P. infestans that was simply inherited and easy to select for in breeding programmes. Breeding for general resistance was then generally discontinued. After the introduction of varieties with what is termed specific resistance, virulent races of P. infestans were identified which overcame this resistance. Specific resistance proved to be effective against one or more races of the pathogen depending on the number of genes for resistance which were introduced into the host. However, it was of no use against other races for which the host lacked the corresponding gene for resistance. When race-specific resistance proved unreliable, emphasis was again placed on breeding for general resistance. The assumption was that the more genes that govern resistance, the more long-lasting it will be. Breeding for general resistance necessitated the screening of a much larger number of seedlings for a greater number of traits, than breeding for specific resistance. As a result, and as relatively inexpensive and reliable chemical treatments became available, some programmes gave up screening for resistance to late blight altogether.

Over the years, breeding for resistance to vine downy mildew has involved intercrossing American and European vines followed by continuous back-crossing to European varieties to transfer high resistance from the American species (Matthews, 1981). Complete immunity is rare in the Vitaceae. Resistance commonly takes the forms of prevention of germ tube penetration, hypersensitive reactions, and restriction of mycelial growth in the host.

1.3.3 Chemical Control

Chemical treatments currently available for the control of

vine downy mildew and potato late blight are commonly divided into two categories : the residual fungicides and the systemic fungicides.

a) Residual fungicides

Until the last 10 years or so, chemical control of vine downy mildew (Schwinn, 1981) and potato late blight (Staub & Hubele, 1980) relied on the use of residual fungicides. Examples of this type of chemical include the dithiocarbamates such as thiram, maneb, mancozeb; copper compounds such as Bordeaux mixture; triphenyl tin compounds such as fentin hydroxide and fentin acetate; phthalimides such as folpet, captafol and dichlofluanid and phthalonitriles such as chlorothalonil. All are broad spectrum fungitoxicants which lack the ability to penetrate plant surfaces in effective concentrations, and so cannot reach the target pathogen once it has infected the host tissue. Additionally, they are subject to weathering and require frequent application.

In spite of these shortcomings, the residual fungicides still dominate the market for Oomycete fungicides (Staub & Hubele, 1980).

b) Systemic fungicides

Systemic fungicides for the control of vine downy mildew and potato late blight have been introduced within the past decade, and are considered to be technically superior to the residual fungicides for several reasons (Staub & Hubele, 1980). First, with their curative properties the systemic fungicides can inhibit the development of the target fungus within the host tissue, whereas the residual fungicides would be completely ineffective. Second, it may no longer be necessary to apply a continuous fungicide film over the plant parts to be protected, since the systemic products are distributed in a fairly predictable way throughout the plant. Third, the suitability of the systemic fungicides for use in connection with disease warning systems, together with low use rates and extended spray intervals, offer greater

possibilities for disease control strategies than the residual fungicides. Additionally, the hazards due to ecological and human exposure are lower. In practice, and for reasons given below, systemic and residual fungicides are frequently combined in commercial mixtures.

Cyanoacetamide - oxime (Cymoxanil), manufactured by Du Pont, acts selectively against members of the Peronosporales, and is recommended for the control of vine downy mildew and potato late blight (Serres & Carraro, 1976). The chemical has both contact and local systemic activity, is strongly curative for 3 - 4 days after infection occurs, but weakly persistent. For this reason it is usually used in mixtures with residual fungicides such as copper, folpet or mancozeb (Klopping & Delp, 1980).

Ethyl phosphites were discovered by Rhône-Poulenc (Williams & Beach, 1977). The most widely used compound within this group of fungicides, phosethyl-Al, is highly toxic to most species in the Peronosporales, including P. viticola. It is however, relatively inactive against P. infestans (Schwinn, 1983).

Early studies proposed that the spectrum of activity of phosethyl-Al was governed in part by the host plant, or by the interaction between the host plant and the chemical (Vo et al., 1979; Raynal et al., 1980). More recently, it has been demonstrated that the postulation of a unique control mechanism by the chemical, other than direct inhibition of growth and sporulation, was not necessary (Fenn & Coffey, 1984 & 1985; Rohrbach & Schenck, 1985).

Phosethyl-Al is readily translocated throughout the plant to new growth, but since this is usually at the expense of the concentration in older sprayed parts, the chemical is recommended in mixtures with residual fungicides (Marais & van der Walt, 1978).

Acylalanines were first discovered by Ciba-Geigy (Urech et al., 1977). They have strong protective and curative activity,

and are taken up readily by the plant and translocated through the apoplastic system. The most effective molecule is metalaxyl (Schwinn, 1983). Other commercially available compounds in the group are furalaxyl, ofurace* (formerly known as milfuram) (Lukens et al., 1978), and benalaxyl (Bergamaschi et al., 1981).

Interference with RNA synthesis was proposed as the mechanism of action of acylalanine fungicides in several studies. A comprehensive review of this subject was provided by Davidse (1984).

Schwinn (1983) noted investigations into the effect of metalaxyl on different stages of the life-cycle of target pathogens. These revealed that the fungicide was effective against fungi within the host plant, and over a longer time-scale than residual fungicides. Nevertheless, to broaden their spectrum of activity and to reduce the risk of fungicide resistance, acylalanines are now used in mixtures with residual fungicides (Staub & Sozzi, 1984).

1.4 Fungicide Resistance

One of the problems with the use of fungicides is resistance to the toxicant in the target fungus.

1.4.1 Practical Experiences

In practice, resistance to the residual fungicides is rare, resistance to organomercurials in Pyrenophora avenae Ito & Kuribay being one of the exceptions (Greenaway & Cowan, 1970). In contrast, serious resistance problems have arisen since the advent 20 years ago of the

* Metalaxyl, furalaxyl and benalaxyl are acylalanine fungicides because their chemical structure incorporates an alanine methylester. In ofurace, this moiety is in the cyclic butyrolactone form. Therefore, this chemical is not an acylalanine. Nevertheless, the above fungicides display similar effects on the biology of target fungi. For the purposes of the present study, ofurace and related chemicals are also described as 'acylalanine fungicides'.

biochemically site-specific, and usually systemic, fungicides, and Dekker (1982a) noted some of the 'notorious cases' which have occurred. Perhaps the best known of these is resistance to the benzimidazoles which are normally effective against a wide taxonomic range of plant pathogens. Unfortunately, Georgopoulos (1977a) considered it easier to name those fungi in which resistance was not known. Both Dekker (1976) and Georgopoulos (1977b) provided lists of references to reports of benzimidazole resistance. Dekker (1982b) concluded that the phenomenon was usually stable (i.e. remained unchanged in the absence of the fungicide) and resistant strains were frequently as fit (competitive) as sensitive wild strains.

The dicarboximide fungicides have been widely used to control plant diseases caused by taxonomically related pathogens within the genera Botrytis, Monilinia and Sclerotinia. Laboratory studies indicated that resistant strains may be as fit as wild strains, and after many years of use, loss of disease control by these fungicides occurred in the field (Leroux & Fritz, 1984).

Ergosterol biosynthesis inhibitors (EBI's) comprise a heterogeneous group of broad spectrum fungicides toxic to representatives of the Ascomycetes, Deuteromycetes and Basidiomycetes (Fuchs & de Waard, 1982). Surveys during several years in the United Kingdom have revealed a gradual decrease in sensitivity to EBI's in the barley powdery mildew (causal organism Erysiphe graminis D.C. ex. Mérat f.sp. hordei) population (Wolfe et al., 1984; Heaney et al., 1984). Despite the lack of a statistically significant correlation with poor disease control (Heaney et al., 1984), the trends observed emphasised the need for care in the use of EBI's to reduce unnecessary selection for resistance (Wolfe et al., 1984). In cucurbits, intensive use of EBI's led to a clear loss of disease control due to resistant strains of Sphaerotheca fuliginea Schlecht. (ex.Fr.) Poll (Schepers, 1983).

Resistance to the acylalanine fungicides has been comprehensively reviewed by Davidse (1982). First reports described loss of disease control due to resistance in Pseudoperonospora cubensis (Berk. & Curt.) Rostow. on cucumbers during the 1979-80 growing season in Southern Greece and Crete (Georgopoulos & Grigoriu, 1981), and in Israel (Reuveni et al., 1980; Katan & Bashi, 1981). Soon afterwards, during 1980, resistance in P. infestans from potatoes was detected in The Netherlands (Davidse et al., 1981) and Eire (Dowley & O'Sullivan, 1981).

There are now numerous accounts of acylalanine resistance in the field and glasshouse. Table 3 lists many of the relevant references. Although this list is extensive, it was recognised that other cases of acylalanine resistance could always occur (Staub & Sozzi, 1981). These authors indicated that in all cases, significant loss of disease control due to resistance had been associated with the repeated and exclusive use of metalaxyl alone under environmental conditions highly favourable to the pathogen. The use of mixtures of acylalanines with residual fungicides in geographically separate but epidemiologically comparable locations did not lead to resistance problems. Thus, in the United Kingdom, where during 1980 a mixture of metalaxyl and mancozeb was used to control potato late blight, no cases of resistance were reported (Carter et al., 1982). However, in the same year in The Netherlands, where metalaxyl was used exclusively, serious cases of resistance occurred (Davidse et al., 1981).

Other important examples of fungicide resistance were presented as case studies in the book, 'Fungicide Resistance in Crop Protection'. They were Cercospora beticola Sacc. of sugar beet, (Georgopoulos, 1982a) Venturia of pome fruits and Monilinia of stone fruits (Gilpatrick, 1982), Pyricularia oryzae Cav. of rice (Uesugi, 1982), powdery mildews of barley and cucumber (Brent, 1982), and Penicillium decay of citrus fruits (Eckert, 1982).

Table 3 Reports of resistance to acylalanines under field and glasshouse conditions

Pathogen	Crop	Country	Reference
<u>P. tabacina</u>	Tobacco	Nicaragua Cuba	Staub & Sozzi (1981) S P Heaney (1985, personal communication)
<u>Phytophthora capsici</u> L.	Sweet pepper	Brazil	Reifschneider (1982)
<u>P. infestans</u>	Potato	Netherlands Eire Northern Ireland South America (several countries) England New Zealand Israel Scotland	Davidse <u>et al.</u> (1981, 1983) Dowley & O'Sullivan (1981) Cooke (1981) Staub & Sozzi (1981) Carter <u>et al.</u> (1982) Hartill <u>et al.</u> (1983) Cohen & Reuveni (1983) Holmes & Channon (1984)
	Tomato	Greece	Pappas (1985)
<u>P. viticola</u>	Vine	South Africa France	Staub & Sozzi (1981) Moreau <u>et al.</u> (1983)
<u>P. cubensis</u>	Cucurbits	Israel Greece & Crete Japan	Reuveni <u>et al.</u> (1980) Katan & Bashi (1981) Georgopoulos & Grigoriu (1981) Staub & Sozzi (1981)
<u>Pythium aphanidermatum</u> (Edson) Fitzp.		USA	Sanders (1984)

1.4.2 Genetical and Biochemical Background

Some of the biochemical mechanisms by which resistance to a fungicide could develop were described by Dekker (1984) and Georgopoulos (1982b). These included modification of the biochemical site at which the fungicide exerts its toxic effect, bypass of the site due to the operation of an alternative biochemical pathway, decreased uptake or increased secretion of the toxicant through the cell membrane, detoxification of the fungicide, and decreased conversion of an initially non-toxic compound into a toxic compound within the fungus. Georgopoulos (1982b) considered the first two mechanisms to be important in the development of resistance to the site-specific, usually systemic fungicides which affect a limited number of biochemical processes. Additionally, this author related the remaining three mechanisms to site-specific and multisite residual fungicides. Since all of these mechanisms are under genetic control, the effectiveness of chemicals may be reduced or completely lost because of mutations in target fungi (Georgopoulos, 1977a).

In contrast to the site-specific fungicides, where a single mutation at a target site may confer resistance to a toxicant, mutations at all sites involved leading to resistance to multisite fungicides was considered to be unlikely or impossible (Dekker, 1976). The same author noted that significant increases in resistance due to changes in membrane permeability or detoxification of the fungicide seemed to occur infrequently.

1.4.3 Predicting Resistance to Fungicides

Resistance to a once effective fungicide may only be detected following a failure in disease control under field and glasshouse conditions (Dekker, 1982b). By then, crop losses have usually occurred and anti-resistance strategies are more difficult to implement.

Dekker (1982c) considered the search for new fungicides with low

risk for development of resistance an important aim, but as a corollary stated that "the widespread use of a new fungicide, which has not been tested with respect to the possibility of development of fungicide resistance should be discouraged" (Dekker, 1976).

By reference to previous experiences, Dekker (1982b) showed that the artificial selection of resistant mutants could be of value in predicting the risk of resistance arising in practice. The same author considered that when it appears impossible to obtain resistance in vitro, even using mutagens, development of resistance in the field is unlikely. If resistant mutants are obtained, investigation of their fitness relative to wild strains may provide useful information about the characteristics of similar resistant strains which could occur naturally.

Monitoring of the pathogen population should be started before the introduction of a new type of fungicide in order to detect naturally occurring resistant strains (Staub & Sozzi, 1984), and to establish 'baseline sensitivity data' i.e. the range of sensitivity demonstrated by isolates of a pathogen never previously exposed to the new fungicide (Wade, 1982; Staub & Sozzi, 1983 & 1984). This enables subsequent changes in the sensitivity of fungal strains to be detected early, and appropriate anti-resistance strategies to be implemented.

In practice, extrapolation from laboratory and glasshouse studies to practical situations can be subject to many uncertainties (Staub & Sozzi, 1984). For example, carboxin is used as a seed treatment against smut in cereals. Resistant mutants were easily obtained in vitro, but after many years of use there were no reports of carboxin resistance under field conditions (Dekker, 1982 b). Conversely, significant loss of disease control due to resistance has occurred to some fungicides originally thought not to be at risk. For example, one of the first attempts to predict the likelihood of resistance arising in a target pathogen to the acylalanines was described by Staub et al. (1979). This relied on the

selection of resistant mutants of P. infestans without the use of mutagenic techniques. The failure to obtain isolates which were resistant in vivo gave the impression that acylalanine resistance would not be a problem (Davidse, 1982). Subsequent studies with other members of the Peronosporales, some of which employed mutagens, were more successful. Thus, metalaxyl-resistant mutants which were as fit as wild strains were obtained using adaptation procedures (which involved the transfer of propagules to increasing doses of chemical) (Bruin & Edgington, 1981); ultra-violet (UV) irradiation (Bruin & Edgington, 1982) and N-methyl-N'-nitro-N-nitrosoguanidine (NTG) mutagenesis (Davidse & Krüse, 1981; Davidse, 1981). These investigations also revealed that resistance to metalaxyl could confer resistance to structurally related compounds such as furalaxyl, ofurace and benalaxyl. This is known as cross-resistance, and is due to a change in one genetic factor conferring resistance to different fungicides. Unfortunately, these latter studies were published at about the same time as serious cases of metalaxyl resistance were already occurring under field and glasshouse conditons.

Despite the difficulties, Wade (1982) argued that agrochemical companies needed to increase their efforts in assessing the risks of resistance developing to a new fungicide before its introduction.

1.5 Benzamide Fungicides

The term 'benzamide' is used to describe a group of chemicals discovered through speculative syntheses by chemists in ICI.

The compounds are protected in the major territories of the world by patents. British patents pertaining to the individual compounds described in this thesis, GB 2 095 237 A, GB 2 094 786 A and GB 2 107 308 A, were filed in the United Kingdom patent office in February and September

1982, and April 1983 respectively, with I T Kay, D Bartholomew, E G Williams and R A Noon as inventors.

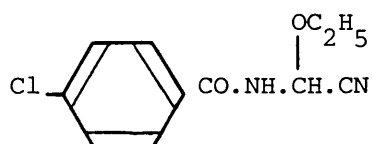
Early benzamides demonstrated herbicidal activity, and some were also highly fungicidal, primarily against plant pathogens within the fungal order Peronosporales.

Three main series of compounds evolved from the preliminary chemical syntheses and glasshouse screening, namely the 'ethers', the 'furans' and the 'pyrazoles' (for examples, see Figure 1). Further experimentation revealed that compounds with a single 4-substituent in the benzene ring were less phytotoxic. As a result, following their selection from glasshouse tests, in 1982 the activity of several leading benzamides was examined under field conditions. The ether, R157001, exerted particularly good control of P. viticola on vines in France that year.

Field trials continued in subsequent years with R157001 and other leading analogues, primarily against vine downy mildew and potato late blight. In some situations the use of mixtures of benzamides with other fungicides resulted in superior disease control compared to the application of the individual components. This was a consequence of combining the systemic properties of the benzamides with the better eradicant and protectant properties of some companion fungicides.

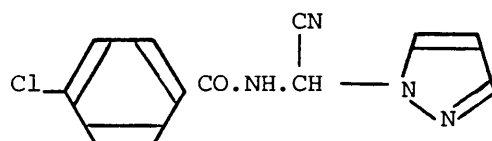
Figure 1 Chemical structures of some benzamide fungicides

a) R157001 (Ether)



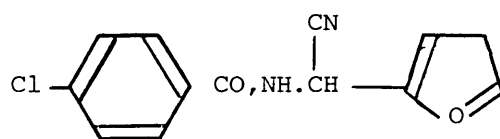
2-(4-chlorobenzoylamino)-2-ethoxyacetonitrile

b) R157846 (Pyrazole)



2-(4-chlorobenzoylamino)-2-(1-pyrazolyl)-acetonitrile

c) R153588 (Furan)



2-(4-chlorobenzoylamino)-2-(2-furyl)-acetonitrile

EXPERIMENTAL APPROACH

P. viticola is the single most important plant pathogen against which the benzamides would be used commercially. Consequently, it was the preferred subject in the present study of the likelihood of resistance developing to the new type of fungicide. However, P. viticola is an obligate parasite, and as such, was not considered as amenable to a resistance-risk-assessment as fungi which could be more readily handled in vitro. Also, there is an absence in the literature of reports of fungicide resistant mutants of P. viticola obtained through artificial selection procedures. In contrast, in vitro studies with Phytophthora spp. have yielded useful data on the risk of resistance developing to acylalanine fungicides (Bruin & Edgington, 1981 & 1982; Davidse, 1981; Joseph & Coffey, 1984). Because P. infestans was the second major target of the benzamide fungicides, and amenable to in vitro studies, it was chosen as the main subject of the present study. Other species of Phytophthora were included in the investigation where they contributed to the overall assessment of the likelihood of resistance arising to the major benzamide targets, P. viticola and P. infestans.

Chapter Two of this thesis describes an in vitro attempt to obtain benzamide and metalaxyl-resistant mutants of Phytophthora megasperma Drechsler f.sp. medicaginis without the use of mutagenic agents. It was based on the work of Davidse (1981), and involved the mass transfer of mycelium to increasing concentrations of fungicide in agar i.e. the procedure known as 'adaptation'.

An investigation of the natural sensitivity of populations of Phytophthora spp. to benzamide fungicides and metalaxyl is presented in Chapter Four. Besides establishing the range of sensitivity to benzamides in populations of target pathogens never previously exposed to these

chemicals, isolates which were inherently resistant to the benzamides were sought.

Chapter Six describes attempts to obtain resistant mutants of P. megasperma f.sp. medicaginis and P. infestans following UV irradiation or NTG mutagenesis. Both techniques were used with particular success by other workers to isolate mutants of various members of the Peronosporales which were highly resistant to metalaxyl.

In Chapters Three and Five, the chemical nature of the benzamides and their mode of action against Phytophthora spp. was examined. These aspects were believed to influence the ease and extent to which resistance might develop.

In Chapter Seven, the findings of the experimental work were used to predict the likelihood of resistance to benzamides occurring in target pathogens. Recommendations for the application of the fungicides under field and glasshouse conditions were also presented.

2 ADAPTATION OF PHYTOPHTHORA MEGASPERMA F. SP. MEDICAGINIS TO R157001 AND METALAXYL

2.1 General Introduction

The capability of a fungus to develop resistance to a fungicide can be tested in vitro using a variety of methods (see Dekker, 1982b). One method, sometimes called 'mass selection', is to transfer spores to an agar medium containing an amount of fungicide just greater than the minimum inhibitory concentration (MIC). Only those cells that have acquired resistance will survive and form a colony. By counting the number of colonies, the frequency of mutation to resistance can be determined. Another method, sometimes called 'adaptation', is to transfer mycelium to an agar plate containing a growth-inhibiting, but sublethal, concentration of the fungicide. Development of resistance in cells of the mycelium may result in more rapidly growing sectors in the colony. After isolation of resistant colonies and exposure of the spores or mycelium to a range of concentrations of the fungicide, the degree of resistance can be assessed.

Selection for resistance to acylalanine fungicides using adaptation and mass selection procedures has been attempted in several laboratories. Staub et al. (1979) cultured isolates of P. infestans on media at sublethal concentrations of furalaxyl. Sporangia and zoospores from isolates with reduced sensitivity were harvested and transferred onto media at higher concentrations. In this way, several isolates were obtained which were from two to more than 100-times less sensitive to furalaxyl than the original isolates. The isolates were cross-resistant to other acylalanines and they maintained their resistance for at least 2 years with repeated subculturing on media without the fungicides. However, these strains had either lost their virulence or they were still fully

sensitive to acylalanine fungicides on tomato or potato plants. Hence in vitro resistance was not necessarily accompanied by in vivo resistance.

Bruin & Edgington (1981) studied adaptation to metalaxyl in several fungi. Following 6-12 transfers in vitro, the sensitivity to metalaxyl of isolates of P. capsici and several Pythium spp. was reduced by 100-3600-times. Some of the metalaxyl-resistant strains retained their high degree of resistance during subculturing for 8 months in the absence of the fungicide. Other strains slowly became less resistant or lost their resistance completely. All strains were usually cross-resistant to other acylalanines. The metalaxyl-resistant strains of P. capsici appeared to retain their pathogenicity on peppers and the disease produced was not controlled by metalaxyl.

With P. megasperma f.sp. medicaginis, resistant isolates were produced by growing the fungus on metalaxyl-amended agar for 2-4 weeks and then transferring mycelium onto media containing metalaxyl at higher concentrations. Mass selection from encysted zoospores on agar amended with metalaxyl was also successful. Both methods yielded mutants with a relatively low degree of resistance. Isolates showed reduced virulence to alfalfa seedlings or were avirulent.

Davidse & Krüse (1981) obtained metalaxyl-resistant isolates of P. megasperma f.sp. medicaginis and P. megasperma f.sp. glycinea using adaptation and/or mass selection procedures. Three mutants of P. megasperma f.sp. glycinea resistant to metalaxyl and furalaxyl were selected from encysted zoospores on agar containing either fungicide. The degree of resistance was high in one mutant selected on the medium containing furalaxyl. This mutant was as virulent as the original isolate and was not controlled on soybean seedlings treated with normally effective doses of metalaxyl.

Recently, it was reported that a metalaxyl-resistant isolate of P. megasperma f.sp. medicaginis had been obtained by the mass selection

of encysted zoospores on metalaxyl-amended agar (Stack & Millar, 1985). This isolate was identical to the parent strain in terms of its morphology and virulence to the host plant.

There are no reports of successful experiments in which in vivo adaptation to metalaxyl was induced. In growth room studies, a field population of P. infestans was exposed to sub-lethal doses of metalaxyl on potato plants for 11 infection cycles, but no isolates with decreased sensitivity were obtained (Staub et al., 1979). These authors, and Bruin & Edgington (1981), were not able to obtain metalaxyl-resistant isolates of P. infestans after several cycles of mass selection with sporangia on metalaxyl-treated potato tuber tissue. A similar procedure with P. parasitica and cabbage seedlings also did not result in loss of sensitivity (Bruin & Edgington 1981).

In vitro adaptation and mass selection procedures enabled Bruin & Edgington (1981) and Davidse (1981) to conclude that development of resistance was likely to occur under field and glasshouse conditions. This view was confirmed by subsequent reports of resistance in several genera of the Peronosporales to acylalanine fungicides (see Table 3).

The success of in vitro studies of adaptation to metalaxyl in members of the Peronosporales prompted a similar investigation, reported here, with the benzamides.

2.2 In vitro Adaptation of *P. megasperma* f.sp. *medicaginis*

2.2.1 Introduction

The transfer of fungal mycelium to agar amended with increasing concentrations of chemical was considered to be one of the simplest ways in which benzamide-resistant mutants might be obtained. The isolate of *P. megasperma* f.sp. *medicaginis* used in the experiments of Davidse (1981) was chosen as the test isolate in the present study.

The adaptation of the fungus to metalaxyl and R157001 was studied in parallel, enabling comparison of the rate at which mutants developed to each fungicide.

2.2.2 Materials and Methods

An isolate of P. megasperma f.sp. medicaginis (coded 7x) was obtained from L C Davidse, The Agricultural University, Wageningen, The Netherlands. The isolate was given the ICI Culture Collection code number K1120 and maintained on V8 agar (See Appendix A) at 25°C.

R157001 and metalaxyl (N-[2,6-dimethylphenyl]-N-[methoxyacetyl]-alanine methylester) were each suspended in acetone and incorporated into clarified V8 agar (See Appendix A) at a range of concentrations. The concentration of acetone in agar never exceeded 1%. Between four and six replicate agar plates (9cm in diameter) were prepared per chemical concentration. Plugs of mycelium, (6mm in diameter) were placed, mycelium downwards, at the centre of test plates. Every 3-6 weeks, plates which supported fast radial growth of mycelium were selected. Plugs from these areas of rapid growth were transferred to newly-amended plates. Untreated controls and higher rates of fungicide were always included. A parallel series of plates was inoculated with K1120, which had not been subjected to fungicide. Plates were incubated at 25°C and radial growth was determined every 7 days during the selection period by measuring two colony diameters per plate at 90° to each other, averaging them, and subtracting the diameter of the inoculum plug. Up to eight periods of selection on R157001-amended and metalaxyl-amended agar were completed.

To study the dosage-response behaviour of a metalaxyl-resistant strain, metalaxyl was added either as the solid, or as a suspension in acetone, to clarified V8 agar at concentrations to 800µg metalaxyl/ml medium. Three replicate agar plates were prepared per chemical

concentration. Plugs (6mm) of mycelium of a metalaxyl-adapted strain and the parallel unadapted strain were placed, mycelium downwards, at the centre of appropriate test plates. Additionally, in the last dosage-response test, a subculture of K1120 which had been maintained independently of the adaptation procedure, was included as a comparative standard.

Plates were incubated at 25°C for 8 days or less. Inhibition of radial growth of mycelium was determined by measuring two colony diameters per plate at 90° to each other, averaging them, and subtracting the diameter of the inoculum plug. Dosage-response curves were then drawn.

2.2.3 Results

a) Adaptation to metalaxyl

The experimental route by which isolate K1120 was subjected to adaptation pressure by metalaxyl is shown in Table 4. The response to metalaxyl changed rapidly from "highly sensitive" (86% inhibition at 0.3125 µg metalaxyl/ml in selection period 1) to "highly resistant" (44% inhibition at 100µg metalaxyl/ml in selection period 6).

Problems occurred when cultures which had adapted to 100µg metalaxyl/ml at selection period 6 were transferred after 4 weeks to newly-amended media. After 14 days, bacterial contamination was observed in some plates. Therefore, as a precaution against losing this strain, a fresh transfer was made from a different plate of the selection period 6 series after 6 weeks (Table 4b). Growth eventually occurred at 100µg metalaxyl/ml on both sets of plates, although initially it was slow in subculture B. The difficulties with subculture A necessitated an interim transfer on unamended medium before selection period 8. In both selection periods 7 and 8, subculture A was less sensitive to metalaxyl than subculture B. No tests were made with metalaxyl above 100µg/ml.

Table 4 Adaptation of *Phytophthora megasperma* f.sp. *medicaginis* isolate K1120 to growth on metalaxyl-amended medium

a) Selection periods 1-6

Selection period ^a	Inhibition (%) ^b of radial growth by metalaxyl (µg/ml) :								
	0.3125	0.625	1.25	2.5	5	10	25	50	100
1	86	90	<u>100</u> ^c	100	100	-	-	-	-
2	-	-	<u>63</u>	<u>81</u>	-	-	-	-	-
3	-	-	-	<u>59</u>	47	<u>53</u>	-	-	-
4	-	-	-	-	-	<u>56</u>	78	<u>100</u>	-
5	-	-	-	-	-	-	29	<u>36</u>	<u>57</u>
6	-	-	-	-	-	-	16	24	<u>44</u>

b) Selection periods 7 and 8

Transfers from selection period 6 after	Selection period	Inhibition (%) of radial growth by metalaxyl (µg/ml) :		
		25	50	100
4 wk (A)	7	33	67	<u>75</u> ^d
	8	39	41	<u>51</u>
6 wk (B)	7	100	100	<u>100</u>
	8	58	50	<u>83</u>

^a Selection periods were of 4 wk duration, except period 1 which lasted 3 wk (but see note d).

^b Assessed 7 days after transfer.

^c Underlined values indicate the cultures from which transfers were made for the next selection period.

^d This selected isolate was grown on agar with 100µg metalaxyl/ml for 4 wk and then on unamended agar for 2 wk before use in selection period 8.

At the completion of selection periods 5,6, and 8, the ED₅₀'s of one of the cultures growing on 100µg metalaxyl/ml, and the parallel culture maintained on unamended medium, were compared in dosage-response experiments. Isolate K1120, maintained as a stock culture, was also included in the test after selection period 8. The ED₅₀'s of this culture and of those transferred on unamended agar throughout the adaptation period were similar (Table 5a), indicating that sensitivity to metalaxyl did not change with repeated subculturing to unamended media. When tested at the completion of selection period 8, the sensitivity of the metalaxyl-adapted strain differed by about 9000-times from that of the unselected strains (Table 5b). The response to metalaxyl of the adapted strain and the parallel culture maintained on unamended medium, at the completion of selection period 8, is shown in Plate 1a.

As K1120 adapted to increasing concentrations of metalaxyl, so its growth on unamended medium decreased relative to the unadapted strain (Table 6). By selection period 4, growth of the adapted strain after 7 days was only 20% of the unadapted strain, although overall, growth rates varied between this value and 84% (subculture B, selection period 8).

It was concluded that P. megasperma f.sp. medicaginis adapted readily to metalaxyl. Adapted cultures had reduced growth rates suggesting that they might be less competitive.

Davidse (1981) indicated that metalaxyl-adapted strains of isolate K1120 were less pathogenic than the parent to the host plant, alfalfa, or were avirulent. This aspect required further study in order to establish whether reduced growth in vitro in the experiments reported here would be manifested as decreased virulence in vivo.

b) Adaptation to R157001

The experimental route by which isolate K1120 was subjected to

Table 5 Inhibition (%) of radial growth of *Phytophthora megasperma* f.sp. *medicaginis* isolate K1120 by metalaxyl, at the completion of periods of selection^a

a) Maintained on unamended medium^b

Metalaxyl concentration ($\mu\text{g/ml}$)	Selection period			
	5	6	8	
			unadapted	stock culture
1	98	-	-	-
0.8	-	-	98	95
0.4	-	-	94	90
0.3	96	85	-	-
0.2	-	-	90	70
0.1	90	44	66	57
0.08	-	-	-	65
0.04	-	-	60	59
0.03	46	22	-	-
0.02	-	-	56	46
0.01	27	0	44	38
0.005	-	-	35	30
0.003	8	0	-	-
0.0025	-	-	0	13
0.00125	-	-	10	8
ED ₅₀ ($\mu\text{g/ml}$)	0.035	0.012	0.017	0.029

b) Transferred to metalaxyl-amended agar (subculture A)^c

Selection period	Metalaxyl ($\mu\text{g/ml}$)										ED ₅₀ ($\mu\text{g/ml}$)
	20	40	60	100	150	200	300	400	600	800	
8	0	19	14	24	43	52	62	95	100	100	149

^a See text for a description of selection periods.

^b Subcultured in parallel with the adapting strain (selection periods 5,6 and 8 [unadapted]) or separately as a stock culture (selection period 8 [stock culture]).

^c See text for description of subculture A.

Plate 1 Response of variants of *Phytophthora megasperma* f.sp. *medicaginis* isolate K1120 to metalaxyl and R157001

a) Metalaxyl

Metalaxyl ($\mu\text{g/ml}$)

Left to right : 0 25 50 100

Top row : Metalaxyl-adapted strain

Bottom row : Unadapted-strain

b) R157001

R157001 ($\mu\text{g/ml}$)

Left to right : 0 2.5 5 10

Top row : R157001-adapted strain

Bottom row : Unadapted-strain

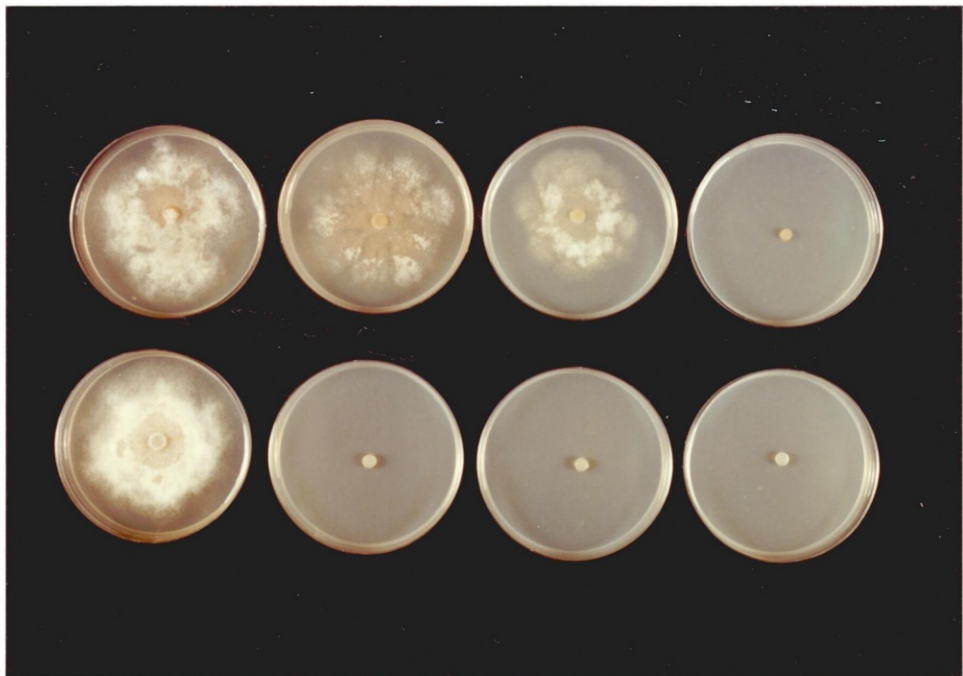
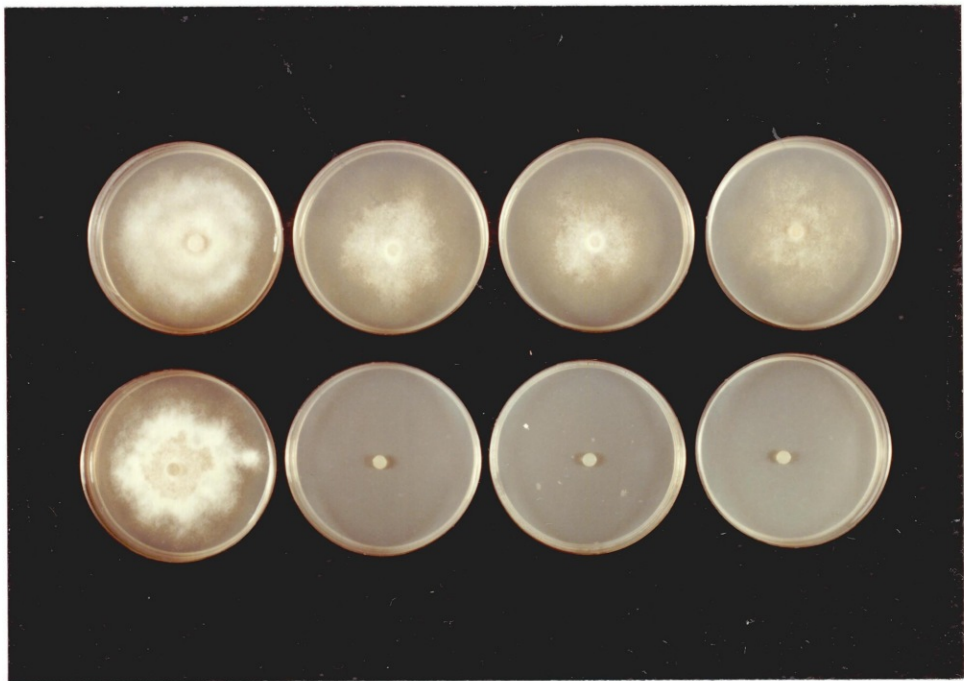


Table 6 Growth of *Phytophthora megasperma* f.sp. *medicaginis* isolate K1120 maintained on unamended agar or transferred to increasing doses of metalaxyl

a) Selection periods 1-6

Selection period	Radial growth (mm) ^a on medium :		Inhibition (%) ^b
	without metalaxyl(1)	with metalaxyl(2)	
1	49	Not applicable	
2	49	27	45
3	48	17	65
4	44	9	80
5	45	14	69
6	52	20	62

b) Selection periods 7 and 8

Subculture ^c	Selection period	Radial growth (mm) on medium :		Inhibition (%) ^b
		without metalaxyl(1)	with metalaxyl(2)	
A	7	49	No visible growth	Not determined
	8	30	12	60
B	7	49	12	76
	8	49	41	16

^a Average colony diameters, measured after 7 days.

^b $\frac{(1) - (2)}{(1)} \times 100$

^c See text for description of subcultures A and B.

adaptation pressure by R157001 is shown in Table 7. The extent of inhibition of the isolate by 1.25 μ g R157001/ml decreased from 80% in selection period 1 to 59% in selection period 2. After 4 weeks, a transfer was made from selection period 2. When, subsequently, no growth was visible on R157001-amended plates, a transfer was made after 14 days from unamended plates. Concomitantly, a fresh transfer was made from a different R157001-amended plate of the selection period 2 series after 6 weeks. Growth eventually occurred at 5 μ g R157001/ml on these sets of plates, but remained fully inhibited by R157001 on the first set to be inoculated. The two subcultures which grew were maintained separately until selection period 5. One of these was not transferred beyond this stage when it failed to grow at 5 μ g R157001/ml (Subculture A). A problem also occurred at selection period 6 when insufficient growth of the remaining subculture (B) was visible after 4 weeks at 5 μ g R157001/ml. Therefore, a transfer was made from a plate amended with 2.5 μ g R157001/ml. Adequate growth eventually occurred at 5 μ g R157001/ml in selection period 6 after 6 weeks. When this subculture (D) was transferred, it failed to grow on R157001 amended agar, and after 2 weeks was transferred from the unamended control plates. Despite these difficulties, this subculture continued to grow at 5 μ g R157001/ml in selection period 7. In contrast, the subculture (C) made from the plate amended with 2.5 μ g R157001/ml in selection period 6, was highly inhibited by 5 μ g R157001/ml in selection periods 7 and 8. Throughout the adaptation process, K1120 was highly inhibited by 10 μ g R157001/ml.

Between selection periods 1-8, isolate K1120 which was maintained on unamended agar was inhibited by 92 - 100% at 2.5 μ g R157001/ml. Additionally, apart from slight growth in selection period 5 at 5 and 10 μ g R157001/ml, the isolate was fully inhibited by 5 μ g R157001/ml. This indicated that the sensitivity of K1120 to R157001 did not alter with repeated subculturing to unamended agar.

Table 7 Adaptation of *Phytophthora megasperma* f.sp. *medicaginis* isolate K1120 to growth on R157001-amended medium

Stage of adaptation ^a	Origin of culture	Selection period	Inhibition (%) ^b of radial growth by R157001 (µg/ml)					
			0.625	1.25	2.5	5	10	20
<u>Selection periods 1 and 2</u>	Not applicable	1	43	80 ^c	92	100	100	-
		2	-	<u>59</u>	<u>100</u>	-	-	-
<u>Selection periods 3-6</u>	Selection period 2 after 6 wk (A)	3	-	51	66	<u>98</u>	-	-
		4	-	-	-	<u>100</u>	100	100
		5	-	-	<u>70</u>	<u>100</u>	100	-
		6	not transferred					
	Selection period 2 after 4 wk on agar with 2.5µg R157001/ml and 2 wk on unamended agar (B)	3	-	54	60	<u>100</u>	-	-
		4	-	-	-	<u>100</u>	100	100
		5	-	-	58	<u>95</u>	100	-
		6	-	44	<u>44</u>	<u>100</u>	100	-
	Selection period 6 after 4 wk on agar with 2.5µg R157001/ml (C)	7	-	-	<u>90</u>	100	100	-
		8	-	-	<u>66</u>	100	100	-
	Selection period 6 after 6 wk on agar with 5µg R157001/ml and 2 wk on unamended agar (D)	7	-	-	<u>90</u>	100	100	-
		8	-	-	<u>66</u>	100	100	-

^a Selection periods were of 4 wk duration, except period 1 which lasted 3 wk, and where indicated.

^b Assessed 7 days after transfer.

^c Underlined values indicate the cultures from which transfers were made for the next selection period.

Throughout the adaptation process, there was no major difference between the MIC of isolate K1120 transferred to R157001-amended agar, and maintained separately on unamended agar. Therefore, dosage-response experiments were not undertaken. However, from comparison of percentage inhibition of growth values in selection period 1 (43% at 0.625µg R157001/ml) and selection period 8 (55% at 2.5µg R157001/ml) it was apparent that adaptation pressure yielded a strain of K1120 with a reduced sensitivity to R157001 of approximately four-times. The colony morphology of this strain differed from that of K1120 maintained on unamended agar. In the former, the colony surface was more convoluted, sectoring was commoner, and the colony margins were "ragged" instead of smooth. The response of both strains to R157001 at the completion of selection period 7 is shown in Plate 1b.

Between selection periods 1-8 the radial growth of K1120, subjected to R157001, was either unaffected or reduced by a maximum of 12%, on unamended agar (Table 8). This suggested that adapted strains would be highly competitive with wild strains in the absence of the fungicide.

These results indicated that limited adaptation occurred to R157001. However, the extent of adaptation was much less than with metalaxyl, and this difference is clearly shown in Figure 2.

The findings of the present investigation are discussed in relation to in vivo studies of the pathogenicity of adapted strains in Section 2.3.

2.3 Pathogenicity of Adapted Strains of *P. megasperma* *f.sp. medicaginis*

2.3.1 Introduction

Davidse (1981) demonstrated that metalaxyl-adapted strains of *P. megasperma* *f.sp. medicaginis* K1120 were less virulent than the parent

Table 8 Growth of Phytophthora megasperma f.sp. medicaginis isolate
K1120 maintained on unamended agar or transferred to increasing
doses of R157001

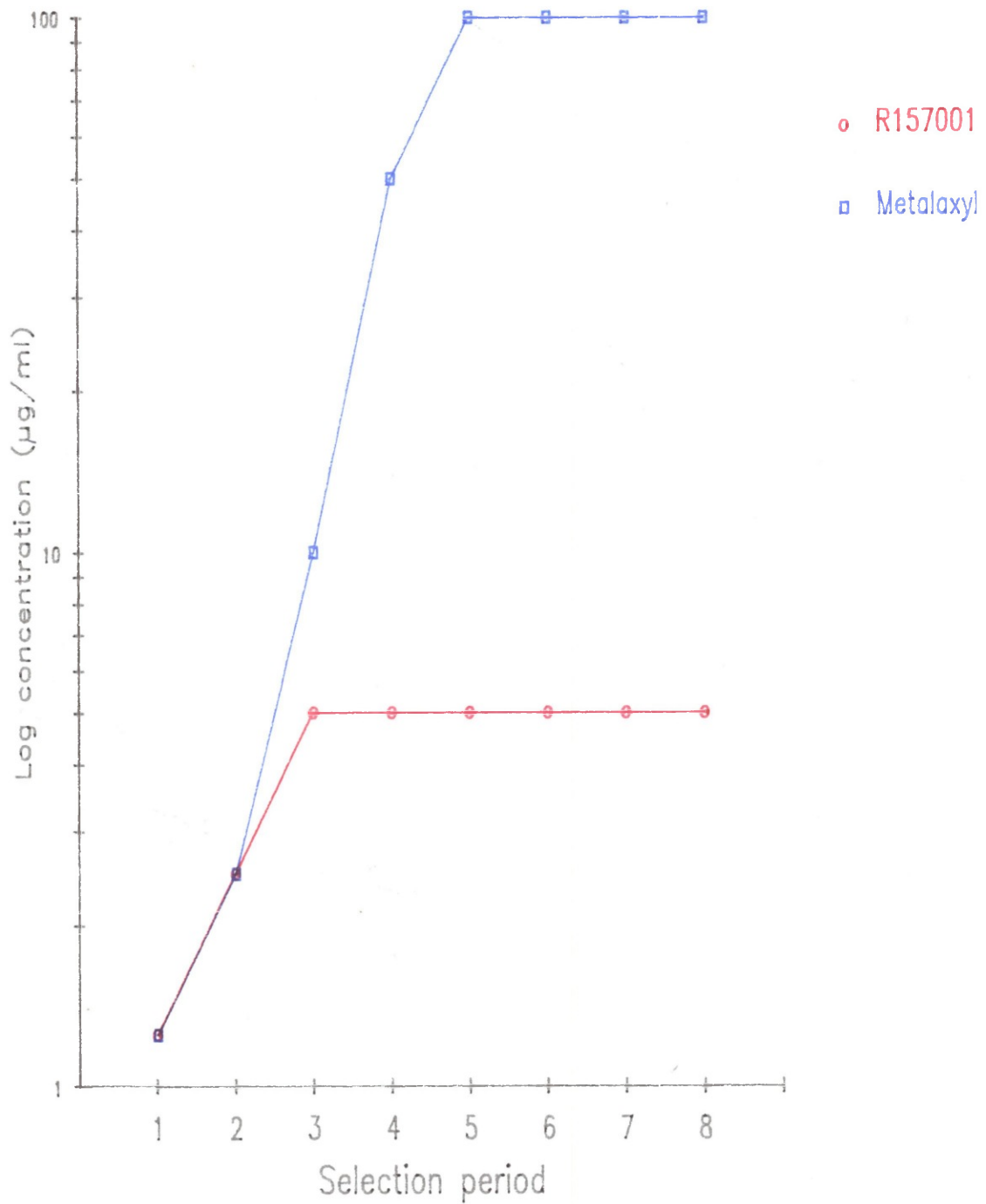
Subculture ^a	Selection period	Radial growth (mm) ^b on medium :		Inhibition(%) ^c
		without R157001(1)	with 1 R157001(2)	
	1	49	Not applicable	-
	2	49	51	0
A	3	46	59	0
	4	49	46	6
	5	52	46	12
	6	Not determined		
B	3	50	57	0
	4	49	55	0
	5	52	57	0
	6	31	41	0
C	7	47	60	0
	8	44	44	0
D	7	44	42	5
	8	Not determined		-

^a See text for a description of subcultures A to D.

^b Average colony diameters (minus inoculum plug diameter), measured after 7 days.

^c $\frac{(1) - (2)}{(1)} \times 100$

Figure 2 Highest concentrations of R157001 and metalaxyl at which *Phytophthora megasperma* f.sp. *medicaginis* isolate K1120 grew, at the completion of succeeding periods of selection



isolate to alfalfa, showed a reduced mycelial growth rate in vitro, and were usually unable to infect alfalfa seedlings treated with metalaxyl. The metalaxyl-adapted strains obtained in the present study also showed a reduced mycelial growth rate, and it was presumed that these would be weakly pathogenic to alfalfa, or avirulent, as were Davidse's strains. It was not known whether the pathogenicity of R157001-adapted strains was likely to have altered during the adaptation procedure. Thus the virulence of metalaxyl and R157001-adapted strains to alfalfa seedlings was investigated as a prelude to potential in vivo studies of fungicide sensitivity.

2.3.2 Materials and Methods

Prior to testing their pathogenicity to alfalfa, fungicide-adapted strains were transferred during selection period 6 to unamended agar. As this experiment was intended to be a preliminary determination of pathogenicity, parallel unadapted strains were not tested. Instead, isolate K1120 which had been maintained independently of the adaptation process was included as the unadapted control.

For each test culture, 0.25g (approximately 105) seeds of alfalfa cv. Euver were sown on 100g John Innes seed compost in a 7cm² plastic pot. Seeds were covered with a further 100g of the same soil mix, watered thoroughly, and placed in a growth room (16h photoperiod [6000 lux], 24°C day, 17°C night). After 7 days, the number of seedlings which grew in each pot was counted. Eight plugs of mycelium (6mm in diameter) from each 7-day old culture were evenly distributed, mycelium downwards, amongst the seedlings. Pots were placed separately on plastic weighing dishes (14cm²) and watered thoroughly so that the soil was saturated. Free water was left in the weighing dishes for the duration of the experiment. The proportion of seedlings killed by each culture was

determined at regular intervals during incubation for 21 days. Seedling death was indicated by the shrivelling of plants in infected pots, and was assessed as follows:-

0	=	0	
1	=	1 - 14	
2	=	15 - 34	
3	=	35 - 64	- percent seedlings shrivelled
4	=	65 - 84	
5	=	85 - 99	
6	=	100	

Numbers of healthy seedlings were not determined after inoculation because of the subsequent difficulty in counting the densely growing plants.

2.3.3 Results

Clear differences between infection of alfalfa seedlings by adapted and unadapted cultures were evident after only 6 days (Table 9). The majority of seedlings inoculated with the unadapted parent were killed by this time, whereas metalaxyl and R157001-adapted strains caused the death of a relatively small number of seedlings. The progress of the infection hardly changed during the next 15 days. The metalaxyl-adapted strain caused the death of a slightly increased number of seedlings, unlike the R157001-adapted strain. The parent isolate succeeded in causing the death of all seedlings, 21 days after inoculation. Uninoculated seedlings remained healthy throughout the experiment. The symptoms of infection at the completion of the test are shown in Plate 2. This shows that the virulence of both the metalaxyl and R157001-adapted strains was low, compared with the parent isolate, K1120.

2.3.4 Discussion

P. megasperma f.sp. medicaginis isolate K1120 adapted readily to metalaxyl following transfer of plugs of mycelium to increasing

Table 9 Disease severity grades^a following inoculation of alfalfa seedlings with metalaxyl and R157001-adapted strains of *Phytophthora megasperma* f.sp. *medicaginis*

Isolate/ subculture	Days after inoculation				
	0	6	9	15	21
Metalaxyl- adapted	0(53) ^b	1	1	2	2
R157001- adapted	0(51)	1	1	1	1
K1120 ^c	0(56)	4	5	5	6
Uninoculated	0(56)	0	0	0	0

^a See text for assessment method.

^b Figures in parentheses were the total number of healthy seedlings in each pot prior to inoculation with the fungus.

^c K1120 was the parent isolate maintained independently of the adaptation process on unamended agar.

Plate 2 Symptoms of infection of alfalfa cv. Euver by strains of
Phytophthora megasperma f.sp. medicaginis isolate K1120

Left to right :	Uninoculated control	Parent isolate	Metalaxyl- adapted strain	R157001- adapted strain
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concentrations of the chemical in agar (see Section 2.2). This corresponded to results obtained by Davidse (1981). In both studies, metalaxyl-adapted strains grew more slowly than the parent isolate on unamended medium and were also less virulent to alfalfa seedlings. Davidse (1981) considered that the strains of P. megasperma f.sp. medicaginis obtained by adaptation were likely to be of the same type as those of P. infestans obtained by Staub et al. (1979). These isolates also showed reduced virulence and/or were fully sensitive to acylalanine treatments on potato and tomato plants. The apparent absence of a relationship between in vitro and in vivo resistance to acylalanines led Staub et al. (1979) to suggest that in vitro resistance to these fungicides was an unsuitable parameter to predict the risk of in vivo resistance. The results of the present investigation support this opinion, so far as adaptation is concerned.

Although adaptation to R157001 was attempted using procedures similar to those for metalaxyl, it was not possible to reduce the in vitro sensitivity of K1120 to R157001 to the same degree as it was to metalaxyl (see Section 2.2). Conversely, the low virulence of R157001-adapted strains was similar to that found in metalaxyl-adapted strains. A reduction in fitness or virulence of resistant strains under laboratory conditions could be expected to coincide with a competitive disadvantage of these strains under field and glasshouse conditions (Dekker, 1982b). In the presence of the fungicide, the resistant strains would have an advantage over the sensitive strains, which are more strongly inhibited by the fungicide. However, even if the R157001-adapted strains obtained in the present investigation had been fully virulent, it is likely that they would still have been controlled by relatively low doses of R157001 under field or glasshouse conditions.

It has been proposed that metalaxyl is fungistatic in its mode of action (Coffey & Young, 1984). This may account for the ease with which

adaptation by P. megasperma f.sp. medicaginis (Davidse, 1981; Section 2.2) and P. infestans (Staub et al., 1979) occurred. In contrast, the limited adaptation of the fungus to R157001, and the inability of the adapted strain to grow beyond a sharply-defined maximum concentration, may indicate a different mode of action for R157001. Work was undertaken to investigate the modes of action of metalaxyl and R157001, and is reported in Chapter Five.

Alternatively, instability of the chemical in agar media might account for the low degree of adaptation attained to R157001. A diminishing concentration during each selection period could have resulted in a reduced selection pressure on the fungus. Consequently, the stability of benzamide fungicides in agar media was examined (see Chapter Three).

Although mutants of P. megasperma f.sp. medicaginis with both a high resistance to R157001 and displaying normal virulence characteristics were not obtained in the present study, it was conceivable that they would be obtained more readily by other means, for example by the use of mutagenic techniques. Mutants of K1120 which were highly resistant to metalaxyl in vitro and in vivo, and as fit as the parent isolate, were obtained using NTG mutagenesis (Davidse, 1981). Similarly, UV irradiation of P. capsici and P. ultimum also resulted in the development of strains which were highly resistant to metalaxyl, and as virulent as the parent isolate (Bruin & Edgington, 1982). Consequently, and subsequent to the adaptation studies reported here, it was decided to attempt the induction of mutants of Phytophthora spp. with a high degree of resistance to R157001 using mutagenic techniques (See Chapter Six).

3 CHEMICAL STABILITY OF BENZAMIDE FUNGICIDES

3.1 General Introduction

Metalaxyl is known to persist for relatively long periods in plant tissues (Schwinn, 1979; Schwinn & Staub, 1981). This is presumably a consequence of the high stability of the fungicide in aqueous conditions (T E M Fraser, personal communication). The degree of stability of benzamide fungicides in aqueous environments was not known when adaptation of P. megasperma f.sp. medicaginis to R157001 was attempted (see Chapter Two). After eight periods of selection on amended media, the fungus adapted to growth at 5µg R157001/ml, but not at 10µg R157001/ml and above. In a similar series of transfers, adaptation to growth at 100µg metalaxyl/ml occurred after five periods of selection. The results suggested that the different fungitoxic effect of R157001 was contributing to the failure of resistant strains to emerge. However, an alternative explanation for the inability of P. megasperma f.sp. medicaginis to adapt to high concentrations of R157001 could be that the chemical decomposed into inactive intermediates in the relatively aqueous conditions of an agar plate, and this resulted in a declining selection pressure on the fungus. The following experiments investigated this possibility by monitoring the concentration of active ingredient in agar during a 4-week period.

