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The role of the oospore in the population dynamics of Phytophthora infestans

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The Role of the Oospore in the Population Dynamics of *Phytophthora infestans*

by

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Summary

The ability of oospores of *P. infestans* to form in the leaves and stems of pot-grown potato plants when the A1 and A2 strains originate from different infector plants within a plot was tested in semi-controlled conditions inside a polythene tunnel. Oospores were found in 9 out of 64 potato plants in the experiment, with the A1 and A2 isolates found intermixed on 17 plants. The resultant blighted plant material was left to decompose from October to April after which the detritus was assayed for any surviving *P. infestans* propagules. Sexual recombinants were detected from three different potato plants from the experiment with a total of 6 recombinant isolates recovered. The effect of the carbamate fungicide propamocarbHCl on oospore formation in leaf disks, in pot-grown potato plants and field-grown potato plants was also assessed. PropamocarbHCl was found to inhibit the formation of oospores at concentrations where asexual reproduction was not affected. In pot-grown potatoes the formation of oospores was found to be dependant on temperature, with oospores formed at 18°C, but not at 5 or 24°C. PropamocarbHCl was found to have no effect on the germination of oospores *in vitro* at concentrations ranging from 0 to 500µg/mL. The inheritance of sensitivity to propamocarbHCl was also studied. The level of sporulation and the latent period of 15 progeny from each of two crosses of *P. infestans* was tested in the presence of propamocarbHCl on 5 different potato cultivars (King Edward, Home Guard, Maris Piper, Tina and Stormont Enterprise) differing in their horizontal resistance to late-blight. It was determined that offspring that are more tolerant to propamocarbHCl than parental isolates could be generated through sexual reproduction, although this was expressed differently on different potato cultivars. Some correlation was found between latent period of offspring in the absence of propamocarbHCl and their propamocarbHCl sensitivity.

Figures and Tables. **Page**

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Chapter 1

General introduction

Phytophthora infestans (Mont.) de Bary is a plant pathogenic pseudofungus, belonging to the kingdom Chromista, phylum Oomycota, order Pythiales and the family Pythiaceae (Dick, 1995a, b). It is the cause of late-blight disease of potato and tomato and occurs worldwide (Erwin & Riberio, 1996).

The oomycete fungi have recently been reclassified and it is now apparent that they are not related to the ascomycete and basidomycete fungi, but are more closely related to the chrysophytes (golden-brown algae) (Gunderson *et al.*, 1987).

Reproduction in *P. infestans* can be either sexual or asexual, with the asexual lifecycle dominating (Dick, 1995a). Asexual reproduction is facilitated through the production of sporangia, which can either germinate directly to produce mycelial growth or indirectly to produce motile biflagellate zoospores that then encyst before producing germtubes (Erwin & Riberio, 1996).

This thesis intends to deal with three questions that remain unanswered within *P. infestans* research.

(1) Is the sexual stage of *P. infestans* able to form when parental isolates originate in independent loci in semi-natural conditions?

(2) Does the carbamate fungicide propamocarbHCl affect the formation, survival and germination of the sexual stage of *P. infestans*?.

(3) Can variation in tolerance to the carbamate fungicide propamocarbHCl be generated through sexual recombination?

Unlike the majority of *Phytophthora* species *P. infestans* is heterothallic. This means that in order to reproduce sexually, two mating types or compatibility types are needed, designated A1 and A2. In addition, *P. infestans* is bisexual with a single isolate of either mating type capable of producing gametangia of both types (oogonia and antheridia). The production of two types of gametangia facilitates both inbreeding and out-crossing, however each isolate is self-sterile, hence the requirement for two mating types for sexual reproduction (Gallegly & Galindo, 1958; Smoot *et al.*, 1958; Galindo & Gallegly, 1960). It was demonstrated by Smoot *et al.* (1958) that oospores could be produced in controlled conditions when the two different mating types were paired on agar or on potato or tomato leaves; no oospores were formed when each mating type was grown in isolation. Smoot *et al.* (1958) were able to produce only two isolates from germinated oospores and demonstrated that they retained their pathogenicity to potato plants.

Self-fertile isolates have also been described (Fyfe & Shaw, 1992; Smart *et al.*, 1998) which formed oospores *in vitro* when isolates generated from single zoospores were grown independently of other isolates.

The centre of origin of *P. infestans* is thought to be the central highlands of Mexico (Toluca Valley). Work conducted in the 1950s revealed the presence of both mating types in nature as well as oospores in potato plant tissue (Niederhauser, 1956; Gallegly & Galindo, 1958; Smoot *et al.*, 1958). Mating types in the Toluca Valley were found to be present in a 1:1 ratio on both native *Solanum* species and in commercial potato crops (Gallegly & Galindo, 1958). This has been confirmed by Niederhauser (1991), who described the distribution of A1 and A2 isolates collected from Mexico from 1950 to 1970. The A1 and A2 mating types were found together only in the high-altitude region of Mexico where wild *Solanum* species proliferate. The A1 mating type was found in other regions of Mexico, but the A2 mating type was absent.

The *P. infestans* population in central Mexico is very diverse in both phenotypic characteristics and selectively neutral markers including allozymes (Tooley *et al.*, 1985b) and DNA fingerprints (Matuzak, 1994). These studies demonstrated that at least half of the isolates collected from central Mexico were of unique genotype, despite the dominance of asexual reproduction, and that alleles in the population were in Hardy-Weinburg equilibrium. The conclusion drawn from the work of Tooley *et al.* (1985b) is that random mating was occurring in the Mexican population.

Late-blight was first detected outside Mexico in Philadelphia, USA in 1843 (Bourke, 1964). It appears that late-blight was first imported into Europe shortly after this time along with tubers from either the USA or Peru which were intended for trials in Belgium (Bourke & Lamb, 1993; Andrivon, 1996; Abad & Abad, 1997). It is recorded that the European potato crop had been suffering from "degeneration" due to viral disease and dry rot, so the intention was to bring in new varieties to rejuvenate the ailing European potato (Bourke & Lamb, 1993). Two varieties were selected and grown in the 1844 season, after which harvested tubers developed "black spots and rotted in storage" (Bourke & Lamb, 1993). There is evidence that blight reached southern England as early as September 1844 remaining confined to eastern Kent until the following season. It was during the latter part of the 1845 season that late-blight progressed as far as southern Sweden, Germany, southern France and the west of Ireland (Bourke & Lamb, 1993).

Owing to the lack of diversity in populations of *P. infestans* outside Mexico it is postulated (Goodwin *et al.*, 1994a) that the route of the first migration in the 1840s followed either one of two routes. Firstly, several clones migrated from Mexico to the USA after which a single clone contained in diseased seed potatoes was transported to Europe. An alternative hypothesis was proposed by Abad & Abad (1997) that potatoes were imported directly into Europe (see above) from Peru where it is believed late-blight had been endemic for centuries. *P. infestans* may have travelled directly in these shipments in 1844 (Goodwin *et al.*, 1994a).