3.2 Measurement of the Concentration of Fungicides in Agar

3.2.1 Introduction

This experiment examined the rate at which benzamide fungicides decomposed, if at all, in agar media.

3.2.2 Materials and Methods

In one experiment, fungicide was added to V8 agar to give final concentrations of 2.5 and 5µg R157001/ml. Concomitantly, the pH of unamended V8 agar was determined. Amended agar was dispensed into 9cm petri dishes and 6mm plugs of mycelium of a benzamide-adapted strain of P. megasperma f.sp. medicaginis were inoculated onto media containing 2.5µg R157001/ml. Plates were incubated at 25°C for 4 weeks. Fungicide was extracted from two separate uninoculated agar plates at 7-day intervals, and from one inoculated plate after 28 days. The contents of each plate were cut into small pieces and these were shaken vigorously on a Gallenkamp flask shaker for 15 min in two changes of 50ml chloroform. The resulting supernatants were combined, rotary-evaporated, and the residue dissolved in 2ml methanol (60%). A sample (approximately 100µl) was analysed by High Performance Liquid Chromatography using standard procedures. The identity of the organic molecules present in the solvent was established from the peaks they traced on linear chart-recording paper. The quantity (percent) of benzamide present was determined from the area enclosed by the relevant peak.

In a second experiment, R157846 was incorporated into V8 agar in 9cm petri dishes at a final concentration of 10µg/ml and the quantity of the chemical present during incubation for 28 days was determined as described for the previous experiment.

In a third experiment, R157001 and metalaxyl were incorporated separately into V8 agar at final concentrations of 1 and 0.1µg/ml, respectively. These media were inoculated with 6mm plugs of mycelium of benzamide-adapted and metalaxyl-resistant (K1121) variants of P. megasperma f.sp. medicaginis isolate K1120. The quantity of fungicide present after 14 days was determined as described for the first experiment.

3.2.3 Results

The concentration of R157001 in V8 agar at an initial pH of 5.6 declined exponentially to 35% during 28 days (Figure 3). The concentration of 4-chlorobenzamide, an intermediate form of R157001, inactive against members of the Peronosporales (ICI, internal information), was found to increase in direct proportion to the reduction in concentration of R157001. After 28 days, the benzamide-adapted variant of P. megasperma f.sp. medicaginis had fully colonised the agar plate containing 2.5µg R157001/ml. Only a trace (less than 1%) of the chemical remained in this plate. In the second experiment, R157846 declined to 84% of its original concentration during 28 days (Figure 4).

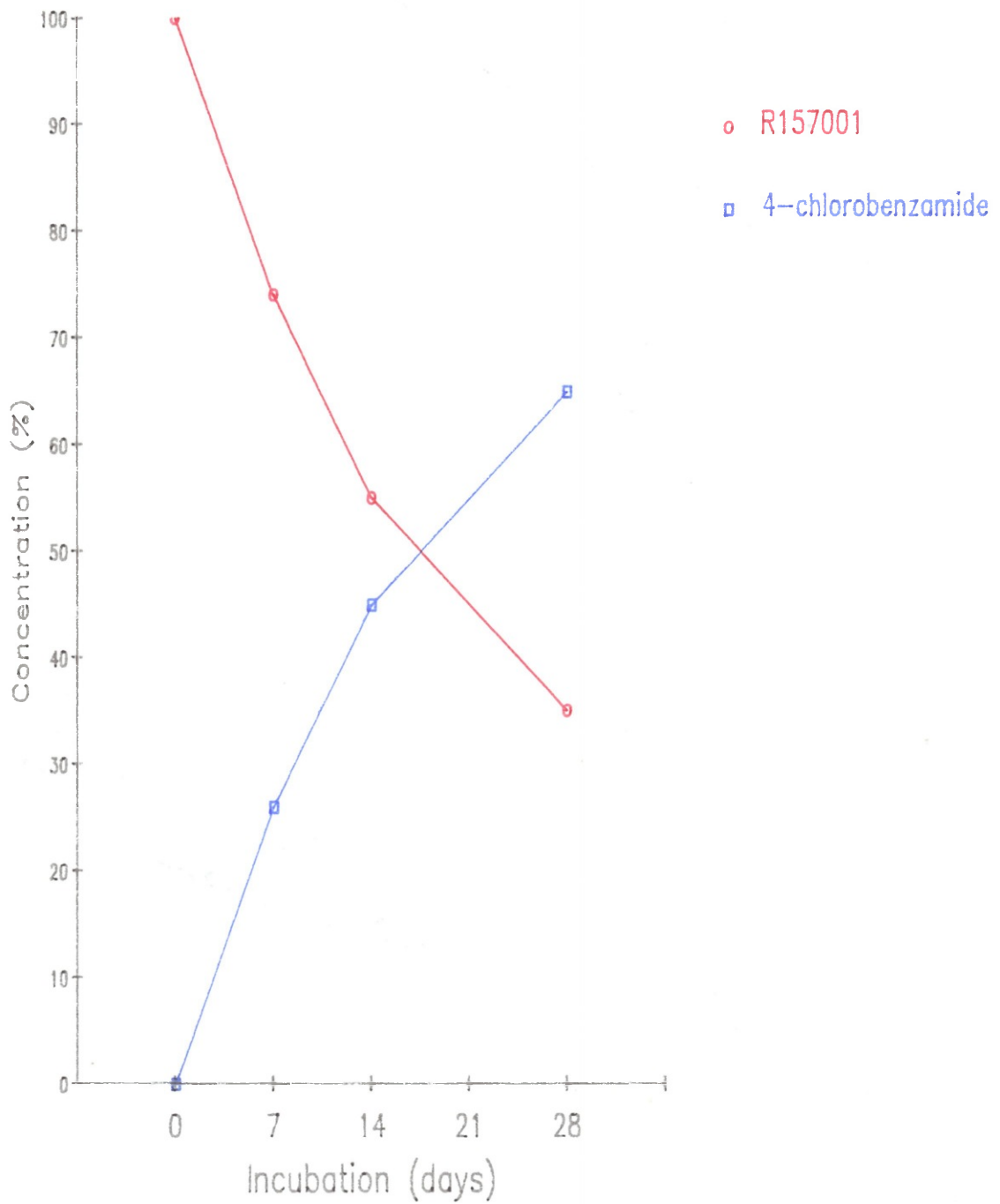
In the third experiment, the concentration of metalaxyl was unchanged in uninoculated medium, but R157001 had decomposed by 56% after 14 days (Table 10). In medium inoculated with the benzamide-adapted variant of P. megasperma f.sp. medicaginis only a trace (0.7%) of the chemical remained in the plate. The concentration of metalaxyl was unaffected by growth of the metalaxyl-resistant strain.

3.2.4 Discussion

The apparent instability of benzamide fungicides implied that these chemicals are unlikely to persist in host plants. Dekker (1981) explained that a fungicide which is very persistent on or in the plant exerted a continuous selection pressure for resistance on the pathogen population. A fungicide with less persistence may be less hazardous in this respect. Thus, resistance may not develop as readily under field and glasshouse conditions to the benzamides, compared with chemicals such as metalaxyl, which are relatively more stable and presumably persistent.

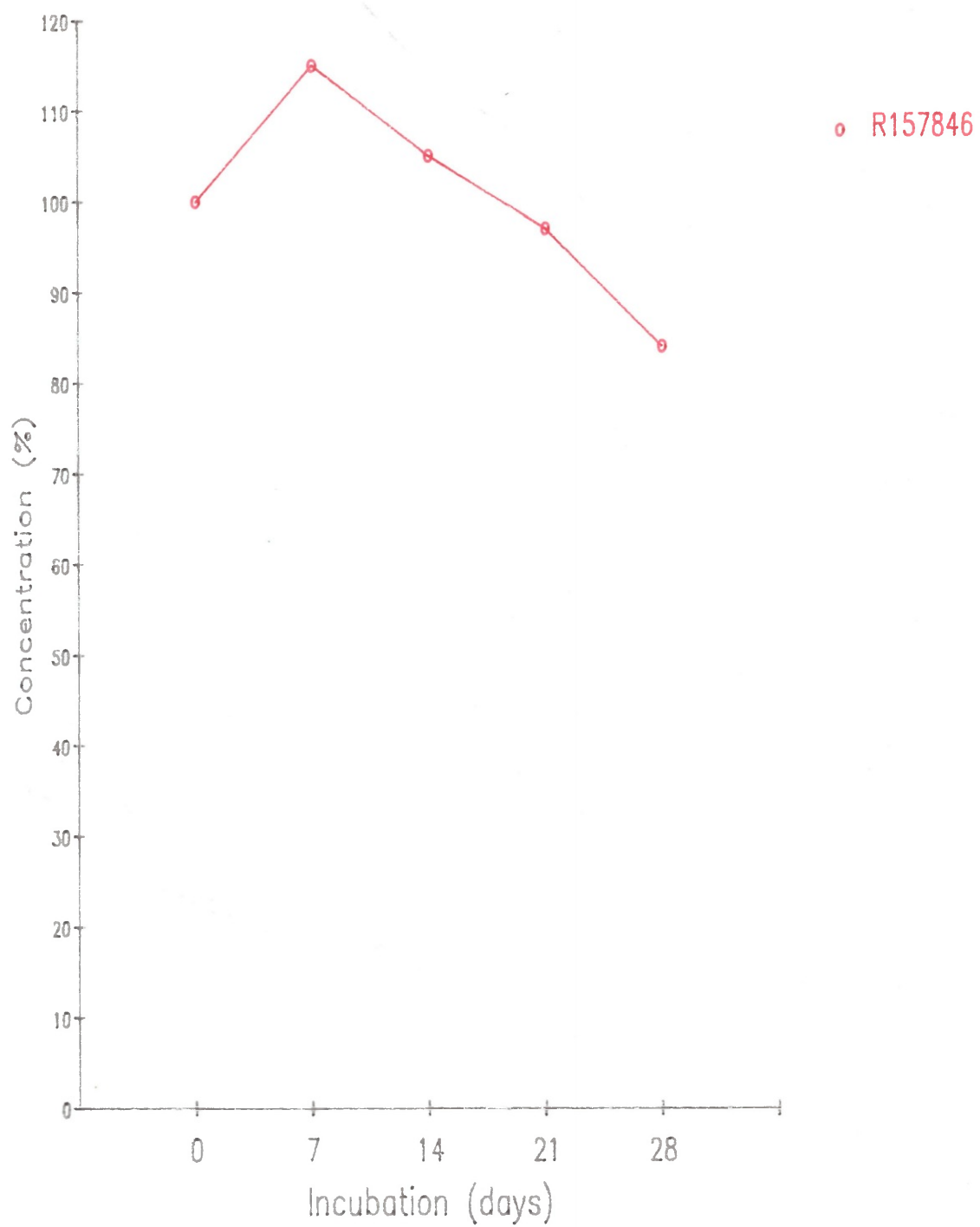
The rate of degradation of R157001 and R157846 was apparently accelerated by the growth of P. megasperma f.sp. medicaginis. Explanations

Figure 3 Relative concentration of R157001 and
4-chlorobenzamide in V8 agar during incubation at 25 C
for 4 weeks



Concentration at day 0 calculated as 100%

Figure 4 Concentration of R157846 in V8 agar during incubation at 25 C for 4 weeks



Concentration at day 0 calculated as 100%

Table 10 Quantity (%) of fungicide after 14 days^a in medium inoculated
with variants of Phytophthora megasperma f.sp. medicaginis
isolate K1120

Variant isolate	Fungicide ^b	
	R157846	Metalaxyl
Uninoculated	56	100
Benzamide-adapted	0.7	-
Metalaxyl-resistant	-	100

^a Quantity at day 0 calculated as 100%

^b Initial concentration was 1µg R157846/ml and 0.1µg metalaxyl/ml

for this result included:-

- i) Change in the pH of the medium. This would result from the normal metabolic processes of the growing fungus. If benzamide stability varied according to pH, an alteration in this parameter might cause an increase in the rate of degradation of the chemical.
- ii) Degradation of the benzamide molecule to inactive intermediates by enzymes produced by the fungus.
- iii) Binding of the benzamide molecule within the mycelium of the fungus as it grew. Subsequent extraction procedures may have been inadequate at recovering the chemical.

The first of these possibilities was examined in the next experiment. The remaining explanations required detailed biochemical investigation which was considered to be outside the scope of the present study.

3.3 Effect of the Growth of *P. megasperma* f.sp. *medicaginis* on the pH of Clarified V8 Broth

3.3.1 Introduction

There was evidence (T E M Fraser, personal communication) that R157001 was most stable in aqueous solution at pH4. The chemical also apparently decomposed by a factor of 10-times with every unit increase in pH-value above 7. Therefore, the effect of *P. megasperma* f.sp. *medicaginis* on the pH of a broth medium was examined in the present experiment. This was then related to the accelerated decomposition of R157001 by the fungus on agar medium (Section 3.2).

3.3.2 Materials and Methods

Thirteen 100ml Erlenmeyer flasks each containing 50ml clarified V8 broth, were sterilised by autoclaving. Six flasks were inoculated with

aliquots (0.5ml) of a suspension of mycelial fragments of P. megasperma f.sp. medicaginis isolate K1120. This suspension was prepared by macerating 10 plugs of mycelium (6mm diameter), cut from a 5-day culture, in 15ml sterile water. Seven flasks were not inoculated.

The pH of the medium in one uninoculated flask was measured on day 0 of the experiment, and the contents were then discarded. The same procedure was adopted for one uninoculated and one inoculated flask on six subsequent occasions.

3.3.3 Results and Discussion

The pH of the uninoculated medium remained at 5.4 during incubation for 16 days at 25°C (Figure 5). However, in flasks with P. megasperma f.sp. medicaginis there was initially a decrease to pH5.0 (day 2) followed by a rapid rise over the next 6 days to pH8.0. From 10-16 days the rise in pH slowed to values likely to be increasingly inhibitory to growth of the mycelium.

Thus, growth of P. megasperma f.sp. medicaginis results in changes in pH to values at which R157001 is rapidly decomposed to an inactive intermediate.

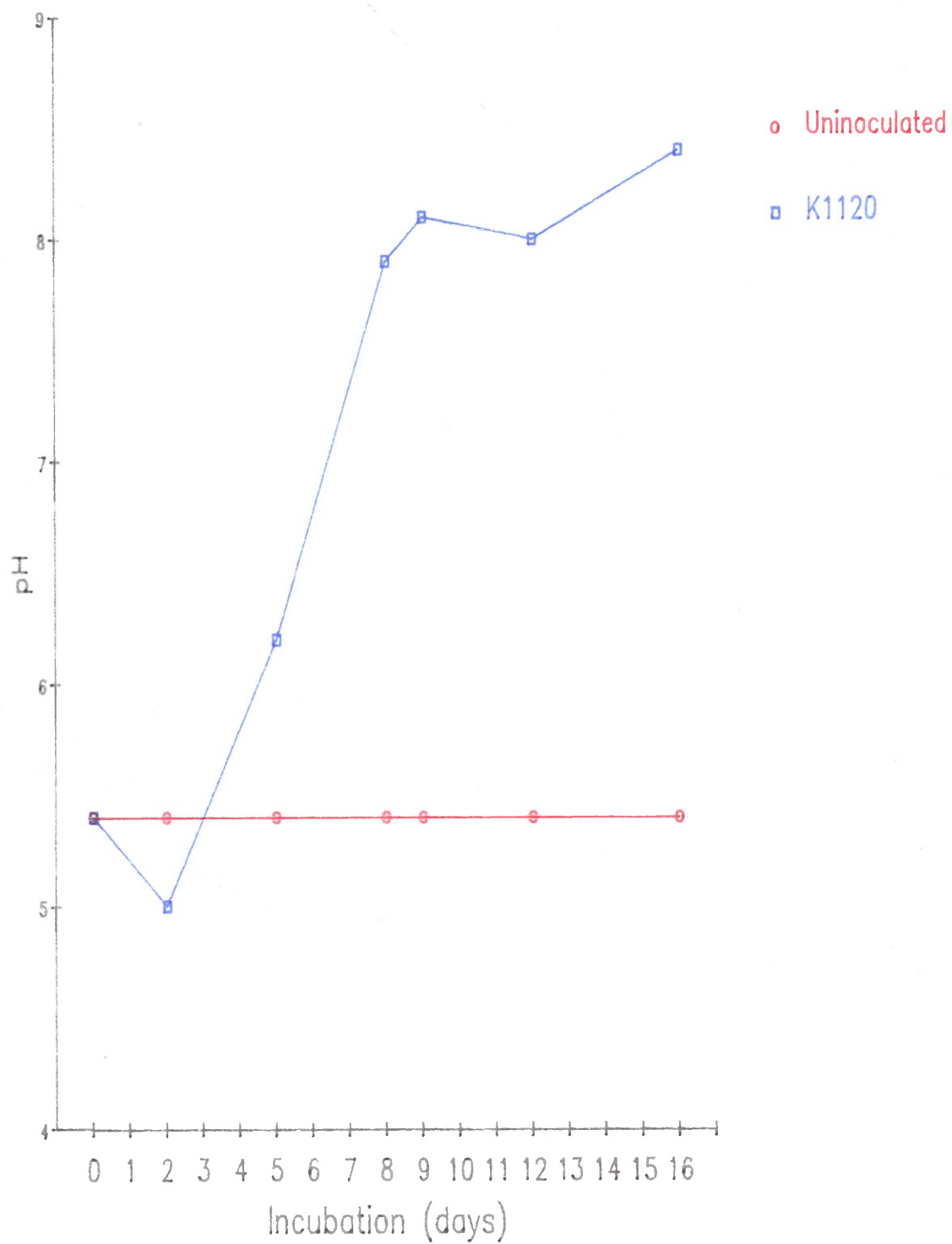
3.4 Use of Buffered Media to Limit the Change in pH During the Growth of P. megasperma

3.4.1 Introduction

A change in pH which accompanied the growth of P. megasperma f.sp. medicaginis could have accelerated the decomposition of R157001 in agar medium (see Section 3.3).

The present experiment investigated the possibility of limiting this change to pH-values of less than 7 using buffered media.

Figure 5 Change in pH of V8 broth during growth of *Phytophthora megasperma* f.sp. *medicaginis* isolate K1120



3.4.2 Materials and Methods

Solutions of McIlvaine's citric acid-phosphate buffer (McIlvaine, 1921) were prepared at approximately pH4 and pH6, respectively (see Appendix B). Each was added separately to clarified V8 broth to final concentrations of 5 and 50% v/v. An additional batch of clarified V8 broth was prepared without buffer. Aliquots (200ml) of each broth were transferred to two 250ml Erlenmeyer flasks. The pH of each broth was then determined on a 10ml sample from one flask of each treatment. These flasks subsequently served as untreated controls. Each of the remaining flasks was inoculated with 10 plugs of mycelium (6mm diameter) cut from 5-day cultures of P. megasperma K1240. The flasks were then incubated on an orbital shaker at 25°C. The pH of 10ml aliquots of broth from each flask was determined at regular intervals during incubation for 15 days.

After the final assessment, mycelium was separated from the broth by filtration, under vacuum, through Whatman no.4 filter paper, partially dried by compression between filter pads, and freeze-dried overnight. As soon as the process was complete, the mycelium was weighed.

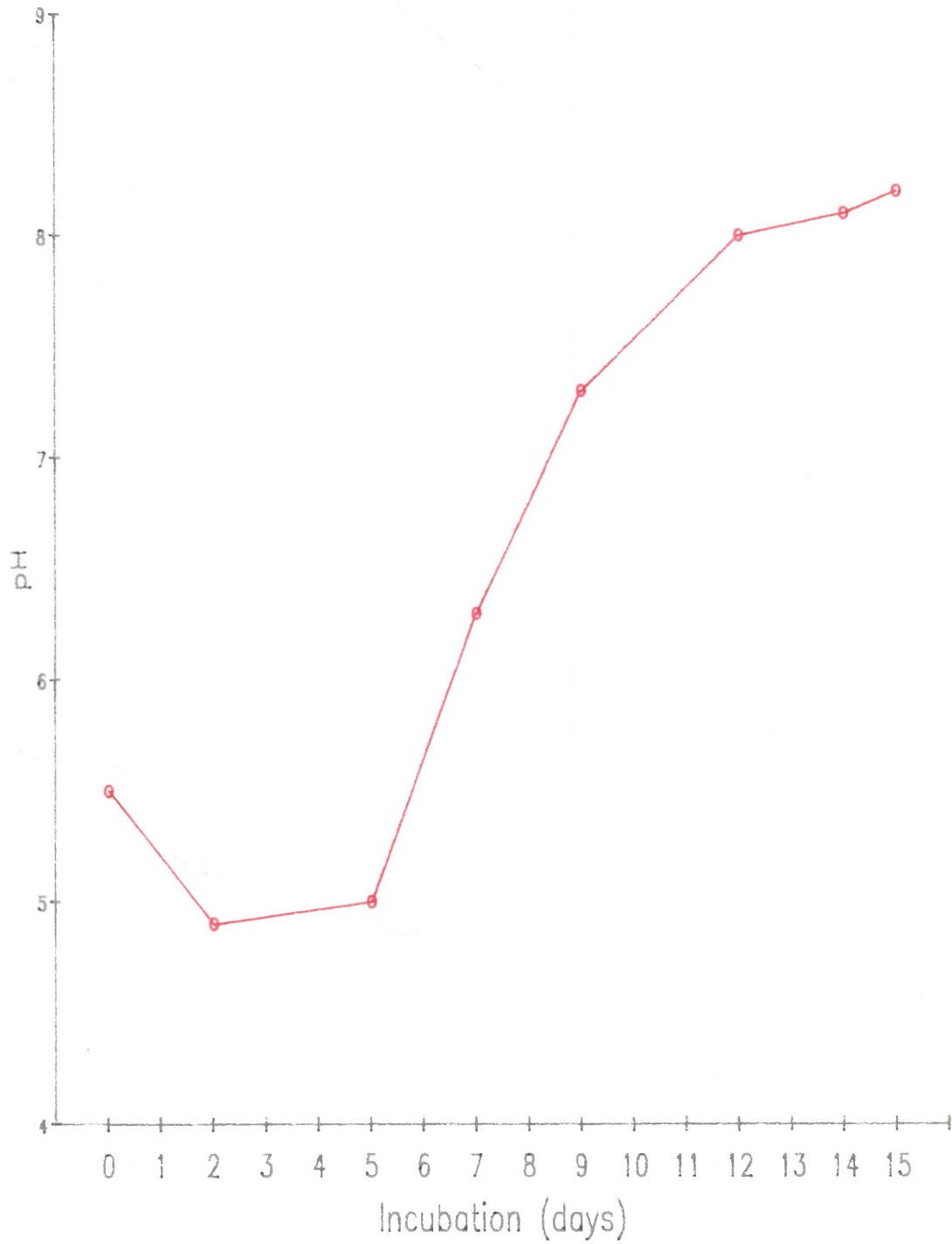
3.4.3 Results and Discussion

The pH-values of freshly-prepared buffer solutions were 4.1 and 6.2. Compared with these, the pH of clarified V8 broths containing buffer varied from no change to a difference of almost one pH-unit.

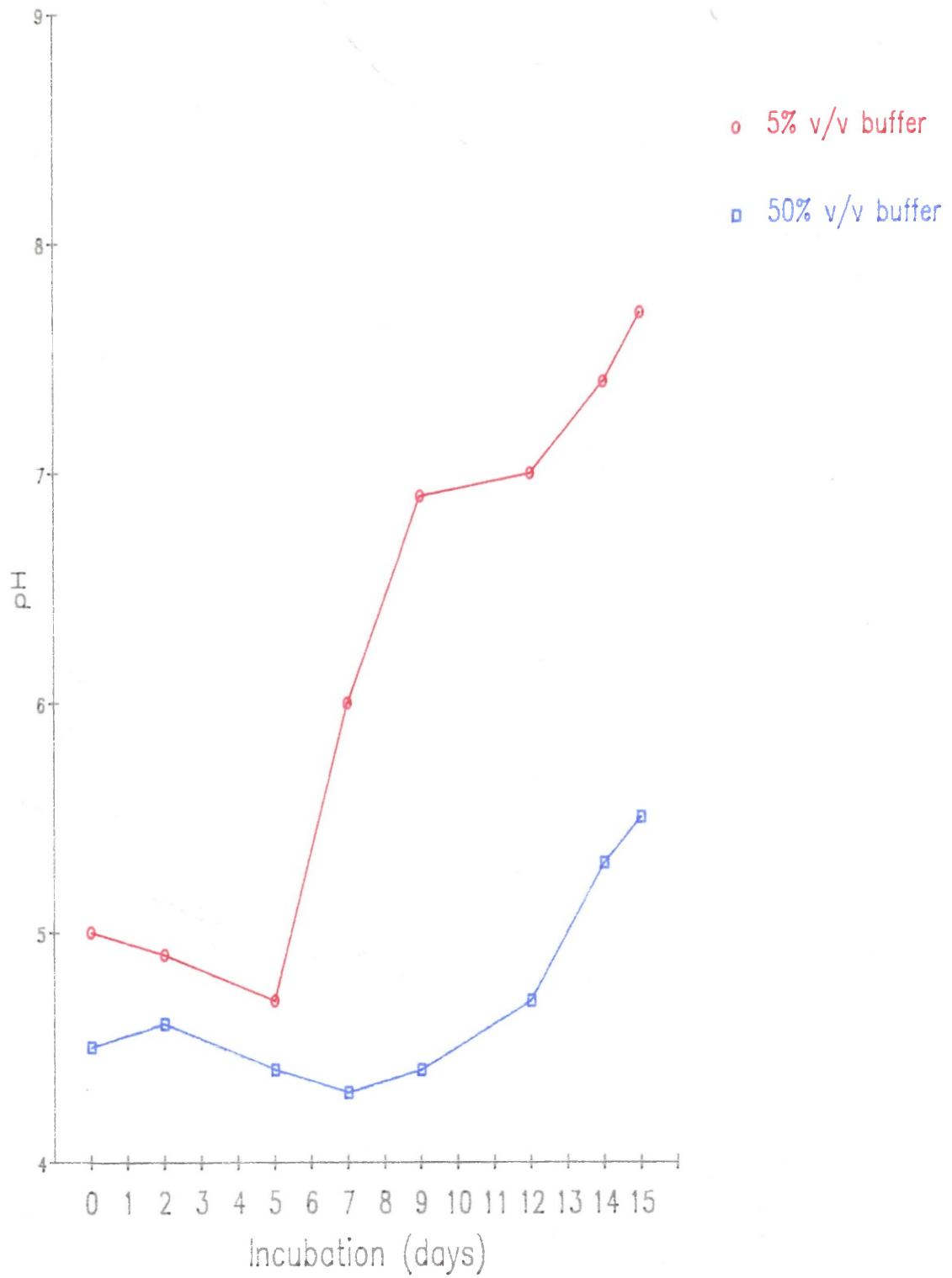
There was no change in the pH of uninoculated broths during the experiment. The pH of inoculated, unbuffered medium, initially 5.5, fluctuated between 4.9-8.2 (Figure 6). Broths containing 5% buffer hardly suppressed this fluctuation and therefore would not be expected to prevent the rapid degradation of R157001 under alkaline conditions. Only broths containing 50% buffer prevented a change to alkaline conditions during the

Figure 6 Changes in pH of V8 broth during growth of
Phytophthora megasperma isolate K1240

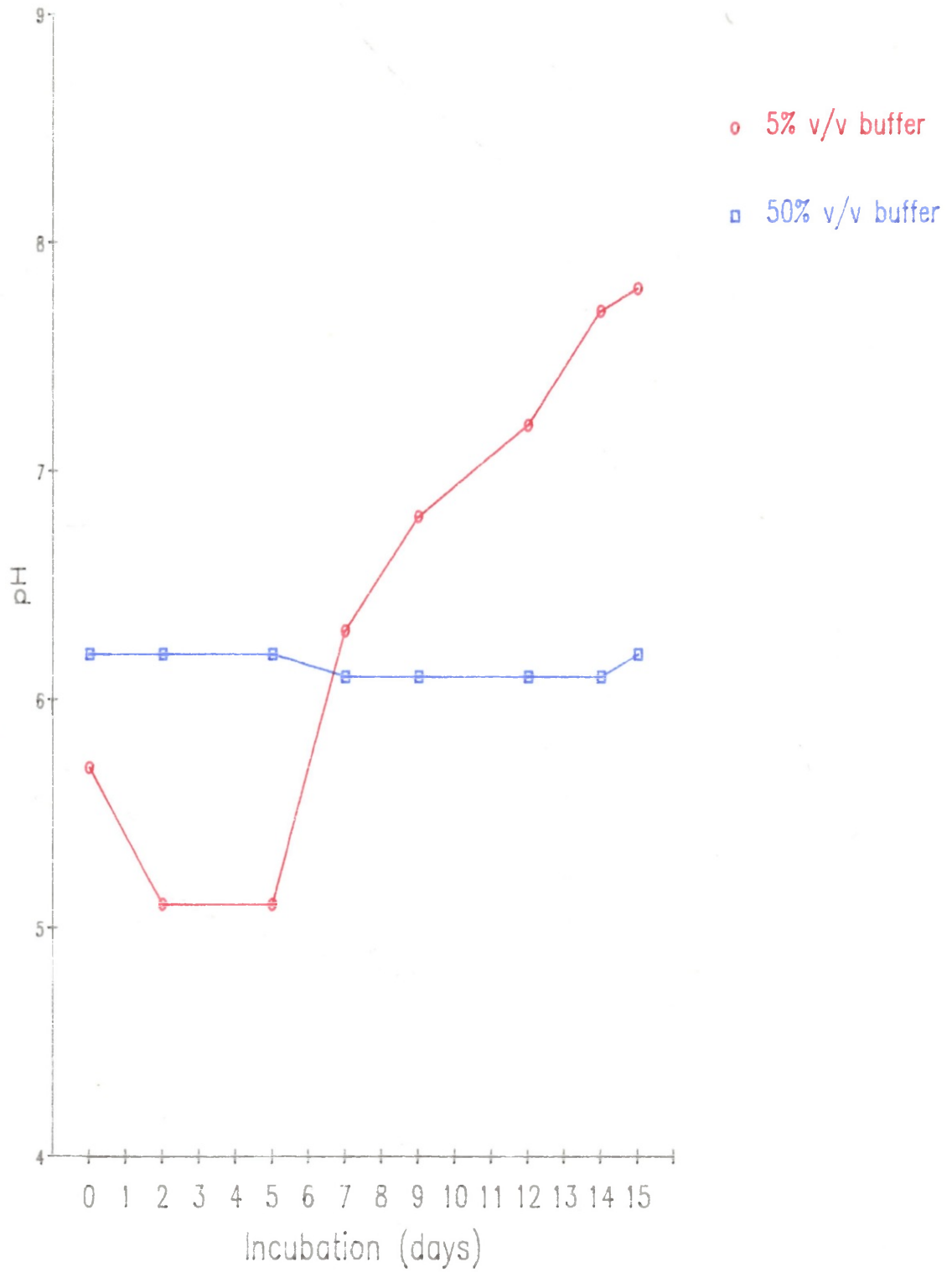
a) Unbuffered medium



b) Buffered medium: pH 4 (nominal)



c) Buffered medium: pH 6 (nominal)



experiment, that at pH6 having most effect. However, this was at the expense of fungal growth (7% final dry weight compared with that from unbuffered medium). Therefore, this medium would be unsuitable for use in the present study.

In addition to preventing a change to alkaline conditions, medium containing 50% of McIlvaine's buffer at pH4 caused an increase of 68% in the final dry weight of mycelium. This was presumably due to metabolism of the buffer by the fungus. In subsequent tests in which this buffer was used instead of water, it was found that the solution had a highly inhibitory effect on zoospore production in P. megasperma f.sp. medicaginis and P. infestans. These results indicated that fungal development was influenced significantly by the buffer. As a precaution against an adverse effect, particularly during the selection of fungicide-resistant mutants, testing and use of buffered media was discontinued. Furthermore, it was recognised, from the results obtained in Section 3.2, that degradation of R157001 would occur in buffered media even under acidic conditions. Instead, it was decided to restrict experiments with R157001 to 10-days duration. The degree of degradation was considered to be acceptable within this timescale. It was also desirable to test the natural sensitivity of populations of target fungi to the leading benzamide, which was R157001. In experiments of longer-term duration, usually those involving the selection of benzamide-resistant mutants after the use of mutagens, R157846 was chosen. The stability of this benzamide would be less affected by the growth of target fungi than R157001.

4 COMPARATIVE SENSITIVITY OF ISOLATES OF PHYTOPHTHORA SPP. TO R157001 AND METALAXYL

4.1 General Introduction

There are no reports of studies which monitored the sensitivity of populations of target fungi before the commercial introduction of a new fungicide. However, Staub & Sozzi (1984) advocated this type of investigation because it provides base-line sensitivity data and enables the detection of resistant strains.

The existence of resistance prior to the introduction of a new fungicide has been claimed when resistant strains could be isolated from locations in which the fungicide had never been applied. For example, strains of Penicillium italicum Wehmer and Penicillium digitatum Sacc. resistant to thiabendazole were found in orchards and packinghouses where the fungicide had not been used (Harding, 1972). Therefore, this author proposed that such strains occurred naturally. However, Hunger et al. (1982) pointed out that the resistant strains might have originated from locations in which thiabendazole or related chemicals were used. They cited the findings of Gutter et al. (1981) who isolated strains of P. digitatum and P. italicum resistant to benzimidazole fungicides (which includes thiabendazole) from orchards where these chemicals had never been applied. The origin of these strains was attributed to packinghouses where benzimidazoles were used. Similarly, metalaxyl-resistant strains of P. infestans were detected on potato crops which had never previously been exposed to acylalanine fungicides (Davidse et al., 1981). Neighbouring crops which had been treated with metalaxyl were considered to be likely sources of these strains.

Once a fungicide is in commercial use, valid studies of the natural sensitivity of target fungi necessarily entail recourse to cultures

collected before the introduction of the chemical. In an in vitro study of the benomyl-sensitivity of three isolates of Verticillium malthousei Ware, Wuest et al. (1974) found that one isolate, collected before the introduction of the fungicide, was resistant to it. More recently, Hunger et al. (1982) surveyed the in vitro sensitivity to metalaxyl of 35 isolates of P. megasperma which were collected from locations throughout the world before the commercial introduction of the fungicide. It was demonstrated that some isolates were naturally highly resistant to metalaxyl.

The following series of investigations examined the sensitivity of isolates of Phytophthora spp. to R157001. Because it has not yet been introduced into commercial use, none of the isolates obtained would previously have been subjected to the chemical. Thus, it was anticipated that the determination of base-line sensitivity data would enable the detection of resistant strains which occurred naturally. The frequency of such strains could also be established. Metalaxyl was included in experiments to detect acylalanine-resistant strains. Thus, cross-resistance to the benzamides and acylalanines was evaluated.

4.2 P. megasperma : In vitro Sensitivity to R157001 and Metalaxyl-1

4.2.1 Introduction

In the experiments of Hunger et al. (1982), sensitivity to metalaxyl in vitro was determined by measuring the radial growth of 35 isolates on agar with and without metalaxyl. On this basis, isolates were placed in one of three sensitivity groups, defined as follows:-

- i) highly sensitive (greater than 95% inhibition at 1 and 10 μ g metalaxyl/ml) : 15 isolates
- ii) moderately resistant (51-90% and 52-63% inhibition at 1 and 10 μ g metalaxyl/ml respectively) : 8 isolates

- iii) highly resistant (27-47% and 29-63% inhibition at 1 and 10 μ g metalaxyl/ml respectively) : 12 isolates

The results showed that in vitro resistance to metalaxyl existed naturally amongst isolates of P. megasperma. Additionally, the authors suggested that if similar resistance occurred in vivo, then in the presence of the fungicide, selection for highly resistant strains could proceed rapidly with concomitant loss of disease control by metalaxyl.

The findings of Hunger et al. (1982) prompted the present investigation of the natural sensitivity of P. megasperma to R157001, using metalaxyl as a comparative standard. Seventeen of the isolates tested by Hunger and his colleagues at Oregon State University, and assigned to P. megasperma, were examined, together with an isolate of P. megasperma f.sp. medicaginis (K1120).

4.2.2 Materials and Methods

Isolates (see Section 2.2.2 and Table 11) were maintained on V8 agar at 25°C.

R157001 and metalaxyl were each suspended in acetone and incorporated into clarified V8 agar at a range of concentrations. The concentration of acetone in agar never exceeded 1%. Three replicate agar plates (9cm diameter) were prepared per chemical concentration. Plugs (6mm) of mycelium were placed, mycelium downwards, at the centre of the test plates which were incubated at 25°C for 5 days. Radial growth was determined by measuring two colony diameters per plate at 90° to each other, averaging them, and subtracting the diameter of the inoculum plug.

Logit relative potency analysis was performed on the data according to the procedure described by Finney (1964). From the resulting means, isolates were differentiated at the 95% probability level by Duncan's (1955) multiple range test.

Table 11 Sources of isolates of *Phytophthora megasperma*

Isolate reference		Host	Location	Isolate group ^b
ICI	Original ^a			
K1150	S1	Alfalfa	Salem, Oregon	A1
K1151	P1	Alfalfa	Corvallis, Oregon	A2
K1152	M1	Alfalfa	Medford, Oregon	A1
K1153	PC3	Alfalfa	Princeton, Oregon	A1
K1154	PC5	Alfalfa	Klamath Falls, Oregon	A1
K1155	S2	Alfalfa	Salem, Oregon	A1
K1156	P3	Alfalfa	Corvallis, Oregon	A2
K1157	B3A	Douglas-fir	Brownsville, Oregon	DF1
K1158	B217	Douglas-fir	Brownsville, Oregon	DF1
K1159	345	Douglas-fir	Brownsville, Oregon	DF1
K1160	336	Douglas-fir	Toledo, Washington	DF2
K1161 ^c				
K1162	508	Alfalfa	Oregon	A2
K1163	510	Alfalfa	Oregon	A2
K1164	509	Alfalfa	Oregon	A2
K1165	304	Douglas-fir	Oregon	DF1
K1166	520	Douglas-fir	Brownsville, Oregon	DF2

^a All isolates were supplied by E M Hansen and P B Hamm, Oregon State University, Corvallis, Oregon, USA

^b A1, A2, DF1, DF2 were the designations of Hansen & Hamm (1983) to describe the virulence of isolates of *P. megasperma* to the host of origin (see Section 4.2.3 for further details).

^c Isolate presumed to be *P. megasperma* at the time of testing, but later identified as *Phytophthora citricola* Sawada (see Section 4.7.2).

4.2.3 Results and Discussion

The results of this study are presented in Table 12.

K1161 demonstrated a significant resistance ($P = 0.05$) to R157001 compared with the 17 remaining isolates, while the least sensitive of the latter group, K1160, was approximately twice as resistant as the most sensitive isolate, K1165. K1161 was over six times more resistant to R157001 than K1160. Additionally, the radial growth of K1161 was only inhibited by 42% at 10 μg R157001/ml, while the radial growth of remaining isolates was inhibited by over 90% at this concentration. This implied that isolates of P. megasperma with a naturally high resistance to R157001 could be detected at a potentially high frequency (6%). Before resistance was invoked as the cause of this difference in behaviour of K1161, a number of alternative explanations required examination. These included an appraisal of the experimental technique and a check on the identity of the isolate. Techniques were reviewed, and cultures of K1161 were both examined microscopically at Jealott's Hill and also sent to the Commonwealth Mycological Institute, Kew, Surrey, for identification by a Phytophthora specialist (see Section 4.7.2).

In contrast to their sensitivity to R157001, isolates displayed a relatively wide range in sensitivity to metalaxyl. K1160 was over 1000-times more resistant to the fungicide than K1155, and furthermore its radial growth was only inhibited by 54% at 100 μg metalaxyl/ml. This suggested that metalaxyl-resistant strains occurred at a higher frequency than R157001-resistant strains.

A relationship was identified in the present study between the sensitivity of isolates to metalaxyl and the isolate groups of Hansen & Hamm (1983). These authors showed that isolates of P. megasperma could be distinguished by their aggressiveness to the host of origin. Thus, one group of Douglas-fir isolates (DF1) caused extensive necrosis of main roots

Table 12 Inhibition (%) of radial growth of Phytophthora megasperma isolates by R157001 and metalaxyl

Isolate	R157001 concentration ($\mu\text{g/ml}$)							ED_{50}^x ($\mu\text{g/ml}$)
	0.03	0.1	0.3	1	3	10	30	
K1161 ^y	0	0	14	15	24	42	80	7.446 a ^z
K1160	3	3	5	41	92	97	-	1.108 b
K1150	0	0	0	0	55	91	-	1.045 bc
K1163	2	2	2	38	100	100	-	1.045 bc
K1156	0	0	2	54	96	96	-	1.045 bc
K1151	0	0	0	59	96	96	-	0.955 bcde
K1120	0	0	0	75	88	91	-	0.935 bcde
K1162	3	3	3	61	100	100	-	0.832 bcde
K1154	3	5	0	65	95	98	-	0.824 bcde
K1153	0	0	0	84	91	93	-	0.783 bcdef
K1164	0	2	5	71	100	100	-	0.730 bcdef
K1166	0	0	20	65	95	100	-	0.695 cdef
K1152	0	0	9	89	93	98	-	0.640 def
K1158	0	0	30	58	99	100	-	0.633 def
K1155	2	4	4	91	93	93	-	0.633 def
K1157	0	3	29	58	99	100	-	0.621 ef
K1159	3	4	30	59	98	100	-	0.615 ef
K1165	2	0	43	65	93	100	-	0.509 f

Isolate	Metalaxyl ($\mu\text{g/ml}$)										ED ₅₀ ($\mu\text{g/ml}$)
	0.003	0.01	0.03	0.1	0.3	1	3	10	100	1000	
K1160	-	0	4	18	33	32	39	54	54	100	5.158 a
K1161	0	0	5	7	38	52	76	79	-	-	0.866 b
K1164	-	3	13	33	50	60	70	66	77	100	0.807 b
K1162	-	0	13	35	52	65	61	70	87	100	0.665 b
K1151	0	3	17	37	54	63	71	74	-	-	0.445 bc
K1166	0	0	26	42	58	74	74	84	-	-	0.271 cd
K1156	0	5	23	43	60	70	80	88	-	-	0.246 cd
K1163	0	0	21	44	59	71	91	97	-	-	0.207 cd
K1159	0	0	9	41	67	91	94	97	-	-	0.168 d
K1157	4	7	13	37	62	96	96	96	-	-	0.156 d
K1165	0	5	8	37	75	95	95	97	-	-	0.153 d
K1158	1	3	10	46	75	90	94	96	-	-	0.140 d
K1120	3	23	43	73	97	100	-	-	-	-	0.030 e
K1154	11	31	54	69	91	97	-	-	-	-	0.026 e
K1153	8	38	48	77	97	100	-	-	-	-	0.025 e
K1152	6	35	65	77	87	100	-	-	-	-	0.020 e
K1150	16	35	52	87	94	97	-	-	-	-	0.018 e
K1155	21	50	97	100	100	100	-	-	-	-	0.005 f

^x ED₅₀-values were derived from logit relative potency analysis of the response (percent inhibition of radial growth) plotted against the dosage (log concentration of R157001).

^y Isolate presumed to be P. megasperma at the time of testing but later identified as P. citricola (see Section 4.7.2)

^z Values with no letters in common are significantly different, $P = 0.05$, according to Duncan's multiple range test.

on 2-year old Douglas-fir trees, while the less aggressive DF2 group was generally confined to smaller lateral roots. One group of alfalfa isolates (A1) caused taproot necrosis on alfalfa, killing most plants and severely affecting survivors, but was avirulent on Douglas-fir. In contrast, other alfalfa isolates (A2) were usually confined to lateral roots of alfalfa, and caused less severe infections than A1 isolates; these also caused some root rot on Douglas-fir. The same isolate groups could be distinguished by certain morphological characteristics such as length of sporangia and diameter of oogonia, and also by their sensitivity to metalaxyl. Thus, the radial growth of alfalfa A1 isolates was completely inhibited by 1 μ g metalaxyl/ml in agar. Inhibition of Douglas-fir isolates (DF1 and DF2) and alfalfa A2 isolates ranged from 44-67% at this concentration. Similarly, in the present study, alfalfa A1 isolates (K1150, K1152, K1153, K1154, K1155) were significantly more sensitive ($P = 0.05$) to metalaxyl than remaining isolates. Douglas-fir DF1 isolates (K1157, K1158, K1159, K1165) were more sensitive than Douglas-fir DF2 and alfalfa A2 isolates, and the DF2 isolate K1160 was significantly less sensitive to metalaxyl than remaining isolates. Isolates could not be differentiated into the isolate groups of Hansen & Hamm (1983) by their sensitivity to R157001, and apart from the relatively high tolerance of K1160 to both R157001 and to metalaxyl, there was no discernible relationship between the order of sensitivity of isolates to both fungicides. These data suggested that there was no cross-resistance of isolates to the benzamides and the acylalanines.

By dividing them into three distinct groups, Hunger et al. (1982) inferred that the distribution of isolates, based on metalaxyl-sensitivity, was discontinuous. The results of the present study showed that this inference could not be justified; the distribution of the same isolates in their response to both metalaxyl and R157001 (with the exception of K1161) was continuous.

4.3 P. megasperma : Pathogenicity to Alfalfa

4.3.1 Introduction

This experiment sought to determine the pathogenicity to alfalfa of isolates of P. megasperma tested in Section 4.2. The intention was to undertake an in vivo study of the fungicide sensitivity of isolates if adequate virulence was observed. By this means any correlation with their in vitro sensitivity could be determined.

4.3.2 Experimental

The procedure described in Section 2.3.2 was used to determine the virulence of isolates to alfalfa cv. Euver.

Infection of all seedlings by alfalfa A1 isolates was evident after 21 days. During the same period, Douglas-fir isolates (DF1 and DF2) and alfalfa A2 isolates caused little or no obvious damage (Table 13, Plate 3). Both groups of Douglas-fir isolates, and alfalfa A2 isolates, were considered insufficiently pathogenic to alfalfa under the conditions of the experiment, for a valid determination of their in vivo sensitivity to metalaxyl. Although alfalfa A1 isolates were clearly highly pathogenic to alfalfa seedlings, in vivo examination of the fungicide sensitivity of these isolates was also not considered to be worthwhile; only five isolates of one isolate group, which displayed a narrow range of sensitivity in vitro to metalaxyl (see Section 4.2), were suitable for testing.

No further in vivo experiments with these or other isolates of P. megasperma were undertaken.

4.4 P. infestans : In vitro Sensitivity to R157001 and Metalaxyl

4.4.1 Introduction

The apparent discovery of in vitro resistance to R157001 in an

Table 13 Disease grades^a following inoculation of alfalfa seedlings with
Phytophthora megasperma isolates

Isolate Group ^b	Isolate	Days after inoculation ^c		
		9	15	21
Alfalfa A1	K1150	4	5	6
	K1152	3	4	6
	K1153	4	4	6
	K1154	3	4	6
	K1155	4	4	6
Alfalfa A2	K1151	0	0	0
	K1156	0	0	0
	K1162	0	0	0
	K1163	0	0	0
	K1164	0	0	0
Douglas-fir DF1	K1157	1	1	1
	K1158	1	1	1
	K1159	1	1	1
	K1165	0	0	0
Douglas-fir DF2	K1160	0	0	0
	K1162	0	0	0
	K1166	0	0	0
-	K1161 ^d	0	0	0
-	Untreated	0	0	0

^a 0 = 0%, 1 = 1-14%, 3 = 35-64%, 4 = 65-84%, 5 = 85-99%
 6 = 100% diseased seedlings

^b Designation of pathogenicity to host of origin (Hansen & Hamm, 1983).

^c All treatments were scored as grade 0 at day 0

^d Isolate presumed to be P. megasperma at the time of testing but later identified as P. citricola (see Section 4.7.3).

Plate 3 Symptoms of disease 21 days following inoculation of alfalfa seedlings with Phytophthora megasperma isolates

a) Isolates K1120 to K1157

<u>Top row</u>	Isolate :	Uninoculated	K1154	K1155	K1156	K1157
Virulence group ^a :	-	A1	A1	A2	DF1	
<u>Bottom row</u>	Isolate :	K1120	K1150	K1151	K1152	K1153
Virulence group :	-	A1	A2	A1	A1	

b) Isolates K1158 to K1166

<u>Top row</u>	Isolate :	Uninoculated	K1163	K1164	K1165	K1166
Virulence group :	-	A2	A2	DF1	DF2	
<u>Bottom row</u>	Isolate :	K1158	K1159	K1160	K1161 ^b	K1162
Virulence group :	DF1	DF1	DF2	-	A2	

^a See Section 4.2.3 for explanation

^b Isolate presumed to be P. megasperma at the time of testing but later identified as P. citricola (see Section 4.7.2)



isolate of P. megasperma never previously exposed to this class of fungicide, prompted a search for similar R157001-resistant strains of P. infestans. Fungicides are used much more frequently to control this pathogen than P. megasperma, and the agrochemical industry regards potato late blight as the more important disease. A detailed investigation was undertaken of the in vitro and in vivo (see Section 4.5) sensitivity to R157001 of a large number of isolates of P. infestans, obtained worldwide. Metalaxyl was included in tests as a comparative standard. Most of the cultures eventually received originated from locations which had been, or could have been, exposed to acylalanine treatments. Some isolates held at Jealott's Hill at the commencement of this study were known to be acylalanine-resistant, and resistance amongst the new isolates was anticipated. Therefore, cross-resistance between the benzamides and acylalanines could be determined. The majority of isolates were examined in vitro only for their sensitivity to R157001 because resistance to metalaxyl had been determined for many of them already (see Table 14). However, during the sensitivity testing programme, 17 isolates were received from Peru, an area of the world which was thought to be free of acylalanine treatments. These were tested for their in vitro response to metalaxyl to determine the inherent sensitivity of isolates unlikely to have been exposed to acylalanines.

4.4.2 Materials and Methods

Most isolates of P. infestans were received as cultures on agar; a few were supplied on potato tuber slices and transferred onto agar at Jealott's Hill (Table 14). Cultures were routinely maintained on V8 agar, and/or Frozen Pea (FP)agar (see Appendix A), depending on which medium supported the better growth of individual isolates. In each experiment, fungicide was added separately to either clarified V8 or clarified FP agar (see Appendix A) at final concentrations to 0.8µg R157001/ml and 100µg

Table 14 Sources of cultures of *Phytophthora infestans*

Isolate Reference		Supplier ^a	Isolation Details			Other Details ^c
ICI	Original		Potato cv. or other host ^b	Location	Date	
<u>ENGLAND</u>						
K1183	2/1	LARS	Pentland Javelin	Avon	1981	(S)
K1184	8/1	LARS	Pentland Crown	Somerset	1981	(S)
K1185	PC 1209	LARS	(Bristol ADAS)		1981	(S)
K1186	1/2	LARS	King Edward	Avon	1981	(R) Race 4
K1187	2/8	LARS	Drayton	Avon	1981	(R) Race 4
K1188	9/5A	LARS	Maris Piper	Somerset	1981	(R) Race 4
K1189	OG050	LARS	(Ciba-Geigy, Cambridgeshire) King Edward	East Anglia	1982	(R) Races 3,4,7
K1190	OG051	LARS	(Ciba-Geigy, Cambridgeshire) Maris Piper	East Anglia	1982	(R) Races 1,3,4,10
K1191	19/3	LARS	Maris Piper	Somerset	1982	(R)
K1192	25/1A	LARS	Croft	Cornwall	1982	(R)
K1193	25/3	LARS	Wilja	Cornwall	1982	(R)
K1194	27/1A	LARS	Croft	Cornwall	1982	(R)
K1195	27/2	LARS	Romano	Cornwall	1982	(R)
K1196	27/6	LARS	Pentland Squire	Cornwall	1982	(R)
K1197	27/6A IV	LARS	Pentland Squire	Cornwall	1982	(R)
K1198	27/6A V	LARS	Pentland Squire	Cornwall	1982	(R)
K1199	34/0 I	LARS	Pentland Crown	Somerset	1982	(R)
K1200	36/0	LARS	Desiree	Avon	1982	(R)
K1201	36/1	LARS	King Edward	Avon	1982	(R)
K1202	37/0 IV	LARS	Pentland Hawk	Cornwall	1982	(R)
<u>WALES</u>						
K964	B25	UCNW			1972	Races 4,11
K1086	22 S	UCNW	Desiree	Bethesda	1978	Races 1,3,4,10,11
K1090	20 S	UCNW	Maris Peer	Bethesda	1978	Races 1,2,3,4,5,10,11
K1133		JHCC	Maris Piper	Cardiff	1982	Patafol treated
K1142		JHCC		S. Glamorgan	1982	Patafol treated

Isolate Reference		Supplier ^a	Isolation Details			Other Details ^c
ICI	Original		Potato cv. or other host ^b	Location	Date	
K1176	12/34	UCNW		Anglesey	1982	
K1177	13/14	UCNW		Anglesey	1982	
K1178	15/1	UCNW		Anglesey	1982	
K1179	29/35	UCNW		Anglesey	1982	
K1180	40/39	UCNW		Anglesey	1982	
K1181	78/4S	UCNW	Home Guard	Anglesey	1978	Race 1,2,3,10,11
K1182	78/5	UCNW	Pentland Crown	Anglesey	1978	Race 3,4,10,11
K1378	P1298	UCAL			1978	Race 10
<u>SCOTLAND</u>						
K1041		SPBS				Race 1,2,3,4,7,8,10,11
K1094		DAFS				Race 4
K1370	P1295	UCAL				Races 1,2,3,4,7
<u>NORTHERN IRELAND</u>						
K1204	1008/81	DANI	King Edward	Dungiven, Co. Londonderry	1981	(R) Fubol treated
K1205	1021/81	DANI	Pentland Dell	Portglenone, Co. Antrim	1981	(S) Fytospore and Fubol treated
K1206	1028/81	DANI	Pentland Dell	Kells, Co. Antrim	1981	(S) Dithane treated
K1207	88/81	DANI	King Edward	Boveva, Co. Londonderry	1981	(R) Dithane and Duter treated
K1208	43A/82	DANI	King Edward	Stewartstown, Co. Tyrone	1982	(R) Dithane and Duter treated
K1209	66/82	DANI	Arran Banner	Maghera, Co. Londonderry	1982	(R) Dithane treated
K1210	71/82	DANI	Kerr's Pink	Ballykeel, Co. Down	1982	(S) Dithane treated
K1211	NFC	DANI		Belfast		(S)
K1212	1029/81	DANI	Wilja	Ballymena, Co. Antrim	1981	(R) Fubol treated

Isolate Reference		Supplier ^a	Isolation Details			Other Details ^c
ICI	Original		Potato cv. or other host ^b	Location	Date	
<u>EIRE</u>						
K1067		OPRC	Kerrs Pink		1980	
K1068		ICI Ireland			1980	
K1102		OPRC			1981	
K1272	14	OPRC		Manor cunningham, Co. Donegal	1983	
K1273	20	OPRC		Piltown, Co. Kilkenny	1983	
K1274	47	OPRC		Dundalk, Co. Louth	1983	
K1275	51	OPRC		Castlebellingham, Co. Louth	1983	
K1276	56	OPRC		Kells, Co. Meath		
<u>NETHERLANDS</u>						
K1098	4	Wageningen			1980	(R)
K1099	31	Wageningen			1980	(R)
K1100	33	Wageningen			1980	(R)
K1101	37	Wageningen			1980	(R)
K1335	29	Wageningen		Weert	1980	(R) Races 4,7
K1336	102	Wageningen		Schmeeda	1981	(R) Races 1,3,4,7
K1337	207	Wageningen		Schmeeda	1981	(R) Races 1,3,4,11
K1338	247	Wageningen		Zeewolde	1981	(R) Races 4,7
K1339	285	Wageningen		Zeewolde	1981	(R) Race 4
K1340	290	Wageningen		Venray	1981	(R) Races 1,3,7,10
K1341	316	Wageningen		Willenstad	1981	(R) Race 4
K1342	390	Wageningen		Renkum	1981	(R) Races 1,3,4,7
K1343		Wageningen (Turkensteen, IPO)			1980	(S)
K1344		Wageningen (Turkensteen, IPO)			1980	(S)
K1349	99	Wageningen		Landemarke	1984	
<u>SPAIN</u>						
K1284		P Froggatt, ICI Valencia	Finca Mari	Valencia	1983	

Isolate Reference		Supplier ^a	Isolation Details			Other Details ^c
ICI	Original		Potato cv. or other host ^b	Location	Date	
USA						
K1214	102; ATCC 48716	Cornell				Race 0
K1215	103; ATCC 48717	Cornell				Race 0
K1216	104; ATCC 46301	Cornell				Race 0
K1217	106; ATCC 48719	Cornell				
K1218	111; ATCC 48720	Cornell				Race 0
K1219	112; ATCC 48721	Cornell				Race 0
K1220	127; ATCC 48723	Cornell				
K1221	128; ATCC 48724	Cornell				
K1372	P1301	UCAL	Tomato	Ventura, California	1980	Race 0
PERU						
K1294	1	IPC	Amarilla	Mayubamba, Huanuco		
K1295	2	IPC	Revolucion	Rosapampa, Huanuco		
K1296	3	IPC	Revolucion	Togropampa, Huanuco		
K1297	5	IPC	Revolucion	Ructun, Huanuco		
K1298	6	IPC	Renacimiento	Utao, Huanuco		
K1299	9	IPC	Ticahuasi	Huayllacan, Huanuco		
K1300	12	IPC	Revolucion	Huasca, Huanuco		
K1301	14	IPC	Renacimiento	Cajamarquilla, Pasco		
K1302	15	IPC	Renacimiento	Yanapampa, Pasco		
K1303	16	IPC	Casablanca	Yanapampa, Pasco		
K1304	18	IPC	Revolucion	La Victoria, Junin		
K1305	19	IPC	Mariva	La Victoria, Junin		
K1306	21	IPC	Yungay	Matahuasi, Junin		
K1307	22	IPC	Revolucion	Viscap, Junin		
K1308	27	IPC	Yungay	Chaglla, Huanuco		
K1309	29	IPC	Revolucion	Cochas, Junin		
K1310	30	IPC	Mariva	Acha, Junin		

Isolate Reference		Supplier ^a	Isolation Details			Other Details ^c
ICI	Original		Potato cv. or other host ^b	Location	Date	
<u>MEXICO</u>						
K1332	P18	Chapingo	Alpha			
K1333	P29	Chapingo	<u>Solanum ehrenbergii</u>			
K1334	P32	Chapingo	<u>Solanum cardiophyllum</u>			
K1374	P1362	UCAL (C Y Shen, BAU)		Toluca	1979	Race 1,2,3
K1375	P1363	UCAL (C Y Shen, BAU)		Toluca	1979	Race 2,3,4
K1376	P1364	UCAL (C Y Shen, BAU)		Toluca	1979	Race 1,3
K1377	P1370	UCAL (C Y Shen, BAU)		Toluca	1980	
<u>NEW ZEALAND</u>						
K1257		DSIR	Ilam Hardy			
K1350	OG3.08	DSIR		Hamilton	1984 (R)	
K1351	PU3.08	DSIR		Pukekohe	1984 (S)	
K1352	PR5 10.09	DSIR	Ilam Hardy	Pukekohe	1984	
<u>UNKNOWN ORIGIN</u>						
K966		JHOC				
K1203	KR4	Hull (Kue, Kentucky)				Race 4
K1222	RO	Hull (Kue, Kentucky)				Race 0
K1223	R4	Hull				Race 4
K1224	DC	Hull				
K1313	SCRI 20	NIAB				

^a Names of suppliers in parentheses were known primary sources of cultures. Abbreviations for names and addresses were:-

ADAS	=	Agricultural Development and Advisory Service, Bristol, England
ATCC	=	American Type Culture Collection, Rockville, Maryland, USA
BAU	=	Beijing Agricultural University, Beijing, China
Chapingo	=	Jorge Galindo A., National School of Agriculture, Chapingo, Mexico
Cornell	=	W E Fry, Cornell University, Ithaca, New York, USA
DAFS	=	S F Carnegie, Department of Agriculture and Fisheries for Scotland, Edinburgh, Scotland
DANI	=	L Cooke, Department of Agriculture for Northern Ireland, Belfast, Northern Ireland
DSIR	=	W F Hartill, Department of Scientific and Industrial Research, Auckland, New Zealand
Hull	=	J Friend, University of Hull, England
IPC	=	Carlos Martin B. International Potato Centre, Lima, Peru
IPO	=	Research Institute for Plant Protection, Wageningen, The Netherlands
JHCC	=	Jealott's Hill Research Station Culture Collection, Bracknell, Berkshire, England
LARS	=	G A Carter, Long Ashton Research Station, Bristol, England
NIAB	=	P Gans, National Institute of Agricultural Botany, Cambridge, England
OPRC	=	L J Dowley, Oak Park Research Centre, Carlow, Eire
SPBS	=	H E Stewart, Scottish Plant Breeding Station, Pentlandsfield, Edinburgh, Scotland
UCAL	=	M D Coffey, University of California, Riverside, California, USA
UCNW	=	R C Shattock, University College of North Wales, Bangor, Wales
Wageningen	=	L C Davidse, Agricultural University, Wageningen, The Netherlands

^b P. infestans was isolated from normal cultivars of S. tuberosum unless otherwise indicated.

^c Letters in parentheses indicate acylalanine sensitivity as determined by supplier where (S) = sensitive, (R) = resistant

metalaxyl/ml. Treated agar was dispensed into 9cm petri dishes. Plates were inoculated with 6mm plugs of mycelium from 10-14 day cultures and incubated at 19°C in the dark, usually for 7 days. Radial growth was determined by measuring two colony diameters per plate at 90° to each other, averaging them, and subtracting the diameter of the inoculum plug. Subsequent statistical analysis of results was undertaken as described in Section 4.2.2.

4.4.3 Results

The in vitro sensitivity of 112 isolates of P. infestans to R157001 and/or metalaxyl was examined in 10 separate experiments (Tables 15-20).

a) Sensitivity to R157001

Over half of the isolates of P. infestans were fully inhibited, and the remainder were inhibited by at least 71%, at 0.8µg R157001/ml. ED₅₀-values fell within a relatively narrow range from 0.042-0.749µg R157001/ml. In Experiment 2 (Table 15) and Experiment 8 (Table 18), 0.05µg R157001/ml was considerably more inhibitory to mycelial growth of many isolates than some higher concentrations of the chemical. In Experiment 10 (Table 20) the converse effect was observed; mycelial growth of most isolates was increased at 0.01 and 0.1µg R157001/ml. In these experiments, the isolates used as standards also gave similar atypical results, suggesting that there was some experimental variation between tests. However, further tests with the other isolates used in Experiments 2, 8 and 10 were not undertaken because there were no differences ($P = 0.05$) between the ED₅₀-values of individual isolates and remaining isolates. ED₅₀'s based on growth at several concentrations of fungicide were considered to be a more accurate guide to the relative sensitivity of isolates than their response to a single concentration of the chemicals. In seven of the eight experiments with R157001, there was less than a six-fold difference

Table 15 Inhibition (%) of radial growth of *Phytophthora infestans* isolates by R157001

Isolate	R157001 concentration (µg/ml)					ED ₅₀ ^y (µg/ml)
	0.05	0.1	0.2	0.4	0.8	
<u>EXPERIMENT 1</u>						
K1176	0	15	48	69	91	0.245 a ^z
K1094	0	10	50	73	92	0.241 a
K1178	2	20	44	63	98	0.239 a
K1098	0	0	50	93	98	0.212 ab
K1100	0	0	48	96	100	0.211 ab
K1179	7	20	45	76	96	0.207 ab
K1180	2	14	64	76	90	0.201 ab
K1101	0	0	54	100	100	0.200 ab
K1133	0	20	59	82	100	0.185 abc
K1142	0	15	68	85	100	0.182 abc
K966	2	20	67	85	98	0.171 bcd
K1090	5	27	59	83	98	0.171 bcd
K1068	2	25	80	88	100	0.157 bcde
K1086	5	18	73	94	98	0.156 bcde
K1181	18	35	60	83	100	0.144 cde
K1099	0	24	78	98	100	0.141 cde
K1102	18	47	61	82	97	0.132 de
K964	2	39	76	100	100	0.122 e
<u>EXPERIMENT 2</u>						
K1181	0	0	0	46	100	0.466 a
K1177	37	0	29	54	71	0.393 ab
K1176	59	0	43	59	77	0.380 abc
K966	47	0	28	64	81	0.340 abcd
K1142	6	2	28	57	91	0.330 abcde
K1182	47	6	55	66	80	0.274 bcdef

Isolate	R157001 concentration ($\mu\text{g/ml}$)					ED_{50}^y ($\mu\text{g/ml}$)
	0.05	0.1	0.2	0.4	0.8	

EXPERIMENT 2 (Contd)

K1194	45	0	31	81	95	0.266 cdefg
K1197	0	0	19	90	100	0.266 cdefg
K1195	34	0	55	61	93	0.264 cdefg
K1191	0	0	19	90	100	0.264 cdefg
K1184	69	22	39	68	85	0.260 cdefg
K1189	0	0	47	96	100	0.258 cdefg
K1198	0	0	35	83	100	0.254 defg
K1201	0	0	25	91	100	0.251 defg
K1183	73	20	41	55	100	0.251 defg
K1188	36	0	60	91	100	0.236 defgh
K1187	31	0	56	74	95	0.233 defgh
K1186	32	3	45	82	98	0.232 defgh
K1192	0	6	61	94	98	0.232 defgh
K1202	2	0	36	98	98	0.266 efgh
K1200	0	0	45	92	100	0.218 fgh
K1185	82	18	50	73	100	0.213 fgh
K1199	0	0	45	98	100	0.206 fgh
K1041	48	0	55	100	100	0.189 fgh
K1193	0	0	65	96	98	0.187 fgh
K1190	0	0	72	93	97	0.179 gh
K1196	5	16	66	96	100	0.163 h

EXPERIMENT 3

K1176	0	0	3	48	91	0.404 a
K1213	2	16	25	82	86	0.343 ab
K1222	0	0	34	64	100	0.295 bc
K1224	0	4	35	67	100	0.275 bcd
K1209	0	0	41	72	100	0.252 cde
K1207	2	15	40	66	100	0.249 cde
K1203	0	10	35	75	100	0.246 cde

Isolate	R157001 concentration (µg/ml)					ED ₅₀ ^y (µg/ml)
	0.05	0.1	0.2	0.4	0.8	
<u>EXPERIMENT 3 (Contd)</u>						
K1210	2	7	48	73	98	0.230 cdef
K1280	0	7	47	70	100	0.227 def
K1212	0	6	52	81	100	0.205 efg
K1204	0	0	50	83	96	0.199 efg
K1206	6	20	50	76	100	0.198 efg
K1223	0	24	50	74	100	0.197 efg
K1205	0	17	55	88	100	0.183 fg
K1211	7	26	70	81	100	0.160 g
<u>EXPERIMENT 4</u>						
K1213	2	10	22	46	76	0.402 a
K1220	12	0	34	64	74	0.324 ab
K1216	0	11	21	55	100	0.316 bc
K1219	0	16	22	57	96	0.302 bcd
K1067	0	0	29	82	100	0.271 bcd
K966	0	9	34	69	100	0.266 bcd
K1214	0	18	29	82	100	0.263 bcd
K1215	0	16	39	65	94	0.260 cd
K1207	11	18	33	58	98	0.252 d
K1218	0	14	40	71	93	0.246 d
K1142	2	16	58	78	100	0.195 e
K1176	0	18	63	78	100	0.190 e
K1221	9	27	56	71	96	0.188 e
K1217	2	16	55	97	98	0.176 e

^y ED_{50} -values were derived from logit relative potency analysis of the response (percent inhibition of radial growth) plotted against the dosage (log concentration of R157001)

^z Values with no letter in common are significantly different, $P = 0.05$, according to Duncan's multiple range test.

Table 16 Inhibition (%) of radial growth of *Phytophthora infestans* isolates by R157001 (Experiment 5) and metalaxyl (Experiment 6)

Isolate	R157001 concentration ($\mu\text{g/ml}$)					ED_{50}^x ($\mu\text{g/ml}$)
	0.05	0.1	0.2	0.4	0.8	
K1303	0	0	0	46	86	0.470 a ^y
K1297	0	0	11	43	83	0.445 ab
K1300	0	0	24	52	87	0.372 bc
K1220	0	6	33	53	75	0.363 bc
K1305	0	0	26	62	87	0.344 cd
K1217	0	0	27	62	95	0.324 cde
K1299	4	11	28	61	89	0.299 cdef
K1296	5	17	24	51	88	0.295 cdefg
K1306	0	9	35	65	96	0.281 defgh
K1295	21	8	29	60	83	0.281 defgh
K1176	0	0	56	75	78	0.268 defgh
K1301	0	0	34	74	100	0.267 defgh
K1302	2	12	29	66	98	0.261 efgh
K1309	14	17	34	63	91	0.258 efghi
K1294	0	10	45	69	93	0.244 fghi
K1298	10	13	53	63	80	0.243 fghi
K1308	0	5	36	82	100	0.236 fghij
K1304	5	22	51	68	80	0.228 ghij
K1307	0	0	59	78	97	0.222 hijk
K1272	0	10	63	78	90	0.210 ijkl
K1284	0	8	48	92	100	0.206 ijkl
K966	0	9	66	84	97	0.188 jklm
K1275	0	0	78	89	95	0.177 klmn
K1276	3	3	83	91	97	0.167 lmn
K1273	0	3	89	97	94	0.154 mn
K1310	17	29	67	81	98	0.152 mn
K1274	0	11	94	97	97	0.139 n

Isolate	Metalaxyl ($\mu\text{g/ml}$)						ED ₅₀ ($\mu\text{g/ml}$)	
	0.001	0.01	0.1	1	10	100		
K1294	-	-	0	0	0	17	ND ^z	
K1304	-	-	11	5	2	23	ND	
K1310	-	-	6	2	12	46	240	a
K1309	-	-	0	18	13	50	187	a
K1296	-	-	14	7	28	33	176	a
K1307	-	-	9	0	25	49	89	ab
K1302	-	-	4	4	49	55	48	abc
K1133	-	-	7	29	57	38	31	bcd
K1303	0	0	15	10	60	-	12	cde
K1297	-	-	9	26	71	63	8.67	de
K1306	-	-	0	31	64	75	8.61	de
K1298	-	-	28	28	72	75	2.74	ef
K1300	-	-	17	36	77	75	2.62	ef
K1305	-	-	0	58	78	69	2.34	ef
K1295	-	-	22	44	74	71	2.34	ef
K1301	-	-	20	55	82	73	1.26	fg
K1299	-	-	33	63	77	77	0.69	fg
K1308	0	15	53	70	68	-	0.35	gh
K1220	0	26	67	81	79	-	0.09	h
K1217	0	63	89	93	96	-	0.009	i
K966	11	61	95	93	98	-	0.004	i

^x ED₅₀-values were derived from logit relative potency analysis of the response (percent inhibition of radial growth) plotted against the dosage (log concentration of R157001).

^y Values with no letter in common are significantly different, $\underline{p} = 0.05$, according to Duncan's multiple range test.

^z ND = Value could not be determined because of inadequate inhibition of radial growth by metalaxyl.

Table 17 Inhibition (%) of radial growth of *Phytophthora infestans* isolates by R157001 and metalaxyl (Experiment 7)

Isolate	R157001 concentration ($\mu\text{g/ml}$)					ED_{50}^y ($\mu\text{g/ml}$)
	0.05	0.1	0.2	0.4	0.8	
K1332	17	14	31	9	31	0.749 a ^z
K1334	22	25	16	28	97	0.287 b
K1133	6	13	41	74	100	0.164 c
K1335	0	0	32	97	100	0.164 c
K1342	0	2	57	98	98	0.137 cd
K1142	11	30	45	84	100	0.121 cde
K1337	0	0	77	100	100	0.116 cdef
K1340	0	0	81	98	98	0.115 cdef
K1220	47	42	51	70	98	0.092 cdefg
K1344	46	48	46	75	96	0.088 cdefg
K1338	16	19	97	100	100	0.076 defgh
K1339	14	26	97	100	100	0.069 efgh
K1343	59	59	56	77	100	0.063 fgh
K1272	65	59	61	83	100	0.053 gh
K1341	50	15	95	100	100	0.046 h
K1336	71	66	50	95	100	0.045 h
K1333	73	73	67	80	98	0.042 h

Isolate	Metalaxyl ($\mu\text{g/ml}$)						ED ₅₀ ($\mu\text{g/ml}$)	
	0.001	0.01	0.1	1	10	100		
K1340	0	0	2	5	0	7	$>10^5$	a
K1342	5	0	0	0	0	7	$>10^5$	ab
K1335	0	0	0	0	3	18	$>10^5$	bc
K1337	0	0	0	0	9	18	$>10^5$	c
K1133	5	8	6	10	16	10	$>10^5$	d
K1338	0	0	9	22	28	38	41000	e
K1339	23	14	23	29	26	46	504	f
K1334	41	50	47	47	44	41	44	fg
K1332	14	40	40	40	40	51	17	gh
K1341	15	25	65	70	40	55	1.29	h
K1344	50	48	68	71	82	90	0.001	i
K1220	53	49	70	77	84	86	<0.001	ij
K1272	46	52	80	83	87	91	<0.001	ij
K1336	61	61	79	79	89	89	<0.001	jk
K1343	52	61	81	85	93	93	<0.001	jk
K1333	71	71	87	89	91	93	<0.001	k

^y ED₅₀-values were derived from logit relative potency analysis of the response (percent inhibition of radial growth) plotted against the dosage (log concentration of R157001).

^z Values with no letter in common are significantly different, $\underline{p} = 0.05$, according to Duncan's multiple range test.

Table 18 Inhibition (%) of radial growth of *Phytophthora infestans* isolates by R157001 and metalaxyl (Experiment 8)

Isolate	R157001 concentration ($\mu\text{g/ml}$)					ED_{50}^x ($\mu\text{g/ml}$)	ED_{90} ($\mu\text{g/ml}$)
	0.05	0.1	0.2	0.4	0.8		
K1350	0	0	0	37	100	0.506	2.031 a ^y
K1352	63	0	0	0	100	0.412	1.652 ab
K1296	0	0	45	64	100	0.275	1.102 abc
K1351	0	30	28	85	100	0.274	1.099 abc
K1133	0	8	46	86	100	0.215	0.861 abc
K1374	28	0	13	97	100	0.210	0.842 bcd
K1376	51	0	9	84	100	0.209	0.840 bcd
K1370	68	0	32	64	98	0.182	0.729 cd
K1373	62	0	0	100	100	0.179	0.720 cd
K1375	26	0	58	95	95	0.161	0.646 cd
K964	76	0	38	76	100	0.145	0.582 cd
K1272	55	20	61	88	100	0.112	0.448 d
K1372	36	0	97	100	100	0.101	0.405 d
K1349	45	7	97	100	100	0.094	0.377 d
K1377	62	16	66	100	100	0.093	0.374 d

Isolate	Metalaxyl concentration ($\mu\text{g/ml}$)						ED ₅₀ ($\mu\text{g/ml}$)	ED ₉₀ ($\mu\text{g/ml}$)
	0.001	0.01	0.1	1	10	100		
K1296	0	0	0	0	0	0	ND ^z	ND
K1133	0	2	6	8	11	3	14934	>10 ⁵ a
K1350	0	0	0	0	67	74	29.532	17229 b
K1351	0	0	0	15	68	73	21.473	12526 b
K1372	0	6	50	56	61	61	2.084	1216 c
K1349	0	24	48	55	72	72	0.746	435 cd
K1374	0	44	47	53	63	78	0.534	311 cd
K1376	4	42	56	58	65	69	0.310	181 d
K1375	0	26	47	58	79	89	0.261	153 d
K1352	0	0	75	81	75	75	0.224	131 d
K1272	0	45	64	64	66	70	0.215	125 d
K1377	0	57	81	79	85	85	0.026	15 e
K1370	0	40	87	87	100	100	0.025	14 e
K964	0	60	83	86	86	81	0.020	12 e
K1373	0	46	77	85	92	100	0.019	11 e

^x ED₅₀ and ED₉₀-values were derived from logit relative potency analysis of the response (percent inhibition of radial growth) plotted against the dosage (log concentration of R157001).

^y Values with no letter in common are significantly different, $P = 0.05$, according to Duncan's multiple range test.

^z ND = Value could not be determined because of inadequate inhibition of radial growth by metalaxyl.

Table 19 Inhibition (%) of radial growth of *Phytophthora infestans* isolates by metalaxyl (Experiment 9)

Isolate	Metalaxyl ($\mu\text{g/ml}$)						^x ED ₅₀ ($\mu\text{g/ml}$)
	0.001	0.01	0.1	1	10	100	
K1142	0	0	2	0	0	0	ND ^z
K1296	0	0	2	0	0	0	ND
K1309	0	0	0	0	0	4	ND
K1133	0	10	2	11	6	8	ND
K1273	6	0	6	13	6	0	87532 a ^y
K1295	0	2	0	0	48	43	1421 b
K1275	0	17	28	22	22	17	590 c
K1272	0	30	52	48	68	84	0.54 d
K1220	5	24	76	70	73	78	0.31 d
K1090	7	51	66	66	66	68	0.15 d
K966	0	36	71	75	75	68	0.12 d

^x ED₅₀-values were derived from logit relative potency analysis of the response (percent inhibition of radial growth) plotted against the dosage (log concentration of R157001).

^y Values with no letter in common are significantly different, $P = 0.05$, according to Duncan's multiple range test.

^z ND = Value could not be determined because of inadequate inhibition of radial growth by metalaxyl.

Table 20 Inhibition (%) of radial growth of *Phytophthora infestans* isolates by R157001 (Experiment 10)

Isolate	R157001 concentration ($\mu\text{g/ml}$) :				
	0.01	0.1	1	10	100
K1332	0(+54) ^a	0(+23)	51	100	100
K1296	0(+6)	3	100	100	100
K1142	0(+19)	0(+30)	100	100	100
K1217	0(+29)	0(+33)	100	100	100
K1220	0(+11)	0(+25)	100	100	100
K1333	0(+3)	0(+31)	100	100	100
K1334	0(+11)	0(+29)	100	100	100

^a Figures in parentheses indicate percentage increase in radial growth

in the ED₅₀-values of the least sensitive (K1350) and the most sensitive (K1377) isolates. Even allowing for variation between individual experiments, as indicated by values obtained for the standard isolates, this range contrasted markedly with an 18-fold difference between the least (K1332) and most (K1333) sensitive isolates observed in Experiment 7 (Table 17). This was due to the resistance of two isolates, K1332 and K1334, which differed significantly ($P = 0.05$) from each other and from the other 15 isolates. A further experiment (Experiment 10, Table 20), confirmed the resistance of K1332, but not of K1334, to R157001. The mycelial growth of K1332 was reduced to 51% at 1µg R15001/ml whereas the other six isolates were fully inhibited at this concentration. However, K1332 was itself fully inhibited at 10µg R157001/ml, indicating that its resistance to the chemical was limited.

The radial growth of some isolates was increased by low doses (around 1µg/ml) of R157001. This effect is shown for two isolates (K1296 and K1376) in Plate 4.

In one experiment (Experiment 8, Table 18) ED₉₀-values were determined for the inhibition of isolates by R157001. These revealed that the radial growth of isolates was almost completely inhibited at concentrations which were approximately four-times higher than those which inhibited growth by 50%. This result indicated that R157001 was active over a relatively narrow range of concentrations, and inhibited radial growth to a high degree at low doses.

b) Sensitivity to metalaxyl

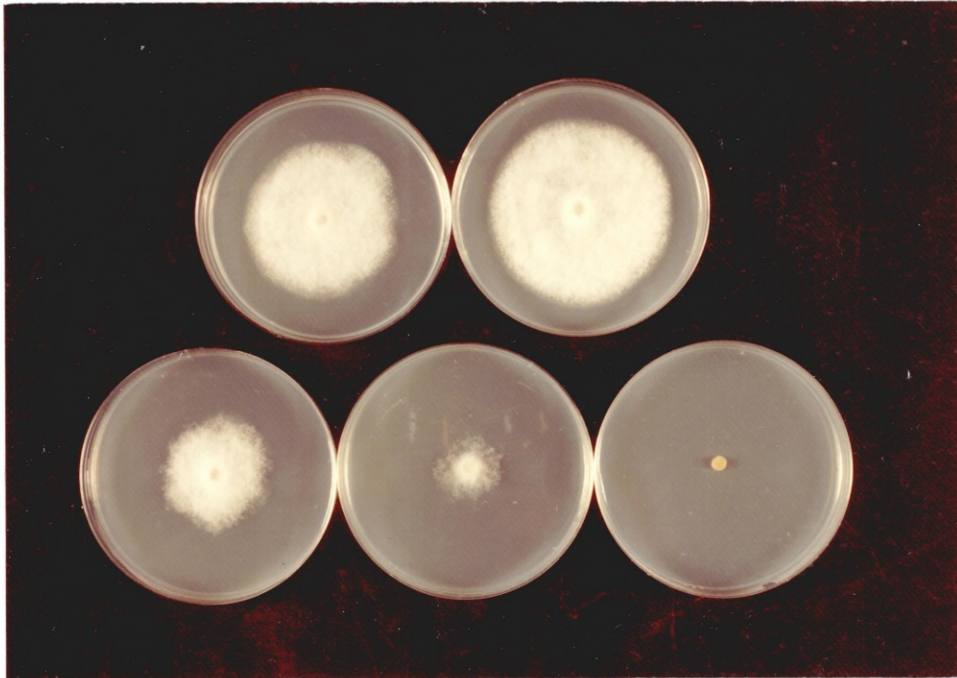
The in vitro sensitivity of 51 isolates of P. infestans to metalaxyl was examined in Experiments 6 - 9 (Tables 16-19).

In Experiment 8, the growth of two isolates (K1133 and K1296) was largely unaffected by 100µg metalaxyl/ml. These isolates were clearly resistant to metalaxyl in vitro. Of the remaining isolates, only two (K1370 and K1373) were completely inhibited at this concentration. The

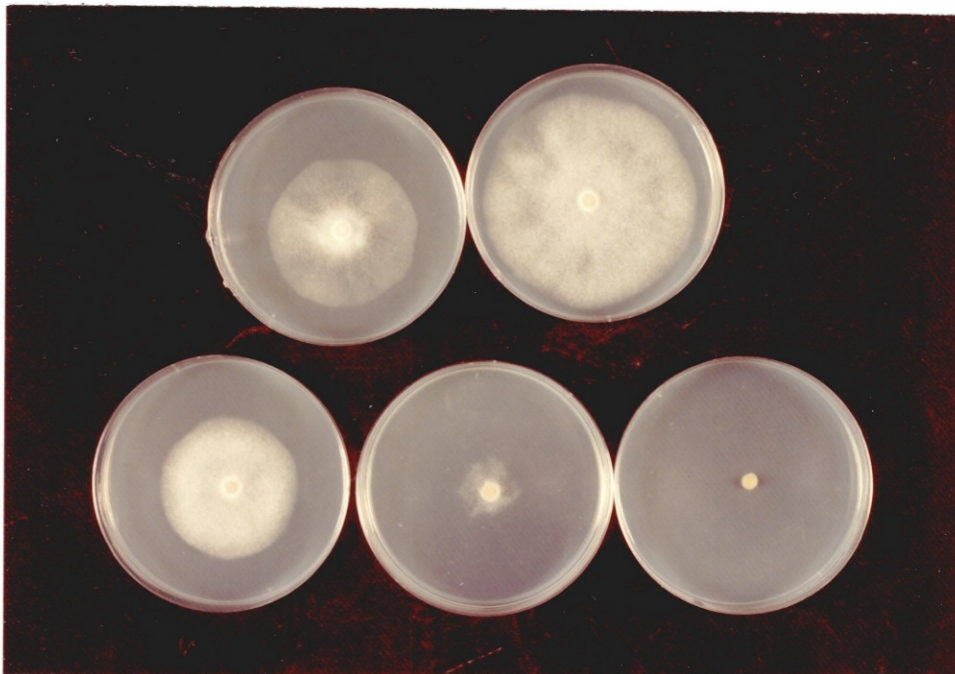
Plate 4 Inhibition of radial growth of isolates of *Phytophthora*
infestans by R157001

Top row (left to right) : 0, 0.1 $\mu\text{g/ml}$
Bottom row (left to right) : 0.2, 0.4, 0.8 $\mu\text{g/ml}$

a) K1296



b) K1376



others showed varying degrees of inhibition from 61-89%. Other isolates which appeared to be highly resistant to metalaxyl (less than 10% inhibition at 100µg metalaxyl/ml in at least one experiment) were K1340 and K1342 (Experiment 7, Table 17), and K1142, K1273 and K1309 (Experiment 9, Table 19). Additional examples of resistant isolates (ED_{50} -values greater than 10,000µg metalaxyl/ml in at least one experiment) included K1294, K1304, K1335, K1337, K1338, K1350 and K1351. Remaining isolates displayed intermediate resistance or were relatively sensitive, in vitro, to metalaxyl. Examples of highly sensitive isolates (ED_{50} -values less than 1µg metalaxyl/ml in at least one experiment) included K966, K1090, K1217, K1220, K1272, K1299, K1308, K1333, K1336, K1343, K1344. Examples of the response of sensitive (K1370) and resistant (K1296) isolates to metalaxyl are shown in Plate 5, where an increase in the radial growth of the resistant isolate on metalaxyl-amended medium is also illustrated. Similar effects were observed in some other isolates, particularly at lower concentrations (around 0.1µg metalaxyl/ml). Generally, a continuum of sensitivity to metalaxyl was apparent between highly sensitive and highly resistant isolates, despite ED_{50} -values between the two extremes differing by a factor of several thousands. A 600-times difference between ED_{50} and ED_{90} -values for the inhibition of mycelial growth of isolates was determined in one experiment (Experiment 8, Table 18). From this result it was concluded that relatively high concentrations of metalaxyl would be required to completely prevent mycelial growth, compared with those concentrations which inhibited growth by 50%.

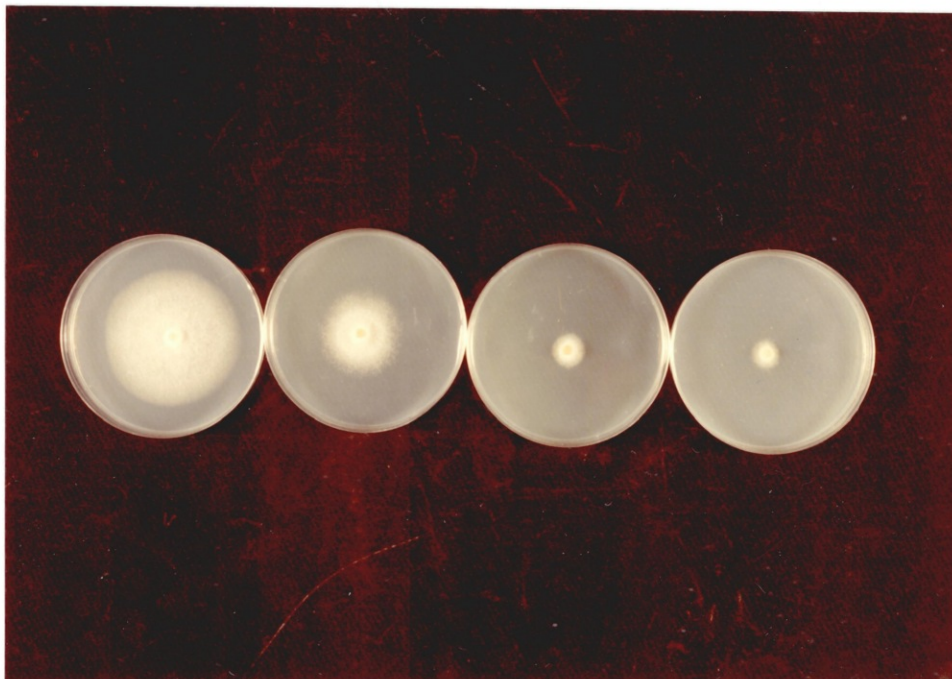
4.4.4 Discussion

No isolates with a high degree of resistance to R157001 were detected even though many of them displayed resistance to metalaxyl. This contrasted with the apparent detection of a naturally resistant strain of

Plate 5 Inhibition of radial growth of isolates of *Phytophthora*
infestans by metalaxyl

Left to right : 0, 0.1, 1, 10 $\mu\text{g/ml}$

a) K1370 (metalaxyl-sensitive)



b) K1296 (metalaxyl-resistant)



P. megasperma in Section 4.2. It was concluded that there was no cross-resistance amongst isolates to R157001 and metalaxyl. Also, it appeared that the benzamides might be at a lower risk than metalaxyl from the development of resistance. However, the high frequency of metalaxyl-resistant isolates in the present study was probably due, in part, to their selection under field conditions prior to isolation. There had been no previous selection pressure for benzamide-resistance. It was possible that a much larger number of isolates would have to be examined for their sensitivity to R157001 before it could be concluded that benzamide-resistant strains of P. infestans were unlikely to occur under field and glasshouse conditions.

Metalaxyl was shown to be relatively poor at causing complete inhibition (as determined by ED₉₀-values) of any isolate. This suggested that the chemical was fungistatic over a wide range of concentrations because true fungicidal behaviour would be manifested as complete inhibition. Conversely, R157001 caused complete inhibition as its concentration was increased by relatively small amounts from non-inhibitory doses. The implication of these observations for the field performance of R157001 and metalaxyl is discussed in Section 4.5.4.

4.5 P. infestans : In vivo Sensitivity to R157001 and Metalaxyl

4.5.1 Introduction

Although measuring mycelial growth on agar is a relatively easy method of determining the sensitivity of a target fungus to a fungicide, it cannot be assumed that the results will necessarily coincide with those obtained in vivo, as past experience with metalaxyl and P. infestans shows (Staub et al., 1979; Staub & Sozzi, 1984). Thus, an in vivo study with R157001 and metalaxyl was undertaken in parallel with the in vitro study

described in Section 4.4. As with the latter, the aims were to establish whether there was cross-resistance to R157001 by metalaxyl-resistant strains, and to seek strains naturally resistant to R157001.

Bioassays to detect acylalanine resistance in P. infestans on potatoes have been successfully conducted on whole plants (Dowley & O'Sullivan, 1981; Hartill et al., 1983; Cohen & Reuveni, 1983), detached leaves (Cooke, 1981; Cohen & Reuveni, 1983; Holmes & Channon, 1984), and leaf discs (Davidse et al., 1981; Carter et al., 1982; Cohen & Reuveni, 1983).

The leaf disc method was adopted in the present study. Only a relatively small quantity of leaf tissue was required for each experiment, and preliminary tests indicated that the technique gave reliable results.

4.5.2 Materials and Methods

Each isolate of P. infestans was grown on V8 or FP agar for 10 days. The mycelium was covered with chilled deionised water, and the surface of the culture rubbed thoroughly with a glass rod to release sporangia, which were harvested by filtration through a layer of muslin. The number of sporangia in the suspension was estimated from counts using an Improved Neubauer haemocytometer. The sporangial suspension was adjusted to 30,000 sporangia/ml before placing it at 10°C for about 2h to encourage zoospore differentiation and release.

R157001 and metalaxyl were prepared separately in deionised water at concentrations to 100µg/ml and 10ml aliquots of each concentration were dispensed into petri dishes (5cm diameter). Dishes containing deionised water alone, served as untreated controls. Five discs (12mm diameter) were cut from leaves of potato cv. Majestic grown in a growth room (16h photoperiod [6000 lux], 21°C day, 18°C night). The leaf discs were floated abaxial surface uppermost on the test solution in each petri

dish. They were inoculated centrally with a 10 μ l droplet of sporangial suspension, using a micropipette, and incubated for 7 days in the growth room described as above. The severity of infection by P. infestans on each leaf disc was determined by the following scale (Carter et al., 1982) :-

- 0 = no visible symptoms
- 1 = necrotic flecks under inoculation droplet
- 2 = necrosis confluent under droplet and extending slightly beyond
- 3 = area of discolouration spreading from droplet and extending to as much as half disc diameter
- 4 = weakly necrotic area covering most of disc. Some sporulation
- 5 = heavy sporulation over entire leaf disc

The typical symptom represented by each grade is shown in Plate 6. A completed bioassay is illustrated in Plate 7.

4.5.3 Results

The sensitivity of 77 isolates of P. infestans to R157001 and metalaxyl in vivo was determined in 11 experiments.

A further 35 isolates could not be evaluated because 11 of them failed to sporulate on agar, and 24 of them failed to produce adequate infection on untreated leaf discs, or leaf discs treated with low concentrations of either R157001 or metalaxyl. Attempts to improve the pathogenicity of the latter isolates by passaging them through tuber slices were largely unsuccessful; the cultures obtained still did not produce sufficient symptoms.

For each isolate, the grades of five replicate leaf discs per treatment were averaged to give a disease severity index (DSI) (Table 21). Dosage-response curves, based on the DSI's of isolates in individual

Plate 6 Symptoms of infection by *Phytophthora infestans* of leaf discs of potato cv Majestic. Represented as a disease grading ^a, 7 days after inoculation

Top row : 0 1 2

Bottom row : 3 4 5

^a See text for details of scale

Plate 7 Infection of potato (cv. Majestic) leaf discs floating on fungicide solutions, by a metalaxyl-resistant isolate of *Phytophthora infestans*, K1197

Top row : 0.016 0.08 0.4 2 100 µg metalaxyl/ml

Middle-left dish : Untreated control

Bottom row : 0.39 1.56 6.25 25 100 µg R157001/ml

Note the satisfactory control of disease by low doses of R157001. Inadequate control was achieved by high doses of metalaxyl.

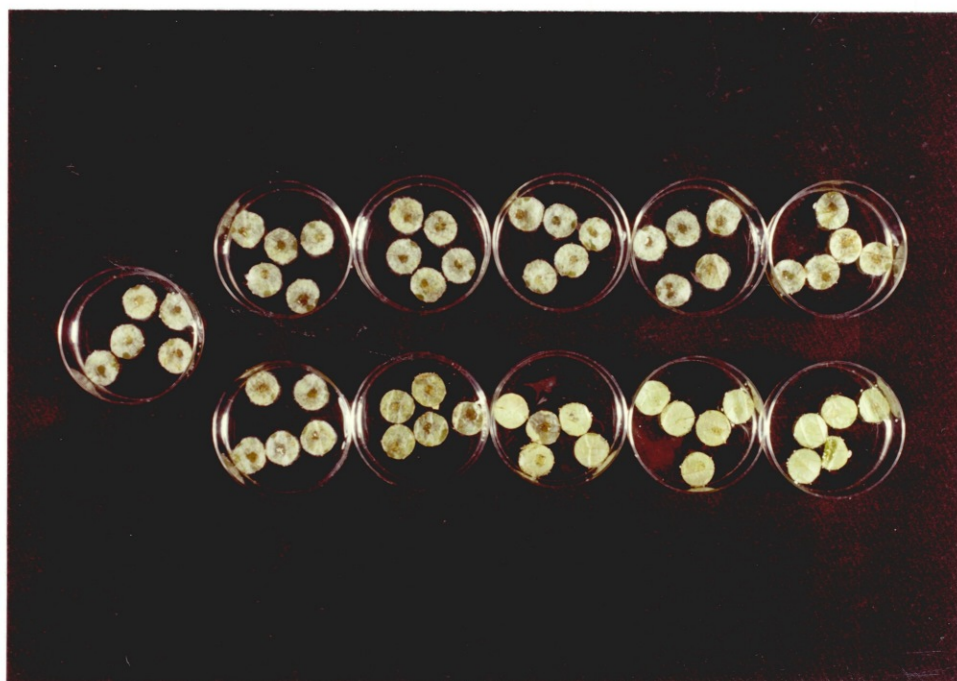
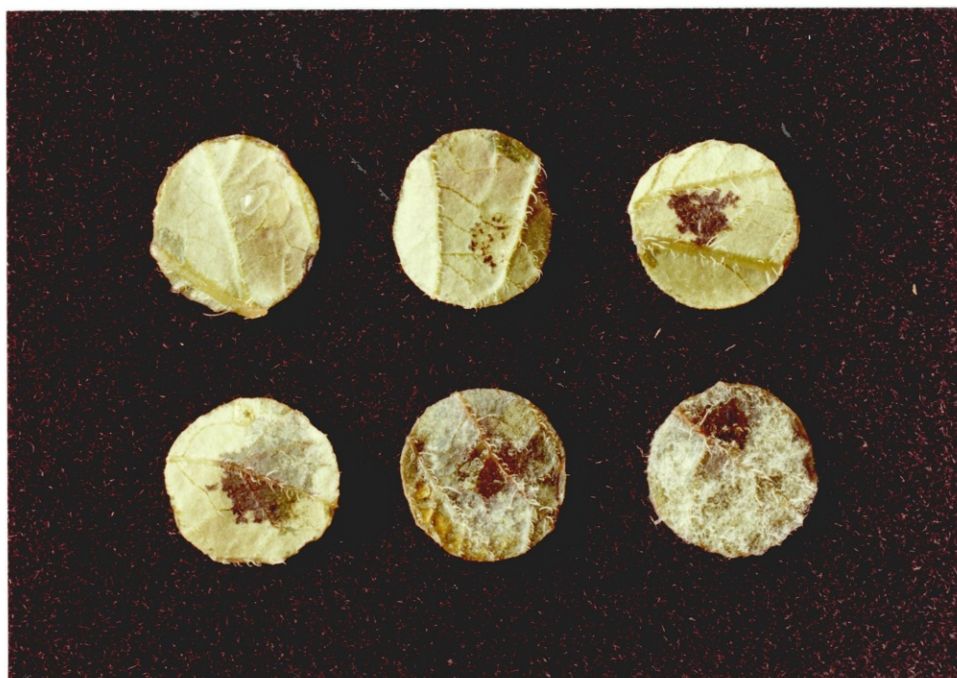


Table 21 Disease severity indices^a following infection by *Phytophthora infestans* isolates of potato (cv. Majestic) leaf discs treated with R157001 and metalaxyl^b

Isolate	Untreated		R157001				Metalaxyl					Metalaxyl sensitivity ^c
		0.39	1.56	6.25	25	100	0.016	0.08	0.4	2	100	
<u>ENGLAND</u>												
K966	5.0	5.0	5.0	1.8	1.4	1.0	5.0	3.8	1.8	1.8	1.8	S
K1183	5.0	5.0	3.6	2.2	1.4	0.6	5.0	3.8	3.0	2.0	1.8	S
K1184	5.0	5.0	4.8	1.8	0.8	0.0	4.8	3.6	2.2	1.8	0.0	S
K1186	5.0	5.0	4.8	1.2	0.6	0.4	5.0	5.0	5.0	4.0	5.0	R
K1187	5.0	5.0	4.6	1.4	1.0	0.0	5.0	5.0	5.0	5.0	5.0	R
K1188	5.0	5.0	3.0	1.2	1.2	0.0	5.0	5.0	4.8	5.0	5.0	R
K1189	5.0	4.2	1.0	0.8	0.2	0.2	4.6	5.0	4.0	5.0	0.8	s1.R
K1190	5.0	5.0	1.2	0.4	0.2	0.0	4.0	5.0	5.0	2.0	5.0	R
K1191	5.0	5.0	3.2	1.8	0.4	0.4	5.0	5.0	5.0	5.0	5.0	R
K1192	5.0	4.0	3.4	2.4	1.0	1.2	5.0	4.0	5.0	5.0	5.0	R
K1193	5.0	5.0	3.2	2.4	0.6	0.4	4.6	4.0	5.0	4.0	4.8	R
K1194	5.0	5.0	4.4	0.8	0.6	0.2	5.0	3.6	2.8	2.2	2.0	S
K1195	5.0	5.0	4.0	1.4	0.6	0.2	5.0	5.0	5.0	5.0	5.0	R
K1196	5.0	5.0	2.4	1.2	0.6	0.6	5.0	5.0	4.0	5.0	4.6	R
K1197	5.0	5.0	5.0	1.8	0.8	0.0	5.0	5.0	5.0	5.0	5.0	R
K1198	5.0	5.0	3.4	1.4	0.8	0.4	5.0	4.0	5.0	5.0	5.0	R
K1199	5.0	4.0	4.6	1.4	0.6	0.6	5.0	5.0	5.0	4.0	5.0	R
K1200	5.0	5.0	5.0	1.6	0.6	0.2	5.0	5.0	5.0	5.0	5.0	R
K1201	5.0	5.0	4.4	1.8	0.4	0.0	5.0	5.0	5.0	5.0	5.0	R
K1202	5.0	5.0	1.2	0.6	0.4	0.6	5.0	2.8	4.6	5.0	4.6	R
<u>WALES</u>												
K1086	5.0	5.0	4.6	1.8	1.0	0.0	5.0	3.2	1.0	0.8	0.8	S
K1090	2.4	1.4	1.2	0.2	0.2	0.0	1.6	0.6	1.0	0.4	0.2	ND
K1133	5.0	5.0	3.4	2.0	1.0	0.0	5.0	5.0	5.0	5.0	5.0	R
K1142	5.0	5.0	4.2	1.0	1.0	0.0	5.0	5.0	5.0	5.0	4.8	R
K1176	5.0	5.0	5.0	0.6	1.0	0.8	5.0	2.6	1.2	1.2	1.2	S
K1177	5.0	5.0	2.6	1.0	1.0	0.8	5.0	3.2	1.2	1.4	1.2	S
K1178	5.0	5.0	4.8	1.0	1.0	0.6	4.6	1.8	1.2	1.0	1.8	S

Isolate	Untreated		R157001				Metalaxyl					Metalaxyl sensitivity ^c
		0.39	1.56	6.25	25	100	0.016	0.08	0.4	2	100	
<u>WALES (Contd)</u>												
K1179	5.0	5.0	5.0	1.6	1.0	0.8	5.0	3.2	2.2	1.2	0.8	S
K1180	3.2	3.8	4.2	0.2	0.2	0.4	4.2	0.8	1.2	0.6	0.4	S
K1181	0.8	0.6	1.0	0.8	1.0	1.0	0.8	0.8	0.2	0.2	0.6	ND
K1182	5.0	5.0	3.4	0.8	0.2	0.0	5.0	3.0	1.0	0.8	0.8	S
K1378	3.2	— ^d	—	—	—	—	—	—	—	—	—	ND
<u>SCOTLAND</u>												
K1094	4.0	5.0	2.0	0.2	0.2	0.4	3.8	1.2	0.8	0.8	0.4	S
<u>NORTHERN IRELAND</u>												
K1204	5.0	5.0	4.8	1.0	1.2	0.2	5.0	5.0	5.0	5.0	5.0	R
K1205	5.0	5.0	3.4	1.0	0.8	0.6	1.2	4.6	1.0	0.6	1.0	S
K1206	4.0	4.2	3.8	0.6	0.6	0.6	0.4	2.2	0.4	0.8	0.6	S
K1207	5.0	5.0	5.0	1.4	0.6	0.6	5.0	5.0	5.0	5.0	5.0	R
K1208	5.0	5.0	4.0	1.2	1.0	0.8	5.0	5.0	5.0	5.0	5.0	R
K1209	5.0	5.0	4.0	1.2	0.8	0.6	5.0	5.0	5.0	5.0	5.0	R
K1210	5.0	5.0	0.4	0.6	0.4	0.4	1.0	0.6	0.4	0.8	1.0	S
K1211	5.0	3.0	2.8	0.8	0.6	0.6	1.0	1.2	0.4	0.6	1.2	S
K1212	5.0	5.0	4.2	1.0	0.4	0.4	5.0	5.0	5.0	5.0	5.0	R
<u>EIRE</u>												
K1067	5.0	5.0	4.2	1.6	0.6	0.4	5.0	5.0	5.0	5.0	4.6	R
K1068	5.0	5.0	5.0	1.8	1.0	1.0	5.0	5.0	5.0	5.0	5.0	R
K1272	5.0	5.0	4.0	1.4	0.8	0.8	5.0	4.8	1.8	0.8	0.8	S
K1273	5.0	4.0	4.2	0.6	0.4	0.4	5.0	4.6	1.6	1.0	0.8	S
K1274	4.0	3.0	3.6	0.4	0.4	0.6	5.0	0.4	0.8	0.8	1.0	S
K1275	5.0	5.0	4.0	1.4	0.8	1.0	5.0	4.4	3.4	2.0	1.0	S
K1276	5.0	5.0	3.4	2.0	1.0	1.0	5.0	5.0	3.4	1.2	1.4	S

Isolate	Untreated	R157001					Metalaxyl					Metalaxyl sensitivity ^c
		0.39	1.56	6.25	25	100	0.016	0.08	0.4	2	100	
<u>NETHERLANDS</u>												
K1098	5.0	5.0	4.0	2.8	0.4	0.0	4.0	5.0	5.0	5.0	5.0	R
K1100	5.0	5.0	4.8	2.8	1.0	0.6	5.0	5.0	5.0	5.0	5.0	R
K1101	1.2	0.8	1.0	0.8	0.8	0.8	0.8	0.8	0.2	0.8	0.6	ND
K1335	5.0	5.0	4.6	2.2	0.0	0.0	5.0	4.6	5.0	5.0	4.8	R
K1336	4.0	3.0	3.8	3.6	2.0	1.2	2.8	1.8	0.6	0.4	0.6	S
K1338	4.0	3.0	3.0	2.0	0.2	0.0	4.8	3.2	3.2	4.0	4.0	R
K1339	5.0	1.0	1.1	0.2	0.2	0.2	5.0	4.0	1.0	0.2	0.6	S
K1341	5.0	4.2	3.0	0.8	0.8	1.0	5.0	4.2	4.8	1.6	1.0	S
K1342	0.4	0.6	3.4	1.4	0.8	1.0	1.8	0.8	0.6	0.4	1.6	ND
K1344	2.0	1.2	1.4	0.4	0.6	0.4	1.2	0.8	0.8	1.0	0.8	ND
K1349	5.0	5.0	4.2	1.0	1.0	1.0	4.0	1.8	0.6	0.8	0.6	S
<u>SPAIN</u>												
K1284	4.0	4.0	3.2	1.6	1.0	1.0	5.0	2.6	1.0	1.0	1.0	S
<u>USA</u>												
K1214	3.4	4.0	4.6	0.6	1.2	0.6	2.0	1.0	0.8	0.8	0.4	S
K1215	5.0	5.0	4.4	2.4	1.4	1.2	5.0	3.8	2.4	1.8	1.2	S
K1216	5.0	5.0	5.0	1.6	1.2	0.4	3.2	2.2	1.8	1.4	1.6	S
K1217	5.0	5.0	3.6	1.2	0.8	0.8	4.2	1.6	1.4	1.0	0.4	S
K1218	4.8	5.0	3.2	1.0	0.8	1.4	3.0	1.4	1.4	1.2	0.6	S
K1219	0.8	1.2	0.2	0.4	0.2	0.6	0.2	0.4	0.0	0.2	0.4	ND
K1220	5.0	5.0	5.0	1.8	1.0	0.8	5.0	4.4	1.6	1.0	1.2	S
K1221	5.0	5.0	5.0	1.2	1.0	1.0	5.0	3.4	1.6	1.0	0.8	S
K1372	0.8	—	—	—	—	—	—	—	—	—	—	ND

Isolate	Untreated	R157001					Metalaxyl					Metalaxyl sensitivity ^c
		0.39	1.56	6.25	25	100	0.016	0.08	0.4	2	100	
<u>PERU</u>												
K1294	5.0	5.0	4.0	2.6	1.0	0.8	5.0	5.0	5.0	4.0	5.0	R
K1295	5.0	5.0	4.0	3.4	1.0	0.8	5.0	5.0	5.0	4.0	1.6	s1.R
K1296	5.0	5.0	3.6	1.2	1.0	1.0	5.0	5.0	5.0	5.0	5.0	R
K1297	5.0	5.0	4.0	2.2	0.4	0.8	5.0	5.0	5.0	5.0	5.0	R
K1298	1.8	2.0	0.2	0.2	0.2	0.6	1.6	0.6	1.4	0.8	0.4	ND
K1299	5.0	3.0	3.6	0.8	0.8	1.0	5.0	5.0	5.0	5.0	0.8	s1.R
K1300	4.0	4.0	2.8	1.8	0.2	0.2	4.0	2.2	4.2	1.0	0.6	S
K1301	4.0	4.0	0.8	0.4	0.2	0.0	5.0	1.0	3.0	1.4	0.4	S
K1302	5.0	5.0	4.0	2.0	1.0	1.0	5.0	4.1	5.0	5.0	1.0	s1.R
K1303	3.0	3.0	2.2	0.4	0.2	0.4	4.0	5.0	3.2	3.2	1.0	S
K1304	2.0	3.0	0.0	2.2	0.2	0.0	2.2	0.0	1.9	2.0	2.6	ND
K1305	5.0	4.0	5.0	2.8	1.0	0.8	5.0	5.0	5.0	5.0	1.0	s1.R
K1306	5.0	5.0	4.6	1.0	1.0	0.2	5.0	5.0	5.0	4.2	1.2	s1.R
K1307	4.0	5.0	3.6	1.8	0.6	0.6	4.0	3.0	2.0	1.0	3.6	S
K1308	2.6	0.4	0.4	0.2	0.2	0.2	0.2	0.0	0.0	0.0	0.0	ND
K1309	4.0	4.0	4.0	0.4	0.2	0.4	4.0	4.0	2.4	0.2	0.2	S
K1310	5.0	4.4	1.4	1.0	0.4	0.6	3.0	2.0	2.0	1.8	1.8	S
<u>MEXICO</u>												
K1332	0.4	1.2	0.8	0.6	0.8	1.0	1.0	0.6	0.4	0.2	0.0	ND
K1333	0.6	0.4	0.4	0.6	0.4	0.0	1.0	0.4	0.2	0.2	0.0	ND
K1334	0.6	0.4	0.4	0.2	0.4	0.2	0.4	0.4	0.4	0.4	0.2	ND
K1375	0.4	-	-	-	-	-	-	-	-	-	-	ND
K1376	1.0	-	-	-	-	-	-	-	-	-	-	ND
K1377	0.6	-	-	-	-	-	-	-	-	-	-	ND

Isolate	Untreated	R157001					Metalaxyl					Metalaxyl sensitivity ^c
		0.39	1.56	6.25	25	100	0.016	0.08	0.4	2	100	
<u>NEW ZEALAND</u>												
K1257	1.4	1.0	1.0	1.0	1.2	1.0	1.0	1.0	1.0	1.2		ND
K1350	2.4	-	-	-	-	-	-	-	-	-		ND
K1351	2.8	-	-	-	-	-	-	-	-	-		ND
K1352	1.6	-	-	-	-	-	-	-	-	-		ND
<u>UNKNOWN ORIGIN</u>												
K1222	3.4	1.0	0.2	0.0	0.0	0.0	0.0	0.4	0.2	0.0	0.0	ND
K1223	0.4	1.8	0.6	0.6	0.6	0.4	0.4	0.6	0.6	0.4	0.6	ND
K1224	0.6	0.8	1.0	0.8	1.0	0.8	1.0	0.8	0.8	0.8	1.0	ND
K1213	5.0	4.0	2.2	0.2	0.4	0.8	2.2	1.0	0.8	0.0	0.2	S

^a See Section 4.5.2 for explanation of disease severity index (DSI)

^b Results of separate experiments

^c Determined from dosage-response curves (see, for example, Figure 7)
S = sensitive, sl.R = slightly resistant, R = highly resistant to metalaxyl
ND = not determined

^d Not tested

experiments, were drawn for those isolates which gave a DSI of at least 4 on untreated leaf discs. With some isolates, leaf discs treated with the lowest concentrations of either R157001 or metalaxyl had a DSI of at least 4 whereas the corresponding untreated discs had a DSI of less than 4.