Goodwin *et al.* (1994b) proposed that from the 1840s until the 1980s the world outside of Mexico was dominated by a single clonal lineage. This lineage, designated US-1, is defined as isolates of the A1 mating type, that differ by only one of the allozyme alleles, *Pep* 92/100 or *Gpi* 86/100 or differ by only one of the 24 defined DNA fingerprint bands (Goodwin *et al.*, 1992; Fry *et al.*, 1993). For example US-1.5 has the *Pep* 92/100 and *Gpi* 86/100 alleles, but band 18 of the DNA fingerprint is present in this strain, whereas it is absent in US-1. This type of variation in a clonal population may be brought about by the loss of alleles from heterozygous loci during mitotic crossing over or by mutation (Goodwin *et al.*, 1994a). All isolates collected outside of Mexico before the 1980s fit these criteria. This supports the theory that late-blight migrated from Mexico via the USA before being transported to Europe.

After entering Europe late-blight was distributed to Asia, South America and Africa sometime after the 1850s, through the world trade in seed potatoes centred in Europe, in which shipments undoubtedly contained diseased tubers (Cox & Large, 1960).

In the early 1980s the A2 mating type was first found outside Mexico, in Switzerland (Hohl & Iselin, 1984) and subsequently in the UK and throughout Europe and the Middle East (Malcolmson, 1985; Shaw *et al.*, 1985; Tantius *et al.*, 1986; Daggett *et al.*, 1993; Cooke *et al.*, 1995). There have been several hypotheses put forward to explain the appearance of the A2 mating type. Ko (1994) suggested that the A2 mating type occurred outside of Mexico through mutation from an A1 isolate, brought about by the use of fungicides. Shaw (1987) suggested that the A2 mating type may have been present for many years, but undetected whilst, Spielman *et al.* (1991), argued that the A2 mating type was introduced by migration.

The overwhelming evidence from population studies indicates that the occurrence of the A2 mating type outside Mexico arose by migration (Shaw *et al.*, 1985; Tantius *et al.*, 1986; Fry *et al.*, 1991; Spielman *et al.*, 1991; Sujkowski *et al.*, 1994; Drenth *et al.*, 1994; Goodwin *et al.*, 1998). Goodwin and Drenth (1997) have examined populations by cluster analysis and shown that the first A2 isolates detected outside of Mexico were unrelated to the previously dominant US-1 clone. Thus, it is concluded that the A2 mating type, along with new A1 genotypes appeared in Europe and indeed throughout the rest of the world through migration by trade in potatoes between Mexico and other countries (Fry *et al.*, 1992).

It is postulated that there have been two major migrations of *P. infestans* out of Mexico since the initial migration of the 1840s. It is thought that the first of these migrations in the late 1970s into Europe, may have been due to the importation of 25 000 tonnes of potatoes directly into Western Europe from Mexico in 1976 (Niederhauser, 1991). This importation of potatoes for processing and consumption was to offset a shortage of home-produced tubers due to a hot and dry growing season that year.

The new A1 and A2 genotypes that have appeared in Europe exhibit an increase in diversity in allozyme markers, mitochondrial-DNA haplotypes (Carter *et al.*, 1991; Day & Shattock, 1997; Griffiths & Shaw, 1998), nuclear DNA fingerprints (Carter *et al.*,1990, 1991; Goodwin, 1991; Drenth *et al.*, 1993), an increase in virulence diversity (Drenth *et al.*, 1994) and a raised level of aggressiveness (Day & Shattock, 1997). This large increase in diversity suggests that a migration of more than one genotype into Europe from Mexico occurred along with the appearance of the A2 mating type (Goodwin & Drenth, 1997).

Currently the old, pre-1980s population has been almost completely displaced by the new population. This displacement appears to have occurred quickly in the UK where the old, US-1 clone has only been found once since 1986 (Day & Shattock, 1997). Data from isolates collected in the Netherlands from 1951 to 1978 show

the population to be exclusively made up of old-population isolates (based on allozyme and DNA markers), but after 1982 these isolates had been completely replaced by the new population (Drenth *et al.*, 1993). Similarly, in Poland between 1985 and 1987 the population of *P. infestans* consisted only of the old clonal type but by 1989 this had also been completely displaced by the new population (Spielman *et al.*, 1991). The exact reasons for such a rapid displacement of the old population are unclear. It has been suggested (Day & Shattock, 1997) that an increase in aggressiveness in the new population, and a lack of resistance to the commonly-used class of systemic fungicides, the phenylamides, in the old population, were contributing factors.

Based on the fact that diversity decreases as successive migrations occur (Hedrick, 1984), it would seem that the spread of the new population throughout the world had its origin in Western Europe, specifically in The Netherlands. A much larger number of different genotypes have been found in The Netherlands than in other regions (Drenth *et al.*, 1993). In Japan, Brazil, and Israel only one or two clones of the new population have been found, although collections from these countries may not be large enough to represent the full diversity of these particular regions (Fry *et al.*, 1992).

In Brazil both the A1 and A2 mating types are present, but each mating type comprised a single clonal strain (Goodwin *et al.*, 1994a). All Israeli isolates characterised by Fry *et al.* (1992) have the same allozyme genotype. In the Philippines the DNA fingerprints and allozymes variations were assessed in 27 isolates over two years but only two genotypes were found and all isolates were of the A1 mating type (Fry *et al.*, 1992).

The most recently detected migration occurred in the late 1970s and 1980s from Mexico to the USA and Canada (Goodwin *et al.*, 1994a). Until this time populations of *P. infestans* in the USA and Canada were dominated by the US-1 clonal lineage as was the rest of the world until the 1970s. The new North

American population was first detected in 1990 on potatoes from Washington and then in 1991 on tomatoes from Florida (Fry *et al.*, 1992). Since then many other new genotypes have been detected in the USA and Canada (Goodwin *et al.*, 1998) with each new individual genotype classified using the US-*n* nomenclature.

The US-6 genotype (A1 mating type) has been identified in culture collections dating back to the early 1980s and is thought to have entered the USA as early as 1979. US-6 was found most commonly in California in 1979, when the first outbreaks of late-blight on tomatoes occurred for 32 years (Goodwin *et al.*, 1994b).

The first A2 mating types were found in the USA in 1992 when genotypes US-7 and US-8 were discovered (Goodwin *et al.*, 1995). US-7 was widespread in 1993 and detected on both potatoes and tomatoes, but has been found to be more pathogenic on tomatoes (Legard *et al.*, 1995). Between 1994 and 1996 the US-8 genotype was found to be most common in North America with US-7 detected rarely (Goodwin *et al.*, 1998). Isolates have been collected from northern Mexico that are identical to US-7 and US-8; thus it is thought that these genotypes are direct migrants from this region (Goodwin *et al.*, 1998).

US-8 is pathogenic on potatoes, but not aggressive on tomatoes (Deahl *et al.*, 1991). It has caused major epidemics throughout the USA and Canada and continues to be a major threat to growers. The apparent distinction between isolates found on tomatoes and potatoes is echoed in Europe. Lebreton and Andrivon (1998) revealed that in France in 1995 and 1996 the A2 isolate was only found on tomatoes, although these isolate were not of the US-8 genotype. This is supported by the recent collection of the first A2 isolate found in Jersey, in the Channel Islands, UK, on a tomato plant, although the DNA fingerprint is different from that of the French isolates (J. P. Day, pers. comm.).