Dosage-response curves for these isolates were also drawn.

a) Sensitivity to metalaxyl

From the slope of its dosage-response curve (see, for example, Figure 7a), each isolate was assigned to one category of sensitivity as follows:-

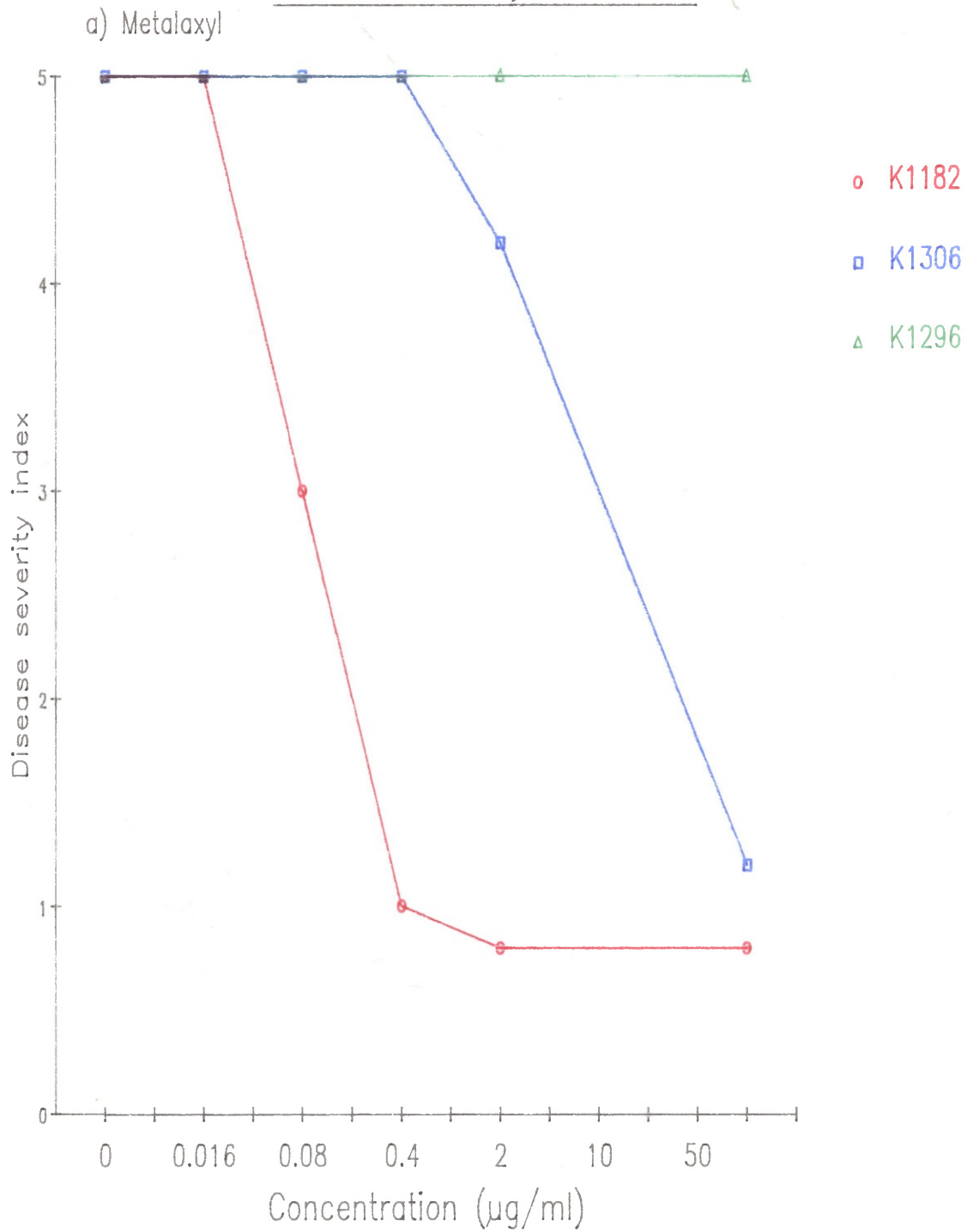
- i) sensitive to metalaxyl : DSI less than 4 at 2µg metalaxyl/ml
- ii) slightly resistant to metalaxyl : DSI at least 4 at 2µg metalaxyl/ml and less than 4 at 100µg metalaxyl/ml
- iii) highly resistant to metalaxyl : DSI at least 4 at 100µg metalaxyl/ml

On this basis, 40 isolates of P. infestans were "sensitive", six were "slightly resistant" and 31 were "highly resistant". Resistant isolates were identified from several geographically separate locations, for example, the United Kingdom, Northern Ireland, Eire, The Netherlands and Peru. However, the effect of acylalanine treatments on the frequency with which they occurred could not be deduced from the relatively small numbers of isolates obtained from each area of the world.

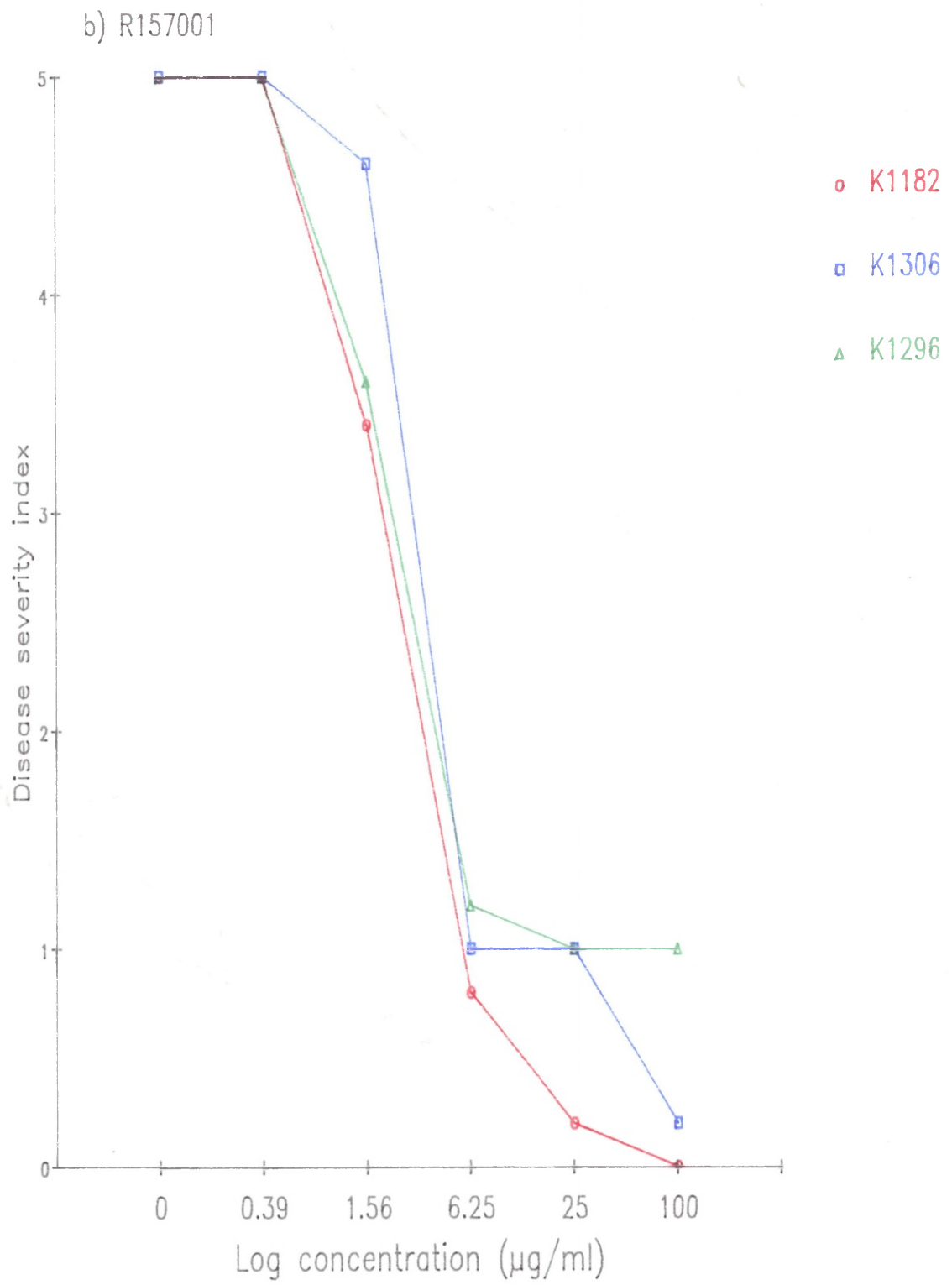
Five of the 14 isolates from Peru were categorised as "slightly resistant" to metalaxyl. Only one isolate of the remaining 62 isolates (K1189 from England), was similarly "slightly resistant". From this it was concluded that isolates of P. infestans with intermediate levels of resistance to metalaxyl might exist at a higher frequency in Peru than in other parts of the world.

All but one isolate (K1184) of the 40 isolates which were designated as "sensitive" to metalaxyl caused limited necrosis, near the inoculum droplet, on leaf discs treated with 100µg metalaxyl/ml. This

Figure 7 *In vivo* response of isolates of *Phytophthora infestans* to metalaxyl and R157001



Results of separate experiments



Results of separate experiments

indicated that inhibition of these isolates probably occurred after penetration of the host tissue.

b) Sensitivity to R157001

The sensitivity of isolates to R157001 was determined on a similar basis to their sensitivity to metalaxyl. From the slope of its dosage-response curve (see, for example, Figure 7b) each isolate which demonstrated a DSI of less than 4 at 5 μ g R157001/ml was designated as "benzamide-sensitive". Conversely, a DSI of at least 4 at 5 μ g R157001/ml meant that the isolate was "benzamide-resistant".

None of the 77 isolates of P. infestans successfully tested in vivo was resistant to R157001. The dosage-response of isolates was steep, usually ranging from no inhibition at 0.39 μ g R157001/ml to complete or almost complete inhibition at 6.25 or 25 μ g R157001/ml. There were no symptoms of infection by 14 isolates at 100 μ g/ml and by one of these (K1335) at 25 μ g/ml R157001. The remaining 63 isolates caused limited necrosis of leaf discs near the inoculum droplet, and this was reflected by little or no change in the DSI from 6.25 or 25 μ g/ml to 100 μ g/ml R157001. For these isolates, it was concluded that R157001 only inhibited development of the fungus once it had penetrated the host tissue.

c) Validity of procedure for classifying isolates

Statistical analyses of DSI's could not be undertaken in the present study due to the variability between some replicate treatments. However, the procedure for classifying isolates based on symptoms of infection appeared to be reliable. This was deduced from a comparison of the results of the present study, and those of original studies (see Table 14) with the same isolates. Only four isolates (K1194, K1336, K1339 and K1341) from a total of 36 tested for their in vivo response to metalaxyl gave opposing results. The four isolates which gave ambiguous results were designated as sensitive in the present study whereas originally they were recorded as being resistant. It was possible that these strains, on

fungicide-free media, had been selected from an initially mixed population of sensitive and resistant strains.

4.5.4 Discussion

The 37 isolates of P. infestans which were slightly or highly resistant to metalaxyl in vivo were controlled by R157001 to the same degree as metalaxyl-sensitive strains. This result indicated different modes of action for the two types of chemical. It also established that there would be no cross-resistance, under field and glasshouse conditions, to the benzamides and acylalanines. Clearly, the benzamides offered an alternative to acylalanines for the control of P. infestans, especially where acylalanine-resistant strains were present.

The detection of resistant isolates of P. infestans in locations as far apart as Peru and Europe provided further evidence that such strains existed naturally before the introduction of acylalanines.

Necrotic lesions resulted from the penetration by P. infestans of potato leaf tissue treated with high concentrations of metalaxyl. The same effects were reported by Staub et al. (1980) with P. viticola on grape leaves and P. infestans on tomato leaves treated with metalaxyl. It was argued from these results that host plant reactions were contributing to the inhibition and death of the fungal hyphae inside the host tissue. Commenting on this report, Fisher & Hayes (1982) considered that host response was unlikely to play more than a minor role in overcoming fungal attack. This opinion was based on their finding that metalaxyl was highly toxic in vitro to species of Phytophthora and Pythium. However, in another study of the in vivo response of P. infestans to metalaxyl, one isolate was completely inhibited by 10µg/ml on potato cultivar Arran Victory, and yet as much as 300µg/ml was required to reduce sporulation on cultivar Kerr's Pink (Coffey & Young, 1984). This result suggested that a stronger host resistance reaction was triggered in Arran Victory treated with metalaxyl.

Further evidence for a host reaction initiated by the presence of metalaxyl was the discovery of an isolate of P. infestans which was unaffected by the chemical in vitro by as much as 350µg/ml, yet in vivo it was controlled by 1µg/ml (Bruck et al., 1980). Similar results were obtained with five isolates (K1273, K1275, K1309, K1310 and K1339) in the present study. The ED₅₀-values for the in vitro inhibition of mycelial growth were greater than 100µg metalaxyl/ml (Section 4.4.3), yet these isolates were designated "sensitive" to metalaxyl in vivo (Section 4.5.3).

In contrast to these results, the in vitro and in vivo response to metalaxyl of other isolates often coincided. Some isolates which were amongst the most highly resistant to metalaxyl in vitro (K1133, K1142, K1294, K1296, K1335 and K1338) were also resistant to the chemical in vivo. Similarly, five isolates (K966, K1217, K1220, K1272, K1336) which were amongst the most sensitive to metalaxyl in vitro, were also sensitive to the chemical in vivo. The results supported the claim by Coffey & Bower (1984) that the major effect of a systemic fungicide such as metalaxyl was in the prevention of mycelial growth within the host plant. It is not yet known if the in vitro and in vivo response to metalaxyl coincides for isolates of other members of the Peronosporales.

The in vitro (Section 4.4) and in vivo (this study) response of isolates to R157001 appeared to coincide. As with metalaxyl, this indicated that the primary effect of R157001 was in the prevention of mycelial growth of the fungus within its host.

It was demonstrated that R157001 was more inhibitory in vitro at high concentrations (around the ED₉₀-value) than metalaxyl (Section 4.4). Similarly, 100µg R157001/ml prevented symptoms of infection on potato leaf tissue by more isolates (14) than 100µg metalaxyl/ml (one isolate), out of a total of 77 isolates. These results indicated that R157001 was likely to be more effective than metalaxyl at inhibiting the development of infection by P. infestans. Other isolates caused necrosis of leaf discs treated with

high concentrations of R157001. A host resistance mechanism may be involved here, which is similar to that proposed to explain the response of isolates to metalaxyl. This finding would be clarified by analysis of the chemical composition of affected cells.

4.6 P. infestans : In vivo Sensitivity of Field Isolates to R157001 and Metalaxyl

4.6.1 Introduction

A severe late blight epidemic caused by metalaxyl-resistant strains of P. infestans resulted in considerable crop losses for many potato growers in the Netherlands during 1980 (Davidse et al., 1981). 'Ridomil' (a commercial formulation of metalaxyl) had been used to control the pathogen. During the same season, and prompted by reports of resistance, a survey for metalaxyl-resistant strains of P. infestans was undertaken in south-west England (Carter et al., 1982). All of the isolates tested proved to be highly sensitive to metalaxyl. Furthermore, the incidence of disease was very low in crops treated with the only acylalanine-containing product available, 'Fubol' (a commercial formulation of metalaxyl plus mancozeb). During the following (1981) growing season, acylalanine-resistant strains of P. infestans were detected during field surveys in England and Wales (Carter et al., 1982) and Scotland (Holmes & Channon, 1984). In these countries, good control of both sensitive and resistant strains was attributed to the effect of the residual partner in the commercial fungicide mixtures.

The surveys of Carter et al. (1982) and Holmes & Channon (1984) led the latter authors to propose that acylalanine-resistant strains were present in the endemic population of P. infestans. This would account for their detection over a wide area in a short period of time. Both sets of authors concluded that the wide incidence of resistant strains could not

have been caused simply by wind dispersal of sporangia or distribution of infected seed tubers.

Field surveys of the occurrence of resistant strains of P. infestans since the commercial introduction of acylalanine-containing fungicides have necessarily involved the testing of isolates which could have been subjected to these fungicides. Therefore, they cannot provide unequivocal data about the origins and frequency of resistant strains. With the benzamides, a unique opportunity was presented to determine the base-line sensitivity of field populations of P. infestans to a new type of fungicide which had not yet been introduced into commercial use. Consequently, during the 1983, 1984 and 1985 growing seasons, it was arranged that samples of potato late blight would be collected from field infections in the United Kingdom and sent to Jealott's Hill. Here, samples were tested for their in vivo sensitivity to R157001 and metalaxyl (to identify acylalanine-resistant strains).

4.6.2 Materials and Methods

Samples of potato late blight were collected by ICI Plant Protection Division sales and technical personnel. Foliage bearing young, freshly sporulating lesions was selected and placed in a clean polythene bag. This was inflated, sealed and placed in a strong cardboard box. Samples were posted by first class mail or Datapost to Jealott's Hill with details of the crop history. Infected tubers were wrapped in paper and posted in a strong box. Occasionally, samples were brought to Jealott's Hill by hand.

On arrival in the laboratory, each sample was placed in a seed tray (57 x 29 x 8cm) lined with moist blotting paper, which was then enclosed in a polythene bag. This was incubated in a growth room (16h photoperiod [6000 lux], 21°C day, 18°C night) for 24 or 48 h to encourage sporulation of P. infestans. Sporangia were gently removed from the

foliage using a dissecting needle and transferred to a few ml of chilled, deionised, water. A count was made of the number of sporangia using an Improved Neubauer haemocytometer, and the suspension was adjusted to 30,000 sporangia/ml. Potato leaf discs floating on aqueous solutions of R157001 (5µg/ml) and metalaxyl (2 and 100µg/ml) were each inoculated with 10µl droplets of the sporangial suspension. This procedure, and the subsequent conditions under which discs were incubated was described in Section 4.5.2.

4.6.3 Results and Discussion

The origin of isolates of P. infestans obtained from the field during the 1983-5 growing seasons in the United Kingdom is shown in Table 22. During 1983 a total of 22 samples of potato foliage and one tuber (no. 22) infected with P. infestans were received. Five of these (nos. 3, 6, 8, 11 and 18) were in very poor condition and attempts to recover the pathogen failed. P. infestans from four other samples (nos. 1, 14, 17 and 20), also in poor condition, was passaged through fresh, detached potato leaves. The aim was to increase the quantity of inoculum for sensitivity tests. However, isolates from these samples subsequently failed to infect leaf discs adequately and were lost. It was determined that this loss of infectivity was due to bacteria and/or leaf exudates washed from the leaves into the sporangial inoculum, which inhibited the germination of the sporangia. Because of this, isolates received during 1984 and 1985 were passaged, when necessary, through tuber slices instead of detached leaves. The inoculum obtained in this way was free of contaminants, and no isolates were lost.

The number of samples received during the three growing seasons was lower than had been expected, given that the network of sales and technical personnel extended throughout the United Kingdom. There were probably several reasons for this. First, a low incidence of late blight

Table 22 Potato late blight samples collected from field infections in the United Kingdom during 1983-5

Sample Number	Date Sampled	Location	Potato cultivar	% blight ^a	Fungicide treatments ^b
<u>1983</u>					
1	14 June	Lakenheath, Suffolk	Pentland Crown	0.1	Patafol
2	15 June	Rendlesham, Suffolk	Pentland Javelin	Not known	Fubol
3	17 June	Spalding, Lincs	Maris Piper	0.5	Patafol, Maneb
4	23 June	Romney Marsh, Kent	Wilja	5.0	Patafol
5	23 June	Romney Marsh, Kent	Pentland Javelin	20.0	Patafol
6	28 June	Soham, Cambs	Maris Piper	0.5	Fubol, Dithane, Maneb
7	29 June	Bury St Edmunds, Suffolk	Maris Piper	5.0	Patafol, Sanspor
8	29 June	Ely, Cambs	Maris Piper	0.5	Fubol, Maneb
9	30 June	Peterborough, Lincs	Maris Piper	0.1	Sanspor, Brestan
10	30 June	Peterborough, Lincs	Maris Piper	1.0	Patafol, Sanspor, Maneb
11	27 July	Grantham, Lincs	King Edward	5.0	Patafol
12	3 August	Thorney, Cambs	Maris Piper	2.0	Patafol, Fubol
13	3 August	Thorney, Cambs	Maris Piper	5.0	Patafol, Fubol
14	4 August	Lincoln	King Edward	0.1	Patafol, Ourzate
15	4 August	Lincoln	King Edward	0.1	Patafol, Ourzate
16	8 August	Blunham, Beds	Wilja	1.0	Patafol, Duter
17	1 September	Alton, Hants	Maris Piper	0.1+pf	Patafol, Maneb, Supertin
18	4 September	Knutsford, Cheshire	Wilja	Not known	Sanspor
19	5 September	Kings Lynn, Norfolk	Maris Piper	20.0	Patafol
20	5 September	Wisbech, Cambs	King Edward	Not known	Patafol
21	7 September	Kinross, Edinburgh	Wilja	0.1	Patafol
22	7 September	Kinross, Edinburgh	Wilja	1.0	Patafol
<u>1984</u>					
1	7 August	Holbeach, Lincs	Maris Piper	5.0	Patafol Plus
2	8 August	South Kyme, Lincs	Wilja	5.0	Fubol
3	16 August	Kings Lynn, Norfolk	Maris Piper	1.0	Patafol Plus, Sanspor
4	18 August	Wisbech, Cambs	Wilja	1.0	Patafol Plus, Dithane
<u>1985</u>					
1	10 July	Kings Lynn, Norfolk	Bintje	1.0	Patafol Plus, Sanspor, Brestan
2	11 July	Penzance, Cornwall	Maris Bard	1.0	Patafol Plus
3	11 July	Penzance, Cornwall	Clustar	1.0	Patafol Plus
4	15 July	Kings Lynn, Norfolk	Homeguard	5.0	Patafol Plus
5	17 July	Ely, Cambs	Maris Piper	10.0	Patafol Plus
6	17 July	Bumt Fen, Norfolk	Maris Piper	4.0	Patafol Plus, Captafol
7	22 July	Ely, Cambs	Pentland Javelin	5.0	Fubol
8	23 July	Methwold, Norfolk	Maris Piper	2.0	Patafol Plus
9	14 August	Ely, Cambs	Maris Piper	0.01	Patafol Plus, Captafol
10	19 August	Looe, Cornwall	Pentland Hawk	0.01	Patafol Plus

^a Determined according to the assessment scale devised by The British Mycological Society (1947)
pf = primary focus of infection

^b 'Patafol (Plus)' and 'Fubol' are acylalanine-containing products.
'Ourzate' is the trade name of cyanoacetamide-oxime.
Remaining products contained residual fungicides as the sole active ingredient.

was observed during the hot, dry summers of 1983 and 1984. This also limited the number of forays undertaken by the author for diseased material. Second, there was often a reticence by sales and technical personnel to seek late blight infections actively, particularly when there were no complaints by users about the performance of the ICI products, 'Patafol' (1983) or 'Patafol Plus' (1984 and 1985), commercial formulations of ofurace and a dithiocarbamate, vondozeb. Third, the acylalanine-containing products demonstrated good control of late blight, even during the very wet summer of 1985.

The results of the present study are shown in Table 23. This reveals that 13 samples in 1983, four in 1984, and 10 in 1985, were successfully tested. Most of these originated from eastern counties of England. Two samples came from Scotland (1983) and three from south-west England (1985). The majority of samples were obtained from crops which had been treated with 'Patafol' or 'Patafol Plus'. This coincided with the extent of the contact that sales and technical personnel had with users of these ICI products.

Assessment of the sensitivity of isolates to R157001 and metalaxyl was based on the procedure described in Section 4.5.2. Isolates were differentiated as follows:-

- i) sensitive to R157001 or metalaxyl : DSI less than 4 at 5µg R157001/ml or 2µg metalaxyl/ml
- ii) slightly resistant to metalaxyl : DSI at least 4 at 2µg metalaxyl/ml and less than 4 at 100µg metalaxyl/ml
- iii) resistant to metalaxyl : DSI at least 4 at 100µg metalaxyl/ml
- iv) resistant to R157001 : DSI at least 4 at 5µg R157001/ml

On this basis, none of the isolates tested were resistant to R157001. Additionally, none of the isolates tested in 1983 and 1984 were resistant to metalaxyl. In 1985, three isolates (nos. 2, 8, 10) were clearly resistant to metalaxyl. One isolate (no. 1) gave an ambiguous

Table 23 Disease severity indices^a following infection by *Phytophthora infestans* isolates^b of potato leaf discs treated with R157001 and metalaxyl

Isolate ^c	Untreated discs	Fungicide (µg/ml)			Metalaxyl sensitivity ^d
		R157001	Metalaxyl		
		5	2	100	
1983					
2	4.0	0.4	0.2	1.8	S
4	5.0	1.0	0.4	1.0	S
5	4.0	0.0	0.2	0.0	S
7	5.0	0.4	1.6	1.2	S
9	5.0	0.0	1.4	0.6	S
10	5.0	0.2	0.0	0.2	S
12	5.0	0.8	2.0	1.4	S
13	5.0	0.0	0.2	0.4	S
15	5.0	0.0	0.0	0.0	S
16	5.0	0.6	1.4	1.2	S
19	5.0	1.0	1.8	1.6	S
21	5.0	0.0	0.0	0.0	S
22	4.0	0.2	0.0	0.2	S
1984					
1	5.0	1.2	1.0	0.8	S
2	5.0	0.8	0.8	0.0	S
3	4.6	0.0	0.4	0.0	S
4	5.0	0.2	0.8	1.0	S
1985					
1	5.0	0.6	1.6	3.0	S/R ^e
2	5.0	1.0	5.0	5.0	R
3	5.0	1.0	1.2	1.0	S
4	5.0	1.0	1.0	1.0	S
5	5.0	1.4	2.0	2.0	S
6	5.0	1.2	2.0	2.0	S
7	5.0	1.2	1.0	1.0	S
8	5.0	1.0	5.0	5.0	R
9	5.0	1.0	1.2	1.2	S
10	4.2	0.2	5.0	4.4	R

^a Mean of five replicates. Graded as follows:-
 0 = no infection; 1,2,3, = necrosis within area of inoculum droplet, extending slightly beyond inoculum droplet, extending to half disc diameter, respectively; 4,5 = discolouration plus sporulation over most, and the whole, of the leaf disc, respectively

^b Obtained from infected potato crops in the United Kingdom (see Table 22)

^c Isolates were evaluated in separate experiments, in which known metalaxyl-sensitive and resistant isolates were included as standards

^d S = sensitive, R = resistant to metalaxyl, according to the assessment scale described in Section 4.5.2.

^e Isolate which gave variable results

result which was due to variability between replicates. A similar result was obtained when the test with this isolate was repeated. It was possible that a mixed population of sensitive and resistant sporangia was responsible for the complete infection of some metalaxyl-treated leaf discs, and the poor infection of others.

In the present study, the detection of metalaxyl-resistant strains of P. infestans in 1985 but not in previous years does not necessarily indicate that such strains may now be detected more readily in the United Kingdom. Insufficient numbers of isolates were received to determine the frequency of such strains. For the same reason, the existence of strains which are naturally resistant to R157001 cannot be excluded.

4.7 P. megasperma : In vitro Sensitivity to R157001 and Metalaxyl-2

4.7.1 Introduction

A request for cultures of P. megasperma in addition to those already tested (Section 4.2) resulted in the provision by E M Hansen and P B Hamm of Oregon State University of a further 27 isolates which had never previously been exposed to metalaxyl under field and glasshouse conditions. These were examined for their in vitro sensitivity to R157001 and metalaxyl alongside selected isolates from the first experiment. Isolates were sought which were inherently resistant to R157001, as K1161 appeared to have been in Section 4.2.

4.7.2 Experimental

Details of test isolates are provided in Tables 11 and 24. Experimental procedures were described previously in Section 4.2.2.

a) Sensitivity to R157001

K1161 was significantly more resistant ($P = 0.05$) to R157001

Table 24 Sources of isolates of *Phytophthora megasperma*^a

Isolate Reference		Host	Location	Other Details
ICI	Original			
K1228	-	Cabbage	Oregon	
K1229	-	Cauliflower	Oregon	
K1230	29-4-1	(W Wilcox) Apricot	California	
K1231	WC-1	(J Stack) White Cockle	New York	
K1234	24-4-9	(S Mircetich) Apple	California	
K1235	13-1-5	(S Mircetich) Lilac	California	
K1236	088	(Jeffers) Apple	New York	
K1237	24-3-8	(S Mircetich) Kiwi	California	
K1238	Car 2 (86)	(J Stack) Wild Carrot	New York	
K1239	411	(C R Grau) Soybean	Wisconsin	Race 8
K1240	PB	(Y Nagai) Rose	Japan	
K1241	PA	(Y Nagai) Rose	Japan	
K1242	T47; IMI 147131	(CMI) Apple	New Zealand	
K1243	T56; IMI 56348	(CMI) <u>Brassica</u> sp.	United Kingdom	
K1244	107	(R G Pratt) Clover	Mississippi	
K1245	909	(C R Grau) Soybean	Wisconsin	Race 3
K1246	908	(C R Grau) Soybean	Wisconsin	Race 1
K1247	19-2-1; K 1	(S Mircetich) Grape soil	Napa County, California	
K1248	12-3-1; K3	(S Mircetich) Almond	Red Bluff, California	
K1249	6-1-9; K1	(S Mircetich) Mahaleb cherry	Stockton, California	
K1250	T28; ATCC 28765	(ATCC) <u>Populus robusta</u>	United States	
K1251	275 C-1	(S Mircetich) Cherry	California	
K1252	029	(Jeffers) Apple	New York	
K1253	075	(Jeffers) Apple	New York	Race 8
K1254	4-1.5	(S Mircetich) Apricot	California	
K1255	055	(Jeffers) Apple	New York	
K1256	20-3-9	(Jeffers) Apple	New York	

^a All isolates were supplied by E M Hansen & P B Hamm, Oregon State University, Corvallis, Oregon, USA
Names in parentheses were known primary sources of cultures
CMI = Commonwealth Mycological Institute, Kew, Surrey, England
ATCC = American Type Culture Collection, Rockville, Maryland, USA

than 33 other isolates (Table 25), including six tested previously in Section 4.2. There was slightly more than a four-fold difference in ED_{50} values ($0.325 - 1.341 \mu\text{g/ml}$) between the least and the most sensitive of the group of 33 isolates, and a seven-fold difference ($1.341 - 9.614 \mu\text{g/ml}$) between K1161 and the least sensitive isolate of this group, K1254.

Over a third of the isolates were completely inhibited by $10 \mu\text{g}$ R157001/ml and the others were inhibited by at least 86%. All except K1161 responded to R157001 over a relatively narrow range of concentrations. This was exemplified by six isolates (K1164, K1236, K1240, K1241, K1244, K1245) which were not inhibited at one concentration of R157001, but were highly inhibited (>84%) by a three-fold increase in concentration. The comparative response of three isolates (K1161, K1234 and K1240) to R157001 is shown in Plate 8. From these results it was concluded that no other isolates apart from K1161 were resistant in vitro to R157001. R157001 at relatively low concentrations ($1 \mu\text{g/ml}$) caused macroscopically visible colony deformations in some isolates, for example K1165 (Plate 9).

The colony morphology and ultrastructure of the resistant isolate, K1161, was compared with other isolates included in this study; K1161 produced radiate growth with narrow lanceolate-shaped sectors, whereas remaining isolates were slightly radiate with more abundant aerial hyphae (see, for example, Plate 10). Sporangia of K1161 were frequently distorted in shape, and no proliferation of sporangiophores inside empty sporangia was evident. In other isolates, the sporangia were regularly ovoid, and internal proliferation was common, both characteristics of P. megasperma (Waterhouse & Waterston, 1966). The suspicion that K1161 was an incorrectly-labelled species of Phytophthora was confirmed when it was subsequently identified at the Commonwealth Mycological Institute as Phytophthora citricola Sawada. It remained to be demonstrated that other

Table 25 Inhibition (%) of radial growth of *Phytophthora megasperma*
isolates by R157001

Isolate ^x	R157001 concentration (µg/ml)						ED ₅₀ ^y (µg/ml)
	0.1	0.3	1	3	10	30	
K1161	–	0	9	22	31	40	9.614 a ^z
K1254	0	0	62	90	95	91	1.341 b
K1253	0	0	35	95	100	100	1.250 bc
K1255	0	4	56	85	93	93	1.168 bcd
K1252	0	0	59	93	93	93	1.114 bcd
K1256	0	5	46	95	95	95	1.104 bcd
K1250	0	0	52	88	88	94	1.079 bcde
K1228	0	3	55	93	95	98	1.061 bcdef
K1120	0	11	75	86	89	89	0.993 bcdefg
K1234	0	10	48	95	98	98	0.973 bcdefg
K1153	0	21	79	86	86	90	0.857 bcdefg
K1237	0	29	71	88	88	88	0.829 bcdefg
K1251	0	0	78	96	93	96	0.820 cdefg
K1229	6	9	60	94	97	100	0.807 cdefg
K1150	14	22	69	89	89	89	0.750 defgh
K1164	0	0	84	94	97	100	0.725 defgh
K1236	0	0	87	97	97	100	0.673 efghi
K1230	0	23	67	93	100	100	0.671 efghi
K1235	0	4	84	96	100	100	0.664 efghi
K1247	0	12	81	96	100	100	0.660 efghi
K1242	0	5	81	100	100	100	0.643 ghi
K1249	0	10	80	97	100	100	0.632 ghi
K1248	0	7	85	96	100	100	0.618 ghi
K1243	0	24	80	92	96	96	0.613 ghi
K1162	0	13	83	100	100	100	0.611 ghi
K1238	0	52	71	92	99	99	0.462 hij
K1231	0	51	69	93	99	100	0.461 hij
K1165	4	56	76	89	99	99	0.419 ij
K1239	0	59	96	96	96	96	0.325 ik
K1246	0	50	97	100	100	100	0.319 jk
K1240	0	85	95	98	98	100	0.217 kl
K1245	0	85	100	100	100	100	0.190 l
K1241	0	94	100	100	100	100	0.172 l
K1244	0	100	100	100	100	100	0.159 l

Isolate	Metalaxyl concentration ($\mu\text{g/ml}$)										ED ₅₀ ($\mu\text{g/ml}$)	
	0.003	0.01	0.03	0.1	0.3	1	3	10	100	1000		
K1234	-	0	0	9	15	15	23	30	26	96	37.749	a
K1256	-	0	2	14	22	24	25	29	25	98	27.052	a
K1254	-	0	0	16	20	22	31	31	29	100	22.506	ab
K1242	-	0	0	0	30	27	42	50	42	100	9.701	bc
K1255	-	0	3	15	27	33	45	48	45	97	6.442	cd
K1252	-	0	0	25	31	38	53	66	63	-	2.965	de
K1251	-	0	18	35	53	56	59	59	59	94	1.345	ef
K1250	-	0	0	19	46	62	70	68	68	-	1.307	ef
K1161	-	0	0	2	35	65	76	83	81	-	1.197	ef
K1228	-	0	6	35	46	59	69	76	74	98	0.892	fg
K1162	-	0	12	36	48	61	67	67	73	-	0.855	fg
K1164	-	0	10	33	50	65	69	75	19	-	0.707	fgh
K1253	-	0	18	39	48	64	76	79	79	-	0.563	fghi
K1235	-	0	0	27	53	60	63	67	90	-	0.422	ghij
K1243	-	0	13	41	59	44	63	93	93	-	0.372	ghijk
K1248	-	0	0	19	70	81	89	93	96	-	0.307	hijk
K1165	0	0	0	22	60	89	96	97	-	-	0.273	hijkl
K1237	-	0	18	44	58	78	89	89	84	-	0.244	ijkl
K1249	0	0	15	48	68	80	88	90	90	-	0.223	ijkl
K1247	-	3	10	42	55	65	90	87	90	-	0.201	jklm
K1229	-	0	20	42	60	78	96	98	98	-	0.167	jklm
K1238	-	0	10	42	63	84	93	96	96	-	0.153	jklm
K1230	-	0	24	43	67	81	95	95	95	-	0.153	jklm
K1246	-	0	0	18	68	91	100	100	100	-	0.144	klmno
K1150	-	16	32	39	84	84	89	89	89	92	0.108	lmno
K1236	-	0	24	68	85	73	93	95	93	-	0.076	mnop
K1231	-	0	17	56	85	97	99	100	100	-	0.074	mnop
K1245	-	0	0	23	77	86	95	95	95	-	0.073	mnop
K1239	-	0	12	73	88	96	100	100	100	-	0.057	nop
K1153	0	6	34	84	94	97	97	-	-	-	0.050	op
K1241	0	0	27	87	100	00	100	100	100	-	0.041	op
K1240	0	8	55	84	88	96	96	98	98	98	0.038	p

^x All isolates were presumed to be P. megasperma at the time of testing. K1161 was later identified as P. citricola.

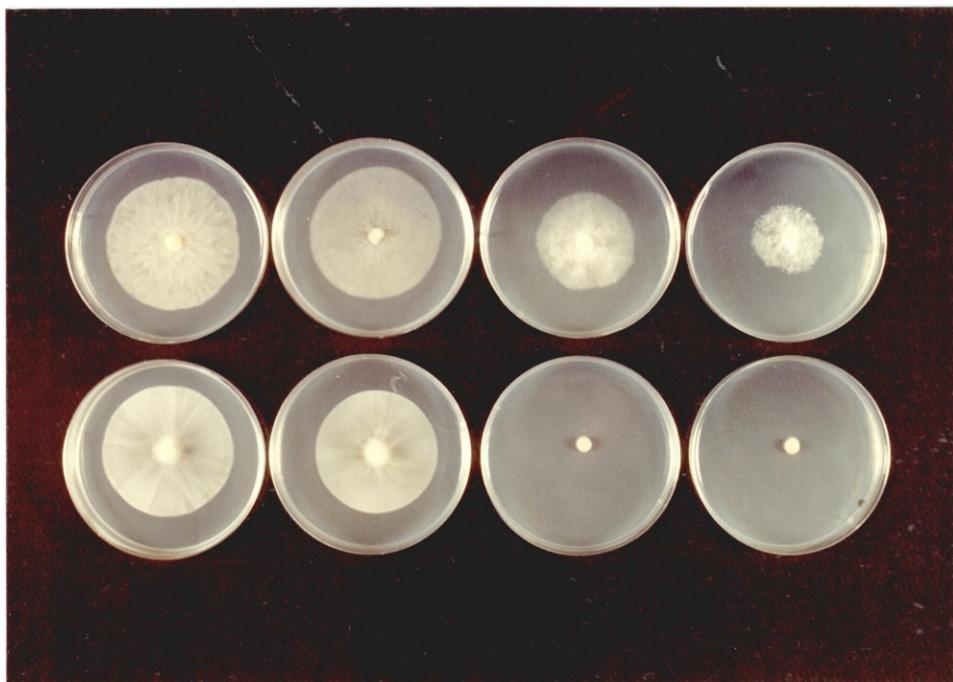
^y ED₅₀-values were derived from logit relative potency analysis of the response (percent inhibition of radial growth) plotted against the dosage (log concentration of R157001)

^z Values with no letters in common are significantly different, $P = 0.05$, according to Duncan's multiple range test

Plate 8 In vitro response of isolates of *Phytophthora* spp. to R157001Left to right : 0, 0.3 3, 30 $\mu\text{g/ml}$

a) Top row : K1161

Bottom row : K1234



b) Top row : K1161

Bottom row : K1240

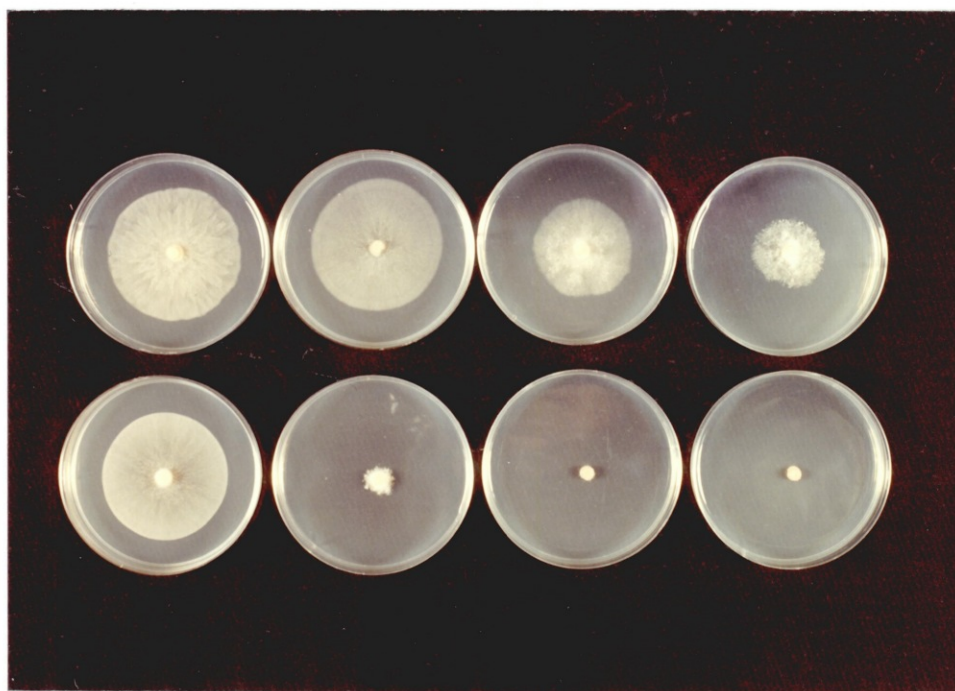
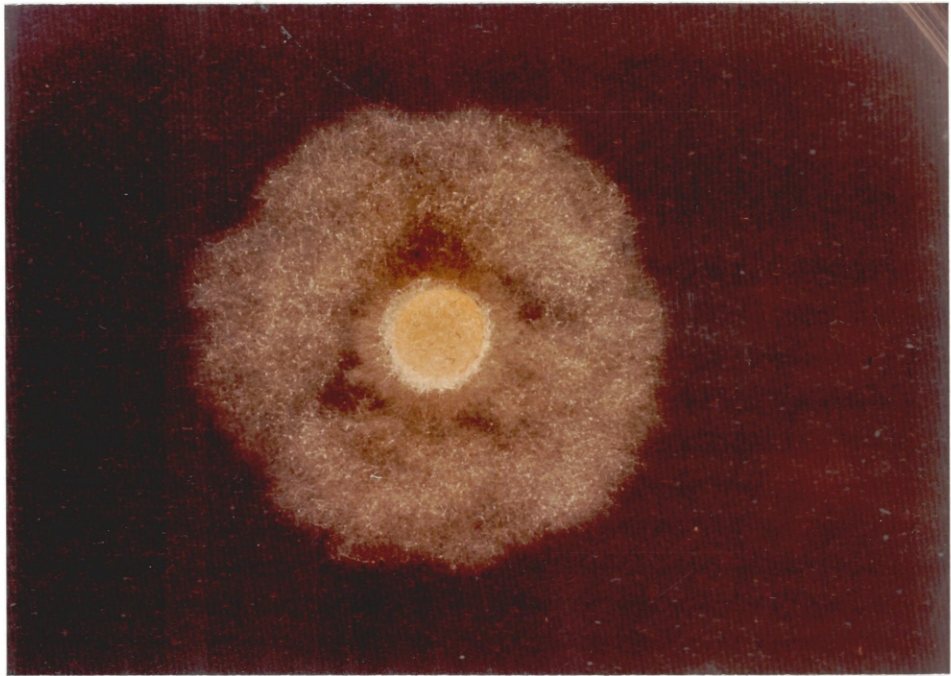


Plate 9 Colony appearance of Phytophthora megasperma isolates at 1µg
R157001/ml

a) K1234 (typical)



b) K1165 ('coagulated')

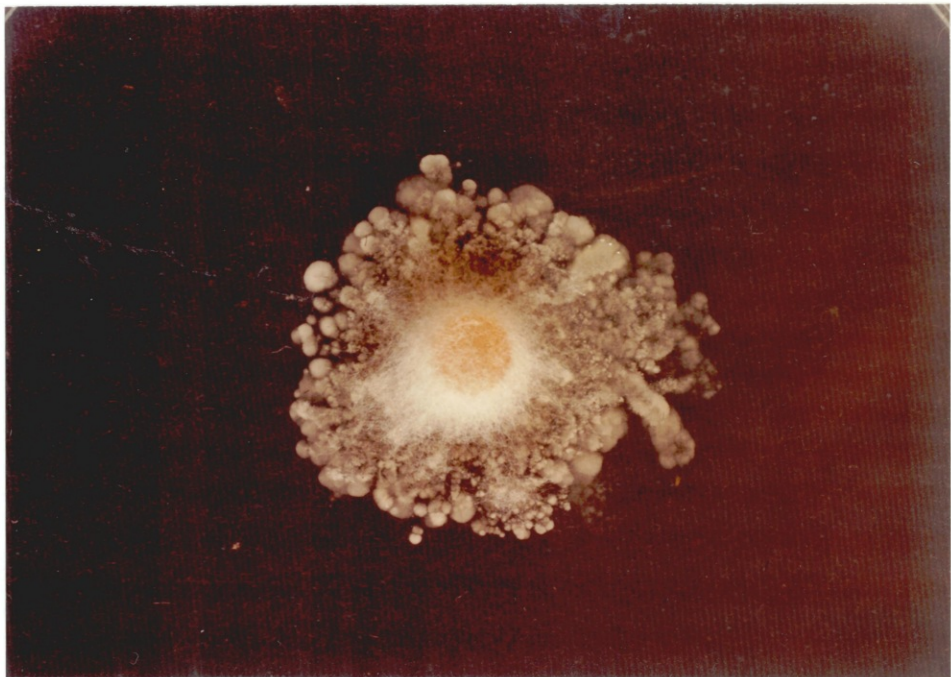
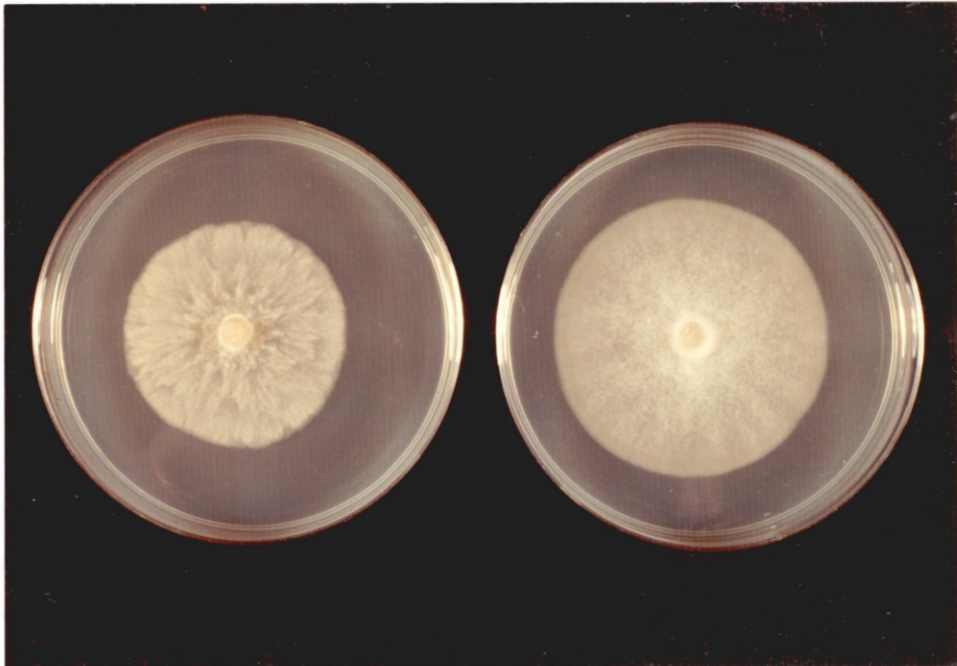


Plate 10 Appearance of colonies of Phytophthora spp.

Left to
right :

Isolate K1161

Isolate K1163



isolates of P. citricola were inherently more resistant to R157001 than isolates of P. megasperma, and that isolates of P. megasperma could be found which were significantly resistant ($P = 0.05$) to R157001.

b) Sensitivity to metalaxyl

The wide variation in the in vitro sensitivity to metalaxyl, already noticed amongst isolates in Section 4.2, was also apparent when the second consignment of isolates from Oregon State University was tested (Table 25). The ED_{50} of the isolate least sensitive to metalaxyl, K1234, was $37.75\mu\text{g/ml}$, making it approximately 1000-times less sensitive to the chemical than the most sensitive isolate, K1240 ($ED_{50} = 0.04\mu\text{g/ml}$).

The majority of isolates continued to grow at $100\mu\text{g}$ metalaxyl/ml. K1234 was only inhibited by 26% at this concentration. Furthermore, seven out of nine isolates (K1150, K1228, K1234, K1240, K1251, K1255, K1256) grew slowly at $1000\mu\text{g}$ metalaxyl/ml. In contrast to these findings, the growth of six isolates (K1153, K1231, K1239, K1240, K1241, K1246) was inhibited by more than 90% at $1\mu\text{g}$ metalaxyl/ml or less. The wide difference in the sensitivity of isolates to metalaxyl is exemplified by comparison of the response of two isolates to the chemical in Plate 11.

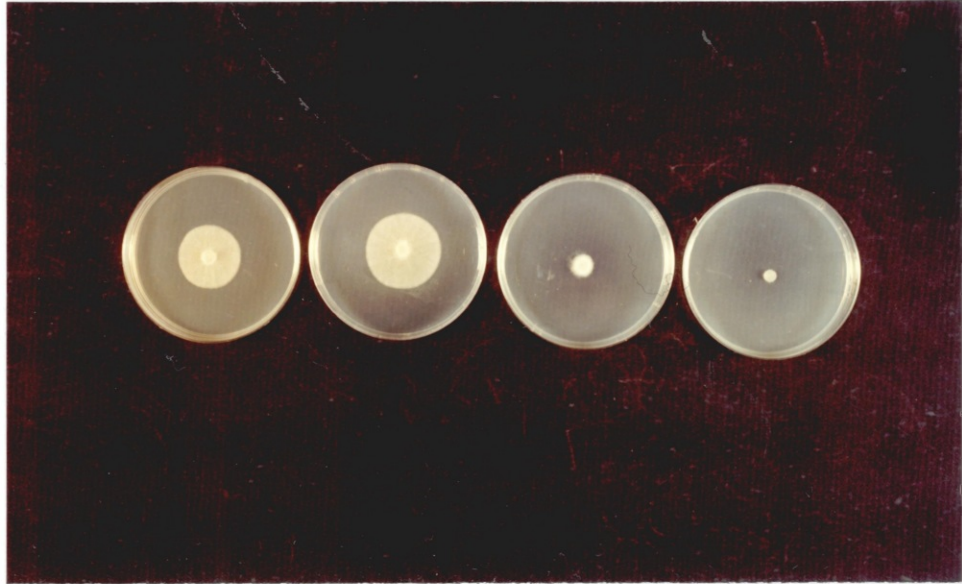
4.7.3 Discussion

The order of sensitivity of some isolates to R157001 and metalaxyl coincided (Table 25). For the least sensitive isolates this was related to the host from which they were obtained. Excluding K1161, four of the five isolates least sensitive to R157001 (K1252, K1254, K1255, K1256) isolated from apple or apricot, were amongst the group of six isolates which were least sensitive to metalaxyl. The latter group also included another two isolates (K1234 and K1242) from apple. Isolates originated from separate geographical locations, namely New York, California and New Zealand (apple isolates) and California (apricot

Plate 11 In vitro response of isolates of *Phytophthora megasperma*
to metalaxyl

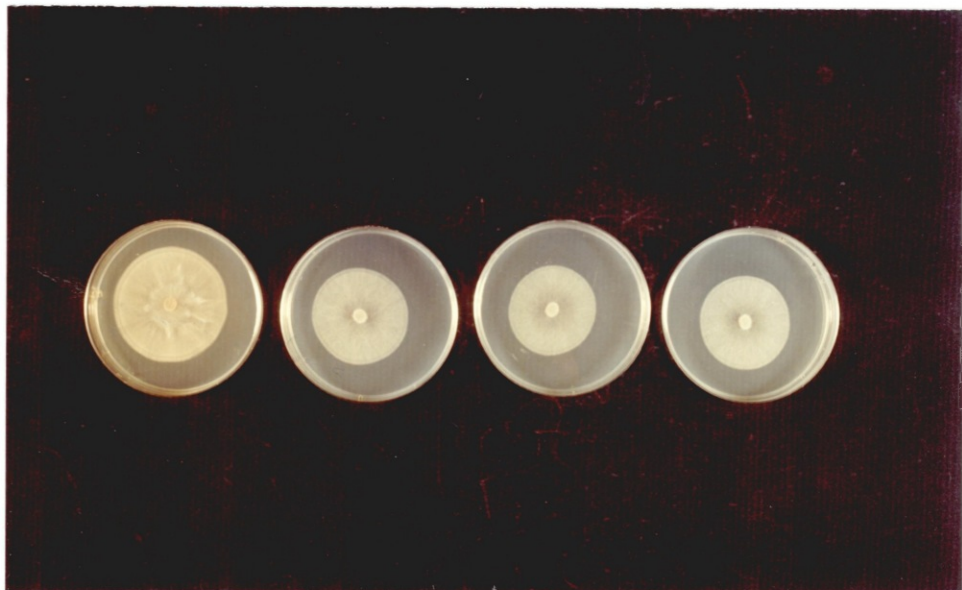
a) K1245

Left to right : 0, 0.1 1, 10 $\mu\text{g/ml}$



b) K1256

Left to right : 0, 1, 10, 100



isolate). A similar relationship was determined for five of the seven isolates most sensitive to R157001 (K1231 from White Cockle, K1239 and K1245 from soybean, and K1240 and K1241 from rose), excluding K1244 which was tested for R157001-sensitivity only. These were amongst a group of six isolates which were most sensitive to metalaxyl. It has been demonstrated that cross-resistance does not occur between the benzamides and acylalanines (Sections 4.2, 4.4, 4.5). Therefore, it was likely that the relationship observed was due to a mechanism other than modification of the biochemical site(s) of action of the fungicides. For example, decreased membrane permeability might have resulted in a reduced uptake of both, unrelated, toxicants.

Host-specific subgroups of P. megasperma have been described which differ in morphological characteristics (Hansen & Hamm, 1983). This was probably the consequence of the partial isolation afforded populations of P. megasperma by host specialisation and limited means of dispersal. It may also explain the high resistance to R157001 and metalaxyl of isolates originating from apple and apricot.

Apart from this relationship, the results confirmed previous work (Section 4.2) which indicated that there was no cross-resistance to R157001 and metalaxyl in isolates of P. megasperma. Additionally, there was good agreement between the results of the present study and a similar investigation with isolates of P. infestans (Section 4.4) which suggested that in vitro resistance to R157001 occurs, if at all, at a lower frequency than resistance to metalaxyl.

If in vitro and in vivo sensitivity to metalaxyl coincided amongst isolates of P. megasperma, loss of disease control due to strains which were inherently resistant to the fungicide would be expected to occur under field and glasshouse conditions. Under the same circumstances, good control of metalaxyl-sensitive and -resistant strains would probably be achieved by low doses of R157001.

4.8 Phytophthora spp : In vitro Sensitivity to the Benzamides R157001 and R157846, and Metalaxyl

4.8.1 Introduction

In two experiments (Sections 4.2 and 4.7) an isolate of P. citricola was more resistant ($P = 0.05$) to R157001 than 44 isolates of P. megasperma. From this result it was deduced that species of Phytophthora differed in their response to R157001. Furthermore, the ability of R157001 to control different Phytophthora spp. in the field and glasshouse might be affected. The present study sought to clarify the situation by comparing the in vitro sensitivity of isolates of P. citricola, Phytophthora cryptogea Peth. & Lafferty, P. infestans and P. megasperma, to two benzamides (R157001 and R157846) and metalaxyl.

4.8.2 Experimental

Origins of test isolates are provided in Tables 11, 24 and 26. Experiments were undertaken according to the procedures described for P. megasperma (see Section 4.2.2).

a) Comparative sensitivity to R157001 and metalaxyl

In Experiment 1 (Table 27), nine isolates of P. citricola were more resistant ($P = 0.05$) to metalaxyl than two isolates of P. megasperma. The same isolates of P. citricola were also more resistant ($P = 0.05$) to R157001 than one of the isolates of P. megasperma, K1165. However, whereas the isolates of P. citricola were inhibited by between 84-91% at 100 μ g R157001/ml, both isolates of P. megasperma were completely inhibited at this concentration.

Three groups of isolates were identified in Experiment 2, each significantly different ($P = 0.05$) in their response to metalaxyl (Table 27). There were six isolates of P. citricola and two isolates of P.

Table 26 Sources of isolates of *Phytophthora* spp

Isolate reference		Supplier ^a	Host	Location	Date	Other Details
ICI	Original					
<u>P. citricola</u>						
K1161	089	OSU				Originally presumed to be <u>P. megasperma</u>
K1285	84/1	GORI	<u>Lomatia tintoria</u>	United Kingdom	1984	
K1286	73/1/R	GORI	<u>Thiia plicata zabrina</u>	United Kingdom	1973	
K1287	74/77/R	GORI	<u>Chamaecyparis lawsoniana</u> 'Pottent'	United Kingdom	1974	
K1288	74/80/St	GORI	<u>Penethya mueronata</u>	United Kingdom	1974	
K1289	74/121/SB	GORI	<u>Cytisus praecox</u> 'Allgold'	United Kingdom	1974	
K1290	74/14/S3	GORI	<u>Scimmia japonica rubella</u>	United Kingdom	1974	
K1291	P2; IMI 21173T	GORI	<u>Citrus sinensis</u>			
K1292	77/4; IMI 211638	GORI	<u>Rhododendron</u> hybrid 'Sappho'	United Kingdom	1977	
<u>P. cryptogea</u>						
K1315	75/5/St	GORI	<u>Chamaecyparis lawsoniana columnaris glauca</u>	United Kingdom	1972	A2 mating type
K1316	77/6	GORI	Tomato	United Kingdom	1977	A1 mating type
<u>P. megasperma</u>						
K1317	77/19/3/R	GORI		United Kingdom	1977	DF2 ^b
K1318	220	DSRI	Soil	New Zealand	1981	
K1319	221	DSRI	Alfalfa	New Zealand	1981	
K1320	270	DSIR	Soil	New Zealand	1983	
K1321	261	DSIR	Soil	New Zealand	1983	
K1322	C17	OSU	Douglas Fir	Elkton, Oregon		

^a Abbreviations used for names and addresses were:-

DSIR = B Hawthorne, Department of Scientific and Industrial Research, Auckland, New Zealand

GORI = P M Smith, Glasshouse Crops Research Institute, Littlehampton, Sussex

OSU = E M Hansen & P B Hamm, Oregon State University, Corvallis, Oregon, USA

^b DF2 was the isolate group described by Hansen & Hamm (1983)

Table 27 Inhibition (%) of radial growth of Phytophthora isolates by R157001 and metalaxyl

Phytophthora species	Isolate	R157001 concentration (µg/ml)								ED ₅₀ ^y (µg/ml)
		0.01	0.1	0.3	1	3	10	30	100	

EXPERIMENT 1										
citricola	K1291	-	0	8	27	32	62	78	84	6.321 a ^z
citricola	K1161	-	0	17	34	34	59	80	90	5.068 a
citricola	K1287	-	10	28	50	55	73	80	90	1.950 b
citricola	K1290	-	11	34	62	68	81	83	87	1.134 c
megasperma	K1163	-	0	0	48	94	98	98	100	0.995 c
citricola	K1285	-	13	37	59	70	81	87	91	0.946 c
citricola	K1286	-	19	41	63	71	84	83	88	0.823 cd
citricola	K1288	-	19	45	71	76	83	84	93	0.590 de
citricola	K1292	-	10	49	75	78	84	86	92	0.588 de
citricola	K1289	-	20	52	78	78	86	86	92	0.456 de
megasperma	K1165	-	0	52	72	96	100	100	100	0.290 f

EXPERIMENT 2										
citricola	K1286	0	0	-	29	-	68	-	68	7.700 a
citricola	K1161	0	0	-	22	-	71	-	86	5.316 ab
citricola	K1285	0	0	-	52	-	73	-	82	3.266 bc
citricola	K1287	0	0	-	64	-	77	-	80	2.660 bc
citricola	K1290	0	0	-	47	-	78	-	88	2.239 cd
megasperma	K1317	2	0	-	48	-	93	-	98	1.234 de
megasperma	K1160	0	7	-	46	-	95	-	98	1.088 de
citricola	K1288	0	13	-	71	-	81	-	84	1.006 ef
megasperma	K1319	0	0	-	57	-	98	-	100	0.879 ef
megasperma	K1322	0	0	-	70	-	93	-	96	0.829 ef
megasperma	K1318	4	4	-	61	-	100	-	100	0.692 ef
megasperma	K1163	5	3	-	69	-	95	-	95	0.692 ef
megasperma	K1320	0	0	-	69	-	97	-	100	0.606 efg
cryptogea	K1315	3	5	-	71	-	98	-	100	0.540 efg
megasperma	K1165	1	10	-	73	-	100	-	100	0.458 fg
cryptogea	K1316	0	12	-	76	-	96	-	100	0.457 fg
megasperma	K1321	9	14	-	91	-	95	-	100	0.271 g

Phytophthora species	Isolate	Metalaxyl concentration (µg/ml)					ED ₅₀ ^y (µg/ml)
		0.01	0.3	3	30	100	

EXPERIMENT 1							
citricola	K1291	3	5	41	65	84	3.873 a
citricola	K1161	0	2	54	73	88	2.219 ab
citricola	K1285	4	27	49	69	76	1.946 ab
citricola	K1287	8	28	44	70	77	1.777 ab
citricola	K1289	7	20	50	70	82	1.688 ab
citricola	K1290	0	25	55	75	78	1.475 b
citricola	K1292	3	28	52	72	84	1.408 b
citricola	K1288	7	29	47	74	84	1.277 b
citricola	K1286	7	24	55	76	79	1.222 b
megasperma	K1163	8	50	81	88	81	0.221 c
megasperma	K1165	0	40	86	90	96	0.200 c

EXPERIMENT 2							
citricola	K1286	0	11	54	75	86	7.700 a
citricola	K1285	0	0	18	73	82	6.882 ab
megasperma	K1160	0	17	49	61	61	4.688 bc
citricola	K1287	0	0	34	73	80	4.465 bc
citricola	K1290	0	12	50	73	85	2.055 cd
citricola	K1288	0	8	52	79	83	1.942 cd
citricola	K1161	0	0	73	88	92	0.911 de
megasperma	K1317	2	25	73	84	82	0.692 de
megasperma	K1165	0	39	87	94	96	0.195 f
cryptogea	K1315	11	57	75	94	97	0.142 f
megasperma	K1319	10	60	86	88	88	0.128 f
megasperma	K1163	10	60	84	88	93	0.125 f
megasperma	K1322	64	64	86	93	89	0.101 f
megasperma	K1318	0	61	86	100	100	0.088 f
cryptogea	K1316	18	57	86	92	96	0.083 f
megasperma	K1321	36	86	91	95	100	0.014 g
megasperma	K1320	34	83	93	97	97	0.013 g

^y ED_{50} -values were derived from logit relative potency analysis of the response (percent inhibition of radial growth) plotted against the dosage (log concentration of R157001)

^z Values with no letters in common were significantly different, $P = 0.05$, according to Duncan's multiple range test.

megasperma in the first group (ED_{50} 's 0.692-7.700 $\mu\text{g/ml}$), five isolates of P. megasperma and the two isolates of P. cryptogea in the second group (ED_{50} 's 0.083-0.195 $\mu\text{g/ml}$) and two isolates of P. megasperma in the third group (ED_{50} 's 0.013-0.014 $\mu\text{g/ml}$). In the same experiment, the five isolates of P. citricola which were least sensitive to R157001 were also more resistant ($P = 0.05$) to the chemical than seven of the nine isolates of P. megasperma and both isolates of P. cryptogea. Also, whereas the six isolates of P. citricola were inhibited by between 68-88% at 100 $\mu\text{g/ml}$ R157001, the other 11 isolates were inhibited by between 95-100% at this concentration.

The conclusion from Experiments 1 and 2 was that isolates of P. citricola could be differentiated from isolates of P. megasperma and P. cryptogea by their different response to R157001 and metalaxyl. However, isolates of P. megasperma and P. cryptogea could not be differentiated from each other on this basis.

b) Sensitivity to R157846

Four out of five isolates of P. citricola were less sensitive to R157846 than three out of four isolates of P. megasperma (Table 28). The overlap between species was due to P. megasperma K1160, which was one of the isolates of this species least sensitive to R157001 in previous experiments (Sections 4.2, 4.7). The higher resistance of P. citricola to R157846 compared with P. megasperma also agreed with the results of the study with R157001 (Section 4.7)

The five isolates of P. citricola were significantly more resistant ($P = 0.05$) to R157846 than the five isolates of P. infestans. The latter were more sensitive to R157846, though not significantly so, than the isolates of P. megasperma.

4.8.3 Discussion

The variation in sensitivity to metalaxyl in vitro amongst

Table 28 Inhibition (%) of radial growth of Phytophthora isolates
by R157846

Phytophthora species	Isolate	R157846 concentration ($\mu\text{g/ml}$)					ED ₅₀ ^y ($\mu\text{g/ml}$)
		0.001	0.01	0.1	1	10	
citricola	K1287	0	3	38	71	92	0.285 a ^z
citricola	K1286	0	2	47	77	91	0.220 ab
citricola	K1285	0	6	49	78	94	0.158 abc
citricola	K1288	0	7	59	75	94	0.138 bc
megasperma	K1160	0	2	37	98	100	0.136 bc
citricola	K1289	0	4	66	80	93	0.120 bcd
megasperma	K1154	0	0	55	94	97	0.106 cd
megasperma	K1153	0	0	80	93	93	0.069 de
megasperma	K1155	0	9	87	96	96	0.040 ef
infestans	K1308	0	0	100	100	100	0.033 fg
infestans	K1296	0	10	100	100	100	0.024 fgh
infestans	K1300	0	19	100	100	100	0.020 gh
infestans	K1220	0	27	97	100	100	0.018 gh
infestans	K1294	0	34	100	100	100	0.013 h

^y ED₅₀-values were derived from logit relative potency analysis of the response (percent inhibition of radial growth) plotted against the dosage (log concentration of R157846)

^z Values with no letter in common are significantly different, $P = 0.05$, according to Duncan's multiple range test.

isolates of Phytophthora spp. has been described by several workers (Hunger et al., 1982; Coffey et al., 1984; Coffey & Bower, 1984). In these studies the response of isolates, based on inhibition of linear growth of mycelium, was initially tested at one or two concentrations. Selected isolates were then examined for their sensitivity to a range of several concentrations of metalaxyl. One of the conclusions of Coffey & Bower (1984) was that metalaxyl appeared to be useful, especially at low concentrations around 0.1µg/ml, as an aid in the identification of Phytophthora spp. Thus, isolates of Phytophthora parasitica Dastur were more resistant to 0.1µg metalaxyl/ml than isolates of Phytophthora citrophthora (Sm. & Sm.) Leonian, although these differences were not apparent at 1.0µg metalaxyl/ml. Similarly, in the present study, Phytophthora spp. could be discriminated by single concentrations of R157846; isolates of P. citricola were more resistant to 1µg R157846/ml (71-80% inhibition of growth) than isolates of P. megasperma (93-96% inhibition of growth) (Table 28). Furthermore, the latter were more resistant to 1µg R157846/ml than isolates of P. infestans (100% inhibition of growth). These differences were not apparent when the concentration of R157846 varied by 10-times around 1µg/ml. Apart from this finding, the present study differed from previous investigations in its emphasis on the response of all isolates tested to a range of concentrations of the fungicide. Subsequent determination of ED₅₀-values enabled a more accurate assessment of the comparative sensitivity of isolates.

Coffey & Bower (1984) claimed that the major effect of a systemic fungicide such as metalaxyl was to prevent mycelial growth of the fungus within its host. As evidence they demonstrated that the radial growth of isolates of P. cinnamomi was highly inhibited by the chemical. Since isolates of P. citricola were not controlled to the same degree, it was also suggested that metalaxyl treatments in the field and glasshouse would be less effective against P. citricola than against P. cinnamomi. In

the present study, P. citricola was generally more resistant to metalaxyl, and the two benzamides R157001 and R157846, than P. megasperma. Therefore, in the field and glasshouse, higher doses of each fungicide would probably be required to control P. citricola to the same degree as P. megasperma. Additionally, R157846 was more active against P. infestans than P. citricola and P. megasperma. This also may coincide with the relative effectiveness of benzamide fungicides under field and glasshouse conditions against these pathogens.

5 NATURE OF THE INHIBITORY EFFECTS OF BENZAMIDE FUNGICIDES

5.1 General Introduction

Coffey & Young (1984) suggested that metalaxyl has a fungistatic effect on the physiology of target pathogens. In support of their argument they demonstrated that there were no visible changes in the ultrastructure of metalaxyl-treated zoospores or sporangia of P. infestans. They also cited a previous study in which there were no obvious changes in the fungal cytoplasm, following metalaxyl application, in the interaction between Peronospora pisi Sydow and Pisum sativum L. (Hickey & Coffey, 1980). Other evidence was the continued development of P. infestans when metalaxyl was used as a curative treatment 48 h after infection (Coffey & Young, 1984). In the present study (Chapter Four), the majority of isolates of P. infestans grew, in vitro, at relatively high concentrations of metalaxyl (>100µg/ml). This included isolates which were designated 'sensitive' to metalaxyl, and collected before the commercial introduction of the fungicide.

The fungistatic mode of action of metalaxyl may have contributed to the rapidity with which resistance developed in target fungi (Coffey & Young, 1984). The converse, a fungicidal mode of action, would presumably militate against the development of resistance, because there would be no viable nuclei from which resistant strains could be selected.

The fungicide phosethyl-Al is highly systemic and relatively persistent in the host plant (Williams & Beach, 1977). These characteristics probably favour the development of resistance (Dekker, 1981). However, unlike metalaxyl, after many years of use there are no reports of resistance under field and glasshouse conditions to phosethyl-Al. The latter is degraded to phosphorous acid in plant tissues (Vo et al., 1979). Phosphorous acid was shown to exert a lethal effect on mycelium of P. cinnamomi and P. parasitica when treated plugs of mycelium

failed to grow following their transfer to unamended medium (Rohrbach & Schenck, 1985). The implication of this finding is that the ultimately fungicidal effect of phosethyl-Al may have contributed to the prevention of resistance in target pathogens. However, the mode of action of a fungicide is probably also an important determinant of the ease or difficulty with which resistance may develop. Modification of a biochemical process resulting in resistance is likely to occur more easily to some fungicides than to others.

It was demonstrated in the present study (Chapter Two) that P. megasperma f.sp. medicaginis did not adapt to growth at high concentrations of R157001. This, and the complete inhibition of adapted strains by concentrations around 10µg R157001/ml suggested that the chemical was exerting a fungicidal effect. The hypothesis was that such a characteristic might significantly reduce the likelihood of benzamide-resistant strains of target fungi arising under field and glasshouse conditions. Accordingly, in vitro experiments were undertaken to determine whether benzamide fungicides were fungistatic or fungicidal in their effect on Phytophthora spp.

5.2 Reversibility of Inhibition of Growth

5.2.1 Introduction

Rooke & Shattock (1983) described a procedure for determining the reversibility of antibiotic inhibition of P. infestans. They placed 'weft' cultures (mycelium grown across cellophane squares) in liquid medium amended with chloramphenicol or streptomycin for different periods of time. The treated cultures were washed free of antibiotic, transferred to antibiotic-free medium for 14 days and weighed. From the results they concluded that chloramphenicol was fungistatic because the inhibitory

effect of as much as 640µg/ml for 48 h could be fully reversed. Streptomycin was assumed to be fungicidal because exposure to as much as 64µg/ml for 96 h resulted in diminished recovery approaching 85% inhibition.

The procedures were modified in the present study to determine the reversibility of inhibition of P. infestans by R157846. Metalaxyl and mancozeb were included as standard chemicals which were considered to be fungistatic and fungicidal respectively in their mode of action.

5.2.2 Materials and Methods

In a preliminary experiment, 6mm plugs of mycelium of P. megasperma f. sp. medicaginis isolate K1120 were transferred from V8 agar amended with 2.5µg R157001/ml or 5µg metalaxyl/ml to unamended V8 agar. At the time of transfer, slight growth of the fungus was evident on metalaxyl-amended agar but none on R157001-amended agar. Six replicate plates were inoculated, and re-growth from the inoculum plugs was determined during 14 days at 25°C.

In a second experiment, aliquots (8ml) of sterile aqueous suspensions of R157846, metalaxyl and mancozeb were transferred separately to 5cm-diameter petri dishes. Three 10mm plugs of mycelium were cut from 5-day cultures of P. infestans K1272. These were inverted in each fungicide solution, and also in a petri dish containing sterile water. Dishes were incubated at 22°C for 48 h. The plugs of mycelium were recovered from each dish and agitated thoroughly in two changes of 30ml sterile water on a Whirlimixer (Fisons Scientific Apparatus, Loughborough, Leicestershire). The washed plugs were transferred to separate 100ml Erlenmeyer flasks containing 50ml aliquots of clarified FP broth. Flasks were incubated for 12 days on an orbital shaker. Fungal mycelium, including the inoculum plug, was harvested from each flask by filtration

through Whatman no. 4 filter paper under vacuum. Mycelium was partially dried by compression between filter pads, freeze-dried overnight and weighed.

5.2.3 Results and Discussion

In the preliminary experiment, growth occurred from the plugs of mycelium previously subjected to 5 μ g metalaxyl/ml. No growth occurred from plugs which had been treated with 2.5 μ g R157001/ml.

In the second experiment there were large differences in the amount of mycelium produced between some replicate treatments (Table 29). Thus, the dry weights of mycelium derived from untreated plugs varied between 68-173mg. However, assessment of the overall effects of the three fungicides was possible. Metalaxyl at 100 μ g/ml failed to prevent the re-growth of mycelium, although the rate of growth was slower than that of the untreated control. Growth of mycelium previously subjected to 1 μ g metalaxyl/ml was only slightly faster than that subjected to 100 μ g metalaxyl/ml. These results supported the findings of previous studies (Hickey & Coffey, 1980; Coffey & Young, 1984) which indicated that metalaxyl has a fungistatic effect on target pathogens.

Slightly faster re-growth occurred from mancozeb-treated plugs of mycelium than metalaxyl-treated plugs. There was no difference (in terms of average dry weights of mycelium) in the recovery of mycelium from the effects of 1 and 100 μ g mancozeb/ml. In the present study, mancozeb apparently exerted a fungistatic effect on the mycelium. This result was somewhat unexpected, since the chemical is generally assumed to exert a lethal effect in vivo. It could be that as mancozeb is not systemic (Schwinn, 1981), it may not have been able to penetrate into the deep-seated mycelium within the inoculum plug to kill the fungus.

R157846 was the most effective of the three fungicides in

Table 29 Growth of mycelium of *Phytophthora infestans* treated with fungicides and subsequently transferred to unamended medium

Fungicide treatment		Dry weight (mg) ^a in unamended medium after 12 days			
Fungicide	Concentration (ug/ml)	Individual weights			Average weight
R157846	10	0	0	0	0
	0.1	12	11	8	10
Metalaxyl	100	23	17	11	17
	1	29	31	42	34
Mancozeb	100	75	38	15	43
	1	51	31	46	43
Untreated	0	122	173	68	121

^a Dry weight of inoculum plug (10mg) deducted

preventing the re-growth of mycelium from treated plugs. Growth was completely prevented by 10µg R157846/ml, and was highly inhibited by 0.1µg R157846/ml. It was concluded that, depending on its concentration, R157846 was probably fungicidal to the growth of mycelium.

Rohrbach & Schenck (1985) deduced that phosphorous acid exerted a lethal effect on P. cinnamomi and P. parasitica because treated plugs of mycelium failed to grow when transferred to unamended medium. However, renewed mycelial growth in both their experiments and the present study might have been prevented by the presence of highly inhibitory concentrations of phosethyl-Al or benzamide, respectively, in the inoculum plugs. The second experiment in the present study aimed to exclude this possibility by washing the plugs of mycelium free of fungicide. It was not known if this was successful.

The results suggest that under field and glasshouse conditions, relatively high concentrations of benzamide would cause an irreversible inhibition of growth, with concomitant reduction in the selection of resistant strains. In contrast, metalaxyl-resistant strains would appear to be selected more readily because of the fungistatic nature of the chemical at high concentrations. At the low concentrations which may prevail under field and glasshouse conditions, the inhibitory effect of benzamides may apparently be reversed, and the selection of resistant strains thereby increased.

Ultimately, the degree of selection probably depends on the mode of action of the benzamides, which has yet to be elucidated, rather than simply their fungistatic or fungicidal effect on metabolism.

5.3 Observations on the Effect of Fungicides on Spore Germination

5.3.1 Introduction

For systemic fungicides, Staub et al. (1980) argued that it was

important to know their site of action on or in treated plants in order to design the most effective methods of application. They used light and electron microscopy to examine the effect of metalaxyl on the development of P. viticola and P. infestans in their host plants. It was demonstrated that release and germination of zoospores and formation of appressoria were not affected by concentrations of metalaxyl many times higher than those required for effective disease control. Further development of the fungus appeared to be inhibited after the first haustorium was formed. Unlike metalaxyl, disease control by the residual fungicide, maneb, could be attributed entirely to its inhibitory effect on sporangia and zoospores. Necrotic microlesions were associated with the inhibition of the fungi within their hosts by metalaxyl, which suggested there was a combined action of metalaxyl and host plant responses against the pathogens.

Stössel et al. (1982) used both light and electron microscopy to examine the interaction between soybeans and P. megasperma Drechs. var. sojae Hildeb. following treatment of seedlings with metalaxyl. The fungicide greatly reduced hyphal branching and spread in the host. Slow growth in treated seedlings after 12 h provided further evidence for a fungistatic mode of action. The authors noted that glyceollin or other inhibitory factors of host origin could have contributed to the observed changes in the ultra-structure of the fungus. They proposed that metalaxyl acted primarily on the fungus and that necrosis and glyceollin production associated with metalaxyl treatment was a secondary effect. Coffey & Young (1984) used electron microscopy to determine whether metalaxyl was fungistatic or fungicidal to P. infestans. Because they found no visible changes in the ultrastructure of zoospores treated with 100µg/ml or sporangia treated with 50µg/ml they concluded that metalaxyl had a fungistatic action on this fungus. Light microscopy was employed in the present study to evaluate the in vitro effect of R157001 on spore germination in P. megasperma f.sp. medicaginis and P. infestans.

5.3.2 Materials and Methods

a) P. megasperma f.sp. medicaginis

Zoospore production. Isolate K1120 was incubated on plates of V8 agar for 4 days at 25°C. Agar was removed as a ring (approximately 1cm wide) from the edge of growing colonies. Each plate was flooded with 15-20ml sterile deionised water and incubated overnight at 15°C. The resulting zoospore suspension was collected in a sterile Erlenmeyer flask.

Spore germination in aqueous solutions. Equal volumes of zoospore suspension and deionised water or aqueous solutions of fungicide were mixed separately in 50ml glass beakers. Droplets of each suspension were dispensed singly into ringed slides using a Pasteur pipette and the slides were incubated for 4 h at 25°C in a spore germination tray lined with damp filter paper. Photomicrographs were taken to record the effects of the fungicides on zoospore germination.

Spore germination on agar. Nine ml aliquots of zoospore suspension were transferred to six empty 9cm petri dishes and 1ml undiluted clarified V8 broth (Appendix A) was added to each suspension to induce encystment and attachment of zoospores to the bottom of the dish. After 1 h the liquid was decanted from each dish and replaced with 15ml clarified V8 agar, unamended or containing 5µg/ml of either R157001 or metalaxyl. Plates were incubated at 25°C for 5 days. Photomicrographs were taken of fungal growth in the plane of view at the bottom of each dish.

b) P. infestans

A suspension containing 2×10^6 sporangia/ml of isolate K1220 was prepared in chilled deionised water. Aliquots of this suspension were mixed separately with equal volumes of aqueous suspensions of metalaxyl (as the commercial formulation 'Ridomil'), R157001 and mancozeb to a final concentration of 50µg a.i./ml. Droplets were transferred to microslides and these were placed in a damp chamber (as in a) above), first at 10°C for 3 h to encourage the differentiation and release of zoospores, and then at

22°C. The effects of the fungicides on spore germination and subsequent development were examined at 6 h and 24 h and photomicrographs were taken of appropriate treatments.

5.3.3 Results

a) P. megasperma f.sp. medicaginis

Incubation for 4 h in the presence of R157001 at 10µg/ml, and metalaxyl at 10 and 100µg/ml, failed to prevent the germination of zoospores of P. megasperma f.sp. medicaginis (Plate 12). In plates with unamended agar after 5 days, mycelia had ramified throughout the medium, hyphal branching was common, and the origin of mycelium could not be traced to individual zoospores (Plate 13a). However, in plates with fungicide, hyphal growth was severely reduced. Metalaxyl induced curling of the hyphae (Plate 13c) but R157001 caused more extensive malformations (Plate 13b). Hyphae treated with R157001 were frequently swollen and distorted; vacuolation and general cellular disorganisation was also common.

b) P. infestans

Germination of zoospores and sporangia of P. infestans was prevented by 50µg R157001/ml and 10 or 50µg mancozeb/ml after 6 h, but not by as much as 50µg metalaxyl/ml (Table 30). Crystals in suspensions treated with 50µg R157001/ml demonstrated that the chemical was somewhat insoluble under aqueous conditions.

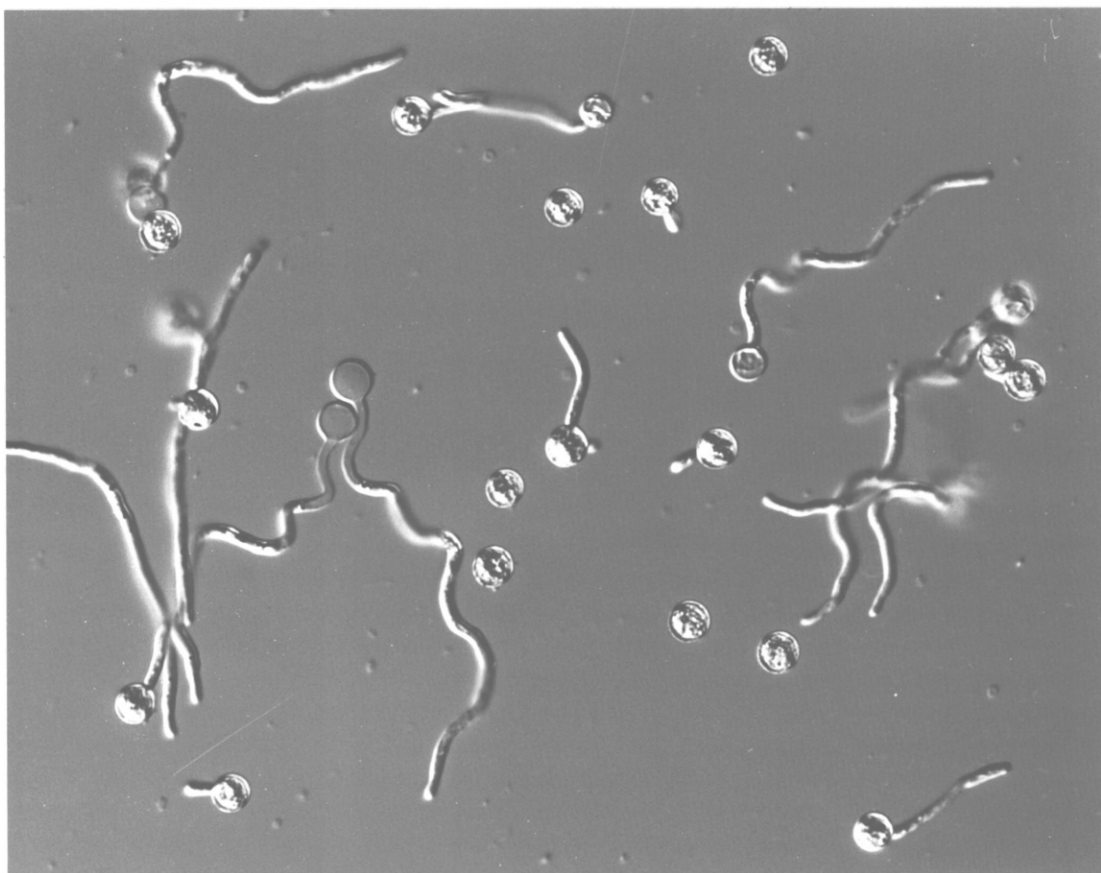
After 24 h long germ tubes were produced by sporangia (direct germination) and zoospores in untreated suspensions (Plate 14a). Mancozeb at 10µg/ml and 50µg/ml continued to prevent the germination of spores, whereas at 50µg R157001/ml it was now occurring (Table 30). However, hyphal extension was severely inhibited by both 10 and 50µg R157001/ml (Plate 14c & d). Abnormalities were common, and included hyphal swelling, vacuolation and general cytoplasmic disorganisation. Long germ tubes were produced at 10 (Plate 14b) and 50µg metalaxyl/ml, although by fewer spores

Plate 12 Germination of zoospores of *Phytophthora megasperma* f.sp. medicaginis in suspensions containing R157001 or metalaxyl

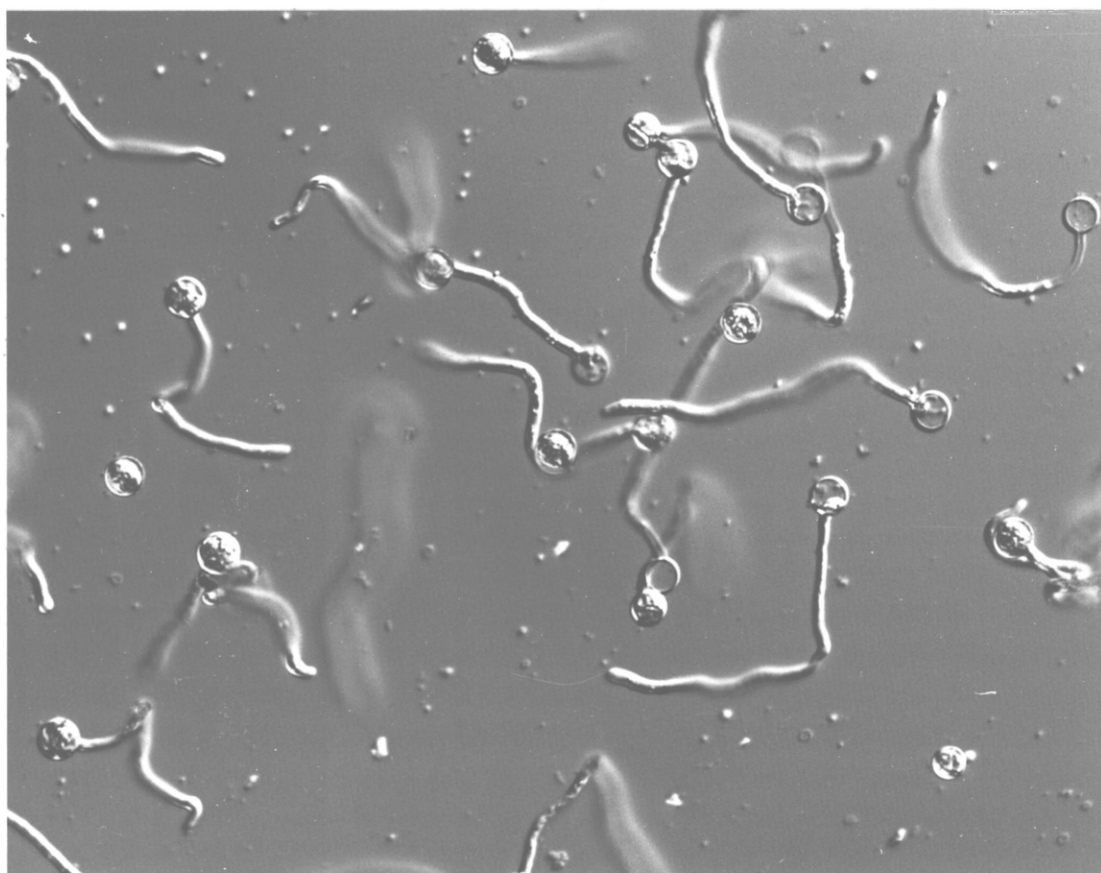
(4 h after treatment; x400)

- a) Untreated
- b) 10 μ g R157001/ml
- c) 10 μ g metalaxyl/ml
- d) 100 μ g metalaxyl/ml

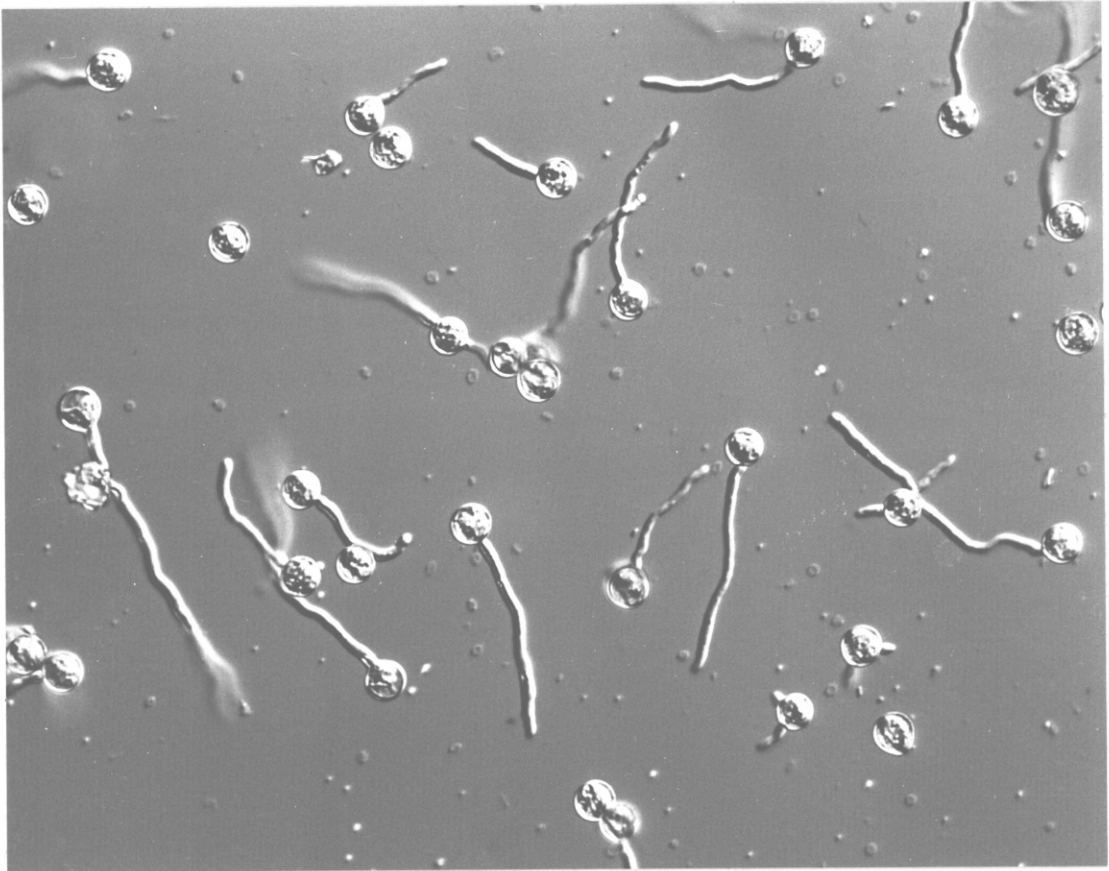
a



b



C



d

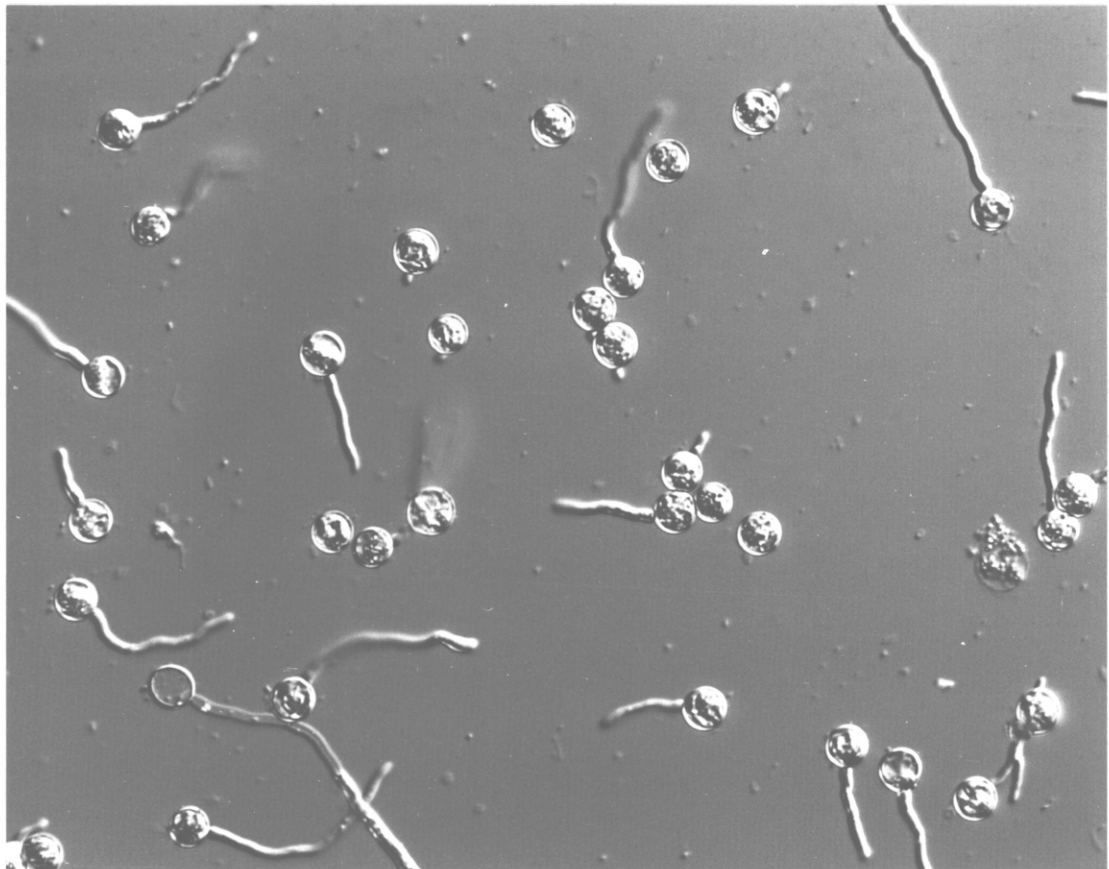


Plate 13 Zoospore germination and subsequent development of *Phytophthora*
megasperma f.sp. *medicaginis* in contact with agar containing
R157001 or metalaxyl

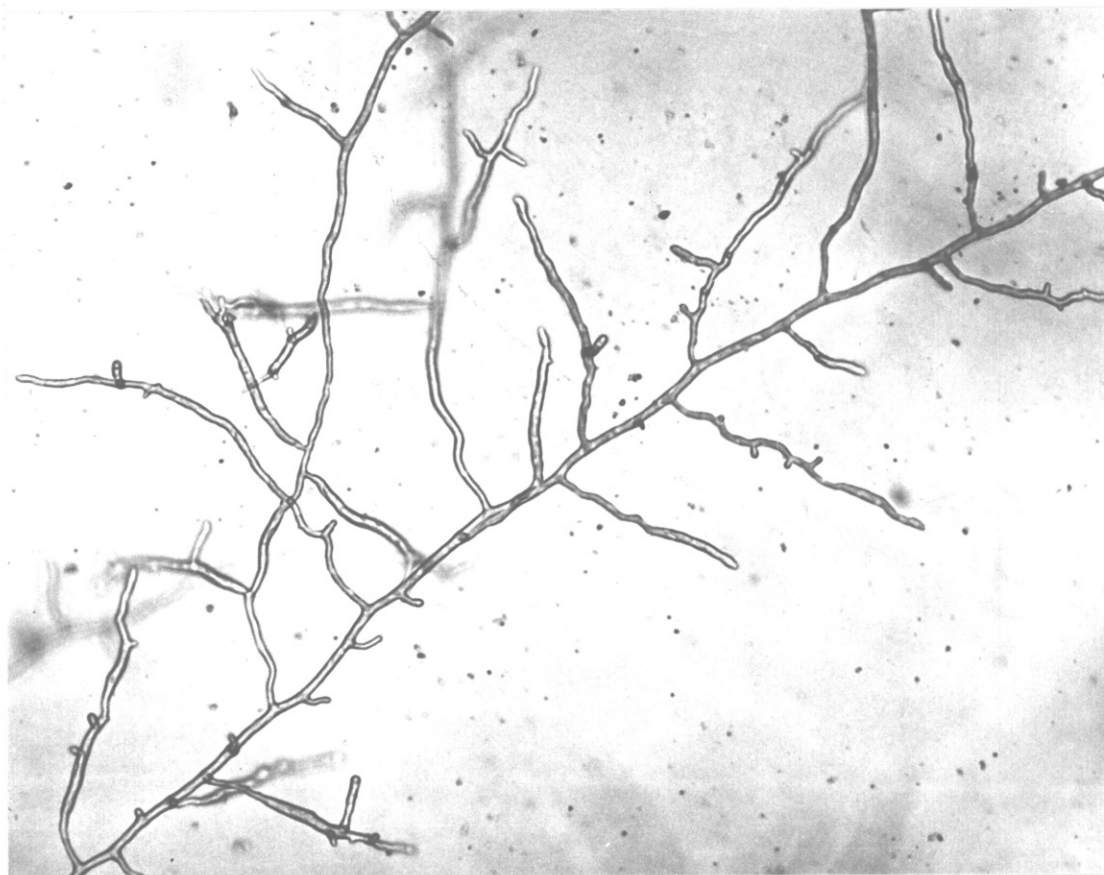
(5 days after treatment; x200)

a) Untreated

b) 5 μ g R157001/ml medium

c) 5 μ g metalaxyl/ml medium

a



b



C



Table 30 Germination of sporangia and zoospores of *Phytophthora infestans* treated with R157001, metalaxyl and mancozeb

Fungicide treatment		Production of germ tube ^a during incubation (h)	
Fungicide	Concentration (μ g active ingredient/ml)	6	24
R157001	50	-	+
	10	+	+
	2	+	+
Metalaxyl	50	+	+
	10	+	+
	2	+	+
Mancozeb	50	-	-
	10	-	-
	2	+	+
Untreated	0	+	+

^a + = formation

- of germ tube

- = no formation

Plate 14 Germination of sporangia and zoospores of *Phytophthora infestans*
in suspensions containing R157001 or metalaxyl

(24 h after treatment; x500)

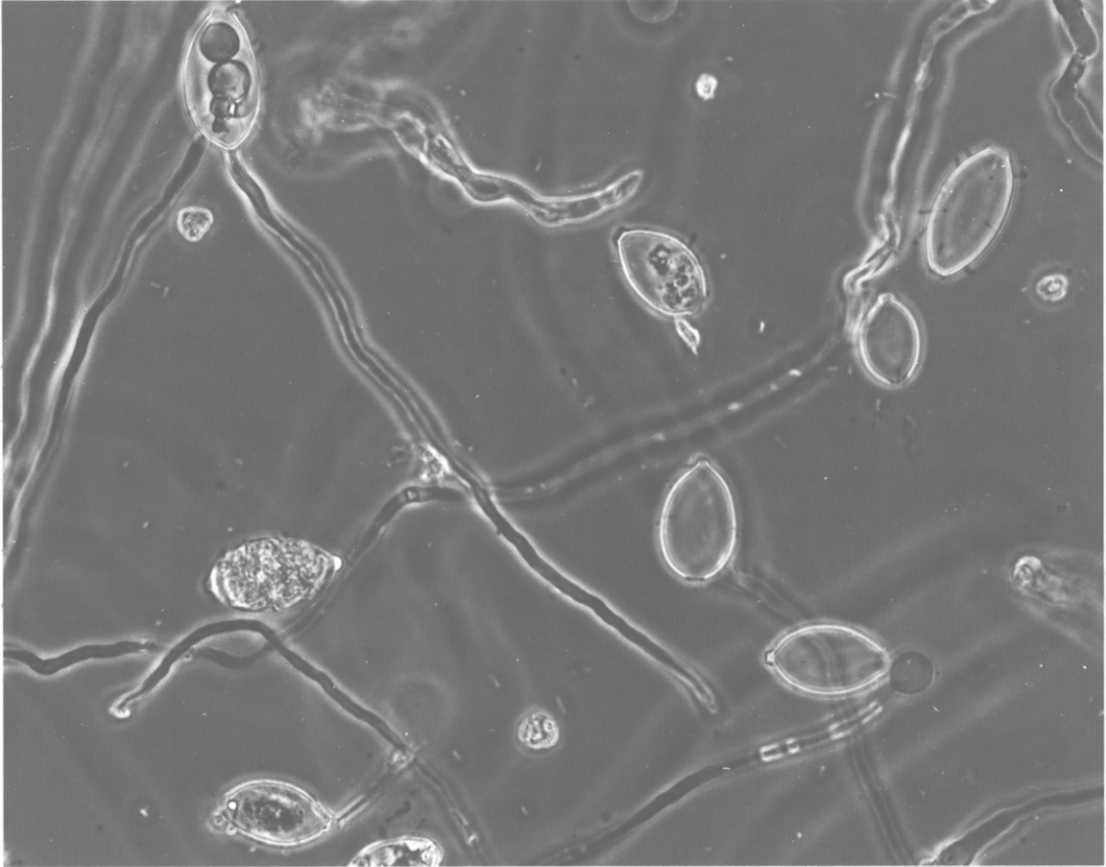
a) Untreated

b) 10 μ g metalaxyl/ml

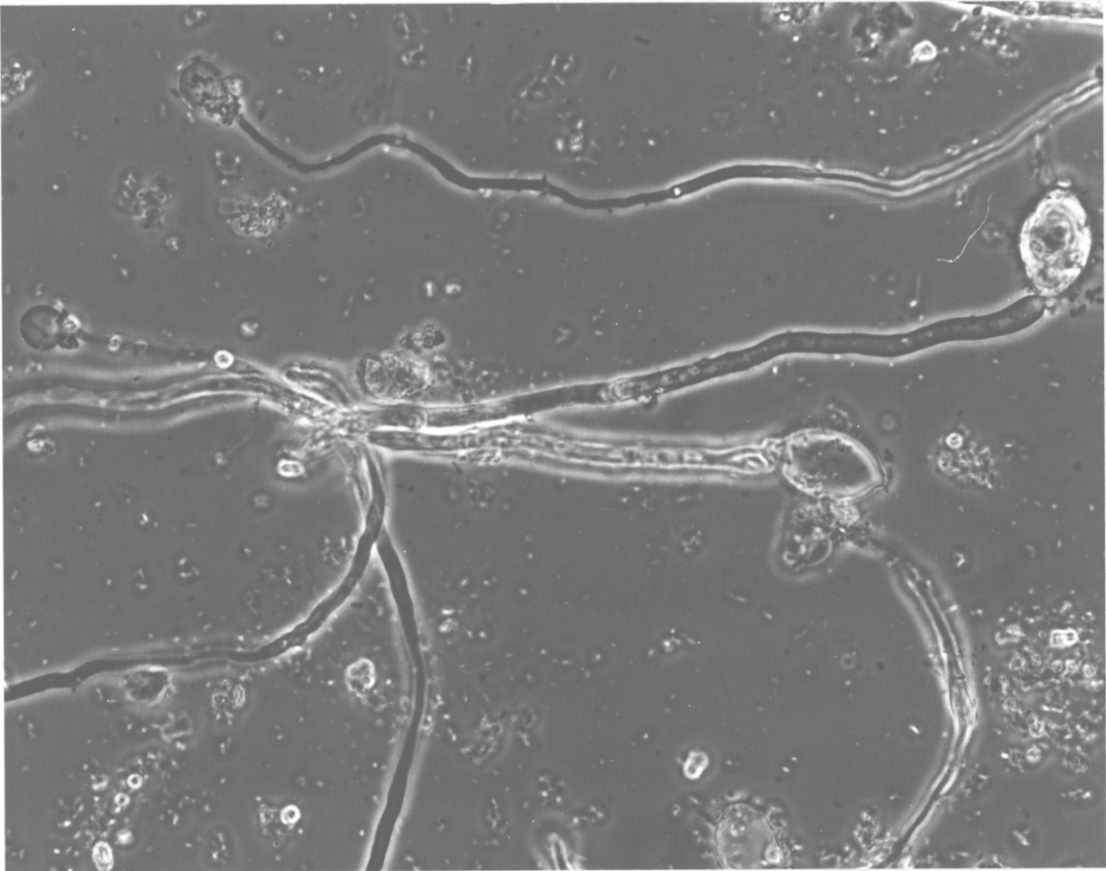
c) 10 μ g R157001/ml

d) 50 μ g R157001/ml (arrow indicates crystal of R157001 -
see text)

a



b



C



d



at 50µg/ml. Hyphal curling was induced by the chemical, but there were no other obvious abnormalities.

5.3.4 Discussion

The ability of spores of P. infestans and P. megasperma f.sp. medicaginis to germinate in the presence of 50 or 100µg metalaxyl/ml respectively, apparently without disruption of their cell contents, corresponded with the results of previous studies (Staub et al., 1980; Staub & Young, 1980; Farh et al., 1981; Coffey & Young, 1984). This finding supported conclusions that the chemical was fungistatic in its effect on metabolism (Coffey & Young, 1984; Section 5.2). A fungistatic mode of action may partly explain the relative ease with which P. megasperma f.sp. medicaginis adapted to high concentrations of the chemical (Chapter Two), and the development of resistance under field and glasshouse conditions.

The germination of zoospores and sporangia of P. infestans in the presence of as much as 50µg R157001/ml, was evidence that the formation of necrotic microlesions on potato leaf discs treated with relatively high concentrations of R157001 (see Section 4.5) resulted from inhibition of the fungus after it had penetrated the host tissue. The high degree of inhibition of hyphal extension, and considerable cellular disorganisation of germinated zoospores and/or sporangia of P. infestans and P. megasperma f.sp. medicaginis, tended to support evidence (see Section 5.2) for a fungicidal mode of action of R157001. This may partly explain the inability of P. megasperma f.sp. medicaginis to adapt to high concentrations of R157001 (see Chapter Two). Additionally, a fungicidal mode of action may reduce the selection for R157001-resistant strains of target pathogens under field and glasshouse conditions.

Mancozeb completely prevented the germination of zoospores and sporangia of P. infestans at 10µg/ml and above, and this suggested that the chemical was probably exerting a fungicidal effect.

6 SELECTION OF FUNGICIDE-RESISTANT MUTANTS USING MUTAGENIC TECHNIQUES

6.1 General Introduction

The isolation in laboratory experiments of strains of target pathogens which are resistant to a fungicide, could provide useful information about the likelihood of similar resistance developing under field and glasshouse conditions. Strains of P. megasperma f.sp. medicaginis with slightly reduced sensitivity to R157001 were obtained using adaptation procedures (Chapter Two). However, since these were not only fully inhibited in vitro by relatively low concentrations of the chemical but also avirulent, it was concluded that they were of little practical significance.