New (non-US-1) populations of *P. infestans* have been detected throughout the world, except in a few regions, including Australia where the A2 mating type remains absent (Fry *et al.*, 1992). Using characterisation of isolates based on DNA fingerprint (using the multilocus fingerprinting probe RG57) the new European populations are comprised of many different strains, whilst the US-1 clone is only found occasionally (Spielman *et al.*, 1991; Drenth *et al.*, 1993; Sujkowski *et al.*, 1994; Day & Shattock, 1997). Even though the USA is currently dominated by new genotypes, between 1994 and 1996 8% of isolates collected were found to be of the US-1 clonal lineage, a level much higher than that in Europe where displacement of the old population occurred quickly and almost completely.

The impact of new populations of *P. infestans* on the nature of late-blight has been varied. One striking change has come with the emergence of phenylamide resistance within a few years of the first record of the new populations (Davidse *et al.,* 1981, 1989; Cooke, 1991; Bradshaw & Vaughn, 1996). Resistance to these fungicides is possibly a direct result of variability within the migrant isolates of *P. infestans* (O'Sullivan *et al.*, 1995).

The appearance of the A2 mating type along with an increased number of variant isolates in the UK and many other countries opens the possibility for the generation of a highly plastic population capable of adapting to environmental changes and the establishment of a sexually reproducing population outside of Mexico for the first time (Spielman *et al.*, 1991; Fry *et al.*, 1993). The exact implications of this are still not fully understood, but studies have been undertaken to elucidate any consequences of such marked changes in the structure of populations outside of Mexico.

Since the spread of the A2 mating type across the world, it has been shown that oospores of *P. infestans* can form in different plant parts. Oospores have been shown to form in leaf disks, leaflets (Drenth *et al.*, 1995) and stems (Frinking *et al.*, 1987; Drenth *et al.*, 1995) of potato when A1 and A2 isolates are applied in a

mixed sporangial suspension. In addition oospores have also been found to form naturally in the field in potato crops (Gallegly & Galindo, 1958; Grinberger *et al.*, 1989; Dagget *et al.*, 1993; Drenth *et al.*, 1994; Chycoski & Punja, 1996).

Many environmental factors have been shown to exert an influence on oospore production *in vitro* and *in planta* in species of *Phytophthora*, influencing both the numbers of oospores produced and their viability.

In *P. infestans* the optimal temperature for sexual reproduction has been shown to be the same as that suitable for vegetative growth both *in vitro* (Shaw, 1987) and *in planta* (Drenth *et al.*, 1995; Cohen *et al.*, 1997). Temperature pre-treatments of oospores prior to germination also plays an important role in determining the rate at which oospores will germinate, with differing responses seen in different species of *Phytophthora*. Pre-treatments ranging from 25°C to 33°C have been shown to increase the rate of oospore germination in *P. drechsleri* (Klisiewicz, 1970), *P. megasperma* (El-Hamalawi, 1986) and *P. infestans* (Duncan, 1977). Fay and Fry (1997) documented that oospores did not germinate after 2h treatments at or above 46°C, or after 12h treatments at 40°C. In *P. drechsleri* (Klisiewicz, 1970), *P. cactorum* (Blackwell, 1943) and *P. infestans* (Romero & Erwin, 1969) pre-treatments of 1-5°C have increased the rate of oospore germination, but a temperature of –80°C suppressed oospore formation in *P. infestans* (Fay & Fry, 1997).

In addition to *in vitro* studies, the effect of soil temperature on oospore germination has been investigated. The percentage germination of oospores of *P. porri* was severely reduced after incubation for 5h at 45°C, and totally absent after 5h at 55°C in soil in the Netherlands (Smilde *et al.*, 1996). It was also found that soil solarization for six weeks during May and June 1992 raised the soil temperature at 5 cm depth above 45°C for as long as 17h. This was not shown to reduce the initial level of disease in a leek trial planted in soil infested with *P. porri* oospores (Smilde *et al.*, 1996). Drenth *et al.* (1995) recorded that oospores

of *P. infestans* from soil exposed to temperatures of 40°C or higher were not infectious, but oospores survived exposure to soil temperatures of between 35°C and -80°C for 48h.

The optimal incubation temperature for oospore germination *in vitro* has been investigated in some *Phytophthora* species. For example, Hord and Ristaino (1991) showed that oospores of *P. capsici* germinated *in vitro* at an optimal temperature of 24°C. Oospores of *P. megasperma* have been found to germinate most rapidly at 27°C (Erwin & McCormick, 1971; Schechter & Gray, 1987). Smoot *et al.* (1958) found germination of oospores of *P. infestans* germinated at temperatures ranging from 12°C to 25°C. The optimal temperature for germination of oospores of *P. infestans* is within this range, but germination is inconsistent and a conclusive optimal temperature has not been established and may be different for oospores from matings involving different parents (Shattock *et al.*, 1986; Pittis & Shattock, 1994; Al-Kherb *et al.*, 1995).

It has been demonstrated that light is important in the formation of oospores of *Phytophthora* species. Harnish (1965) and Brasier (1969) both found that light inhibited the formation of oospores in species of *Phytophthora* whilst stimulating sporangia formation. Different species of *Phytophthora* are affected by the presence of light in different ways.

Germination of oospores of *P. megasperma* (Schechter & Gray, 1987) and *P. nicotianae* (Ann & Ko, 1988) has been found to be adversely affected by the presence of light. Oospores of *P. cactorum* (Shaw, 1967) exhibited an opposite response to light with germination being stimulated. Similar results have been found in *P. infestans* (Chang & Ko, 1991) where a 70% germination rate has been achieved after 20 day incubation under lights.

Oospores of *P. infestans* are able to germinate on water agar, but the rate of germination is increased by the addition of nutrients (Smoot *et al.,* 1958; Al-Kherb

et al., 1995). Smoot *et al.* (1958) used several nutrient supplements to enhance the rate of oospore germination in *P. infestans*: horse dung infusion, a mixture of nine vitamins and potato leachate. Not all nutrient supplements have a positive effect on oospore germination. Glucose, fructose and sucrose have all been shown to have an inhibitory effect on oospore germination in *P. cactorum* and *P. megasperma* (Weste, 1983).

The observed level of germination of oospores of *P. infestans* has been shown to vary between different crosses. Pittis and Shattock (1994) found that the mean number of oospores formed in different crosses ranged from 586 to 7274 per 4-mm diameter agar plug. The highest level of germination achieved was 13.4%; one cross examined failed to produce oospores completely. Fay and Fry (1997) found that oospores produced from matings between US strains of *P. infestans* varied in the percentage germination. Of 35 crosses only 3 produced oospores with sufficient germination for the intended assay.

Longevity studies with oospores of *Phytophthora* species have been conducted and revealed that oospores of *P. cactorum* survived for a year in soil (Malajczuk, 1983). In Mexico it has been shown that oospores of *P. infestans* can survive in the soil for up to three years (Perches & Galindo, 1969). In Europe it has been demonstrated that oospores produced *in vitro* and then buried can survive for at least 8 months (Pittis & Shattock, 1994). Drenth *et al.* (1995) were able to show that oospores of *P. infestans* produced *in planta* can overwinter in the soil and then infect healthy potato plants in the following season.