The use of mutagenic agents is generally assumed to increase the frequency with which fungicide-resistant strains of target pathogens are obtained (Dekker, 1982c). However, many early attempts to isolate mutants of Phytophthora spp. by treatment of asexual spores with a mutagen were described by Elliott & MacIntyre (1973) as singularly unrewarding. As examples, they cited studies with Phytophthora cactorum (Leb. & Cohn) Schroet using UV irradiation (Buddenhagen, 1958), with P. infestans using UV irradiation and the chemical mutagen, ethyl-methane-sulphonate (EMS) (Clarke & Robertson, 1966), and P. capsici and Phytophthora drechsleri Tucker using UV irradiation (Castro et al., 1971). Auxotrophic mutants were sought in all these investigations, and Swiezynski & Jankowska (1970) suggested that the difficulty with which they were obtained was associated with the diploidy of Phytophthora. Thus, most mutations to auxotrophy, being recessive, would not be expressed in a diploid nucleus (Clarke & Robertson, 1966). In contrast, drug resistance was shown to be dominant in P. drechsleri (Shaw & Khaki, 1971), and seemed more likely to be expressed. Swiezynski & Jankowska (1970) obtained mutants of P.

infestans by mass selection which grew vigorously on medium amended with streptomycin and acriflavine. The mutants retained their pathogenicity after being passaged on unamended medium. In a subsequent study, Shattock & Shaw (1975) obtained three mutants of P. infestans following NTG mutagenesis of zoospore suspensions. Of two which were resistant to streptomycin, one mutant was dependent on the antibiotic. A third mutant was resistant to chloramphenicol. The resistance to both streptomycin and chloramphenicol appeared to be stable, since it was not lost during repeated subculturing of the mutants on unamended medium. Additionally, the mutants retained their virulence to potato tissue.

Attempts to obtain acylalanine-resistant mutants of Phytophthora using mutagenic procedures have met with varying degrees of success. UV irradiation of zoospores and mycelium of P. capsici resulted in strains which were highly resistant to metalaxyl (Bruin & Edgington, 1982). Some of these remained highly resistant during subculturing on unamended medium and also retained their pathogenicity to pepper (Capsicum frutescens L). Davidse (1981) obtained mutants of P. megasperma f.sp. medicaginis using UV irradiation with a relatively low degree of resistance to metalaxyl, all of which were less virulent than the parent isolate. However, mutants of P. megasperma f.sp. medicaginis and P. megasperma f.sp. glycinea with a high degree of resistance to metalaxyl were isolated following NTG mutagenesis (Davidse & Krüse, 1981; Davidse, 1981). Many of these were fully virulent on appropriate host plants, and were not controlled by doses of metalaxyl which prevented infection by the parent isolate. The resistance to metalaxyl appeared to be stable, in the absence of the fungicide, since it was retained during in vivo subculturing. More recently, Joseph & Coffey (1984) obtained mutants of P. citricola which were highly resistant to metalaxyl, using both UV irradiation and NTG mutagenesis. Some of these were also resistant in vitro, and retained their resistance in the absence of metalaxyl.

Dolgova & Nikulina (1983) obtained metalaxyl-resistant mutants of P. infestans using N-methyl-N-nitrosomethylurea (NMU). After treating two isolates, which were completely inhibited by 0.5 and 5µg 'Ridomil/ml' respectively, with 0.005% NMU for 18 h, mutants were isolated which grew at 15µg 'Ridomil/ml'. Unfortunately, these authors did not evaluate further the response of these mutants to metalaxyl, so the significance of their work to the present study cannot be judged.

Because of the success with which mutagenic treatments had been used previously to isolate metalaxyl-resistant mutants, it was decided to renew attempts to obtain benzamide-resistant mutants of Phytophthora using similar techniques. Initial efforts concentrated on P. infestans, since this was the most important species of Phytophthora against which the benzamides would be used.

6.2 UV Irradiation of P. infestans : Test Development

6.2.1 Choice of Spore Type

a) Introduction

Zoospores of Phytophthora spp. have been used more frequently in studies with mutagens than any other material (see, for example, Clarke & Robertson, 1966; Elliott & MacIntyre, 1973; Shattock & Shaw, 1975). This is presumably because a mutation for resistance in the single nucleus of a zoospore may be expressed more readily than a similar mutation in, for example, the mycelium, where the expression of resistance may be influenced by surrounding sensitive nuclei (Bruin & Edgington, 1982). Consequently, the induction and subsequent yield of zoospores from cultures of P. infestans were investigated in the following experiment.

b) Materials and methods

Suspensions of sporangia in deionised water were prepared separately from 9, 16 and 23-day cultures of K966 and K1142 maintained on

FP agar. The number of sporangia present was determined from counts using an Improved Neubauer haemocytometer, and the suspensions were adjusted to approximately 10^5 sporangia/ml. Each suspension was divided equally into two amounts, one of which was placed at 4°C , and the other at 10°C , for approximately 2.5 h. The relative numbers of sporangia and zoospores in each suspension were determined from further counts using the haemocytometer.

In another experiment, sporangia were centrifuged and resuspended in deionised water, and then handled as described for the unwashed sporangia.

c) Results and discussion

The highest number of zoospores was obtained from 9-day cultures at 10°C (Table 31), confirming similar work by R C Shattock (personal communication). Fewer or no zoospores were produced in the remaining suspensions. A significant proportion of sporangia remained intact in all suspensions. The numbers of ungerminated sporangia were similar in the experiment where sporangia from cultures were washed by centrifugation. This suggested that nutrients from the agar medium in the first experiment had little effect on zoospore differentiation.

Since sporangia of P. infestans can germinate directly by germ tubes (see, for example, Plate 13), it seemed advantageous to use mixtures of sporangia and zoospores in selection experiments. A similar approach appeared to be adopted by Swiezynski & Jankowska (1970), who incubated sporangia from 14-day cultures of P. infestans at 9°C until zoospores appeared. Sporangia (presumably intact) were recorded in higher numbers than zoospores in three out of six selection experiments, and in large numbers in all their experiments. Mixed suspensions of sporangia and zoospores were then used to obtain antibiotic-resistant mutants. However, a mutagenic agent was not employed in these experiments and so the use of a mixture of spore types was probably not critical. In contrast, experiments

Table 31 Numbers ($\times 10^{-5}$) of spores produced by isolates of *Phytophthora infestans* following incubation in deionised water at 4°C or 10°C

Incubation		Isolate K966		Isolate K1142	
Temp.	Days	Sporangia	Zoospores	Sporangia	Zoospores
4°C	9	1.38(71%) ^b	0	1.55(81%)	0.15
	16	1.20(75%)	0	1.70(85%)	0.13
	23	2.48(38%)	0	1.58(62%)	0
10°C	9	1.40(36%)	1.15	1.58(55%)	1.65
	16	1.58(70%)	0	1.85(84%)	0.60
	23	2.00(51%)	0	1.38(89%)	0

^a Age of culture at time of preparation of spore suspensions

^b Percentage of intact sporangia in parentheses

involving the use of a mutagen usually necessitate a preliminary evaluation of its effect on the spore population. To ensure that reproducible results are obtained, a single spore-type should presumably be the subject of the investigation. Consequently, it was decided to attempt the selection of benzamide-resistant mutants of P. infestans from sporangia. Thus, the maximum number of nuclei, in a uniform spore type, would be subjected to mutagenic treatment.

6.2.2 Experimental Medium

a) Introduction

Most selection experiments have involved the isolation of fungal mutants on solid media, one advantage being that individual resistant colonies frequently originate from single spores, and therefore mutation frequencies can be calculated (Dekker, 1982b). Clarke & Robertson (1966) claimed that a specially-developed medium, Pectin Glucose Agar, supported the growth of colonies of P. infestans from widely-spaced zoospores, and so would be suitable for mutation studies. However, their source reference (Clarke, 1966) indicated that relatively few zoospores (2-3% to 25-30% depending on the isolate tested) developed into visible colonies on this medium. Shattock & Shaw (1975) encountered difficulties using the same medium, and other agar media, and so used rye broth to select antibiotic-resistant mutants of P. infestans. They subsequently obtained three mutants resistant to chloramphenicol or streptomycin (see Section 6.1).

In the present study, agar and broth preparations of three media were evaluated to find the one on which most colonies of P. infestans developed from individual sporangia.

b) Materials and methods

The first experiment examined the development of colonies from sporangia on solid media. Sporangia were washed from 9-day cultures of

isolates K1220, K1303 and K1304 with deionised water at room temperature. Suspensions were adjusted to 10^5 sporangia/ml, and 10-fold dilutions were made to 10 sporangia/ml. Aliquots (0.2ml) of selected dilutions were spread onto four plates of clarified FP, clarified V8 or Rye A agar (see Appendix A) and these were examined regularly for 11 days at 22°C.

The second experiment evaluated the growth of isolate K1220 in broth media. A suspension containing 10^5 sporangia/ml was diluted serially by 10-fold amounts to 10 sporangia/ml. Aliquots (1 ml) of selected dilutions were transferred separately to 250 ml Erlenmeyer flasks each of which contained 100 ml clarified FP, Rye B (see Appendix A) or clarified V8 broth. Three replicate broths were inoculated per treatment and incubated on an orbital shaker at 22°C for 8 days. Mycelium was harvested from each flask, freeze-dried overnight and weighed.

c) Results and discussion

In the first experiment, no colonies developed on plates inoculated with 20 or 2 sporangia even after incubation for 11 days. Growth of the three isolates from inoculations with 200 sporangia/plate varied considerably. Colonies of K1220 were apparent on clarified FP and Rye A agar after 5 days, and by 11 days there was confluent growth on these media (Table 32). No colonies of K1303 or K1304 developed on clarified FP or V8 agar after 5 days, and by 11 days only K1303 on V8 had produced many colonies. Colonies of all isolates grew reasonably well on Rye A agar, but on this and the other media growth was asynchronous, small diffuse colonies being frequently engulfed by larger, faster-growing colonies. Shattock & Shaw (1975) reported a similar asynchronous development of colonies from zoospores.

In the second experiment, K1220 grew best in clarified FP broth, even an inoculation of 10 sporangia resulting in measurable growth after 8 days (Table 33).

It was concluded that clarified FP, either as a broth or

Table 32 Growth of colonies of *Phytophthora infestans* isolate K1220 from sporangia^a on agar media

Isolate	Incubation period (days)	Number of colonies ^b on agar medium:		
		Frozen pea	Rye A	V8
K1220	5	8	6	1
	11	confluent	confluent	2
K1303	5	0	1	0
	11	1	6	8
K1304	5	0	1	0
	11	1	4	0

^a Each plate was inoculated with 200 sporangia

^b Average of four replicates

Table 33 Yield of Phytophthora infestans isolate K1220 from broth media inoculated with sporangia

Broth medium ^a	Dry weight (mg) of mycelium which grew from inoculum of sporangia (number per broth)		
	10	10 ³	10 ⁵
Frozen pea	8 ^b	42	244
Rye	0	2	67
V8	0	0	14

^a See Appendix A

^b Average of three replicates

solidified with agar, would be a suitable medium for mutation studies.

6.2.3 Viability Determination in Broth and Agar Medium

a) Introduction

Lack of synchrony in colony development from propagules can be a problem in mutation studies, as Shattock & Shaw (1975) found in their work with P. infestans. After several unsuccessful attempts to introduce synchronous growth by manipulating the composition of the medium, they adopted the dilution end-point method (Fisher & Yates, 1963) to determine the number of viable zoospores before and after treatment with NTG.

The possibility of using this method with suspensions of sporangia before and after UV irradiation was examined in the following experiment.

b) Materials and methods

A suspension of isolate K1220 containing 10^5 sporangia/ml was diluted by 10-fold amounts to one sporangium/ml. Aliquots (0.2ml) of each dilution were spread onto seven clarified FP agar plates or added to seven test tubes each containing 4ml clarified FP broth.

A 9cm petri dish with 20ml of the original suspension was placed in a purpose-built UV irradiation cabinet. The UV lamp (Sylvania Ltd., wavelength 254nm) was set at 40cm above the dish and switched on for 4 min. The suspension was gently agitated during the period of exposure to subject the sporangia uniformly to irradiation. At intervals, 1ml aliquots of the suspension were diluted serially to 10^{-5} and used to inoculate plates or broth as described above.

The agar plates and tubes of broth were incubated at 22°C in the dark (to prevent photorepair of DNA damaged by UV irradiation) and the number in which there was visible growth of P. infestans was counted after 12 days. From the data the numbers of viable sporangia were calculated using the equation:-

$$\log \lambda = x \log a - K \quad (\text{Fisher \& Yates, 1963})$$

where

λ = number of sporangia per tube at the highest concentration of the dilution series which were able to develop into macroscopically visible colonies.

x = mean fertile level, calculated by dividing the total number of tubes with mycelium present by the total number of tubes per dilution (seven).

a = serial dilution factor ($\times 10$)

K = a constant, obtained from Table VIII₂ in Fisher & Yates (1963) using the value of x . Alternatively, K may be determined from the mean sterile level, y , which is calculated by subtracting x from the number of dilutions (five)

The procedure for determining the numbers of viable sporangia is exemplified by the following calculation :

If 24 broths contained visible growth of P. infestans, and seven tubes were inoculated at each dilution, x is $24 - 7 = 3.429$. Therefore, y is $5 - 3.429 = 1.571$. From the value of y , K is calculated as 0.736.

These data are used in the equation to give

$$\log \lambda = (3.429 \times \log 10) - 0.736$$

$$\log \lambda = 2.693$$

$$\lambda = 493$$

Therefore, the number of viable sporangia/ml in the concentrated suspension is 24, 650.

c) Results and discussion

The data obtained (Table 34) were used to draw a dosage-response curve (Figure 8) of the effect of UV irradiation on viability of sporangia. The results showed clearly that more sporangia grew into visible colonies in clarified FP broth than on agar. Therefore, it was decided to

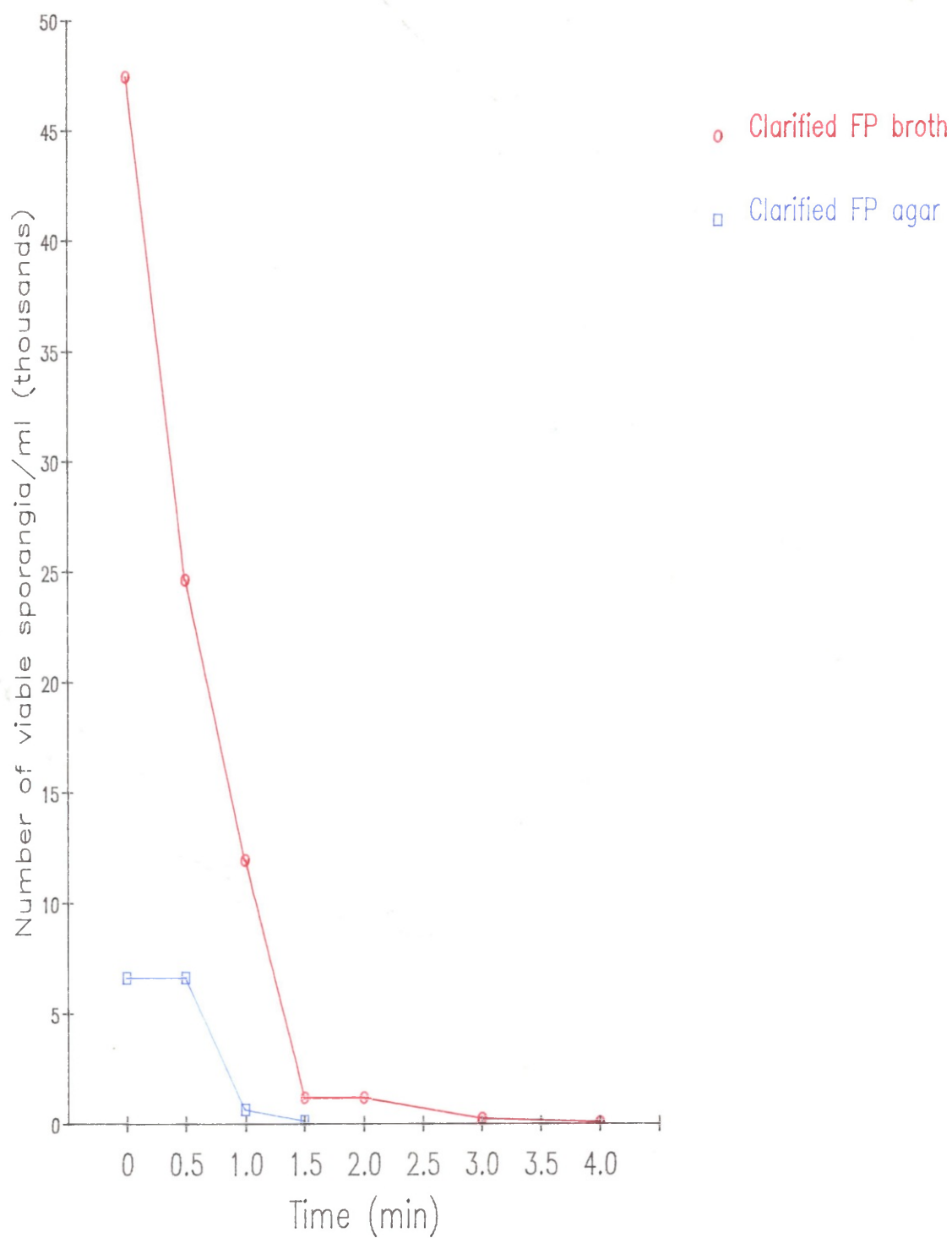
Table 34 Growth^a of *Phytophthora infestans* isolate K1220 in clarified
FP media following UV irradiation for 4 min

UV irradiation (min)	Medium	No. of tubes/plates with visible growth at given dilutions					No. of viable sporangia/ml (thousands) ^b
		10 ⁻¹	10 ⁻²	10 ⁻³	10 ⁻⁴	10 ⁻⁵	
0	Broth	7	7	7	4	1	47.46
	Agar	7	7	5	1	-	6.61
0.5	Broth	7	7	7	3	0	24.65
	Agar	7	7	4	2	1	6.61
1	Broth	7	7	6	2	0	11.94
	Agar	7	4	1	1	-	0.61
1.5	Broth	7	7	1	0	0	1.19
	Agar	6	1	0	0	1	0.09
2	Broth	7	6	2	0	0	1.19
	Agar	0	0	-	-	-	
3	Broth	7	3	0	0	0	0.24
4	Broth	5	1	0	-	-	0.07

^a Determined after incubation at 22°C for 12 days

^b Calculated by the method of Fisher & Yates (1963)

Figure 8 Reduction in the number of viable sporangia of *Phytophthora infestans* following UV irradiation for 4 min



employ the broth medium in both subsequent dilution end-point analyses and attempts to obtain fungicide-resistant mutants.

Shattock & Shaw (1975) chose the death of 50% of zoospores as the degree of kill most likely to yield the highest proportion of antibiotic-resistant mutants of P. infestans. From the present experiment it was deduced that a 50% reduction in the number of viable sporangia was achieved after exposure to UV irradiation for 30s.

6.2.4 Sensitivity to R157846 and metalaxyl

a) Introduction

The purpose of this experiment was to determine the concentration of R157846* and metalaxyl which completely prevented the growth of P. infestans from sporangia in clarified FP broth. Thus, a suitable inhibitory concentration of each fungicide would be identified which permitted the growth of resistant mutants, but prevented the growth of normally sensitive colonies.

b) Materials and methods

R157846 and metalaxyl were suspended separately in acetone and diluted in sterile water to give the range of concentrations shown in Table 35. Aliquots (0.5ml) of each dilution were added to three 250ml Erlenmeyer flasks containing 50ml clarified FP broth. Three unamended broths served as controls. Each flask was inoculated with 1ml of a suspension containing 150,000 sporangia of isolate K1220 per ml.

Flasks were incubated on an orbital shaker at 22°C for 7 days and the mycelium from each was then harvested, freeze-dried and weighed.

c) Results and discussion

Growth of P. infestans was completely prevented by R157846 in all flasks at 1 and 10µg/ml and occurred in only one replicate at 0.1µg

* R157846 was chosen because of its relatively slow breakdown in aqueous solutions (see Chapter Three).

Table 35 Dry weight (mg) of *Phytophthora infestans* isolate K1220
harvested after 7 days from clarified FP broth^a amended
with R157846 or metalaxyl

Replicate	R157846					Metalaxyl			
	0	0.01	0.1	1	10	0.1	1	10	100
1	236	294	4	0	0	123	49	15	5
2	136	61	0	0	0	81	76	11	5
3	82	45	0	0	0	160	38	12	7

^a Inoculated with 150,000 sporangia per broth

R157846/ml. Therefore, 1µg R157846/ml was chosen as the concentration for the selection of benzamide-resistant mutants in subsequent studies.

Growth occurred at all concentrations of metalaxyl tested, so a further experiment was undertaken to find a concentration of the chemical which would completely prevent growth of the sensitive parent strain.

6.2.5 Minimum Inhibitory Concentration of Metalaxyl

a) Introduction

The response of sensitive and resistant isolates of P. infestans to high doses of metalaxyl was determined in the following experiment. A concentration of the fungicide was sought which prevented the growth of the parent isolate, yet permitted the growth of resistant mutants.

b) Materials and methods

Different amounts of 'Ridomil' (25% metalaxyl) were added to four 1000ml Erlenmeyer flasks, each containing 500ml clarified FP agar, to give final concentrations of 100, 200, 400 and 800µg metalaxyl/ml. An additional flask of unamended agar was used to provide controls. The agar from each flask was dispensed into 9cm petri dishes and when set the plates were inoculated centrally with a 6mm plug of mycelium of one isolate of P. infestans. Four metalaxyl-sensitive and two metalaxyl-resistant isolates were used in the experiment and there were three replicate plates for each treatment. Plates were incubated at 22°C for 11 days. The radial growth of mycelium was determined by measuring two colony diameters per plate at 90° to each other, averaging them, and subtracting the diameter of the inoculum plug.

c) Results and discussion

The growth of all isolates was inhibited by 94-100% on media containing 400µg and 800µg metalaxyl/ml, with no apparent difference between sensitive and resistant isolates (Table 36). The resistant isolate

Table 36 Inhibition (%) of radial growth of isolates of *Phytophthora infestans* by high doses of metalaxyl

Isolate ^a	Metalaxyl concentration (µg/ml)			
	100	200	400	800
<u>Sensitive isolates</u>				
K1090	74	80	94	98
K1182	92	97	100	100
K1217	90	97	98	100
K1220	88	89	99	100
<u>Resistant isolates</u>				
K1067	46	30	97	100
K1133	48	85	98	100

^a Metalaxyl sensitivity determined from the in vivo response of isolates to the fungicide (see Section 4.5)

K1067 was inhibited by only 30% at 200µg metalaxyl/ml, but the other resistant isolate K1133 was inhibited by almost as much (85%) as two of the sensitive isolates (K1090, K1220). Only at 100µg metalaxyl/ml was the extent to which the resistant isolates were inhibited (46% - 48%) markedly different from that of the sensitive isolates (74-92%). It was therefore considered that this dose would be suitable for distinguishing resistant mutants.

6.3 UV Irradiation of *P. infestans* : Selection of Fungicide-resistant mutants

6.3.1 Selection Using Erlenmeyer Flasks

a) Introduction

The following section summarises attempts to obtain R157846 and metalaxyl-resistant mutants of *P. infestans* from broth medium in Erlenmeyer flasks, after UV irradiation.

b) Materials and methods

Five, serial, 10-fold dilutions were made from a suspension (80 ml) of each isolate. Aliquots (0.2ml) of the diluted suspensions were added separately to seven test tubes, each containing 4ml clarified FP broth. These served to determine the viability of sporangia before UV irradiation.

Equal amounts (almost 20ml) of concentrated sporangial suspension were transferred to four sterile 9cm petri dishes and irradiated with the lids of the dishes removed. To lessen the chances of contamination the cabinet itself was irradiated for 20 min beforehand. For the irradiation of sporangia, the UV lamp was set at 40cm above the dishes and the suspensions were gently agitated. After irradiation, the suspensions were diluted and aliquots were transferred to tubes of clarified FP broth, as is described for the non-irradiated suspension.

The remaining irradiated suspension in the petri dishes was added separately to four 250ml Erlenmeyer flasks each containing 80ml clarified FP broth amended with either 1µg R157846/ml medium (two flasks) or 100µg metalaxyl/ml medium (two flasks). These were incubated at 22°C on a shaker at low speed for at least 21 days.

c) Results and discussion

Table 37 summarises the results of several experiments which were made in sequence. These results were often erratic. In Experiment 1, K1220 was exposed to UV irradiation for 30s as previous results (Section 6.2.3) indicated that this dosage would kill 50% of sporangia. However, no sporangia apparently were killed in this experiment, and calculated concentrations were higher after irradiation than before irradiation. In Experiment 2, two subcultures of isolate K1220 gave contrasting results. With one culture, 80% of the sporangia were killed by a 1 min exposure, whereas sporangia of the other culture were unaffected by an exposure of 45s. In Experiment 3, the death of 47% of sporangia of isolate K1301 after a 30s exposure was close to the degree of kill which was sought. However, in these three experiments no growth was detected in any broths containing 1µg R157846/ml. Therefore, in subsequent experiments, sporangial suspensions were irradiated for 1 min to increase the degree of kill and so improve the chances of selecting benzamide-resistant mutants. Although a greater degree of kill (70-100%) was achieved, no mutants were obtained which grew at 1µg R157846/ml from approximately 2.6×10^7 sporangia in a total of five experiments. It was concluded that benzamide-resistant mutants occurred, if at all, at very low frequencies. In contrast, colonies of P. infestans frequently developed in metalaxyl-amended broths although they were diffuse and slow-growing. On clarified FP agar the rate of growth of these variant strains was always less than half that of the parent isolate. On agar amended with metalaxyl to 100µg/ml, the degree of inhibition of the variants hardly altered, but was never less than that of

Table 37 Effect of UV irradiation on the viability of sporangia
of *Phytophthora infestans*

Experiment	Isolate	Exposure period (s)	Number of viable sporangia (thousands) ^a		% kill
			Before irradiation	After irradiation	
1	K1220	30	35.0	64.1	0
2	K1220	60	126.5	24.7	80
	K1220 A	45	11.9	12.6	0
3	K966	45	11.9	1.7	86
	K1301	30	1.7	0.9	47
4	K1090	60	6.4	1.7	73
	K1272	60	125.6	3.5	97
	K1301	60	1.7	ND ^b	approx. 100
5	K1272	60	8.7	1.2	86

^a Calculated by the method of Fisher & Yates (1963)

^b Could not be determined

the parent isolate. It was concluded that the response to metalaxyl of the variants was identical to that of mutant strains of P. megasperma f.sp. medicaginis, obtained by Davidse (1981) using UV irradiation. For this reason, and because the selection of metalaxyl-resistant mutants was a secondary aim of the present study, no further work was undertaken with the variant strains of P. infestans. The experimental procedure was modified before continuing attempts to obtain R157846-resistant mutants of P. infestans.

6.3.2 Dosage-response to UV Irradiation in Repli-dishes

a) Introduction

Repli-dishes* were used in this experiment (and subsequent selection experiments) in the hope that the chances of obtaining benzamide-resistant mutants would be increased, and consequently their frequency could be determined.

b) Materials and methods

A suspension containing 10^5 sporangia/ml of isolate K1272A was prepared in sterile deionised water at room temperature. A sub-sample was diluted by 10-fold amounts to one sporangium/ml. Aliquots (0.2ml) of each dilution were transferred separately to seven repli-dish compartments, each containing 3ml clarified FP broth. Aliquots (1ml) of the concentrated sporangial suspension were transferred to several compartments of an empty repli-dish, which was irradiated without its lid for 8 mins, 40cm below a UV lamp. The dishes were gently agitated to ensure that all sporangia were subjected to the same dose of UV irradiation. At various

* sterile, covered plastic dishes (100mm^2) sub-divided into 25 compartments of equal size. Manufactured by Sterilin Ltd., Teddington, Middlesex.

intervals 1ml aliquots of the irradiated suspension were diluted and transferred to clarified FP broth, by the method described for the non-irradiated suspension. All repli-dishes were incubated at 22°C in the dark, and examined regularly for visible growth of P. infestans.

c) Results and discussion

The dosage-response curve (Figure 9) shows that apparently most sporangia were killed within 2 min. Thereafter, the reduction in viability was much less rapid. From the slope of the dosage response curve it was decided to irradiate suspensions in subsequent selection experiments from 30s to 1 min.

6.3.3 Selection using Repli-dishes

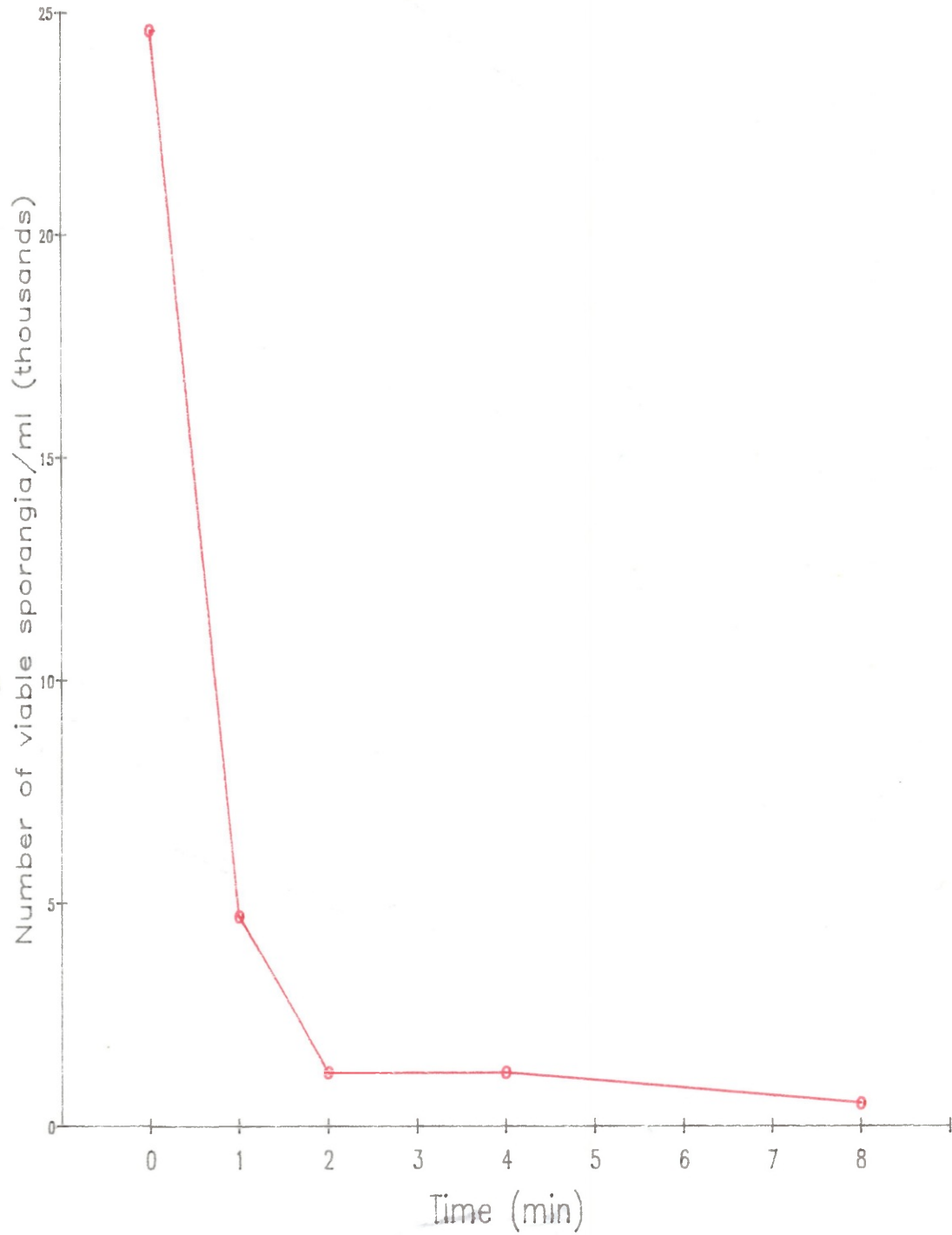
a) Introduction

The following section summarises experiments which attempted to obtain R157846 and metalaxyl-resistant mutants of P. infestans. Suspensions with similar concentrations of sporangia were used throughout, thereby improving between-test comparability.

b) Materials and methods

A suspension containing 10^5 sporangia/ml of isolate K1272 (subculture A or B) was prepared from cultures grown on FP agar at 19°C for 9-13 days. A sub-sample of this suspension was diluted by 10-fold amounts to one sporangium/ml and 0.2ml aliquots of each dilution were transferred separately to seven repli-dish compartments each containing 3ml clarified FP broth. The remaining suspension was transferred as 1ml aliquots to separate compartments of empty repli-dishes without lids, and these were irradiated 40cm below the UV lamp. During irradiation the suspensions were gently agitated. When the procedure was complete, a sub-sample of the irradiated suspension was diluted and transferred to clarified FP broth, as described for the non-irradiated broth. Aliquots (2ml) of clarified FP broth amended with either 1.5µg R157846/ml or 150µg metalaxyl/ml were added

Figure 9 Reduction in the number of viable sporangia of
Phytophthora infestans isolate K1272A during UV irradiation
for 8 min



to prescribed numbers of compartments containing the concentrated, irradiated suspension, to give final concentrations of 1µg R157846/ml and 100µg metalaxyl/ml medium. These repli-dishes were incubated at 22°C in the dark, and examined regularly for visible growth of P. infestans. The viability of sporangial suspensions was determined after approximately 14 days. Fungicide-amended broths were incubated for at least 28 days.

c) Results and discussion

The results of seven experiments are summarised in Table 38. The degree of kill varied widely, despite using sporangial suspensions of uniform density. Thus, in suspensions of K1272A irradiated for 1 min, apparently 81% and 29% of sporangia were killed in Experiments 3 and 4, respectively. Irradiation of separate suspensions of subculture B apparently resulted in the death of 86% and 48% of sporangia after 2 and 4 min, respectively. Table 38 also gives the amounts of sporangial suspensions treated with each fungicide. In the first three experiments, colonies of P. infestans developed at equal rates in all metalaxyl-treated compartments. Because of this, it seemed unlikely that it would be possible to calculate valid mutation frequencies with metalaxyl. Therefore, in subsequent experiments, a small proportion (<15%) of compartments containing irradiated sporangia were treated with metalaxyl, the remainder with R157846. Using the method of Fisher & Yates (1963), it was calculated that a total of 1.3×10^7 sporangia were subjected to selection in metalaxyl-amended clarified FP broth. Because of the development of colonies of P. infestans in all compartments containing the fungicide, and the secondary importance of obtaining metalaxyl-resistant mutants, the growth characteristics of only some colonies were examined in detail. Growth was slow and diffuse, and little aerial mycelium was produced, on unamended medium. The dosage-response of the variants to metalaxyl is described in Section 6.3.4.

Table 38 Viability^a of sporangia in suspensions of *Phytophthora infestans* K1272 before and after UV irradiation

Expt. ^b	Exposure period (min)	Total vol.(ml) of sporangial suspension treated with:		Viable sporangia (thousands/ml)		% kill
		R157846	Metalaxyl	Before irradiation	After irradiation	
1	0.5	53	54	46.3	24.1	48
2	0.75	45	46	120.6	90.2	25
3	1	40	40	64.3	12.4	81
4	1	90	10	90.2	64.3	29
5	1	272	30	32.3	45.4	0
6	4	130	20	32.4	17.0	48
7	2	82	5	6.4	0.9	86

^a Calculated by the method of Fisher & Yates (1963)

^b Subculture A was used in experiments 1-4 and Subculture B in Experiments 5-7

Again using the method of Fisher & Yates (1963), it was calculated that a total of 3.2×10^7 sporangia were subjected to selection in R157846-amended clarified FP broth. However, no colonies of P. infestans developed in any of the benzamide-treated repli-dish compartments. These results appeared to confirm the conclusion reached from previous selection experiments (Section 6.3.1) that benzamide-resistant mutants occurred, if at all, at a very low frequency. Alternatively, the experimental conditions in both sets of experiments may not have been optimal for the induction and/or growth of mutants. In particular, an evaluation of the mutagenic effect of UV irradiation could not be made due to the inability to identify readily, mutants which were highly resistant to metalaxyl. Consequently, a system was sought which would enable an accurate determination of the frequency of mutants highly resistant to metalaxyl. This precondition appeared to be met by a mutagenic procedure described by Davidse (1981). Mutants of P. megasperma f.sp. medicaginis were obtained after treatment with the chemical mutagen, NTG, which were highly resistant to metalaxyl and unchanged in their rate of growth on unamended medium. Because of their faster rate of growth on metalaxyl-amended medium, these mutants could be differentiated from weaker variants. Accordingly, it was decided to attempt the selection of benzamide-resistant mutants of P. megasperma f.sp. medicaginis using Davidse's method (Davidse, 1981). Selection experiments using P. infestans were halted during this investigation.

6.3.4 Dosage-response of Variant Isolates Selected on Metalaxyl-amended Medium

a) Introduction

Colonies of P. infestans isolate K1272 which grew in clarified FP broth amended with 100µg metalaxyl/ml were transferred onto FP agar amended with the same concentration of the fungicide (Section 6.3.3). The

metalaxyl-sensitivity of a selection of these variants was determined in two experiments.

b) Experimental

Metalaxyl was incorporated into molten clarified FP agar at a range of concentrations to 100µg/ml and dispensed into 9cm petri dishes. Three replicate plates were prepared per treatment. Plates were inoculated centrally with a 6mm plug of mycelium cut from agar cultures of the parent isolate, K1272, or variants selected after UV irradiation.

Plates were incubated at 22°C for 7 - 10 days and radial growth of isolates was determined by measuring two colony diameters per plate, at 90° to each other, averaging them and subtracting the diameter of the inoculum plug.

In Experiment A (Table 39a) variant isolates MET-1 to MET-15 were much less inhibited by metalaxyl than the parent isolate, K1272. However, their rate of growth on unamended medium was less than one-third that of the parent isolate.

Growth of most variants in Experiment B was unaffected by 100µg metalaxyl/ml (Table 39b) but on unamended medium, the radial growth of the variants was no more than 58% that of the parent isolate.

c) Discussion

None of 30 variants of P. infestans K1272 was highly resistant, in vitro, to metalaxyl (i.e. with a rate of growth on unamended and metalaxyl-amended medium similar to that of the parent isolate on unamended medium). The inability to identify highly-resistant mutants may, in part, be related to the similar degree of growth demonstrated by sensitive and resistant isolates at high concentrations of metalaxyl (see Section 6.2.5). Consequently, the ability of the system to generate R157846-resistant mutants was not established. This conclusion supported the decision (see Section 6.3.3) to continue attempts to obtain R157846-resistant mutants of Phytophthora using a different system.

Table 39 Radial growth of isolates of *Phytophthora infestans* obtained by UV irradiation and selection from metalaxyl-amended medium^a

a) Experiment A

Isolate reference	Growth on unamended medium(mm)	Inhibition (%) by metalaxyl (µg/ml)					
		0.001	0.01	0.1	1	10	100
K1272	51	4	69	88	92	94	94
MET-1	13	8	23	31	8	15	23
MET-2	14	40	7	36	40	14	21
MET-3	11	0	0	0	0	18	27
MET-4	15	7	7	13	0	47	60
MET-5	10	0	0	0	10	30	20
MET-6	10	0	0	0	0	0	10
MET-7	14	14	29	50	36	36	43
MET-8	13	0	15	15	15	0	0
MET-9	13	0	0	8	23	15	31
MET-10	12	8	8	8	17	33	25
MET-11	13	15	8	23	8	23	31
MET-12	9	0	0	0	0	11	33
MET-13	15	7	20	20	27	40	60
MET-14	13	0	23	15	23	15	15
MET-15	14	0	7	0	0	14	0

b) Experiment B

Isolate reference	Growth on unamended medium(mm)	Inhibition (%) by 100µg metalaxyl/ml
K1272	74	81
MET-16	43	28
MET-17	31	0
MET-18	30	0
MET-19	35	0
MET-20	37	11
MET-21	34	0
MET-22	27	4
MET-23	30	0
MET-24	23	9
MET-25	32	0
MET-26	32	0
MET-27	29	0
MET-28	30	0
MET-29	30	0
MET-30	36	0

^a See Section 6.3.3

6.4 NTG Mutagenesis of *P. megasperma* f.sp. *medicaginis*

6.4.1 Selection of Fungicide-resistant Mutants - 1

a) Introduction

Davidse (1981) stated that metalaxyl-resistant mutants of *P. megasperma* f.sp. *medicaginis* could easily be obtained after treatment of encysted zoospores with NTG, and selection on V8 agar containing 2µg metalaxyl/ml. He described two types of mutant: those which displayed a low degree of resistance and were invariably weakly virulent to host plants and those which were highly resistant to metalaxyl both in vitro and in vivo. On unamended medium, the growth rate and colony morphology of highly resistant mutants was similar to that of the original isolate. ED₅₀-values for inhibition of mycelial growth were well above 100µg metalaxyl/ml. Highly resistant mutants were as virulent to the host plant, alfalfa, as the parent isolate. Metalaxyl, at normally effective concentrations, failed to control the disease caused by resistant mutants. The presence of resistant mutants in mixed populations after 12 infection cycles in the absence of metalaxyl indicated that the fitness of these strains was not significantly reduced. From these findings, it was concluded that development of resistance to acylalanine fungicides was likely to occur under field and glasshouse conditions.

By using the methods described by Davidse (1981), and the same fungal species, *P. megasperma* f.sp. *medicaginis* it was hoped that the efficacy of the system could be quantified. This would provide a better standard for assessing the chances of isolating strains resistant to benzamide fungicides.

b) Materials and methods

Isolate K1120 of *P. megasperma* f.sp. *medicaginis* was chosen for the present study because it had been used successfully by Davidse (1981) to obtain mutants which were highly resistant to metalaxyl. The radial

growth of this isolate was completely inhibited by 1 μ g metalaxyl/ml after 5 days (see Table 12), so it was decided to attempt the selection of strains which would grow at 2 or 20 μ g metalaxyl/ml.

A dosage-response experiment was undertaken to determine a concentration of R157846 which would be suitable for the selection of benzamide-resistant mutants. R157846, suspended in acetone, was added at a range of concentrations, to separate Erlenmeyer flasks containing clarified V8 agar. A flask of V8 agar was left unamended. These media were dispensed into 9cm petri dishes to give three replicate plates per treatment. Each plate was inoculated centrally with a 6mm plug of mycelium of isolate K1120 and incubated at 25°C for 8 days. The radial growth on each plate was determined by measuring two colony diameters per plate at 90° to each other, averaging them, and subtracting the diameter of the inoculum plug.

Fungicide-resistant mutants were selected following NTG treatment of germinating zoospores. Crystals of NTG were suspended in deionised water (adjusted to approximately pH 5.7) at a rate of 30mg chemical/10ml water. On the advice of L C Davidse (personal communication) this solution was left overnight at room temperature to ensure that a sufficient quantity of the mutagen had dissolved. Suspensions of zoospores were prepared as described in Section 5.3.2a. These suspensions were combined in a sterile 500ml Erlenmeyer flask and the concentration of zoospores was assessed using a Sedgewick Rafter counting cell* (Graticules

* 1ml of zoospore suspension was placed in the well of the counting chamber and the glass slide was lowered on top of it. The number of zoospores in 10 diagonal squares of the etched grid was counted and averaged to give the number of zoospores per square. Multiplication by 1000-times gave the number of zoospores per ml.

Ltd., Tonbridge, England). Aliquots (8ml) of zoospore suspension were transferred to 9cm petri dishes and 1ml of undiluted clarified V8 broth was added to each to induce zoospore encystment. This was monitored in one plate. Most zoospores encysted and began to germinate within approximately 1 h (see Plate 15). The addition of 1ml aliquots of NTG solution to each dish was timed. From 10 min exposure to NTG onwards, the liquid was decanted from each plate and replaced with 5-10ml clarified V8 broth. The time required to complete this procedure was recorded. Clarified V8 broth was removed from plates in the same sequence and replaced with 20ml aliquots of V8 agar amended with either metalaxyl to 20µg/ml, or R157846 to 5µg/ml. Medium amended with the different fungicides was added alternately to sets of 10 plates in the order in which they had been subjected to NTG mutagenesis. Control treatments included non-NTG treated, germinating zoospores, to which unamended V8 agar, or V8 agar containing fungicide, was added. Plates were incubated at 25°C for at least 28 days, during which time they were examined regularly for colonies of P. megasperma f.sp. medicaginis.

c) Results and discussion

3µg R157846/ml completely prevented the growth of isolate K1120, whereas slight growth (4%) occurred at 1µg R157846/ml. Consequently, 2 or 5µgR157846/ml was chosen to select for R157846-resistant mutants of the fungus.

A total of 15.95×10^7 zoospores of isolate K1120 were subjected to NTG treatment followed by selection on equivalent numbers of metalaxyl and R157846-amended agar plates (Table 40).

Many colonies developed on metalaxyl-amended medium; only the most vigorous of these was transferred onto newly-amended medium. Mutation frequencies could not be determined because colonies emerged asynchronously between 8 - 25 days. Evaluation of the degree of resistance of isolates to metalaxyl is described in Section 6.4.2.

Plate 15 Stage of development (1 h after encystment) at which
germinating zoospores of *Phytophthora megasperma* f.sp. *medicaginis* were
subjected to NTG mutagenesis

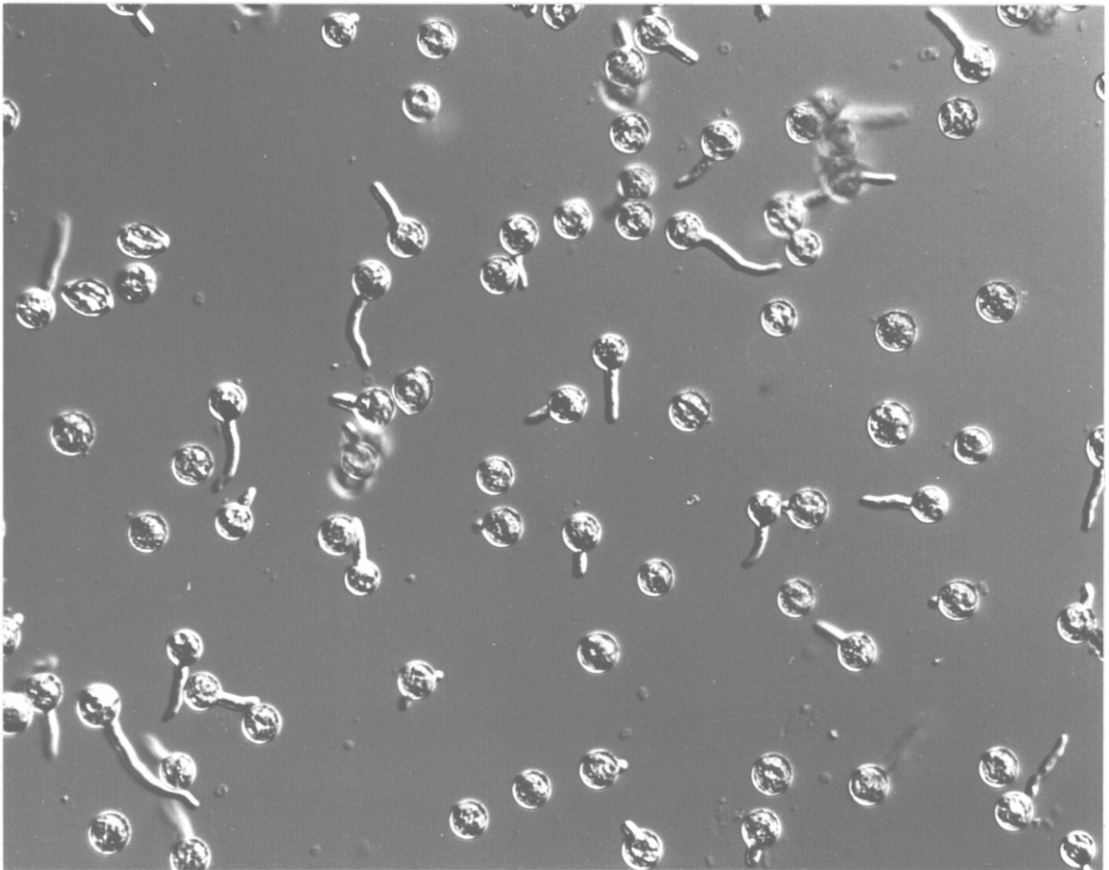


Table 40 Summary of experiments with NTG to select mutants of
Phytophthora megasperma f.sp. medicaginis resistant to
metalaxyl and R157846

Experiment	NTG exposure (min) ^a	No. of zoospores (millions) ^b	Metalaxyl		R157846	
			µg/ml	Number of plates	µg/ml	Number of plates
1	10-16	ND ^c	20	19	5	19
2	10-22	14.3	20	33	5	32
3	10-18	4.2	2	26	2	26
4	10-17	9.7	2	23	2	24
5	10-19	6.5	2	29	2	30
6	10-18	48.0	2	31	2	31
7	18-32	76.8	20	48	2	60

^a Minimum and maximum period of exposure of suspensions in individual petri dishes

^b Calculated using a Sedgewick Rafter counting cell

^c Not determined

Eight colonies grew sparsely on medium amended with 2 μ g R157846/ml. These failed to grow when transferred to newly-amended medium, although development was rapid on unamended medium. These variants were considered to be of little significance because the amount of R157846 used in their selection was only slightly above the minimum inhibitory concentration. The failure to obtain R157846-resistant mutants of P. megasperma f.sp. medicaginis as readily as metalaxyl-resistant mutants provided further evidence that the former were less likely to occur under field and glasshouse conditions.

It was not possible to expose plates containing suspensions of zoospores to NTG for an identical period of time. Additionally, it was observed that encysted zoospores became detached from the base of petri-dishes and were lost during the wash with clarified V8 broth. Those detached zoospores which were retained in the dishes tended to settle and produce confluent growth around the edge of the plates. Therefore, it was decided to modify the system in subsequent attempts to obtain R157846-resistant mutants of P. megasperma f.sp. medicaginis.

6.4.2 Dosage-response of Variant Isolates Selected on Metalaxyl-amended Medium

a) Introduction

Mutant colonies of P. megasperma f.sp. medicaginis isolate K1120 which developed on medium amended with metalaxyl (Section 6.4.1) were transferred to newly-amended medium. The dosage-response of these variants to metalaxyl was determined.

b) Materials and methods

Metalaxyl was suspended in acetone and added to clarified V8 agar at a range of concentrations to 100 μ g/ml. These media and unamended medium were dispensed separately into 9cm petri dishes. Three replicate plates of each medium were inoculated centrally with a 6mm plug of mycelium

of one isolate. The isolates used were mutant strains, and standard metalaxyl-sensitive (K1120) and resistant (K1121) isolates. Plates were incubated at 25°C for 7 days, and radial growth of isolates was determined by measuring two colony diameters per plate at 90° to each other, averaging them, and subtracting the diameter of the inoculum plug.

c) Results and discussion

The dosage-response of 42 isolates of P. megasperma f.sp. medicaginis was examined in two experiments (Table 41). Including the standards, five isolates (K1120, K1121, M2, M9, M11) were tested in both experiments. The identical order in which these isolates responded to metalaxyl indicated that there was a good correlation between tests.

The parent isolate, K1120, was the most sensitive to metalaxyl ($P = 0.05$). The ED_{50} 's of remaining isolates ranged from 0.01 µg/ml to $>10^5$ µg/ml. Generally, it appeared that a continuum of sensitivity to metalaxyl existed amongst isolates. However, these could be arbitrarily differentiated into two types with low or high degrees of resistance to metalaxyl. Highly-resistant mutants (K1121, M2, M4, M7, M12, M21 and M40) grew on medium amended with 100 µg metalaxyl/ml at a similar rate (not greater than 10% inhibition) to the parent isolate on unamended medium. All were presumed to be similar to the mutants with a high degree of resistance obtained by Davidse (1981). Other mutants with low ED_{50} -values or which grew more slowly than the parent isolate on unamended medium, were presumed to possess a low degree of resistance. The response to metalaxyl of mutants with high and low degrees of resistance is shown in Plate 16. The results indicated that mutants of P. megasperma f.sp. medicaginis with a high degree of resistance to metalaxyl could be obtained using the system described by Davidse (1981).

Consideration was given to testing of the pathogenicity and in vivo metalaxyl-sensitivity of mutants. However, since no R157846-resistant mutants had been obtained, and metalaxyl was included as a standard treatment only, no further work was undertaken with these strains.

Table 41 Growth of isolates of *Phytophthora megasperma* f.sp. *medicaginis*
obtained by NIG mutagenesis and selection using metalaxyl-
amended medium

Isolate reference	Source (Expt No) ^a	Unamended medium (mm)	Inhibition (%) by metalaxyl (µg/ml)						ED ₅₀ ^b (µg/ml)	Statistical ^c grouping
			0.001	0.01	0.1	1	10	100		
<u>EXPT A</u>										
K1121	-	56	-	-	0	0	0	0	>10 ⁵	ND
M2	1	42	-	-	2	0	0	0	>10 ⁵	ND
M10	2	39	-	-	0	7	0	0	>10 ⁵	ND
M12	5	43	-	-	0	0	0	0	>10 ⁵	ND
M32	7	40	-	-	0	0	0	5	>10 ⁵	ND
M4	2	45	-	-	2	4	0	0	>10 ⁵	a
M7	2	50	-	-	0	0	2	4	>10 ⁵	b
M1	1	52	-	-	5	0	7	11	97.5x10 ³	c
M6	2	46	-	-	0	2	10	17	25.5x10 ³	d
M11	5	70	-	-	0	1	7	24	21.0x10 ³	d
M16	2	59	-	-	0	0	15	27	8.9x10 ³	de
M31	7	47	-	-	4	14	6	31	2.4x10 ³	ef
M15	4	56	-	-	7	12	21	25	1.2x10 ³	fg
M8	2	42	-	-	7	9	28	42	421.20	g
M5	2	40	-	-	0	20	30	42	355.88	g
M24	6	49	-	-	14	20	26	63	64.16	h
M22	6	47	-	-	0	2	10	17	53.21	hi
M23	6	68	-	-	19	30	39	44	40.71	hij
M27	6	45	-	-	17	28	40	60	25.51	hijk
M14	5	44	-	-	9	20	38	77	24.95	hijk
M13	5	39	-	-	17	33	35	66	18.45	hijk
M28	6	46	-	-	19	28	43	50	13.64	ijk
M9	2	33	-	-	18	36	48	57	11.62	jk
M20	5	47	-	-	25	42	46	59	7.54	kl
M3	1	58	-	-	31	43	50	79	2.76	l
M25	6	61	-	-	44	57	62	73	0.70	m
M26	6	57	-	-	49	56	68	84	0.31	m
K1120	-	47	0	23	55	82	95	-	0.01	n

Isolate reference	Source (Expt No) ^a	Unamended medium (mm)	Inhibition (%) by metalaxyl (µg/ml)						ED ₅₀ ^b (µg/ml)	Statistical ^c grouping
			0.001	0.01	0.1	1	10	100		
<u>EXPERIMENT B</u>										
K1121	—	56	—	—	0	0	0	7	>10 ⁵	ND
M2	2	36	—	—	0	0	0	0	>10 ⁵	ND
M35	6	38	—	—	0	0	0	0	>10 ⁵	ND
M40	6	47	—	—	0	0	0	0	>10 ⁵	ND
M21	6	46	—	—	4	2	0	2	>10 ⁵	a
M36	6	36	—	—	0	0	8	19	70.9x10 ³	b
M11	5	39	—	—	8	21	23	0	4.5 x10 ³	c
M17	1	17	—	—	0	0	18	41	2.3 x10 ⁵	c
M43	7	43	—	—	12	26	35	28	239.83	d
M37	6	34	—	—	0	15	35	50	215.20	d
M42	7	35	—	—	14	20	26	54	139.21	d
M34	6	29	—	—	10	70	34	59	88.53	de
M33	3	39	—	—	31	33	33	54	24.78	ef
M38	6	44	—	—	20	30	41	57	23.10	ef
M41	6	43	—	—	21	30	49	65	14.29	f
M18	2	41	—	—	29	41	46	51	10.57	f
M39	6	43	—	—	23	33	53	65	9.51	f
M30	7	51	—	—	35	33	51	63	6.85	f
M19	5	38	—	—	29	61	74	76	0.49	g
M9	2	27	—	—	52	78	93	100	0.01	h
K1120	—	48	15	33	67	96	98	—	<0.01	i

^a See Section 6.4.1.