Even though oospores have been detected in the field outside of Mexico, and have been shown to be capable of over-wintering when produced in controlled conditions, the likelihood of the full sexual life-cycle occurring naturally in commercial fields and private allotments has not been full assessed. Nevertheless, with the appearance of the A2 mating type in Europe and North America the effects of sexual reproduction on population structure have been investigated.

Drenth *et al.* (1993) used the RG57 probe to study genotypic diversity in 153 isolates of *P. infestans* from the Netherlands. This revealed 35 different genotypes. Previously only 17 genotypes were identified from 205 isolates using allozymes, mating type and metalaxyl resistance as markers (Fry *et al.*, 1991).

In most locations in the US, populations appear to be clonal. However, Goodwin *et al.* (1995) detected the presence of A1 and A2 mating type in a single field in British Columbia along with several unique genotypes. Further to this, A1 and A2 isolates have been found in the USA, in New York State, Texas and California (Goodwin *et al.*, 1998).

With the introduction of a finite number of clones through migration, and subsequent in-depth characterisation of populations, the appearance of large numbers of new genotypes can have only occurred through two mechanisms. It is possible that new undetected migrations into Europe and North America have occurred, through movement across borders of diseased tubers. Alternatively, it is possible, and indeed likely in some regions that sexual recombination is occuring and generating new genotypes. Evidence in support of this hypothesis has been discovered in the USA, where several new genotypes have been detected (US-12, US-13, US-16 and US-17), and where DNA fingerprint patterns may have arisen through sexual recombination between existing clones in the USA (Goodwin *et al.*, 1998).

One major obstacle to the successful establishment of a sexually reproducing population of *P. infestans* may be the routine application of fungicides to crops to reduce the level of disease. This will have the effect of reducing the chance of A1 and A2 isolates coming into contact, so the likelihood of recombination occurring will be reduced.

Unlike some other crops, including wheat and barley, potato varieties such as Maris Piper, developed decades ago are still popular with consumers. Indeed, many large crisp and chip manufactures use few cultivars (e.g. Pentland Dell and Russet Burbank) in the production of particular brands. This results in the maintenance of a reliable market for these cultivars that often have little resistance to late-blight (Anon, 1995). Such cultivars often return a higher profit to growers, as long as the crop remains disease free, so there is not a great incentive to develop new, more blight resistant cultivars when much of the market is dominated by established cultivars. It appears that these cultivars will continue to be planted for the foreseeable future as long as the market remains constant. New technologies, such as the production of transgenic crops, may facilitate the incorporation of durable resistance into the genomes of popular cultivars without altering the characteristics favoured by consumers. Until then the main method for controlling late-blight in main crop potatoes will remain the regular application of a variety of fungicides throughout the growing season and the treatment of seed potatoes.

A typical spray program to protect against late-blight in a potato crop involves a first application of a protectant fungicide such as a dithiocarbamate (e.g. zineb, maneb, and mancozeb), phthalimide (e.g. captan, folpet and captafol) or a novel compound such as chlorothalonil as the potato canopy begins to close, before any disease symptoms are apparent. This is applied on a 3-14 day interval, (at a rates of 1.2-2kg a.i/ha for dithiocarbamates and 200g a.i./ha for phthalimides) depending on the compound used, formulation and disease pressure, with the total number of applications unrestricted (Parry, 1990; Schwinn & Margot, 1991; Egan *et al.*, 1995). The dithiocarbamates have very limited activity and need to be re-applied on a regular basis. These fungicides only inhibit the initial stages of the infection cycle (zoospore release and germination) and are unable to penetrate foliar tissue, so are redundant once infection occurs (Schwinn & Margot, 1991). As a consequence of this, protectants only serve to delay the time when 75% of the foliage is diseased and offer no protection to tubers (Parry, 1990).

An alternative to the use of protectant fungicides to prevent late-blight affecting a potato crop is to use systemic fungicides, which may additionally offer some curative activity. Cymoxanil, a cyanoacetamide-oxime, was the first systemic fungicide to be introduced for use against *P. infestans*. Although this compound is classed as a systemic fungicide it only has good local systemicity with poor apoplastic systemicity and no symplastic systemicity (Schwinn & Staub, 1995). Cymoxanil has short persistence within the plant, restricting its use and so is sold in mixture with residual fungicides. Despite these drawbacks, cymoxanil is commonly used for late-blight control on potatoes, tomatoes and on grapes for the control of downy mildew, mainly because it has been shown to have some curative properties. Cymoxanil can prevent disease development when applied two to three days after artificial inoculation of field-grown potato or tomato plants, but cannot retard established disease (Douchet *et al.*, 1977; Klöpping & Delp, 1980).

Fosetyl-aluminium was introduced in 1977. This compound has good systemic properties and is active against a range of *Phytophthora* species, but its activity against *P. infestans* is somewhat lower (Diriwachter *et al.*, 1987; Anon, 1998). However, there seems to be a great amount of variation in efficiency of the product between different isolates and superior response in older leaves compared to younger ones (Bashan *et al.*, 1990). Nevertheless, it has been reported that fosetyl-aluminium is successfully used in some regions, when applied at 7-11 day intervals (Schwinn & Margot, 1991).

The most important group of systemic fungicides used during the 1980s was the phenylamides, which include metalaxyl, ofurace, benalaxyl and oxadixil. These compounds have characteristically excellent local and apoplastic systemicity, whilst symplastic systemicity is poor (Schwinn & Staub, 1995). Metalaxyl, the most widely used phenylamide has an *in vitro* effect on mycelial growth of ED_{50} between 0.1 and 0.5µg/mL, with sporulation affected at lower concentrations than this (Bruck *et al.*, 1980). Metalaxyl applications of 200-250 g a.i./ha give a protective period of up to 14 days (Bruck *et al.*, 1980; Parry, 1990), but may be as

long as 21 days when applied in mixture with the dithiocarbamate fungicide mancozeb (Cooke *et al.*, 1981).

Initially, metalaxyl was used alone without an application partner on potatoes, but resistance problems quickly arose, and occurred first in the Netherlands and Ireland only two years after commercial introduction (Davidse *et al.*, 1981; Dowley & O'Sullivan, 1981). Subsequently metalaxyl resistant isolates were discovered in the UK (Carter *et al.*, 1982; Holmes & Channon, 1984), Israel (Cohen & Reuveni, 1983) and in the USA and Canada (Deahl *et al.*, 1993a; Deahl *et al.,* 1993b). Since the early occurrence of metalaxyl insensitivity the sale of these compounds in the UK and Ireland has been restricted to formulations containing protectant fungicides such as mancozeb (Fubol 75 WP) in the attempt to prevent further resistance problems (Dowley *et al.*, 1995; Egan *et al.*, 1995).

The carbamate fungicide prothiocarb was the first systemic fungicide to be developed to combat diseases caused by oomycetous pathogens (Sijpesteijn *et al.*, 1974). Initially only used as soil a drench, its potential use as a foliar protectant against late-blight was overlooked. This was because of poor activity against these pathogens *in vitro*. However, it has been established that the activity of carbamate fungicides *in vitro* is highly dependant on pH, working optimally at neutral pH (Sijpesteijn *et al.*, 1974; Guest, 1984).