^b Determined from logit relative potency analysis of the inhibition of radial growth plotted against the concentration of metalaxyl.

^c Isolates without letters in common are significantly different, $P=0.05$, according to Duncan's multiple range test.

ND = isolates which could not be grouped statistically because the growth was insufficiently affected by metalaxyl.

Plate 16 Response of resistant mutants of Phytophthora megasperma f.sp.
medicaginis isolate K1120 to metalaxyl

Metalaxyl concentration ($\mu\text{g/ml}$)

Left to right : 0 0.1 1 10

a) Highly resistant (M40)

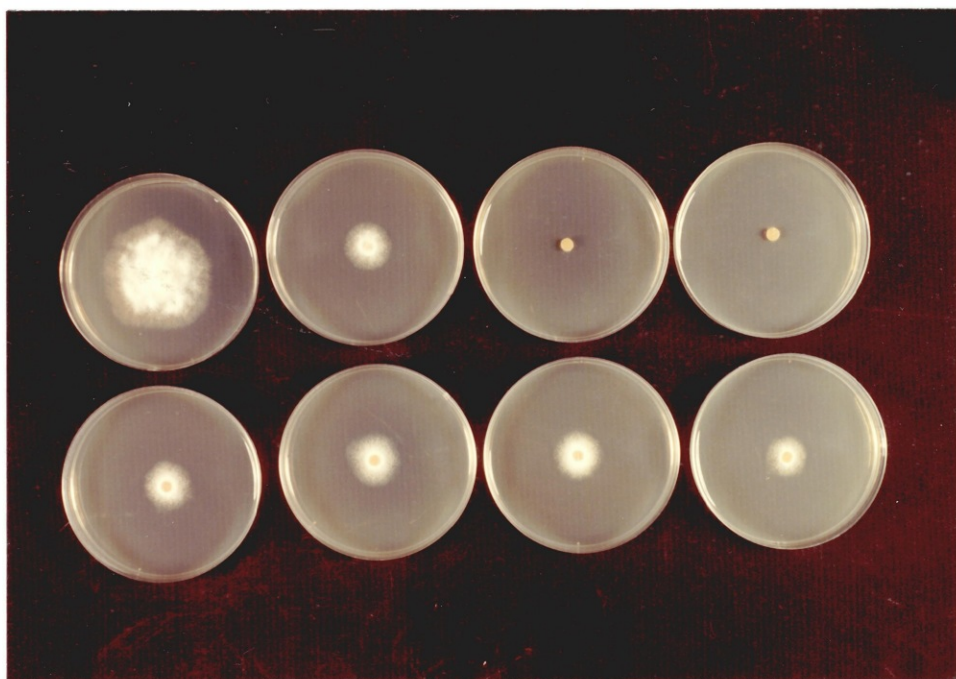
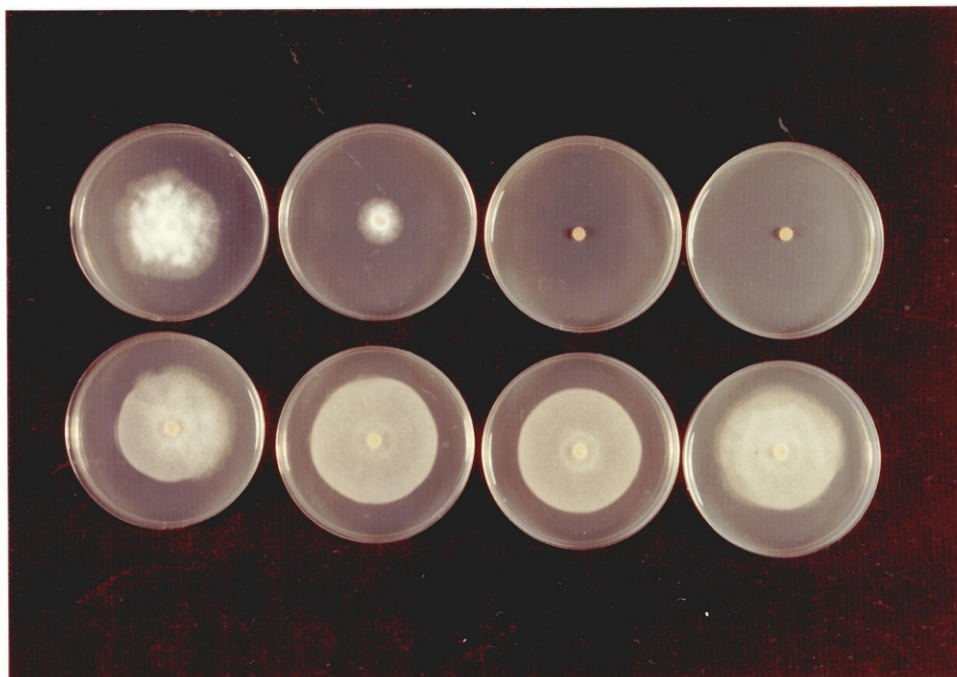
Top row : Parent isolate (K1120)

Bottom row : M40

b) Weakly resistant (M17)

Top row : Parent isolate (K1120)

Bottom row : M17



6.4.3 Effect of Cysteine on the Lethal Action of NTG

a) Introduction

A system was sought in which the lethal effect of NTG on zoospore suspensions of isolate K1120 could be rapidly halted when required, without the loss of inoculum. The isolation of individual fungicide-resistant colonies and calculation of mutation frequencies should also be possible with this system. The use of a chemical antagonistic to the effect of NTG was considered and the thiol, cysteine, appeared to be a likely candidate. It was reported (Slein & Sansome, 1980) that at 30°C and pH 7.4, the half-life of NTG was about 55 min, but in the presence of the thiol (1:1 ratio), the half-life fell to about 1 min.

The use of cysteine to neutralise the effects of NTG on zoospores of P. megasperma f.sp. medicaginis isolate K1120 was examined in the experiments reported here.

b) Materials and methods

In each of two experiments, a zoospore suspension of isolate K1120 was prepared as described in Section 5.3.2a. The suspension was collected in a 250ml Erlenmeyer flask and agitated vigorously on a shaker for 5 min to induce zoospore encystment. The concentration of spores was assessed using a Sedgewick Rafter counting cell.

In the first experiment, 27ml aliquots of a suspension, adjusted to 100 zoospores/ml, were dispensed into four sterile 100ml Erlenmeyer flasks and left for approximately 45 minutes until germination began. An aqueous solution of NTG, which had been incubated overnight, was diluted to give three solutions so that when 3ml of each solution was added separately to three zoospore suspensions, final concentrations of 1, 10, 100µg NTG/ml were obtained. Sterile water (3ml) was added to the fourth suspension which served as a control. Aliquots (1ml) of untreated or NTG-treated suspensions were removed at 0, 3.75, 7.5 15 and 30 min and plated onto clarified V8 agar plates. On each occasion, five replicate plates were

inoculated per NTG treatment. The plates were incubated at 25°C for 7 days and the number of colonies which developed on each plate was counted.

In the second experiment, 9ml aliquots of a suspension adjusted to 500 zoospores/ml were transferred separately to fourteen 100ml Erlenmeyer flasks and left for approximately 45 min until germination began. Dilutions of NTG (prepared overnight) were added in 1ml aliquots to four zoospore suspensions to give final concentrations of 10, 30 and 100µg NTG/ml. After 3.75, 7.5, 15 and 30 min, 1ml aliquots of aqueous solutions of L-cysteine (Koch-Light Laboratories) were added to appropriate treated suspensions. The ratio of cysteine to NTG was always 2:1. Five min after adding cysteine, 0.2ml aliquots of each suspension were plated onto five clarified V8 agar plates. As controls, 2ml sterile water and 1ml sterile water plus 1ml of an aqueous solution of cysteine at 60µg/ml, were added separately to the remaining zoospore suspensions. At the start and completion of the mutagenesis procedure, 0.2ml aliquots of each of these suspensions were plated onto five clarified V8 agar plates. All plates were incubated at 25°C and the number of colonies was counted after 7 days.

c) Results

In Experiment 1, similar colony counts were obtained by plating non-NTG treated zoospores at the start of the experiment and 30 min later (Table 42) indicating that viability was unaffected by incubation in aqueous suspensions when other suspensions were treated with NTG. No colonies developed from suspensions which were treated with 10 or 100µg NTG/ml, but 32-40 colonies developed from zoospores subjected to 1µg NTG/ml. However, the absence of an exponential decline in colonies from the latter suspensions during the increasing period of exposure to 1 µg NTG/ml, suggested that at this concentration the mutagen was exerting a sub-lethal effect on the zoospores.

Table 42 Development of colonies of *Phytophthora megasperma* f.sp. *medicaginis* isolate K1120 from NTG-treated suspensions of zoospores

NTG treatment of suspension (min)	Number of colonies ^a which grew following treatment with NTG ($\mu\text{g/ml}$) :			
	0	1	10	100
0	63	–	–	–
3.75	–	40	0	0
7.5	–	40	0	0
15	–	32	0	0
30	62	39	0	0

^a Average of five replicate plates after 3 days

In Experiment 2, similar colony counts were obtained from suspensions of zoospores which were unamended or amended with cysteine at 60µg/ml, indicating that cysteine did not affect the viability of zoospores. Between 0 and 30 min, the number of colonies apparently increased significantly. This finding could not be explained.

The effect of cysteine on the lethal action of NTG in the second experiment is shown in Figure 10. Zoospore suspensions treated with 10 or 30µg NTG/ml gave rise to colonies on all plates by 3 or 7 days respectively. There was little colony development on plates to which suspensions treated with 100µg NTG/ml were transferred. Comparison of these findings with those of Experiment 1 suggested that cysteine was antagonistic to the lethal effect of NTG.

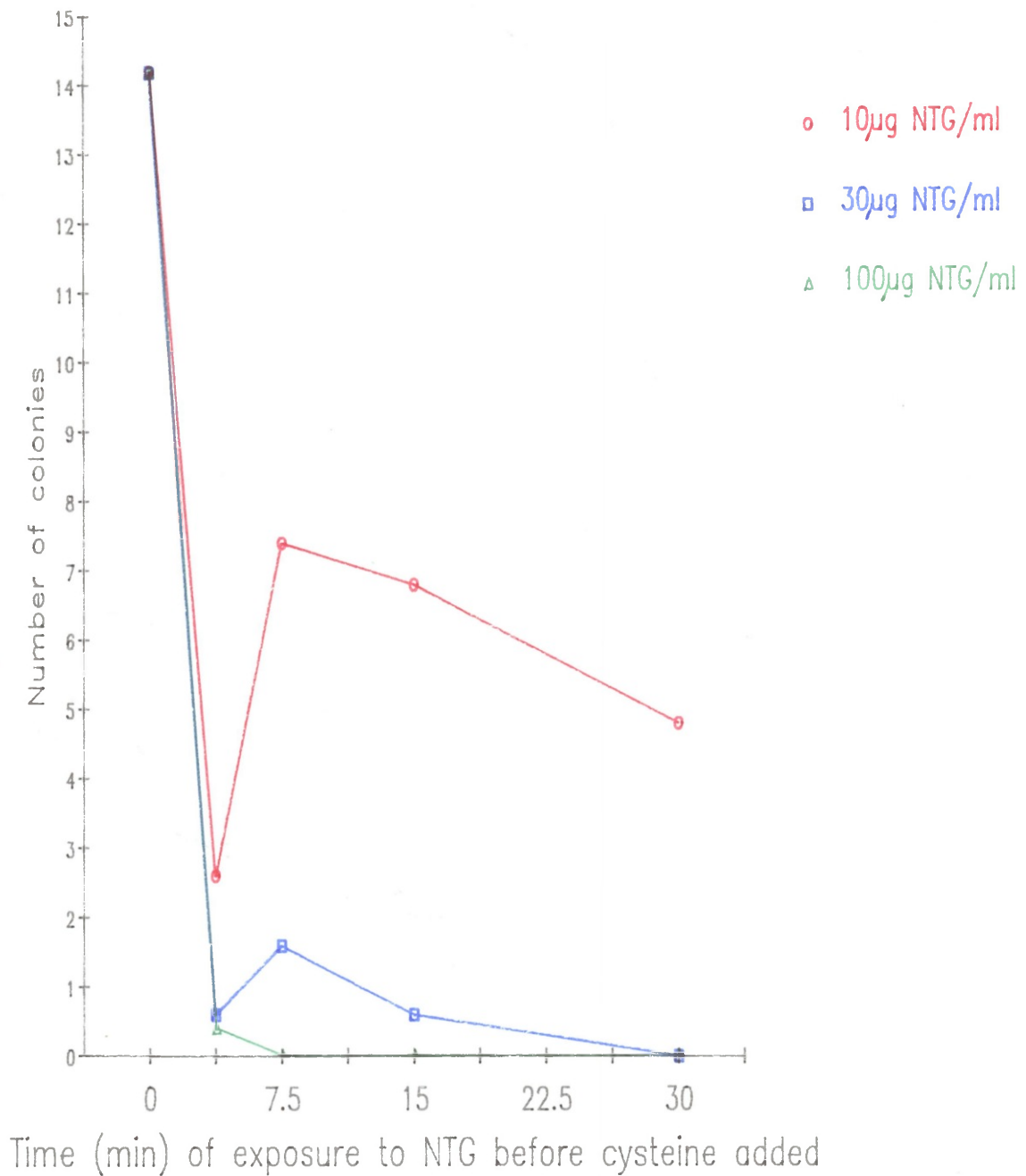
Colonies continued to develop between 3 - 7 days from NTG/cysteine-treated zoospores. Eventually, after 7 days, similar colony counts were obtained from non-NTG treated suspensions, and suspensions treated with 10µg NTG/ml. It appeared that the addition of cysteine reversed the lethal effect of NTG.

d) Discussion

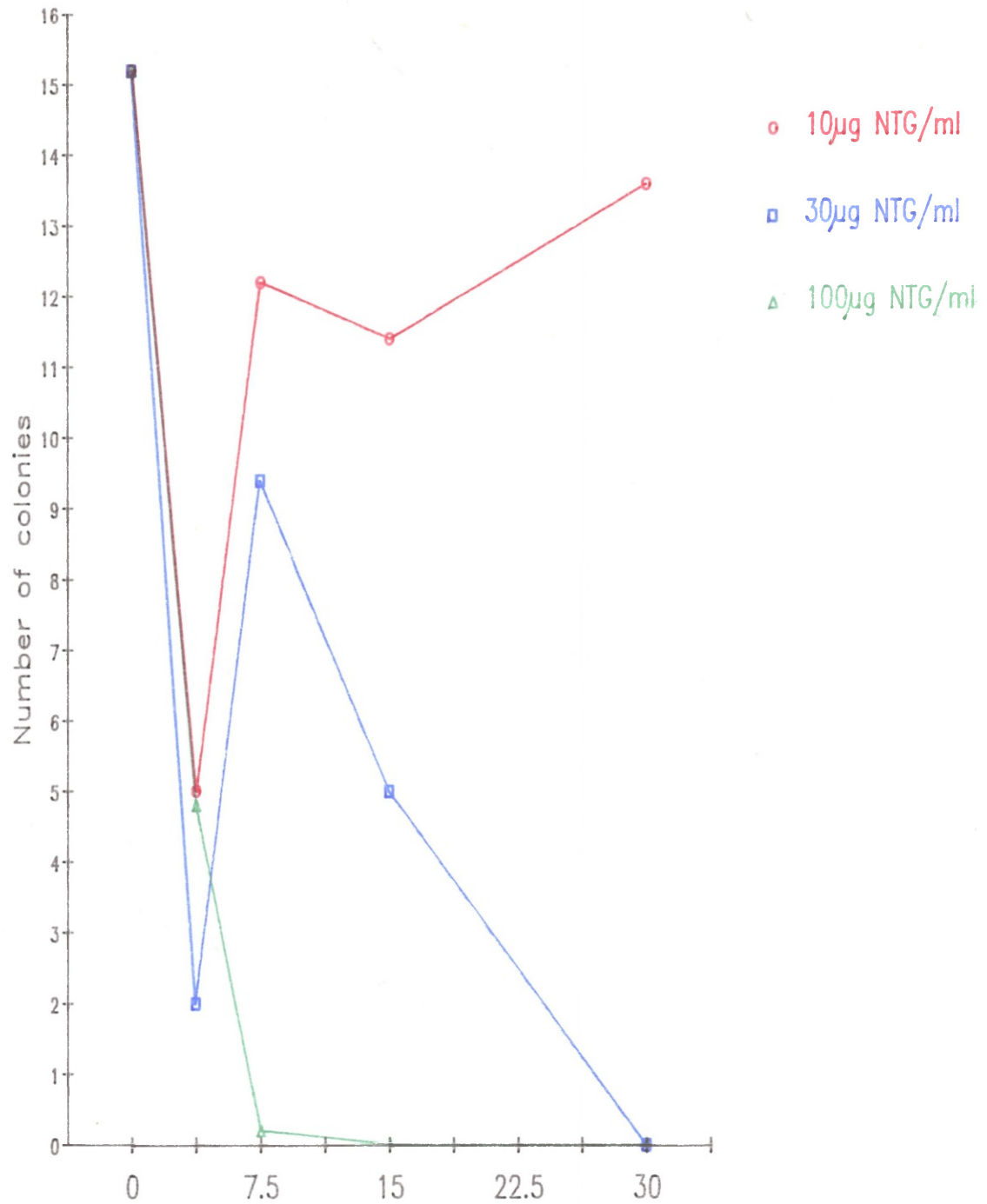
The finding that cysteine was apparently antagonistic to the lethal action of NTG, coincided with the results of studies which demonstrated that cysteine has a protective action against certain types of alkylating agent (Elson, 1963). NTG was not cited in that particular study, but it was also shown to act as an alkylating agent, especially within the vicinity of DNA replication points (Schulz & McCalla, 1969). Further evidence (Elson, 1963) suggested that there was a direct correlation between the reactivity of alkylating agents towards the mutagenic target, and the extent of protection afforded with thiols such as cysteine. This author also indicated that protection might depend on the reaction of free cysteine with alkylating agents within the (mammalian) cell. However, several authors have proposed that the rapid decomposition

Figure 10 Effect of cysteine on the lethal action of NTG applied to zoospore suspensions of *Phytophthora megasperma* f.sp. *medicaginis* isolate K1120

a) Numbers of colonies per plate after 3 days



b) Numbers of colonies per plate after 7 days



Time (min) of exposure to NTG before cysteine added

of NTG by cysteine produces a fragment capable of methylating DNA at an increased rate (Schulz & Mc Calla, 1969; Lawley & Thatcher, 1970; Wheeler & Bowdon, 1972). These findings imply that the mutagenic and possibly also the lethal effect of NTG is enhanced, rather than inhibited, by cysteine. Nevertheless, the use of a NTG/cysteine system in renewed attempts to obtain fungicide-resistant mutants of P. megasperma f.sp. medicaginis was justified from the results of the present investigation.

6.4.4 Selection of Fungicide-resistant Mutants-2

a) Introduction

Based on the results of Section 6.4.3, it was decided to attempt the induction of fungicide-resistant mutants of P. megasperma f.sp. medicaginis isolate K1120 by subjecting germinating zoospores to NTG/cysteine treatment. Two experimental approaches were adopted in the subsequent selection of mutants.

In some experiments, the treated zoospores were to be transferred immediately to medium containing fungicide. This procedure has been frequently used to isolate fungicide-resistant mutants of Phytophthora spp. (Staub et al., 1979; Davidse, 1981; Joseph & Coffey, 1984). In other experiments, zoospores which survived the lethal effect of NTG were to be allowed to develop into macroscopic colonies before they were transferred to medium containing fungicide. It was speculated that R157846-resistant mutants in particular might develop more readily as a consequence of this lag period.

b) Materials and methods

Zoospore suspensions of isolate K1120 were prepared as described in Section 5.3.2a and were distributed in 80-150ml quantities in 250ml Erlenmeyer flasks, which were then agitated vigorously on a shaker for 5 min. The number of zoospores (most of which had encysted) in each suspension was calculated from counts in a Sedgewick Rafter cell.

Suspensions were gently shaken for approximately 45 min until germination began, and then 1ml of a filter-sterilised, aqueous solution of NTG (prepared overnight, pH 5.7) was added to each suspension to give a final concentration of 10 or 30µg NTG/ml. Suspensions were incubated, without shaking, for 30 min and 1ml of a filter-sterilised, aqueous solution of cysteine was added to each suspension at twice the amount of the NTG. Amended suspensions were left for 30 min unless indicated otherwise.

In "direct plating" experiments, 2ml aliquots of NTG/cysteine treated suspensions were plated onto V8 agar amended with either 20µg metalaxyl/ml or 5µg R157846/ml. In "overlaying" experiments, 2ml aliquots were plated onto unamended V8 agar only. After 6 - 14 days, when visible growth of colonies occurred, most plates were covered with an additional layer of V8 agar amended with 20µg metalaxyl/ml or 5µg R157846/ml.

Once they had been subjected to fungicide treatment, inoculated plates were incubated at 25°C for at least 24 days. During this time they were regularly examined for the development of fungicide-resistant colonies. Colonies which grew through overlaid agar were transferred to unamended medium and/or medium amended with fungicide at the concentration present in the selection medium.

c) Results

Germinating zoospores treated with NTG/cysteine were plated directly onto fungicide-amended medium in three experiments (Table 43). In the first, a total of 1.7×10^7 zoospores was subjected to 10µg NTG/ml, and 107 colonies grew on metalaxyl-amended medium. The type of growth which occurred is shown in Plate 17. No colonies developed from the same number of zoospores on R157846-amended medium. Consequently, the dosage of NTG was increased to 30µg/ml in Experiments 2 and 3, with the intention of increasing the degree of mutagenesis. A relationship was observed in these experiments between the numbers of colonies which grew, and the order in

Table 43 Development of resistant mutants of *Phytophthora megasperma*
f.sp. medicaginis from NTG-treated zoospores plated directly
onto metalaxyl-amended medium^a

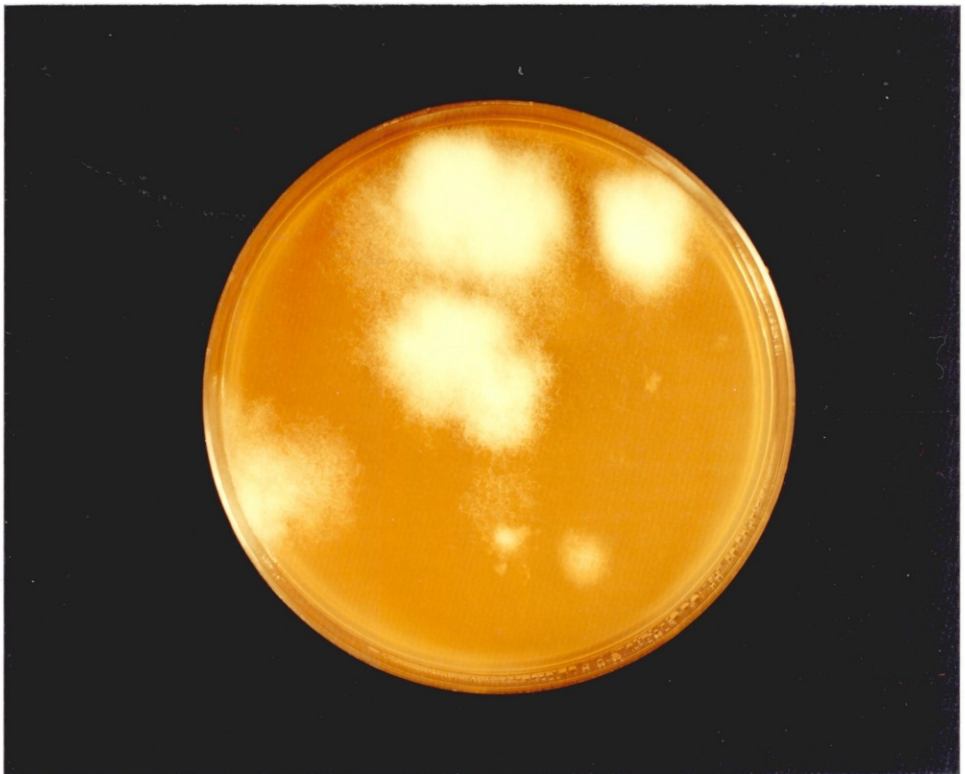
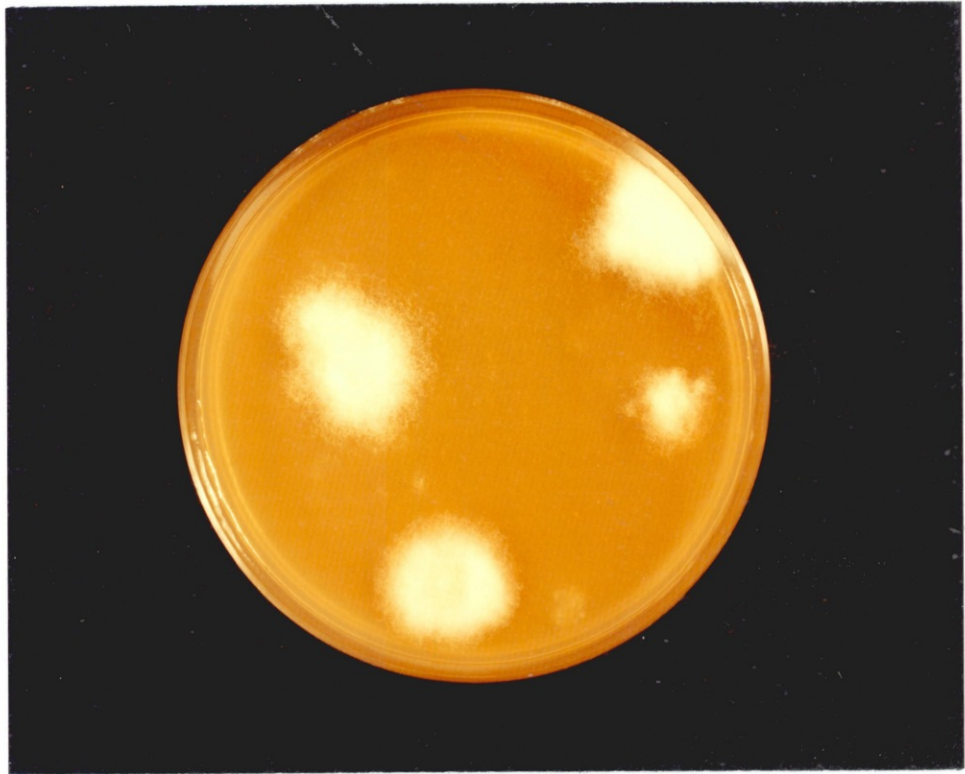
Experiment	NTG ^b ($\mu\text{g/ml}$)	Total no. of zoospores ^c	Inoculation period(days)	No. of colonies	Mutants per 10 ⁷ zoospores
1	10	1.7×10^7	25	107	62
2	30	3.5×10^7	24	126	36
3	30	2.8×10^7	28	6	2

^a Concentration in selection medium was 20 $\mu\text{g/ml}$

^b Zoospore suspensions were subjected to NTG for 30 min prior to the addition of twice the amount of cysteine

^c Calculated using a Sedgewick Rafter counting cell

Plate 17 Development of colonies of Phytophthora megasperma f.sp.
medicaginis isolate K1120 on V8 agar amended with 20µg
metalaxyl/ml following treatment of zoospores with
NTG/cysteine



which zoospore suspension were plated following NTG/cysteine treatment (see, for example, Table 44). Thus, fewer colonies developed from those zoospore suspensions (1- 3) which were plated out within 30 min of the addition of cysteine.

In Experiment 2, 126 colonies, and in Experiment 3, six colonies, grew on metalaxyl-amended medium.

Colonies failed to develop on R157846-amended medium from a total of 8.0×10^7 zoospores subjected to NTG/cysteine treatment in the three experiments.

In four experiments, germinating zoospores treated with NTG/cysteine were plated onto V8 agar and resulting colonies were overlaid with fungicide-amended medium. Relevant experimental details are given in Table 45a.

On unamended medium, the number of colonies which developed was closely related to the order in which zoospore suspensions were plated out. Thus, in Experiment 3 after 14 days, confluent growth of colonies occurred on most plates inoculated with germinating zoospores from suspension 6; few, if any, colonies developed on plates inoculated with zoospores from suspension 1 (see Plate 18).

After 10 days, colonies emerged through metalaxyl-amended medium (see, for example, Plate 19) and fast growing variants could frequently be identified. Since so many colonies developed, only selected isolates were tested further. Sparse-growing colonies also emerged through R157846-amended medium (see, for example, Plate 20), and all were re-examined for their sensitivity to R157846.

Many colonies which were subcultured from metalaxyl-amended medium grew on both unamended medium and medium amended with 20ug metalaxyl/ml (Table 45b, Plate 21). In contrast, although colonies subcultured from R157846-amended medium frequently grew on unamended medium, none grew when subjected to 5ug R157846/ml (Table 45b, Plate 22).

Table 44 Development of colonies of *Phytophthora megasperma* f.sp. medicaginis isolate K1120 from zoospores subjected to NTG/cysteine (Experiment 2)

Original suspension ^b	No. of plates inoculated	Plate number	Number of colonies per plate at day:		
			14	19	24
1	18	-	0	0	0
2	22	1	0	1	1
3	17	-	0	0	0
4	16	1	1	1	4
		2	1	1	1
		3	1	1	1
		4	2	2	FC ^c
		5	3	3	5
		6	1	2	FC
		7	2	2	2
		8	0	3	3
		9	0	1	1
		10	0	3	3
		11	0	2	3
		12	0	0	1
		13	0	0	2
5	23	1	3	3	3
		2	3	4	FC
		3	1	1	1
		4	0	1	1
		5	0	1	1
		6	0	0	1
		7	0	0	1
6	15	1	4	FC	FC
		2	3	7	7
		3	3	8	9
		4	5	6	8
		5	2	4	4
		6	5	8	FC
		7	4	7	FC
		8	5	5	5
		9	3	7	7
		10	3	8	8
		11	1	8	9
		12	0	8	6

^a Zoospore suspensions were exposed to 30µg/ml NTG for 25 min, cysteine was added at 60µg/ml, and 2ml aliquots were plated onto V8-agar amended with 20µg metalaxyl/ml

^b Suspensions were plated during approximately 10 min intervals in the order 1-6

^c FC = full colonisation of agar plate by the fungus.

Table 45 Selection of fungicide-resistant mutants of *Phytophthora megasperma* f.sp. *medicaginis* isolate K1120 by the overlay method

a) Relevant experiment details

Expt.	NTG treatment		Total no. of zoospores subjected to:	
	Conc. ($\mu\text{g/ml}$)	Time (min)	5 μg R157846/ml	20 μg metalaxyl/ml
1	10	30	0.9×10^7	0.9×10^7
2	30	15	2.6×10^7	2.1×10^7
3	30	15	2.9×10^7	1.9×10^7
4	30	15	9.3×10^7	6.9×10^7

b) Further development of colonies transferred from Experiments 1-4

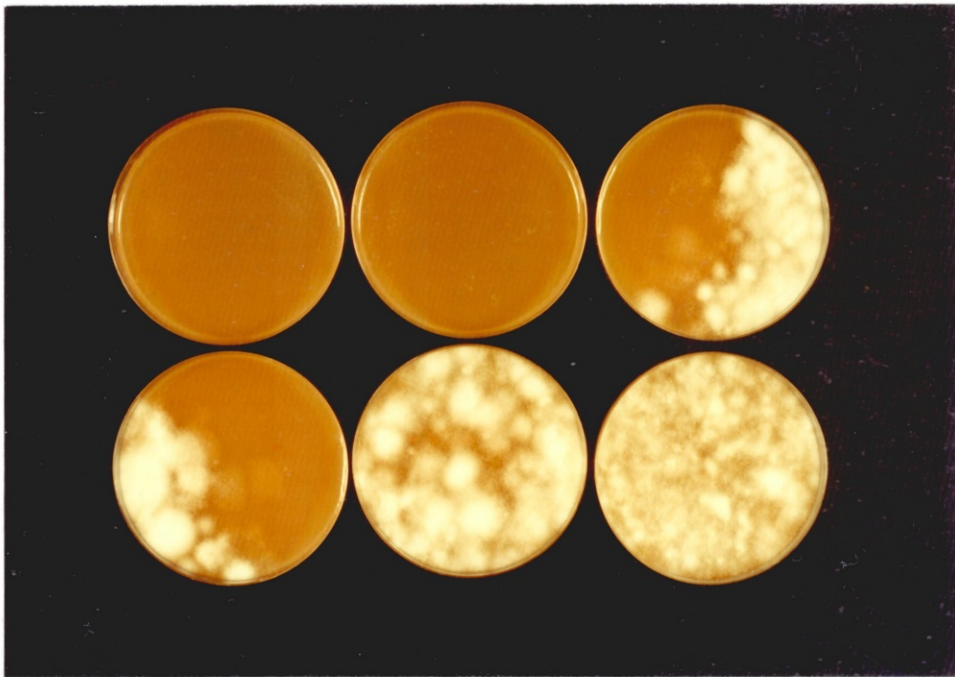
Expt.	from R157846			from metalaxyl		
	No. of colonies transferred	Growth ^a on medium with		No. of colonies transferred	Growth on medium with	
		Unamended	R157846		Unamended	metalaxyl
1	27	7	0	27	NT ^b	19
2	18	17	0	18	NT	4
3	20	20	0	33	32	30
4	14	10	0	33	28	17

^a Recorded as + (macroscopic colony) or - (no visible growth)

^b Not tested

Plate 18 Development of colonies of Phytophthora megasperma f.sp.
medicaginis isolate K1120 on representative plates inoculated
with NTG/cysteine-treated suspensions of zoospores^a

Suspension number			
Top row :	1	2	3
Bottom row :	4	5	6



^a Each suspension was plated out approximately 10 min after its predecessor, and in the order 1 - 6

Plate 19 Development of colonies of *Phytophthora megasperma* f.sp.
medicaginis through an overlay of V8 agar amended with 20 μ g
metalaxyl/ml

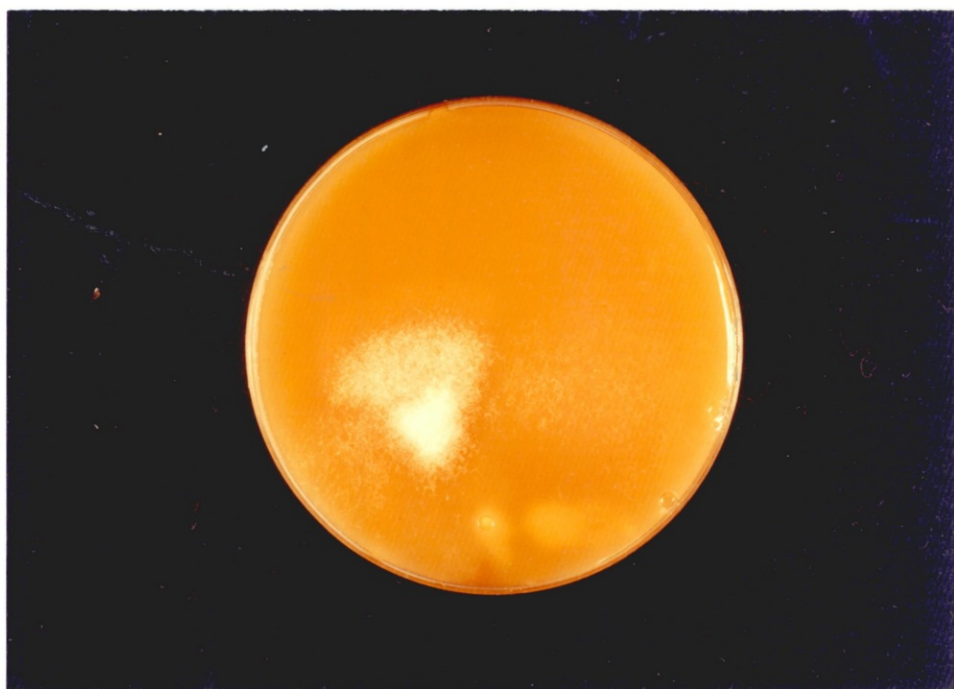
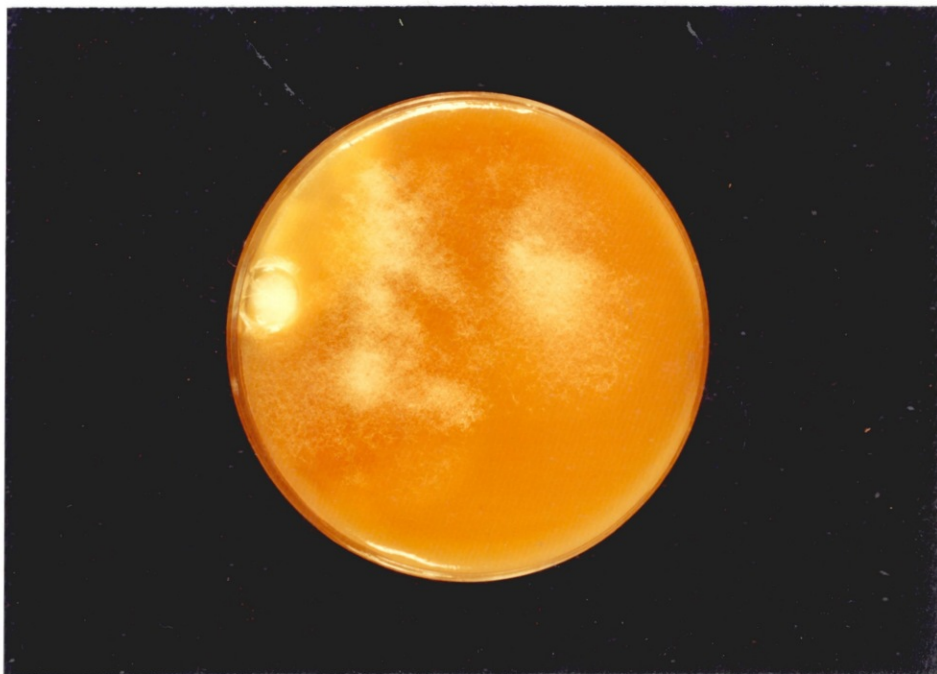


Plate 20 Development of colonies of *Phytophthora megasperma* f.sp.
medicaginis through an overlay of V8 agar amended with 5 μ g
R157846/ml

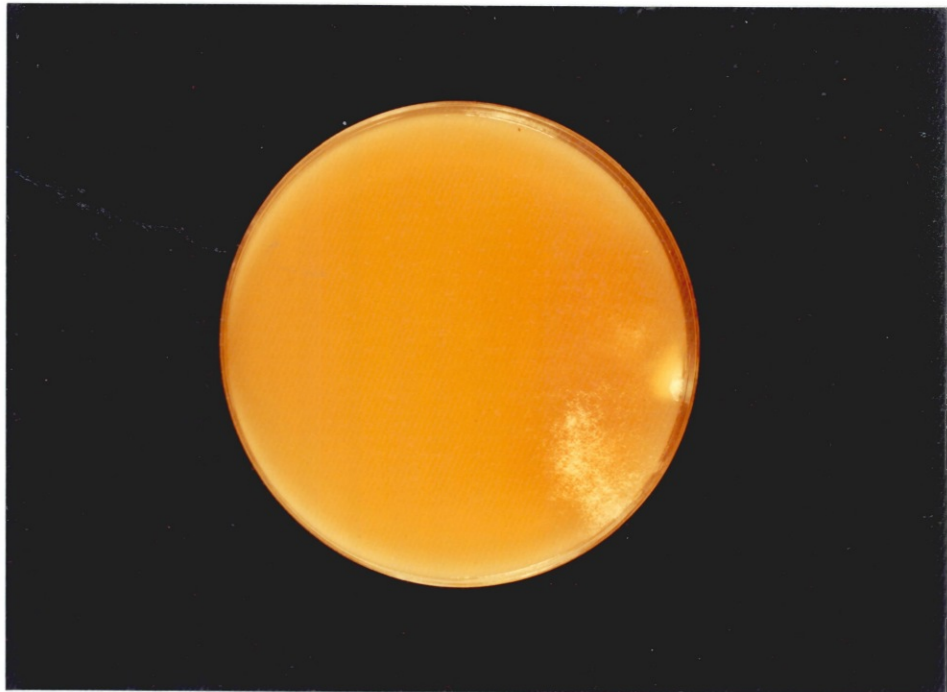
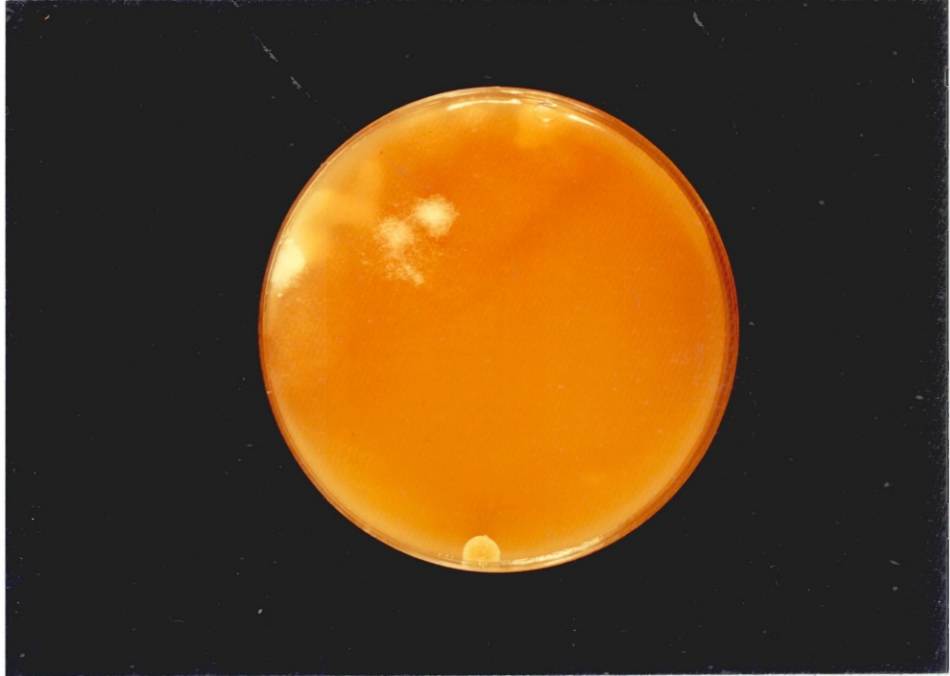


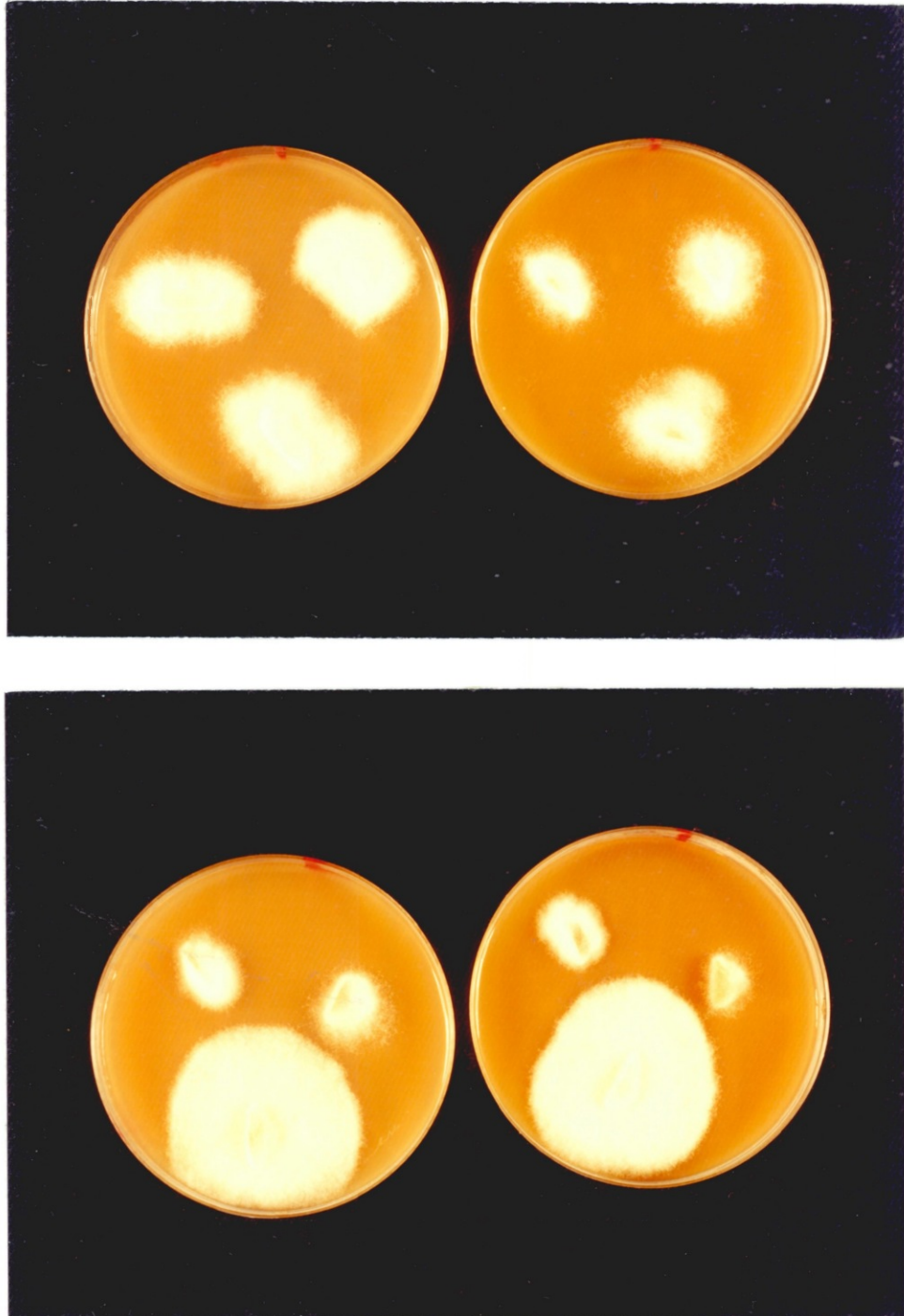
Plate 21 Development of colonies of *Phytophthora megasperma* f.sp.
medicaginis transferred from selection plates (overlay method)
to metalaxyl-amended medium

Metalaxyl ($\mu\text{g/ml}$)

Left to right :

0

20



Fragments of agar in the same position on each set of two plates originated from one colony.

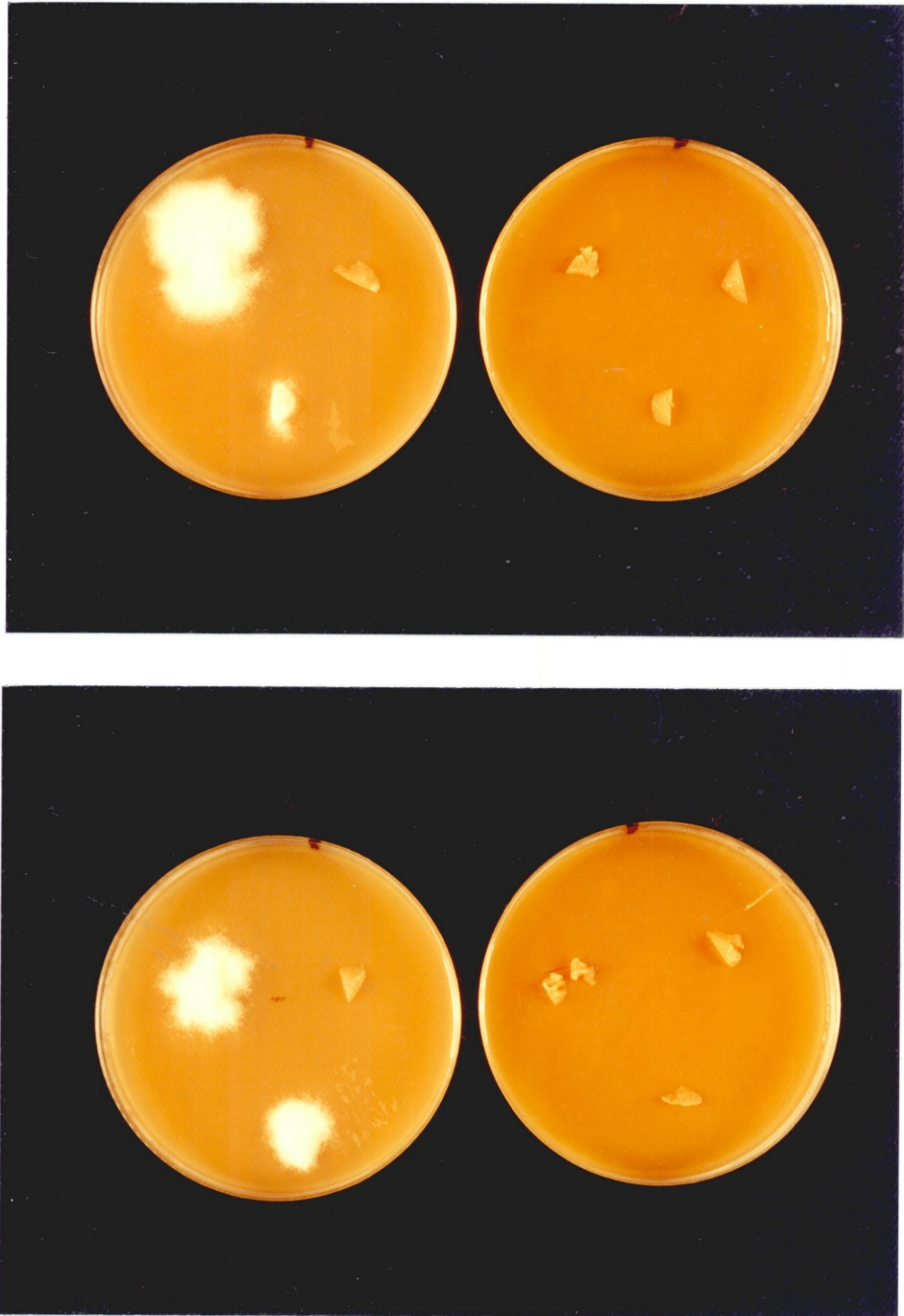
Plate 22 Development of colonies of Phytophthora megasperma f.sp.
medicaginis transferred from selection plates (overlay method)
to R157846-amended medium

R157846 ($\mu\text{g/ml}$)

Left to right :

0

5



Fragments of agar in the same position on each set of two plates originated from one colony.

d) Discussion

A total of 24.1×10^7 germinating zoospores of isolate K1120 were subjected to NTG mutagenesis followed by selection on R157846-amended medium. However, no mutants were obtained which grew, or were able to continue growth, at the relatively low concentration of 5µg R157846/ml. It was concluded that the growth of colonies through overlaid medium amended with 5µg R157846/ml was of little practical significance. Possibly, the growth observed resulted from a breakdown of R157846 by the underlying colony. In contrast, the overlay technique yielded a large number of colonies on metalaxyl-amended medium, and many continued to grow when transferred to newly-amended medium. These, and colonies which developed from germinating zoospores plated directly onto fungicide-amended medium, needed to be characterised for their sensitivity to metalaxyl to identify highly-resistant mutants (see Section 6.4.5). In this way the efficacy of the system could be determined. It was concluded that to obtain a satisfactory number of mutant colonies from all zoospore suspensions, following NTG/cysteine treatment, suspensions had to be incubated for at least 30 min before they were inoculated onto solid medium.

6.4.5 In vitro sensitivity of variant colonies of *P. megasperma* f.sp. *medicaginis*, obtained after NTG/cysteine treatment, to metalaxyl

a) Introduction

The metalaxyl-sensitivity of variant colonies of *P. megasperma* f.sp. *medicaginis*, obtained from the experiments described in Section 6.4.4, was examined. In particular, mutants which were highly resistant to metalaxyl were sought so that the efficacy of the system could be established.

b) Experimental

In separate experiments, clarified V8 agar plates were prepared,

either unamended or amended with 20µg metalaxyl/ml medium. Two replicate plates per treatment were inoculated with 6mm mycelium plugs cut from the edge of variant colonies growing on metalaxyl-amended medium. The parent isolate, K1120, and a standard metalaxyl-resistant isolate, K1121, were included in all experiments. Plates were incubated at 25°C for approximately 8 days.

Two colony diameters per plate were measured at 90° to each other, averaged, and the diameter of the inoculum plug subtracted. When the average radial growth of a variant on metalaxyl-amended medium was not less than 90% that of the parent isolate on unamended medium, it was assumed to be highly resistant to metalaxyl.

The response of 106 mutants to metalaxyl is given in Appendix C. The summary of these results in Table 46 shows that 13 mutants were highly resistant to metalaxyl.

c) Discussion

The mutants of P. megasperma f.sp. medicaginis which were obtained after NTG/cysteine treatment of germinating zoospores varied considerably in their rate of growth on unamended medium and response to metalaxyl. Nevertheless, 13 mutants were obtained which were designated highly resistant to metalaxyl. Consequently, it was established that the system employed in Section 6.4.4 was capable of inducing the desired type of mutation in P. megasperma f.sp. medicaginis to the standard chemical, metalaxyl. The absence of R157846-resistant mutants was probably due to the very low frequency with which they occurred, if at all, and not to inefficiency of the system.

Table 46 Number of mutants of *Phytophthora megasperma* f.sp. *medicaginis*,
obtained by NTG/cysteine treatment, which were highly resistant^a
to 20µg metalaxyl/ml

Selection procedure	Experiment	Total no. of colonies tested	No. of highly resistant mutants ^a
Direct plating ^b	1	27	3
	2	28	1
	3	7	2
Overlaying ^c	1	8	1
	2	20	5
	3	11	0
	4	5	1

^a Inhibition of radial growth on metalaxyl-amended medium was not greater than 10% that of the parent isolate on unamended medium

^b Aliquots of treated zoospore suspension transferred to metalaxyl-amended medium

^c Colonies allowed to develop from treated zoospores on unamended medium prior to being overlaid with metalaxyl-amended medium

6.5 NTG-mutagenesis of *P. infestans*

6.5.1 Effect of NTG on Sporangial Viability

a) Introduction

In experiments (Section 6.2) to select fungicide-resistant mutants of *P. infestans*, aliquots of UV irradiated suspensions of sporangia were added to clarified FP broth amended with either 100µg metalaxyl/ml or 1µg R157846/ml contained in separate compartments of repli-dishes. Mutants resistant to R157846 were sought particularly, the purpose of the tests with metalaxyl being mainly a guide to the ability of the system to identify mutants highly resistant to metalaxyl (i.e. with a growth rate on metalaxyl-amended agar similar to the parent isolate on unamended agar). Ultimately, such mutants could not readily be identified because of the ease with which those with a low degree of resistance grew in the metalaxyl-amended broth. Consequently, the effectiveness of the system at inducing the desired type of mutation to the standard chemical, metalaxyl, could not be established. The inability to obtain R157846-resistant mutants in that investigation meant that it was of increased importance to use a system which was known to induce mutants highly resistant to metalaxyl. Such a system was described in Section 6.4.4 for the selection of fungicide-resistant mutants of *P. megasperma* f.sp. *medicaginis* following NTG/cysteine treatment. This section describes attempts to obtain R157846-resistant mutants of *P. infestans* by combining features of both the selection procedure developed for *P. infestans* (Section 6.2), and the mutagenesis technique devised for *P. megasperma* f.sp. *medicaginis* (Section 6.4.4).