Prothiocarb was phased out after the discovery of its oxygen analogue, propamocarbHCl that was found to have superior anti-fungal activity (Papavizas *et al.*, 1977), and did not exhibit the fetid odour of the related sulphur-containing prothiocarb. PropamocarbHCl is systemic, with good local and apoplastic systemicity in potato (Harris, 1996).

The use of propamocarbHCl was initially overshadowed by the phenylamides. PropamocarbHCL was shown to be less effective in controlling root rot of rhododendron caused by *P. cinnamomi* than the phenylamides when applied as a

soil drench (Englander *et al.*, 1980). The importance of propamocarbHCl only became apparent when resistance to metalaxyl began to appear, and propamocarbHCl was one of a few non-phenylamide systemic fungicides then available for use on potatoes. For this reason propamocarbHCl was specifically used in fields where metalaxyl resistance occurred (Samoucha & Cohen, 1990). Although propamocarbHCl is less effective than some other fungicides against several oomycete diseases (Englander *et al.*, 1980; Hunger & Horner, 1982) and requires application at high rates to achieve control (Samoucha & Cohen, 1990), it has been found that when mixed with mancozeb it gave better control of late-blight than mancozeb alone (Cooke *et al.*, 1981, Samoucha & Cohen, 1990). Synergistic effects have also been recorded in the control of *Py. aphanidermatum* on ryegrass, where propamocarbHCl/mancozeb and propamocarbHCl/fosetyl-aluminium combinations have been found to be a more effective control than the application of the individual fungicides at recommended rates (Couch & Smith, 1991).

Carbamate fungicides have been shown to have activity against a range of related pathogenic fungi: *Pseudoperonospora humuli* on hops (Hunger & Horner, 1982), *Pseudoperonospora sativus* on cucumbers (Cohen, 1979), *Peronospora tabacina* on tobacco, and other species of *Peronospora* on many crops including cabbages (Anon, 1995). The carbamates have good activity against *Pythium* blight on bean (Papavizas *et al.*, 1977) and on turf (Anon, 1995), *Py. aphanidermatum* causing foot rot in many vegetables; *Phytophthora* species causing root and stem rots of vegetable crops, strawberries and ornamentals and *P. nicotianae* causing black shank of tobacco (Anon, 1995). Activity against *P. infestans* on potato and tomato (Diriwachter *et al.*, 1987; Samoucha & Cohen, 1990; Anon, 1995) is also recorded. PropamocarbHCl and prothiocarb have no activity against *Plasmopara viticola* on vines (Anon, 1995). Vines are economically important and often the performance of a fungicide on this crop for disease control can influence whether a compound is commercially viable and therefore made available for use against other diseases such as late-blight of potato and tomato.

PropamocarbHCl is currently available in two formulations for the use on potato crops; as Tattoo (248g/L propamocarbHCl, 301.6g/L mancozeb; applied at 4L/ha at 10-14 day intervals) and Tattoo C (375g/L propamocarbHCl, 375g/L chlorothalonil; applied at 2.7L/ha at 7-14 day intervals).

Initially it was claimed that metalaxyl resistant isolates were also tolerant to propamocarbHCl (Cohen & Samoucha, 1984), but this was later disproved, with metalaxyl-resistant and sensitive isolates showing no difference in overall sensitivity to propamocarbHCl (Samoucha & Cohen, 1990; Bardsley *et al.*, 1996).

The mechanism by which propamocarbHCl inhibits the proliferation of late-blight is not fully understood although it effects are well documented. PropamocarbHCl is fungisatic with activity against mycelial growth *in vitro* (Papavizas *et al.*, 1978) and exhibits a strong inhibitory affect on the sporulation of *P. infestans* (Reiter, 1994). Reiter (1994) hypothesised that propamocarbHCl affects the lipid synthesis of *P. infestans* and also reduces the amount of phospholipid in the organism by 40%. Other workers have shown that sterols may have an important role in the mode of action of propamocarbHCl against oomycetes. Papavizas *et al.*, (1978) found that propamocarbHCl impaired membrane permeability and caused leakage of cellular constituents from the mycelium of eight species of *Pythium*, but this was reversed by the addition of sterols to the growth medium. Furthermore, Papavizas *et al.* (1978) proposed that the reversal of the effects of propamocarbHCl by the addition of sterols may be as a result of the formation of a propamocarbHCl-sterol complex, essentially blocking the effect of propamocarbHCl treatments. It has been suggested that the mode of action of carbamate fungicides may differ between different pythiaceous fungi (Papavizas *et al.*, 1978), possibly accounting for the different hypotheses put forward for the mode of action of propamocarbHCl.

The 1990s has seen the addition of a new and potentially important group of systemic fungicides to those already available to growers to restrict damage to a potato crop by late-blight.

The strobilurins are a new class of commercial fungicide derived from anti-fungal compounds present in the fungi *Strobilurus tenacellus* and *Oudemansiella mucida* the activity of which was first noted in the 1960s and 1970s (Anke *et al.*, 1977). Strobilurins are systemic, but unusually they diffuse through the leaf to reach vascular tissue in the gaseous phase, moving acropetally through the plant (Anon, 1996).

To date three strobilurin fungicides have been developed. They are unusual in their broad range of target fungi, capable of controlling economically important pathogens from the true and pseudo-fungi (Godwin *et al.*, 1992; Anon, 1996). Despite such a broad target range azoxystrobin, first sold in 1996 (Knight *et al.*, 1997), is the only strobilurin that has an application in the control of late-blight, and initially only on tomatoes (Anon, 1998).

Towards the end of a potato-growing season it is common practice to apply protectant fungicides, in particular organotin products such as fentin acetate and fentin hydroxide (Parry, 1990). As well as offering effective protection to the foliage of the potato plant, organotin fungicides provide protection to tubers before lifting, resulting in a yield higher than that offered by dithiocarbamates (Jarvis *et al.*, 1967). These fungicides are solely residual, acting by creating a layer of toxicant in the soil through which *P. infestans* cannot penetrate. The activity is due to the toxicity of tin, inhibiting oxidative phosphorylation, but the organic portion of the molecule may also play a part in the fungicidal effects (Manners, 1992). Like other heavy metal-based fungicides such as Bordeaux mixture and copper oxychloride, organotin compounds are phytotoxic, but this is less problematic with the organotin compounds. However, it is enough of a factor to result in their restricted use as end-of-season protectants (Egan *et al.*, 1995). The

use of organotin fungicides contributes to the observed increase in yield compared with other fungicides based on heavy metals (Jarvis *et al.*, 1967; Manners, 1992).

The overall aim of these studies was to investigate the role that oospores play in the population dynamics of late-blight. It is important to establish whether oospores can form *in planta* under semi-natural conditions. The first study undertaken here examined this question. Until this time it has not been demonstrated whether oospores can form *in planta* when parental isolates have independent origins and also retain their infection potential until the following season. Other workers, as described above, have investigated the role of fungicides in the control of oospore formation but this has not been conducted in pot-grown plants or in the field. It was the aim of this work to establish whether the fungicide propamocarbHCl has an influence on oospore formation in plant tissue and further, on the viability and capacity of oospores to over-winter. Finally, it was an objective of these studies to determine the capacity of sexual recombination to generate diversity in isolate sensitivity to propamocarbHCl. It is important to determine the effect that a sexually reproducing population may have on the sensitivity of *P. infestans* to commercially available fungicides in order to avoid future resistance problems.

Chapter 2

Materials and Methods

General procedures

All isolates used in these experiments were maintained on rye A agar (see Appendix 1) at 18°C in the dark until required.

Table 2.1 Isolates used in experimentation, showing origin, mating type and metalaxyl sensitivity.

Isolate	Origin	Mating type[a]	Metalaxyl sensitivity[a]
95.157.2	Cambridgeshire, Maris Piper tuber	A1	resistant
95.183	Suffolk, King Edward leaf	A1	intermediate
95.194	Dyfed, potato leaf -cultivar unknown	A1	sensitive
IR12.94	Ireland, unknown	A2	sensitive
85.150	Suffolk, Maris Piper tuber	A2	resistant
87.205.4	Cornwall, Pentland Hawk leaf	A2	resistant

[a]see below for method for determination of mating type and metalaxyl sensitivity in *P. infestans*.

Determination of mating type

A 5mm mycelia/agar square from an established culture of the test isolate was placed between 5mm squares from cultures of known A1 (95.194) and A2 (87.205.4) mating types (approximately 20mm away from each) on rye A agar and incubated at 18°C in the dark. Generally four mating type tests were conducted in

a single 9cm diameter Petri dish [strips of agar were cut out to prevent cross contamination between tests]. After 7 days, the zone of mycelial interaction between unknown and tester isolates was examined microscopically and the mating type determined on the basis that oospores are only produced with interaction between isolates of opposite mating type (Smoot *et al.*, 1958; Shattock *et al.*, 1990; Whittaker *et al.*, 1991; Whittaker *et al.*, 1994).

Metalaxyl sensitivity

Sensitivity of isolates to metalaxyl was tested *in vitro* using the method of Shattock *et al.* (1990). Isolates were grown on rye A agar supplemented with 10μg mL^{-1} technical grade metalaxyl or on unamended rye A agar in 9cm Petri dishes as a control. Three unknown isolates were tested in each dish as well as a metalaxyl-insensitive control (AGR1). After 7 days the diameter of the colonies was measured and growth on the metalaxyl-amended plates compared with that on the control plates. Isolates that showed 60% or more growth on metalaxyl-amended plates than on the control plate were scored insensitive. Amended plates were scored as sensitive if they showed growth less than 5% of the control plate. Isolates grown on amended plates that exhibited between 5% and 60% of the growth of controls were scored as intermediate.

Production of oospores on agar

Oospores were produced *in vitro* by mating the A1 and A2 isolates on rye A agar. Squares (5mm) were cut aseptically from established cultures of the A1 and A2 isolates, placed approximately 5cm apart on 9cm Petri dishes [containing rye A agar] and incubated at 18°C in the dark. After one week the first oospore formation was observed. Oospores were harvested after a further two weeks. These were extracted from the rye A agar, germinated and used in the propamocarbHCl tests.

Extraction of oospores from rye agar

Pieces of agar containing oospores were cut out using a flame-sterilised scalpel and transferred to a sterile glass homogeniser (BDH Merk Ltd., Lutterworth, LE17 4XL, UK.) containing 2-3mL of sterile deionized water (SDW). The contents were ground until completely homogenised and filtered through a sterile 20µm-pore nylon membrane to remove excess agar. The residue, containing oospores, was then resuspended in 10mL SDW containing 0.03 NovoZym 234 (Novo Nordic Bioindustries UK. Ltd., Solihull, B91 3DA, UK.) or Lysing Enzymes (from *Trichoderma harzianum*, Sigma, Poole, BH17 7NH, UK) and incubated at 18°C in the dark for 24-36h. The oospore suspension was re-filtered through a 20µm pore nylon membrane to remove the enzymes and washed with approximately 100mL SDW. The retrieved oospores were finally resuspended in SDW and the concentration adjusted to 2 x 10^3 oospores per mL (Whittaker *et al.*, 1991; Pittis and Shattock, 1994).

Germination of oospores

The oospore suspension (2 x 10^3 per mL) was spread on water agar (approximately 1mL per plate), sealed with Parafilm (BDH Merk Ltd.) and incubated at 18°C under lights (16h photoperiod). Each plate was examined every 2-3 days for oospore germination (using a light microscope). Any germinating oospores were cut out with a flame-sterilised scalpel (along with approximately 1mm^2 water agar) and transferred to rye A agar containing 25% of the standard amount of antibiotics (see Appendix 1). When a distinct colony of *P. infestans* could be seen growing into the fresh agar it was sub-cultured and maintained on antibiotic amended rye A agar until required (Whittaker *et al.*, 1991; Pittis and Shattock, 1994).

Potato leaf production

Potato plants for production of leaves were grown in a glasshouse in John Innes no. 1 compost, planted in 5L pots, approximately 10cm deep, and fertilised with 1:1:1 N:P:K (Vitax Ltd, Skelmersdale, WN8 8EF) on a weekly basis. Watering was done using a capillary-mat system. The glasshouse was maintained at a minimum 16°C with mean temperatures of 17-18°C in the winter and 19-21°C in the summer, reaching as high as 29°C on some days; 400-W high-pressure sodium lamps were used to supplement the natural light (16h photoperiod). Leaves used for experiments were harvested at 8 weeks. For the passageing of isolates before experiments the age of the leaves used was not monitored.

Passageing of isolates

Before all experiments involving growth of *P. infestans* on plant material, isolates to be used were first passaged through leaves of potato (cv. Home Guard). Established plates of *P. infestans* were flooded with deionized water to detach sporangia from sporangiophores. This suspension was then transferred to a McCartney bottle and chilled at 10°C for 3-4h to release zoospores. Potato leaves were then spot inoculated with the sporangia/zoospore suspension and damp-incubated in a plastic salad box (Ashwood Plastics Ltd., London, E14 0LN, UK.) at 18°C with a 16h photoperiod for 7 days. Sporangia were washed off the leaves with deionized water ready for experimental use.

Growth and preparation of mycelium for DNA extractions

Mycelium for DNA extraction was grown in pea broth (see Appendix 1) at 18°C in the dark for 10-12 days. The mycelium was harvested by washing in deionized water and filtering through a Buchner funnel [a vacuum line was attached to draw media away from the mycelium] containing a 15cm piece of filter paper (Whatman International Ltd., Maidstone, ME16 0LS, UK.). The mycelium was then placed in a freeze-drier (Modulyo, Edwards High Vacuum International,

Crawley, RH10 2LW, UK.) for up to 48h. When dry, the mycelium was vortexed in a plastic tube containing metal ball bearings for up to 5 min, until a fine powder was produced.