In the following experiment, the period of exposure to different concentrations of NTG which resulted in the death of 50% of sporangia was determined, this degree of kill being considered previously as most likely to yield a relatively high number of mutants (see Section 6.2.3c).

b) Materials and methods

P. infestans isolate K1182 was chosen for this and subsequent experiments because it produced many sporangia. A suspension containing 10^5 sporangia/ml was prepared from 13-day cultures on V8 agar and 1ml of this suspension was used to prepare a 10-fold dilution series to one sporangium/ml. Nine ml aliquots of the original suspension were placed in two sterile plastic universal containers (Sterilin Ltd) and 1ml of filter-sterilised, aqueous NTG was added to give final concentrations of 30 and 100 μ g NTG/ml. After 7.5, 15 and 30 min, 1ml of each treated suspension was diluted serially to 10^{-5} .

Aliquots (0.2ml) of each dilution, prepared before and after NTG treatment, were transferred separately to seven repli-dish compartments, each of which contained 3ml clarified FP broth. The dishes were incubated at 22°C, and after 14 days the number of compartments with visible growth of P. infestans was counted.

c) Results and discussion

The lethal effect of 100 μ g NTG/ml was sufficiently rapid to prevent a determination of the time required to kill half the number of sporangia. However, from a dosage-response curve (Figure 11) drawn from data for 30 μ g NTG/ml, it was calculated that exposure for approximately 10 min would result in the death of 50% of sporangia. Consequently, this regime was chosen for subsequent experiments.

6.5.2 Selection of Fungicide-resistant Mutants - 1

a) Introduction

Attempts to obtain R157846 and metalaxyl-resistant mutants of isolate K1182 following NTG/cysteine treatment are described in the following experiments. The period of exposure of sporangia to NTG prior to the addition of cysteine was initially based on the results of the experiment described in Section 6.5.1.

b) Materials and methods

Similar quantities (100-150ml) of sporangial suspensions of isolate K1182 were placed in several 250ml Erlenmeyer flasks and the number of sporangia in each suspension was calculated using a Sedgewick Rafter cell. Aliquots of a filter-sterilised, aqueous solution of NTG (prepared overnight) were added to each suspension to a final concentration of 30µg/ml. After 10 min, the same volume of a filter-sterilised, aqueous solution of cysteine was added at twice the amount of NTG. Treated flasks were left for 60 min to permit the cysteine to exert a sufficiently antagonistic effect towards the NTG. Aliquots (2ml) of each sporangial suspension were then transferred to individual compartments of repli-dishes each containing 1ml undiluted clarified FP broth (see Appendix A) either unamended (three compartments) or amended with R157846 or metalaxyl to final concentrations of 1µg/ml and 100µg/ml, respectively (see Table 47 for relative numbers of compartments). For the controls, aliquots (1ml) of non-NTG treated suspensions were transferred to sets of three compartments, each containing 2ml undiluted clarified FP broth unamended or amended with fungicide.

Repli-dishes were incubated at 22°C and examined regularly for colonies. When sufficient growth had occurred, colonies were bisected and transferred to unamended V8 agar and V8 agar amended with fungicide at the concentration in the selection medium.

c) Results and discussion

Table 47 summarises the results of three experiments. Growth occurred in all repli-dish compartments containing clarified FP broth amended with 100µg metalaxyl/ml (see, for example Plate 23a). A similar result was obtained during the selection of resistant mutants of several isolates of P. infestans following UV irradiation (Section 6.3). Both

Table 47 Growth of colonies of *Phytophthora infestans* isolate K1182^a in fungicide-amended clarified frozen pea-broth from sporangia subjected to NTG/cysteine treatment^b

Experiment	Suspension number	No. of sporangia (x 10 ⁻⁵)/ml ^c	1µg R157846/ml			100µg metalaxyl/ml		
			No. of compartments of repli-dish	Total no. sporangia (x 10 ⁻⁵)	No. of visible colonies	No. of compartments of repli-dish	Total no. sporangia (x 10 ⁻⁵)	No. of visible colonies
1	1	2.87	32	91.8	0	30	86.1	30
	2	3.44	31	106.6	1	32	110.0	32
	3	2.16	31	67.0	3	30	64.8	30
	4	3.40	32	108.8	0	41	139.4	41
	5	2.84	30	85.2	0	31	88.0	31
2	1	3.62	35	126.7	0	33	119.5	33
	2	4.66	38	177.1	0	37	172.4	37
	3	4.63	30	138.9	0	31	143.5	31
	4	4.76	41	195.2	0	41	195.2	41
	5	2.56	34	87.0	0	32	81.9	32
3	1	1.90	28	53.2	0	26	49.4	26
	2	4.16	30	124.8	0	29	120.6	29
	3	5.18	32	165.0	0	30	155.4	30
	4	2.90	34	98.6	0	31	89.9	31
	5	3.50	29	101.5	0	30	105.0	30

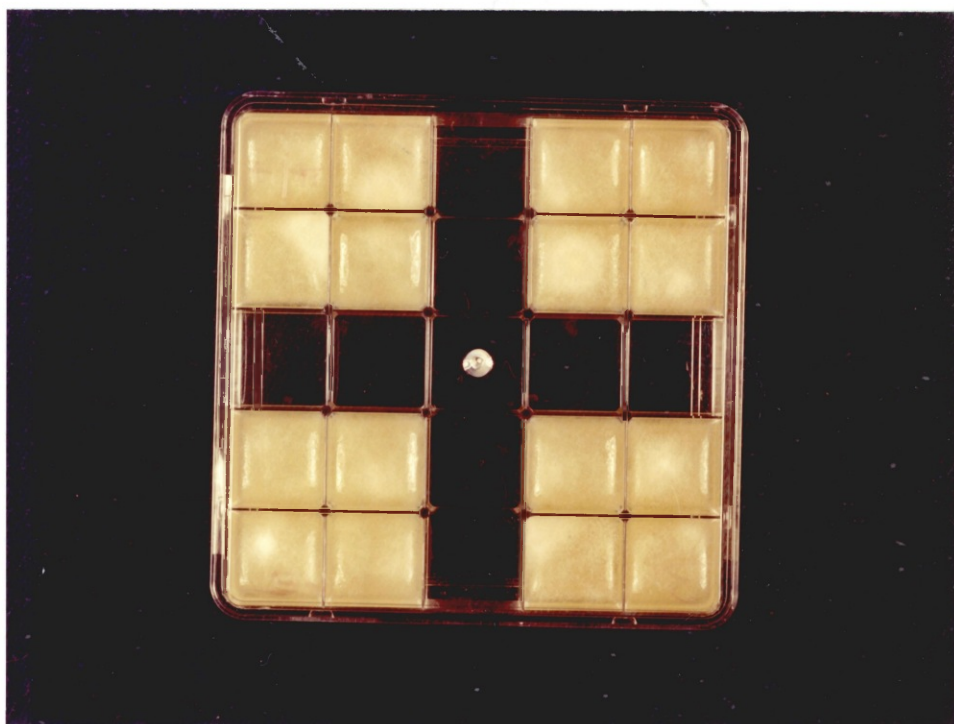
^a Assessed after incubation at 22°C for at least 21 days

^b After 10 min exposure to 30µg NTG/ml, cysteine was added at twice the amount of NTG.

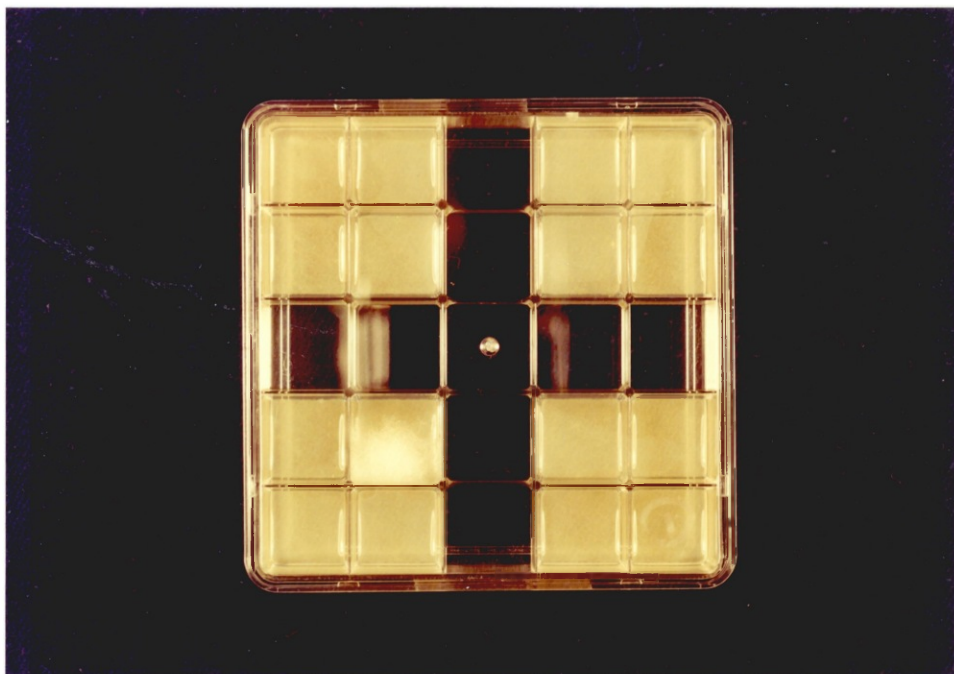
^c Calculated using a Sedgewick Rafter counting cell

Plate 23 Development of colonies of *Phytophthora infestans* isolate K1182
in repli-dish compartments containing clarified frozen pea broth
amended with metalaxyl or R157846

a) 100 μ g metalaxyl/ml



b) 1 μ g R157846/ml



sets of results reflect the ability of the fungus to grow slowly, in vitro, at relatively high concentrations of metalaxyl.

Reasonably vigorous growth occurred in four repli-dish compartments containing clarified FP broth amended with $1 \mu\text{g}$ R157846/ml (see, for example, Plate 23b). A thin layer of growth frequently developed in the remaining compartments containing R157846. This growth consisted of short, interlaced hyphae originating from sporangia which had germinated and then apparently ceased growth. The ability of sporangia of P. infestans to germinate in the presence of R157001, and the apparently lethal effect of R157846 on the mycelium, was described in Chapter Five. It seemed likely that the very weak growth observed in the present investigation was also irreversibly inhibited by R157846. This was confirmed when the mycelia failed to develop further when transferred to unamended agar.

The four colonies which showed most growth in R157846-amended broth grew weakly compared with the parent isolate on unamended agar, and failed to grow on medium amended with $1 \mu\text{g}$ R157846/ml. It was concluded that they were of little practical significance, and although they were stored temporarily, no further work was done with these variants. Thus, no R157846-resistant mutants were obtained following the NTG/cysteine treatment of a total of 1.7×10^8 sporangia of isolate K1182. Many colonies which developed in clarified FP broth amended with $100 \mu\text{g}$ metalaxyl/ml grew on agar medium similarly amended with metalaxyl. A selection of these colonies was retained for further work, described in Section 6.5.3.

It was observed, in the experiments described here, that growth of isolate K1182 from sporangia in unamended clarified FP broth was very slow following NTG/cysteine treatment. Consequently, the antagonistic effect of cysteine towards NTG was investigated in greater detail, before continuing with further selection experiments using P. infestans (see Section 6.5.4).

6.5.3 In vitro and in vivo Response to Metalaxyl of Variant Isolates Selected from Metalaxyl-amended Broth

a) Introduction

Thirteen variants of isolate K1182, obtained after NTG/cysteine treatment and selection in metalaxyl-amended broth (Section 6.5.2) were tested for their in vitro and in vivo sensitivity to metalaxyl. Variants which were highly resistant to the fungicide were especially sought, since their occurrence would provide an indication of the efficacy of the NTG treatment for inducing such mutants.

b) Materials and methods

In two in vitro experiments, sets of 9cm petri dishes were prepared containing V8 agar amended with metalaxyl at a range of concentrations to 300µg/ml. These were inoculated centrally with either variant colonies (MET - 31 to MET - 43), the parent isolate (K1182), standard metalaxyl-sensitive isolates (K966, K1041) or standard metalaxyl-resistant isolates (K1200, K1296). Plates were incubated at 22°C for 9-11 days. Radial growth of isolates was determined by measuring two colony diameters per plate at 90° to each other, averaging them, and subtracting the diameter of the inoculum plug.

The in vivo sensitivity to metalaxyl of variant and standard isolates of K1182 was determined as described in Section 4.5.2. Five discs (12mm diameter) cut from leaves of potato cv. Majestic, were floated abaxial surface uppermost on aqueous solutions of metalaxyl in 5cm petri dishes. These discs were inoculated with droplets (10µl) of a suspension containing 30,000 sporangia/ml, and were incubated in a growth room (16h photoperiod [6000 lux], 21°C day, 18°C night). The degree of infection was assessed after 7 days.

c) Results and discussion

The in vitro response to metalaxyl of variant colonies of isolate K1182 is given in Table 48. Statistical analysis was performed

Table 48 Growth of variants^a of *Phytophthora infestans* isolate KI182 on V8 agar and their inhibition by metalaxyl

Isolate Reference	Growth (mm) on		Inhibition (%) by metalaxyl (µg/ml)							ED ₅₀ (µg/ml) ^b	Statistical grouping ^c
	Unamended medium										
			0.001	0.01	0.1	1	10	100	300		

<u>Experiment A</u>											
MET-38	24	0	0	0	8	0	0	13	>10 ⁴	ND	
K1296	68	1	1	1	3	3	9	46	91.8 x 10 ²	a	
MET-37	24	0	0	0	0	0	0	75	71.5 x 10 ²	b	
MET-35	28	0	0	0	0	0	0	79	35.1 x 10 ²	c	
K1200	60	2	3	2	3	13	30	52	10.7 x 10 ²	d	
MET-34	36	3	3	19	25	25	11	86	1.1 x 10 ²	e	
MET-36	60	0	13	43	47	52	47	72	5.83	f	
MET-31	50	0	24	48	60	58	60	60	3.56	g	
MET-32	44	0	18	41	55	59	64	82	2.11	h	
MET-39	50	0	12	52	64	62	68	74	1.48	h	
K966	25	0	0	44	72	76	88	92	0.50	i	
K1041	66	6	17	52	61	61	85	98	0.45	i	
K1182	50	2	44	66	70	70	78	94	0.12	i	

<u>Experiment B</u>											
MET-33	51		2		39		35	76			
MET-40	26		38		50		50	100			
MET-41	53		6		62		62	79			
MET-42	39		13		23		26	79			
MET-43	42		5		40		40	86			
K1182	50		34		74		96	100			
K1200	65		0		0		8	42			

^a Variants obtained by NIG/cysteine treatment and selection in metalaxyl-amended clarified frozen pea-broth (see Section 6.5.2).
Growth on agar was assessed after incubation at 22°C for 9 days (Experiment A) and 11 days (Experiment B).

^b Determined from logit relative potency analysis of the response (percent inhibition of radial growth) plotted against the dosage (log concentration of metalaxyl)

^c Isolates with no letter in common are significantly different ($P = 0.05$) according to Duncan's multiple range test

ND = Statistical grouping could not be determined because radial growth of the isolate was insufficiently inhibited by metalaxyl

only on the results of Experiment A. These revealed that eight variants were significantly less sensitive ($P = 0.05$) to metalaxyl than the parent isolate and two standard metalaxyl-sensitive isolates (K966 and K1041). Differences between the ED_{50} -values of the most sensitive and the least sensitive variant ranged from $1.48\mu\text{g metalaxyl/ml}$ to $>10,000\mu\text{g metalaxyl/ml}$. One variant (MET-38) was significantly less sensitive ($P = 0.05$) to metalaxyl than the standard metalaxyl-resistant isolate, K1296, and a further two variants (MET-35 and MET-37) were significantly less sensitive ($P = 0.05$) than the standard resistant isolate, K1200. However, it was recognised, from previous work with P. infestans (Sections 4.4 and 4.5) that ED_{50} -values alone were not an adequate guide to the identification of highly-resistant mutants. Consequently, the radial growth of variants was compared with that of the parent isolate on unamended medium. On this basis, five variants (MET-31, MET-33, MET-36, MET-39, MET-41) were identified which on unamended medium grew to at least 90% of the size of the parent isolate. However, all were inhibited by metalaxyl to a much greater degree than the standard resistant isolates.

The results of the inoculations on potato leaf discs (Table 49) indicated that none of the selected variant colonies was resistant to metalaxyl in vivo. Most variants infected and grew abundantly on untreated potato leaf discs but on those treated with metalaxyl at 2 or $100\mu\text{g/ml}$, they induced necrosis only near the inoculum droplet. In the same test, leaf discs treated with metalaxyl to $100\mu\text{g/ml}$ were extensively colonised by the standard resistant isolate, K1201.

Thus, the efficacy of the NTG/cysteine system could not be evaluated because of the inability to identify readily, mutants which were highly resistant to metalaxyl either in vitro or in vivo.

6.5.4 Effect of Cysteine on the Lethal Effect of NTG

a) Introduction

The rate of recovery of sporangia of P. infestans after

Table 49 Infection of potato leaf discs by variants of *Phytophthora*
infestans isolate K1182

Variant ^a	Disease severity index ^b on discs floating on metalaxyl ($\mu\text{g/ml}$) :		
	0	2	100
MET-31	3.8	0.2	0.2
MET-32	5.0	1.4	1.2
MET-33	3.0	1.0	1.0
MET-34	1.4	1.0	0.6
MET-35	4.2	1.0	0.8
MET-36	5.0	1.0	0.8
MET-37	5.0	1.0	0.8
MET-38	5.0	1.0	1.0
MET-39	5.0	2.0	1.6
MET-40	5.0	1.0	1.0
MET-41	5.0	1.0	1.0
MET-42	5.0	1.2	1.0
MET-43	5.0	1.0	0.8
K1182	5.0	1.2	1.0
K1201	5.0	5.0	5.0

^a Induced by NTG/cysteine treatment (Section 6.5.2).

^b Mean of five replicates. Infection was scored as follows:-
0 = no infection; 1, 2, 3 = necrosis - under inoculum droplet, extending slightly beyond inoculum droplet or spreading to up to half the diameter of the disc respectively; 4 = most of leaf disc infected, little sporulation; 5 = heavy, sporulating disease over entire leaf disc

NTG/cysteine treatment appeared to be very low (Section 6.5.2). Consequently, the ratio of cysteine to NTG which resulted in the most rapid development of colonies was investigated in the present experiment.

b) Materials and methods

Aliquots (9ml) of a suspension containing 10^5 sporangia/ml of isolate K1182 were placed in six sterile plastic universal containers. To three of these, 1ml of an aqueous solution of NTG (prepared overnight) was added to give a final concentration of 30 μ g NTG/ml. After 10 min, cysteine was added to two suspensions, using 1ml of two solutions, so that the ratio of cysteine to NTG was 2:1 and 200:1, respectively. One ml sterile water was added to the third suspension, which served as an experimental control.

The suspensions in the three remaining containers served to provide non-NTG-treated experimental controls. Thus, 2ml sterile water were added to one suspension and 1ml sterile water to the other two suspensions, together with 1ml of a solution containing either 600 or 6000 μ g cysteine/ml. Three 1ml samples were taken from each suspension at 30 and 60 min after the addition of cysteine. Each sample was placed in a 250ml Erlenmeyer flask containing 50ml clarified FP broth. Flasks were incubated at 22°C on an orbital shaker. After 11 days the mycelium was harvested from each flask, freeze-dried overnight and weighed.

c) Results and discussion

The results for mean dry weight of mycelium (Table 50) showed that in the absence of NTG, growth from sporangia in suspensions amended with up to 6000 μ g cysteine, was similar to that in unamended suspensions. Thus, this quantity of cysteine had no adverse effect on the development of the fungus.

Growth from sporangia was severely inhibited by 30 μ g NTG/ml and this was not improved by adding cysteine at twice the amount of the mutagen, despite incubation of the reaction mixture for 60 min. This

Table 50 Weight (mg) of freeze-dried mycelium of *Phytophthora*
infestans isolate K1182 harvested from clarified frozen
pea broth^a following NTG/cysteine^b treatment

NTG concentration (μ g/ml)	Ratio cysteine: NTG	Duration (min) of incubation of NTG/ cysteine reaction mixture ^c		
		0	30	60
0	0:0	163 ^d	136	149
	2:1	156	151	171
	200:1	172	156	147
30	0:0	0	8	0
	2:1	0	0	5
	200:1	0	124	157

^a Incubated at 22°C for 11 days.

^b Cysteine added to NTG-treated suspensions of sporangia after 10 min (see text)

^c At the completion of this incubation period, 1ml aliquots of the reaction mixture were transferred to 50ml amounts of clarified frozen pea broth

^d Mean of three replicates

contrasted with the vigorous growth of colonies from zoospore suspensions of P. megasperma f.sp. medicaginis also subjected to NTG : cysteine in a 1:2 ratio for 60 min (see Section 6.4.4).

Mycelium failed to grow from sporangia treated with a 200:1 ratio of cysteine: NTG following immediate transfer to broth medium. However, the yield of mycelium increased as the time of transfer was delayed, and after 60 min, the dry weight of mycelium was similar to that from non-NTG-treated suspensions. These results showed that the addition of a larger quantity of cysteine than had been used previously (Section 6.5.2) and an incubation of the mixture for 60 min were necessary to reverse the lethal effect of NTG on sporangia of P. infestans.

6.5.5 Selection of Fungicide-resistant Mutants -2

a) Introduction

This section describes further experiments designed to obtain R157846-resistant mutants of P. infestans isolate K1182. Modification of the NTG/cysteine system was based on the results given in Section 6.5.4.

b) Materials and methods

Suspensions (100-150ml) of sporangia derived from 11-day cultures of isolate K1182 were put into four or five sterile 250ml Erlenmeyer flasks. The number of sporangia in each suspension was calculated from counts using a Sedgewick Rafter cell. Aliquots (3ml) of a filter-sterilised aqueous solution of NTG (prepared overnight) were added to each suspension to give a concentration of 30µg NTG/ml. After 10 min, the same volume of a filter-sterilised aqueous solution of cysteine was added to each suspension to give a concentration of 6000µg/ml. Sixty min later, 2ml aliquots of the NTG/cysteine-treated suspensions were transferred to repli-dish compartments each containing 1ml undiluted clarified FP broth. In four compartments the

broth was unamended, in a further four compartments the broth was amended with metalaxyl (100µg/ml) and in the remaining compartments (32-72) the broth was amended with R157846 (1µg/ml). Dishes were incubated at 22°C for at least 21 days, and regularly examined for the development of vigorously-growing colonies of P. infestans. All colonies which grew in R157846-amended broths, and selected colonies from unamended broths, were bisected and transferred to unamended FP agar and FP agar amended with 1µg R157846/ml.

c) Results and discussion

In four experiments, a total of 1.4×10^8 sporangia were subjected to NTG/cysteine treatment and 28 colonies subsequently developed in R157846-amended clarified FP broth (Table 51). Vigorous growth of the fungus occurred in all unamended broths and broths amended with 100µg metalaxyl/ml.

Colonies transferred from R157846-amended broths developed on unamended FP agar, but not on FP agar amended with 1µg R157846/ml. Although it appeared, therefore, that these variants were unlikely to be of practical significance, sensitivity to R157846 was re-examined in the next experiment (Section 6.5.6).

6.5.6 Sensitivity to R157846 of Variant Isolates Selected from R157846-amended Broth

a) Introduction

The sensitivity to R157846 of 33 variants of isolate K1182, obtained in Section 6.5.5, was examined in this experiment. Five isolates were selected from unamended clarified FP broth, the remainder from broth amended with R157846.

b) Experimental

Each isolate was grown on two 9cm plates of unamended V8 agar and two plates of V8 agar amended with 1µg R157846/ml.

Table 51 Growth of variants of *Phytophthora infestans* isolate K1182 in clarified frozen pea broth amended with 1 μ g R157846/ml^a

Expt.	Suspension number	No. of sporangia (x 10 ⁻⁵)/ml	No. of compartments	Total no. of sporangia (x 10 ⁻⁵)/ml	No. of visible colonies
1	1	1.88	54	101.5	2
	2	1.26	61	76.9	4
	3	2.48	32	79.4	0
	4	2.04	56	114.2	2
2	1	4.36	55	239.8	1
	2	1.82	52	94.6	0
	3	1.38	72	99.4	2
	4	1.44	60	86.4	2
	5	2.04	52	106.8	0
3	1	0.59	54	31.9	0
	2	1.88	44	82.7	3
	3	0.41	56	23.0	4
	4	1.84	54	99.4	0
	5	0.75	66	49.5	1
4	1	0.74	51	37.7	1
	2	0.87	50	43.5	3
	3	1.07	35	37.5	0
	4	1.11	55	61.1	3
	5	1.42	36	51.1	0

^a Following NTG/cysteine treatment of sporangia (see Section 6.5.5)

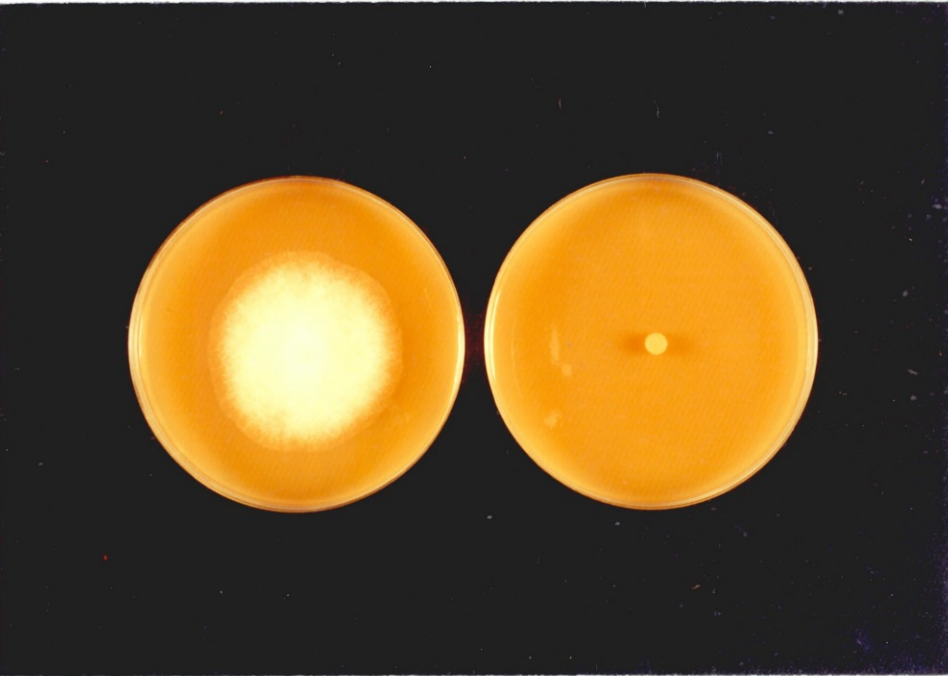
No isolate grew in the presence of 1 μ g R157846/ml. After 7 days, colony diameters (minus the inoculum plug diameter) on unamended V8 agar ranged from 18-67mm for variants obtained originally from broth with R157846 and 48-55mm for variants from unamended broth (see Appendix D and Plate 24).

c) Conclusion

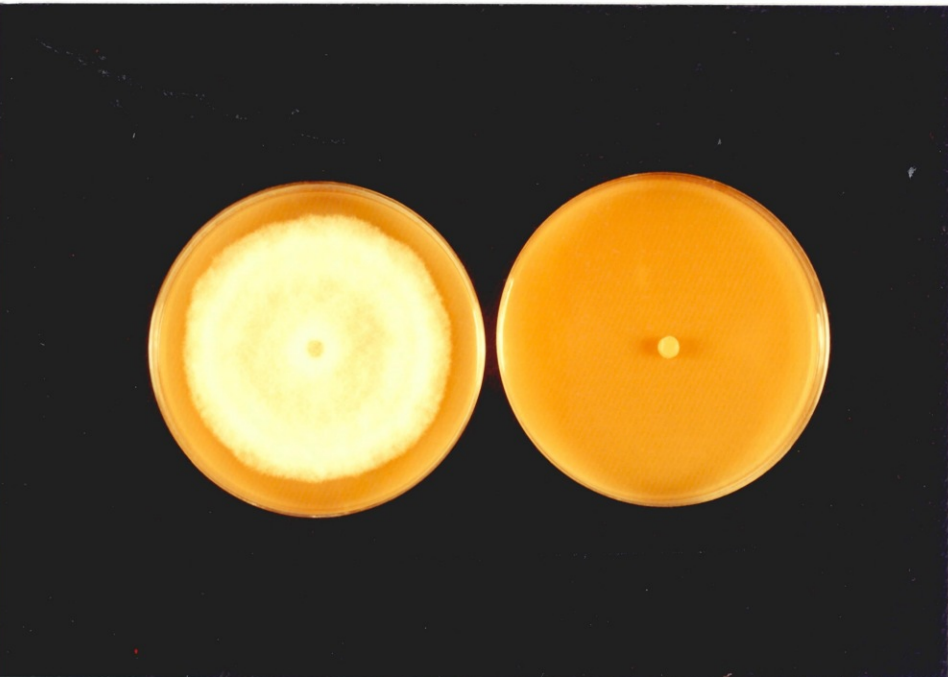
The results reinforced the findings of previous investigations with P. infestans (Section 6.3) and P. megasperma f.sp. medicaginis (Section 6.4) that resistance in target fungi to the benzamide fungicides is likely to occur, if at all, at a very low frequency.

1

a)



b)



7 GENERAL DISCUSSION

7.1 The Significance of Fungicide-resistance to Crop Protection

There are now many publications describing the occurrence of resistance in plant pathogenic fungi to a range of chemicals used as fungicides (see Section 1.4.1).

The development of resistance to metalaxyl in the potato late blight fungus, P. infestans, in The Netherlands, exemplifies the economic importance of fungicide resistance. In 1979, 5% of the potato acreage was treated with metalaxyl (Ridomil) and the expected degree of control of late blight was achieved. In 1980, the usage of metalaxyl had increased to 50% (L C Davidse, personal communication). Weather conditions were conducive to the development and spread of late blight, and during the 1980 growing season, an epidemic occurred which was not controlled by metalaxyl. This was found to be due to strains of P. infestans which were resistant to the fungicide. However, because the grower, advisory service and manufacturer were unprepared for dealing with the breakdown in disease control, the speed and severity of the epidemic resulted in high crop losses (Davidse et al., 1981) and the manufacturer was compelled to withdraw metalaxyl from the market. Consequently, when in 1981 a blight epidemic occurred in The Netherlands, metalaxyl was not available for use, even in those areas where resistance had not been recorded, and where the fungicide might have saved the crop (Dekker, 1981).

The detrimental economic effect of fungicide resistance is experienced in various ways. The grower is confronted with unexpected, and possibly costly, crop losses. Additionally, a once-effective fungicide may be lost, so that the range of chemical control options available to him is reduced. Reduced sales may threaten the financial returns to the manufacturer, who has developed the chemical at high cost.

The consumer may have to pay more for agricultural produce, provided it is available, if losses due to resistant pathogens are high.

Delp (1981) noted that many of the most effective fungicides have a propensity for resistance, and Dekker (1981) indicated that with the development of more selective and efficient fungicides, the danger of development of fungicide resistance will increase in the future.

For agrochemical manufacturers, fungicide-resistance research is, or should be, an integral part of the development of promising new fungicides, and the management of existing products.

7.2 Validity of Predicting the Risk of Resistance to the Benzamides

Previous experiences of fungicide-resistance justifiably cause manufacturers to be cautious about the development and introduction of a new fungicide. Attempts to assess the risk of resistance developing to a novel fungicide have frequently been encouraged (Dekker, 1977; Wade, 1982; Staub & Sozzi, 1984) and in some instances have been implemented (for example, Staub *et al.*, 1979; Davidse, 1981). This information should aid decisions regarding the development and marketing of the fungicide. The present study with the benzamides was based on these principles.

In practice, resistance-risk assessments may not yield reliable information about the likelihood of resistance developing in the field and glasshouse, and resistance may develop contrary to expectations. Thus, with acylalanines, first studies demonstrated that resistance *in vitro* was not expressed *in vivo*, and *in vivo* selection experiments did not yield resistant strains (Staub *et al.*, 1979; Bruin & Edgington, 1981). In contrast, with the same fungicides, and using mutagens, resistant strains were obtained which were as fit as wild strains and not controlled by normally effective doses of acylalanines (Bruin & Edgington, 1981 & 1982; Davidse, 1981). The results indicated that there was a risk of resistance

occurring, and this was confirmed almost simultaneously by reports of resistance under field and glasshouse conditions. It is possible that the availability of the results of the latter studies before the introduction of metalaxyl would have affected the way in which the chemical was marketed. Conceivably, a decision could have been taken not to introduce the chemical in the first instance.

Extrapolations from laboratory studies to field and glasshouse conditions must take into account the nature of the pathogen and the type of disease. Fungicide-resistance seems more likely to build up rapidly in foliar pathogens which have a short life-cycle and sporulate abundantly, than soil-inhabiting pathogens which do not spread readily. Thus, metalaxyl-resistant strains of several air-borne members of the Peronosporales emerged rapidly and reached epidemic proportions in one to two seasons. P. cubensis and P. infestans are notable examples (see Davidse, 1982). Only recently, and after intensive use of the fungicide, has disease control failure due to metalaxyl-resistant strains of soilborne Oomycete fungi begun to be reported. Specifically, the failure of metalaxyl to control Pythium blight on turfgrass was shown to be due to resistant strains of P. aphanidermatum (Sanders, 1984).

Despite the difficulties, the present assessment of the likelihood of resistance developing to the benzamide fungicides had significant advantages over previous risk-assessments. First, a standard chemical (metalaxyl) was available to which resistance under field and glasshouse conditions was known. The relative frequency with which strains resistant to metalaxyl and benzamides could be obtained after selection procedures, was likely to enable a more accurate determination of the incidence of benzamide resistance under field or glasshouse conditions, than obtaining benzamide-resistant strains alone. Since no benzamide-resistant strains were eventually detected, the inclusion of metalaxyl as a standard was especially important. Second, the previous studies with

acylalanines provided information about the problems and benefits of some approaches to obtaining resistant strains using selection procedures. For example, in one study the use of NTG yielded strains of P. megasperma f.sp. medicaginis highly resistant to metalaxyl (Davidse, 1981). In another study, in vivo adaptation of P. infestans to acylalanines was unsuccessful (Staub et al., 1979). Consequently, emphasis in the present study was placed on those techniques which were most likely to yield strains resistant to acylalanines, and potentially, benzamides.

7.3 Likelihood of Development of Benzamide-resistance

Throughout the present study, no strains of Phytophthora resistant to high doses of benzamide fungicides were detected, despite using a variety of methods including in vitro and in vivo sensitivity testing (Chapter Four) adaptation (Chapter Two), UV irradiation and NTG mutagenesis (Chapter Six). Under the same experimental conditions, metalaxyl-resistant strains were detected regularly in large numbers, and many of these displayed a high degree of resistance. The simplest conclusion is that either benzamide-resistance is unlikely to occur, or is likely to occur at a much lower frequency under field and glasshouse conditions than metalaxyl-resistance. Furthermore, the clear lack of cross-resistance to the benzamides means that these fungicides may be used in locations where acylalanine-resistant strains are present.

The inability to detect benzamide-resistant strains after an intensive search for them does not mean that rare resistant strains will not occur under field and glasshouse conditions, where there is a high potential for variation in the pathogen. Nevertheless, there was other evidence from the present study that the risk of benzamide resistance was likely to be low. First, the development of many isolates of Phytophthora spp. was completely inhibited by relatively low doses of benzamide (Chapter

Four), and there were indications that the benzamides were fungicidal in their action (Chapter Five). By killing the fungus at an early stage, the possibility of selecting resistant strains is reduced. Second, the benzamides decompose under aqueous conditions more rapidly than metalaxyl (Chapter Two). As Dekker (1981) pointed out, a fungicide which is very persistent exerts a continuous selection pressure for fungicide resistance on the pathogen population. Metalaxyl is an example of this type of chemical (Schwinn & Staub, 1981). If the benzamides are not persistent in vivo, as is suggested by in vivo field behaviour (ICI, internal information) then they may be less hazardous in this respect.

Although it has yet to be elucidated, the biochemical mode of action of the benzamides is likely to be of great importance in determining the ease or difficulty with which resistance develops.

While site-specific fungicides generally are more likely to succumb to resistance than residual fungicides, field and glasshouse experience suggests that some site-specific modes of action are more readily overcome by the fungus than others. For example, triazole and morpholine fungicides inhibit ergosterol biosynthesis, yet despite intensive use of both fungicides, resistance has only been reported to the triazoles. It may be that the enzyme site inhibited by triazoles is more readily overcome than the site(s) inhibited by morpholines. Similarly, the biochemical mode of action of the benzamides may be such that the risk of resistance is reduced or eliminated.

Some of the other fungicides used to control pathogens belonging to the Peronosporales also seem to be at lower risk from the development of resistance. For example, after many years of usage, there have been no reports of resistance, under field and glasshouse conditions, to either cymoxanil or phosethyl-Al. Both are used to control foliar pathogens with a high reproduction rate, and frequently in the same locations in which acylalanines are used. Cymoxanil is locally systemic in the plant, but its

activity is lost within a few days (Schwinn, 1983), a feature which may partly account for the absence of resistance. In contrast, phosethyl-Al is rapidly translocated in the plant and its activity persists for relatively long periods. Phosethyl-Al resistant strains of P. capsici have been induced under laboratory conditions, which are as fit as the parent strains and not controlled in vivo by normally effective doses of the fungicide (Bower & Coffey, 1985). In the same study, the frequency of phosethyl-Al and metalaxyl-resistant mutants was similar. These findings indicate that the risk of resistance developing to phosethyl-Al might be high, especially considering that in some locations (e.g. against vine downy mildew in regions of France) the fungicide is used in larger amounts than acylalanines (S P Heaney, personal communication). Unlike the acylalanines, which demonstrate high activity at low doses (200-250g ai/ha) against air-borne Oomycete pathogens, phosethyl-Al has to be applied at high rates (approximately 1500g ai/ha) and is generally not considered to be as effective as acylalanines at controlling disease (ICI, internal information). It seems more likely that target pathogens will survive and escape the effect of phosethyl-Al under field and glasshouse conditions than acylalanines. In this way, resistant strains may be prevented from being selected and dominating the pathogen population, and are unable to manifest themselves in disease control failures.

Unlike the acylalanines and phosethyl-Al, the benzamides are a group of fungicides with potentially a low risk for resistance. Dekker (1981) encouraged the search for such types of chemical and observed that it would be valuable to find cases where mutations for resistance would be lethal, thus precluding the emergence of resistance altogether. It is not known if the benzamides may be placed in this category of resistance-risk.

A pragmatic approach to the findings of the present study would be to assume that the benzamides were at low risk from the development of resistance, but at the same time take precautions against the possibility

that rare resistant mutants could exist and be selected under field and glasshouse conditions.

7.4 Anti-resistance Strategies

Staub et al. (1979) inferred that metalaxyl was at a low risk from the development of resistance, and subsequently the fungicide was marketed without the deliberate inclusion of anti-resistance strategies. Such strategies were only implemented after the economically-damaging effects of metalaxyl-resistant strains of target pathogens had been experienced (see Table 3). The Fungicide Resistance Action Committee (FRAC), whose function was described by Delp (1984), has featured predominantly in efforts to develop common anti-resistance strategies between manufacturers of fungicides. Comprised of representatives of the major agrochemical manufacturers (ICI, Ciba-Geigy, Sandoz, Bayer, FBC and Montedison), the United Kingdom Acylalanine Working Group of FRAC decided during 1985 to confirm existing measures, and implement new proposals, to limit the incidence of acylalanine-resistant strains of P. infestans during the 1986 growing season:-

1. Pre-pack mixtures of acylalanine and residual fungicides were to be applied to potato crops to reduce the selection pressure otherwise imposed by the sole use of the site-specific acylalanine component. Where possible, the rate of residual fungicide would be increased to that which would be expected to control disease in the absence of the acylalanine. In this way, resistant strains which were not controlled by the acylalanine would be strongly inhibited by the companion residual fungicide.
2. Despite their eradicant capabilities, fungicide mixtures containing acylalanines were to be applied in a programme of treatments starting

before disease was observed in crops. Thus, selection of resistant strains which might otherwise have become established in the crop would be prevented.

3. A maximum of five treatments of any acylalanine - containing product was to be applied to a crop in one season. The potential increase in the proportion of strains which were resistant, during continuous exposure to the acylalanines, would thereby be limited.
4. Treatment programmes were to be completed by mid- to end-August, when the efficacy of acylalanines declines, and resistant strains might increase in frequency more rapidly than at other times in the growing season.

The International Acylalanine Working Group of FRAC has attempted to impose limits of two to four acylalanine treatments per season in those situations (for example, vine downy mildew in France) where resistance continues to be a severe problem.

Regular monitoring studies will enable the effectiveness of these strategies to be established.

The dramatic experiences with the acylalanines emphasises the importance of incorporating anti-resistance measures into the marketing of even those chemicals which are apparently at low risk. Thus, the benzamides should be marketed in a manner that reduces the likelihood of resistance still further. This desirable aim is not always easy to implement. For example, Delp (1981) and Staub & Sozzi (1984) noted that the agrochemical registration agencies in some countries do not consider the resistance argument as a sufficient reason for registering an anti-resistance mixture.

Some anti-resistance measures are known to be beneficial. For example, the selection of resistant strains is reduced by decreasing disease pressure through cultural practices and the use of resistant

cultivars. Growth room studies (Staub & Sozzi, 1983) and field experience (Davidse, 1982) showed that mixtures of fungicides with different modes of action could slow the build-up of resistant strains. With the acylalanines, cymoxanil and phosethyl-Al, an additional benefit of using them in mixtures with residual fungicides such as mancozeb, folpet and captafol is that the spectrum of activity is increased. Similarly, besides reducing the risk of resistance still further, benzamides should probably be marketed in a commercial mixture with a residual fungicide to increase the range of pathogens which may be controlled. Furthermore, the use of mixtures with more than two components might be justified.

In 1985, ICI introduced a cereal seed-treatment, 'Ferrax', which consists of a mixture of flutriafol (a triazole), ethirimol (a hydroxypyrimidine) and thiabendazole. Both flutriafol and ethirimol are effective against powdery mildews but belong to different chemical groups and have different modes of action. Thiabendazole is included to enhance activity of the product against seed and soil-borne Fusarium species. To date, this mixture has been more effective at controlling mildew populations (which include triazole-resistant and hydroxypyrimidine-resistant strains) than the individual components. Similarly, assuming that a residual fungicide (for example, mancozeb, folpet or captafol) would be included in a mixture with a benzamide, a third, possibly site-specific fungicide, could be included to increase the effectiveness of the product and reduce further the risk of resistance. Because of the problems encountered with resistance in the past, the inclusion of an acylalanine may be undesirable. The incorporation of cymoxanil, phosethyl-Al or its breakdown product, phosphorous acid, is a more attractive proposition. Mixtures containing a benzamide, a residual fungicide and an additional systemic fungicide were under evaluation against vine downy mildew in France during 1985.

It has been demonstrated that the acylalanines (Staub & Schwinn, 1980) and benzamides (ICI, internal information) are capable of eradicating

infections within host plants. However, because of the considerable selection pressure for resistance which could be exerted if the benzamides were applied to diseased crops, it is advisable to use them, like the acylalanines, to prevent the establishment of infection in the first instance.

The implementation of other anti-resistance strategies for the benzamides, similar to those described for the acylalanines, seems less clear. For example, provided that good disease control is maintained throughout a growing season, it is unlikely to be necessary to stipulate a maximum number of treatments which should be applied.

To ensure that satisfactory disease control by the benzamide-containing mixture is being achieved, its efficacy against samples of field or glasshouse populations of target fungi should be tested during successive growing seasons. Ideally, statistically valid surveys should be undertaken at pre-selected sites where the fungicide is being used. Sampling from locations where there is inexplicable loss of disease control should also be undertaken.

The cultures of P. infestans which were collected during the course of the present study should prove useful as reference isolates in assessing the benzamide-sensitivity of potato blight populations. If resistance is detected, appropriate anti-resistance measures may be implemented, hopefully before serious loss of disease control has occurred.

7.5 Further Action

Several aspects of the present study could usefully be subject to further investigation.

The applicability of the NTG/cysteine system to resistance-risk-assessments with other, novel fungicides, should be considered as the need for them arises. Additionally, an explanation for the apparent

reversibility of the lethal effect of NTG on sporangia by cysteine is required.

Almost 200 isolates of Phytophthora spp. were examined for their in vitro and in vivo sensitivity to metalaxyl and representative benzamides. The genetic variation represented by these isolates was undoubtedly small compared with the variation which is likely to exist under field and glasshouse conditions. No isolates were identified within the sample size examined which were naturally highly resistant to a representative benzamide. In theory it is possible that a larger sample size would have revealed resistance. With acylalanines, only 35 of the same or similar isolates had previously had to be tested, for Hunger et al. (1982) to propose that resistance to metalaxyl was a natural feature of some of them. In future monitoring surveys, the possibility of assessing the benzamide-sensitivity of much larger numbers of individuals of target pathogens, more rapidly, should be investigated. Some difficulties can be anticipated already. Like the acylalanines, the benzamides do not prevent the germination of zoospores or sporangia, so that differentiating between benzamide sensitive and resistant spores in vitro may not be feasible. Furthermore, past experience with the acylalanines has shown that in vitro resistance may not be expressed in vivo. Although the dosage-response of isolates to representative benzamides in vitro and in vivo appeared to coincide (Chapter Four), this was not proven statistically, and in vivo experiments using leaf discs or other plant tissue may have to be undertaken to ensure that reliable data are obtained.

A more critical investigation of the conditions under which the benzamides are fungicidal or fungistatic to target pathogens would provide further evidence for the risk of resistance. Comparison of a benzamide with fungicides such as cymoxanil and phosethyl-Al, to which there have been no reports of resistance under field and glasshouse conditions, should aid this determination.

7.6 Recommendations for the Development and Commercial Use of
Benzamide Fungicides

These may conveniently be listed as follows:-

1. The benzamides appear to be at low risk from the development of resistance, and on this basis, their commercial development should proceed.
2. The desirable feature of low risk of resistance could be used commercially to promote the benzamides. However, given that extrapolation from laboratory to field and glasshouse conditions can be uncertain, this may be unwise, at least until sufficient experience of their performance is gained. Nevertheless, there was good evidence that the benzamides were at a lower risk from resistance than the acylalanines, and they could be marketed strongly as an alternative to acylalanines, which are under continuous threat from the development of resistance.
3. Some anti-resistance measures should be included when the benzamides are introduced into commercial use. Mixtures of a benzamide with fungicides which are known to be at low risk from resistance and/or demonstrate no cross-resistance should be marketed, rather than the benzamide alone. Such mixtures should be used to protect crops from infection, rather than to eradicate disease.
4. The performance of the benzamides under field and glasshouse conditions should be monitored so that rapid remedial measures can be implemented if resistance is detected. For this reason, reference isolates of P. infestans from the present study should be maintained to enable changes in the benzamide-sensitivity of field and glasshouse populations of target pathogens to be accurately determined.

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APPENDICES

APPENDIX A : Media used to grow Phytophthora spp.

1. Frozen Pea

'Birds Eye' frozen peas	160g
Deionised water	1000ml
Oxoid no. 3 agar	20g

The frozen peas were macerated in a small quantity of deionised water for 3 min using a blender and filtered through fine muslin gauze.

The filtrate was dealt with as follows:-

- a) frozen pea broth - deionised water was added to the filtrate to a final volume of 1000ml.
- b) clarified frozen pea broth - as described for a), but the macerated peas were centrifuged to remove most of the suspended solids prior to filtering.
- c) undiluted, clarified frozen pea broth - the final volume of the clarified broth was reduced by two-thirds.

Agar was added to broths when a solid medium was required.

Media were sterilised by autoclaving at 15p.s.i. for 20 min.

2. Rye

a) Rye A agar

Rye Seed	60 g
Glucose	20 g
Oxoid no. 3 agar	20 g
Deionised water	1000 ml

Rye seed was soaked in a small quantity of deionised water for 36 h. The grains were then macerated in a blender for 3 min and incubated at 50°C for 3h. This suspension was filtered through fine muslin gauze and deionised water was added to the supernatant to a final concentration of 1000ml. Sucrose and agar were added to the medium which was sterilised by autoclaving at 15 p.s.i. for 20 min.

b) Rye B broth

As for a), but instead of being macerated and then incubated for 3h, grains were boiled in deionised water for 1h. Agar was omitted.

3. V8

Campbell's V8 juice	200 ml
Calcium carbonate	3 g
Deionised water	800 ml
Oxoid no. 3 agar	15 g

Calcium carbonate was mixed with the V8 juice and dealt with as follows:-

- a) undiluted clarified V8 broth - the mixture was centrifuged to remove suspended solids.
- b) clarified V8 broth - as for a), but deionised water was added to the supernatant to a final volume of 1000 ml.
- c) V8 broth - deionised water was added to the mixture to a final volume of 1000 ml.

Agar was added to broths when a solid medium was required.

Media were sterilised by autoclaving at 15 p.s.i. for 20 min.

APPENDIX B : Mc Ilvaine's citric acid-phosphate buffer (Mc Ilvaine, 1921)1. Stock Solutions

- a) Solution A - 21.0 g citric acid was dissolved in 1000 ml deionised water.
- b) Solution B - 35.6 g disodium phosphate was dissolved in 1000 ml deionised water.

2. Buffer Solutions

- a) pH 4 - 61.45 ml Solution A was added to 38.55 ml Solution B.
- b) pH6 - 36.85 ml Solution A was added to 63.15 ml Solution B.

APPENDIX C : Metalaxyl-sensitivity of variants ^a of *Phytophthora megasperma* f.sp. *medicaginis*Isolate K1120

Isolate/ variant	Inhibition(%) of growth ^b on medium:		Isolate/ variant	Inhibition (%) of growth on medium:	
	without metalaxyl	with 20ug metalaxyl/ml		without metalaxyl	with 20ug metaxyl/ml

1. Direct-plating technique

EXPERIMENT 1

K1120	0	100	M59	52	33
K1121	0	0	M60	8	3
M46	44	62	M61 (R)	64	78
M47	48	48	M62	55	67
M48	39	68	M63	46	60
M49	39	52	M64	64	76
M50	18	29	M65	25	36
M51	41	44	M66	39	54
M52	39	35	M67	49	46
M53 (R) ^C	21	8	M68	66	73
M54 (R)	8	8	M69	52	48
M55	44	42	M70	33	37
M56	65	77	M71	60	66
M57	50	30	M72	1	12
M58	64	73			

EXPERIMENT 2

K1120	0	100	M86	43	52
K1121	0	0	M90	49	44
M73	43	32	M91	36	43
M74	60	70	M92	77	87
M75	29	37	M93	38	59
M76	40	38	M94	89	95
M77	40	40	M95	64	77
M78	44	60	M96	36	46
M79	56	24	M97	5	16
M80	37	27	M98	46	66
M81	68	68	M99	79	90
M82 (R)	8	8	M100	49	87
M83	8	37	M101	56	77
M84	54	41	M102	59	64
M85	62	60	M103	46	64

EXPERIMENT 3

K1120	0	100	M104 (R)	20	2
K1121	0	0	M105 (R)	2	5
M87	67	66	M106	23	44
M88	26	11	M107	59	69
M89	66	56			

APPENDIX C : Continued

Isolate/ variant	Inhibition(%) of growth on medium:		Isolate/ variant	Inhibition (%) of growth on medium:	
	without metalaxyl	with 20ug metalaxyl/ml		without metalaxyl	with 20ug metalaxyl/ml

2. Overlay technique

EXPERIMENT 1

K1120	0	100	M148	6	51
K1121	0	0	M149	17	49
M142	43	40	M150(R)	0	0
M146	14	22	M151	42	46
M147	32	82	M152	18	32

EXPERIMENT 2

K1120	0	100	M118	66	80
K1121	0	0	M119 (R)	0	0
M108	61	63	M120 (R)	0	0
M109	34	38	M121 (R)	0	0
M110	6	20	M122 (R)	0	0
M111	75	83	M138	42	79
M112	52	72	M139	26	53
M113	66	81	M140	6	26
M114	67	77	M141	42	65
M115	56	73	M144	64	66
M116	28	39	M145(R)	25	8
M117	58	63			

EXPERIMENT 3

K1120	0	100	M128	52	41
K1121	0	0	M129	18	17
M123	21	27	M130	24	42
M124	35	85	M131	58	76
M125	29	55	M132	41	56
M126	0	12	M133	48	70
M127	47	48			

EXPERIMENT 4

K1120	0	100	M136	38	50
K1121	0	0	M137	71	79
M134	45	62	M143(R)	0	0
M135	76	79			

- a Isolated following NTG/cysteine treatment and selection on metalaxyl-amended V8 agar.
- b Radial growth of daughter colonies on metalaxyl-amended or unamended agar compared with that of the parent isolate on unamended agar.
- c (R) = resistant mutant with a radial growth on metalaxyl-amended agar similar (less than 10% reduction in growth) to that of the parent isolate on unamended agar.

APPENDIX D : Radial growth^a of variants of *Phytophthora infestans* isolate
K1182 on V8 agar

Variant ^b	Radial growth (mm)	Variant	Radial growth (mm)
UNT-1	48	BENZ-12	35
UNT-2	55	BENZ-13	47
UNT-3	53	BENZ-14	32
UNT-4	54	BENZ-15	49
UNT-5	53	BENZ-16	42
		BENZ-17	32
BENZ-1	39	BENZ-18	38
BENZ-2	35	BENZ-19	42
BENZ-3	18	BENZ-20	35
BENZ-4	25	BENZ-21	45
BENZ-5	38	BENZ-22	46
BENZ-6	67	BENZ-23	38
BENZ-7	64	BENZ-24	36
BENZ-8	33	BENZ-25	50
BENZ-9	44	BENZ-26	43
BENZ-10	28	BENZ-27	26
BENZ-11	47	BENZ-28	50

^a mean of two replicates

^b UNT- = variant selected from unamended clarified FP broth

BENZ- = variant selected from R156846-amended clarified FP broth
following NTG/cysteine treatment