DNA extraction

A modified version of that of Raeder and Broda (1985) was used: 3.3mL sterile DNA extraction buffer was added to each centrifuge tube containing ground mycelium, mixed to give a smooth slurry and incubated at 55°C for 10-15 min. to assist re-suspension. Phenol (2.2mL) was then added, mixed and 1mL chloroform/isoamyl alcohol (24:1) added. This was centrifuged at 24,000g for 60 min. at 4°C after which the supernatant was removed and pipetted into a fresh tube. Thirty µL RNAase A (10mg/mL; Promega UK Ltd, Southampton, SO16 7NS, UK.) was added to the supernatant and this was incubated at 37°C for 15 min. Chloroform/isoamyl (3.3mL) alcohol was added, mixed and then centrifuged at 10,000 rpm for 10 min. at 4°C. The supernatant was removed and placed in a fresh tube and centrifuged again at 10,000 rpm for 10 min. The supernatant was again removed and placed in a fresh tube to which 0.54 volumes of 100% isopropanol was added and mixed gently until strands of DNA could be seen precipitating. The DNA suspension was then incubated at room temperature for up to 60 min.

The precipitated DNA was centrifuged at 10,000 rpm for 5 min. at 4°C and the supernatant discarded. Three mL of ethanol (70% v/v) was added to the remaining pellet, mixed and incubated for 5 min. at room temperature then centrifuged at 10,000 rpm for a further 5 min. at 4°C. The supernatant was discarded and the tubes inverted to drain excess liquid and then dried briefly (until the remaining ethanol had evaporated) at approximately 50°C. The DNA pellet was finally

resuspended in 100µL TE buffer [10mM Tris HCl, 1 mM EDTA; adjusted to pH 8.0 with either HCl or NaOH] and stored at -20°C.

Determination of DNA concentration

DNA concentration was determined using a Vikron spectrophotometer. An aliquot (10µL) of DNA was added to 500µL TE buffer in a glass cuvette and placed in the spectrophotometer. An OD_{260} reading of 1 is proportional to 50µg/mL of nucleic acid.

DNA fingerprinting

The multilocus fingerprinting probe RG57 (Goodwin *et al.*, 1992; Drenth *et al.*, 1993) was used for characterisation of isolates.

Genomic DNA (8.0µg) was diluted in TE buffer to a maximum volume of 88µL (amount of DNA and TE buffer was dependant on the DNA concentration) in an Eppendorf tube. Two µL *Eco*R1 (Promega UK Ltd.) restriction enzyme and 10µL 10 x reaction buffer were then added, mixed and incubated at 37°C for 18h.

The DNA was precipitated by adding 10µL of 3.0M sodium acetate (pH 5.2) and 2.5 volumes 100% v/v ethanol (-20°C), mixed and incubated at -20°C for 2-3h. The Eppendorf tubes were then centrifuged at 14,000 rpm for 15 min., the supernatant removed and the DNA pellet washed with 50µL of 70% ethanol. The pellet was dried briefly and resuspended in 20µL TE buffer.

Gel electrophoresis

A 200mL 0.8% agarose gel was prepared in 1 x TAE electrophoresis buffer [121.1g Tris, 0.57 mL Glacial acetic acid, 1mL 0.5 M EDTA - pH 8.0, made up to 500mL with distilled water] with 5µL ethidium bromide (EthBr). Four µL

bromophenol blue gel loading buffer was added to the DNA samples, mixed and loaded into the gel wells. The gel was run at 35V for approximately 18h in at least 1.5 L of 1 x TAE electrophoresis buffer (Sambrook & Russell, 2001).

Southern blotting

A Hybond N+ membrane (Amersham Pharmacia Biotech UK., Buckinghamshire, HP7 9LL, UK.) was placed onto the centre of a vacuum blotter unit following manufacturers instructions (Vacugene XL Vacuum System, Pharmacia Biotech, Hertfordshire, AL1 3AW, UK.) using forceps, lightly wetted with deionized water and a polythene mask placed around the membrane to seal the unit. The gel was placed onto the membrane and the unit secured. The vacuum pump was switched on to a pressure of 5.12kg/cm^2 and approximately 10mL blotting buffer No. 1 [0.25 M HCl] poured onto the top of the gel. This was left for approximately 30 min. (ensuring that there was always sufficient blotting buffer No. 1 to cover the surface) until bromophenol blue dye in the loading buffer turned yellow.

While the vacuum was still running, the blotting buffer No. 1 was removed, the gel rinsed with deionized water, and covered with blotting buffer No. 2 [1.5 M NaCl, 0.5 M NaOH]. This was left for approximately 10 min. until the dye returned back to blue. The gel was again rinsed and the process repeated with blotting buffer No. 3 [1 M Tris HCl, 2 M NaCl, adjusted to pH 7.2] for 10 min., rinsed and covered with 20 x SSC. [3 M NaCl, 0.3 M NaCitrate; pH 7.0] 60 min. Any surplus 20 x SSC was removed, the filter rinsed briefly in 50mL 2 x SSC and baked at 120°C for 30 min. The filter was stored at 4°C until required.

Preparation of the RG57 probe

The RG57 (Goodwin *et al.*, 1992) probe was kindly supplied by N. D. Pipe (University of Wales, Bangor). Between 5 and 10µg of the DNA to be labelled was digested in a volume of 25-50µL and run on a 1.4% agarose gel, the required

band cut out using a flame sterilised scalpel and purified using Promega Wizard Preps (Promega, UK Ltd.) in accordance with the manufacturers instructions.

Probe DNA labelling using digoxigenin (DIG) system

Up to 3µg purified DNA (maximum volume 15µL) was denatured by heating at 100°C for 5-10 min. and placed immediately into an ice bath for 5 min. Two µL of hexanucleotide mixture, 2µL dNTP labelling mixture and 1µL Klenow polymerase (Boehringer Mannheim, Lewes, East Sussex. BN7 1LG, UK.) were added while on ice and incubated at 37°C for 20h. The reaction was stopped by adding 2µL 0.2M EDTA, pH 8.0.

The DNA was precipitated by adding 2µL 4M LiCl and 60µL 100% ethanol (pre-chilled to -20°C), vortexed briefly and incubated at -20°C for 2h. The tubes were centrifuge at 14,000g for 15 min., the supernatant removed and the remaining pellet washed with 50µL 70% v/v ethanol. Finally the labelled probe was resuspended in 50µL TE.

RG57 fingerprinting

All of the following steps were conducted in a sealed incubation bag with as many air bubbles removed as possible at each step following the manufacturers instructions (Boehringer Mannheim, Lewes, East Sussex. BN7 1LG, UK.).

Pre-hybridization (50-100mL) solution (see Appendix 2) was warmed to 55°C in a water bath, added to the filter and incubated at 55°C for 60 min., after which the pre-hybridization solution was discarded. The probe was denatured by boiling at

100°C for 5-10 min. and then snap-frozen by placing the Eppendorf tube onto ice. Fifty mL of hybridization solution (see Appendix 2) was pre-warmed to 55°C and approximately 25ng of freshly denatured DNA probe added, poured into the incubation bag and placed in a water bath for 18h at 55°C. The hybridization solution containing probe was removed and the filter washed twice for 15 min. with 250mL hybridization wash buffer No. 1 (see Appendix 2) on an orbital shaker platform at room temperature. 250mL pre-warmed hybridization wash buffer No. 2 (see Appendix 2) was added and incubated for 15 min. at 55°C in a water bath. This was repeated four times with fresh hybridization wash buffer No. 2. This was followed by 250mL antibody wash buffer No. 1 (see Appendix 2) and incubated for 5 min. on an orbital shaker platform. The filter was then blocked by incubating in 100mL antibody incubation buffer (see Appendix 2) for 30 min. once again on an orbital shaker platform.

Freshly diluted antibody conjugate (see Appendix 2) (4µL into 20mL antibody incubation buffer) was added to the filter for 30 min. on an orbital shaker platform. The filter was washed by 2 incubations of 15 min. in 250mL antibody wash buffer No. 1 and equilibrated for 5 min. in 250mL detection buffer (see Appendix 2).

Twenty-five µL of the chemiluminescent substrate CSPD (Disodium 3-(4-methoxyspiro{1,2-dioxetaane-3,2-(5-chloro)tricyclo[3.3.1.1$^{3.7}$]decan}-4-yl)phenyl phosphate) was diluted into 25mL detection buffer and the filter was incubated for 5 min. at room temperature. The CSPD was poured off and the filter (still in the incubation bag) was incubated at 37°C in the dark for 15 min. XOMAT XR X-ray source film (Kodak Limited, Hemel Hempstead, HP1 1JU, UK.) was placed on to the filter, DNA side up and developed after at least 3h.

Development and analysis of X-ray film

After required exposure, the film was transferred to a dark room and placed into Photosol CD18 X-ray developer solution (Photosol Ltd., Basildon, Essex, UK.)

for up to 5 minutes (agitated gently). The film was then placed into the stop solution (water) and left for 1 min. The film was finally placed into fixing solution, (Photosol Ltd.) for 5 min, removed and left to drip-dry.

Fingerprint patterns were analysed when the film was dry. Bands were numbered from 1 (smallest fragment) to 25 (largest fragment) in accordance with other published work (Goodwin *et al.*, 1992; Drenth *et al.*, 1993).

Chapter 3

Introduction

In planta production, survival and germination of oospores of *Phytophthora infestans*

The formation, survival and infection potential of oospores of many members of the order Pythiales has been investigated in detail by numerous workers: *Phytophthora cactorum* (Horner & Wilcox, 1996); *Phytophthora capsici* (Bowers *et al.*, 1983); *Phytophthora fragariae* (Duncan, 1980; Law & Milholland, 1991); *Phytophthora syringae* (Harris, 1985); *Peronospora farinosa* (Frinking *et al.*, 1985); *Peronospora parasitica* (Moss *et al.*, 1994); *Peronospora viciae* (Van der Gagg *et al.*, 1993; Van der Gagg & Frinking, 1997). In particular, the oospores of *Phytophthora* species have been shown to survive for long periods (Malajczuk, 1983; Weste, 1983), but the amount of research undertaken specifically on the survival and production of oospores of *P. infestans* is limited. This was, in the main, due to the confinement of the A2 mating type to Mexico until the late 1970s (Hohl & Iselin, 1984; Malcolmson, 1985; Shaw *et al.*, 1985). The use of the A2 mating type in field experiments was prohibited until this time because of the lack of naturally occurring local strains.

Oospores of *Phytophthora infestans* have been shown to form in leaf disks, leaflets (Drenth *et al.*, 1995) and stems (Drenth *et al.*, 1995; Frinking *et al.*, 1987) of *Solanum tuberosum* when A1 and A2 strains are applied in a mixed sporangial suspension. In addition, oospores have also been found in the field in potato crops (Grinberger *et al.*, 1989). It has been suggested that in order for oospore formation to occur, a constant and free source of water is required and that leaves are incubated at 50-80% relative humidity. Without free water potato plant material is unable to sustain oospore production (Cohen *et al.*, 1997). This conclusion is in

direct conflict with inferences drawn from other work in the same area (Drenth *et al.*, 1995; Hanson & Shattock, 1998a).

Hanson and Shattock (1998a) investigated the formation of oospores of *P. infestans* in cultivars of potato with differing levels of race non-specific resistance to late blight. Assays were conducted in leaf disks, and with polythene-tunnel-based plants and field-grown plants. The conclusion drawn from this work was that maximum numbers of oospores were produced in foliage of cultivars with medium levels of resistance such as cv. Desiree and cv. Record.

The importance of the oospore of *P. infestans* as a means of survival has been examined in the past by Pittis and Shattock (1994) who found that oospores produced *in vitro* and then subjected to a range of temperatures from 0°C to 20°C, remained viable for 6 months. Pittis and Shattock (1994) found that oospores buried in non-sterile soil survived for up to 8 months under temperate field conditions in Gwynedd, Wales, UK. Mayton *et al.* (1998) conducted similar experiments in the USA on laboratory-produced oospores, burying them in the field at a depth of 32cm, monitoring soil temperature and moisture. The soil moisture ranged from 25% to 50% field capacity; soil temperature ranged from 0°C to 10°C. The proportion of viable oospores detected when subjected to these conditions was reduced over time, falling from 49% in November to 29% by May.

Drenth *et al.* (1995) showed that when potato plants were inoculated with a mixture of A1 and A2 strains and the resultant blighted material mixed into soil in pots and left to over-winter, oospores formed and remained infectious for at least 8 months. When soil containing oospores was subsequently exposed to temperatures above 40°C viable oospores could not be recovered, although oospores did survive when exposed to temperatures ranging from -80°C to 35°C for up to 48 h. The survival of oospores under field conditions was also investigated by Drenth *et al.* (1995). Artificially inoculated, blighted leaves and stems of cultivars Bintje, Irene and Pimpernel containing oospores were mixed into heavy clay soil in September

1992. In May 1993 these plots were planted with cultivar Bintje and in July 1993 soil was collected from each plot and assayed to determine infectivity of the soil. A single recombinant strain was detected (verified using DNA fingerprinting). Similarly, lesions that appeared in the newly developing cv. Bintje plants were analysed and a recombinant strain was detected.

The potential for A1 and A2 strains to interact when originating from different infector plants in a plot has not as yet been investigated. This chapter shows that oospores can be formed *in planta* when the parental strains originate from separate locations. Once formed, oospores are able to survive natural degradation of the haulm and maintain their infection potential for 6 months, until the following season.

The development of foliar disease caused by members of the order Pythiales, spread by airborne asexual spores through a crop has been investigated extensively and models developed and refined to describe such progress (Gregory, 1948; Gregory, 1968; Minogue & Fry, 1983a; Ristaino *et al.*, 1994).

Minogue and Fry (1983a) defined the disease gradient (g) as the slope of the logit of disease severity against distance from source. They suggested that plant disease travels through a crop as a wave of an even velocity, as long as conditions remain constant. This hypothesis was supported with data for the spread of late blight through a crop of potatoes (Minogue & Fry, 1983b). It was observed that in the early stages of development the disease gradient remained steep and within 7-10 days of the first sign of disease the disease gradient became much flatter. It is the aim of the experiment described in this chapter to use the analytical methods developed by Minogue and Fry (1983a, 1983b) to ascertain the distribution of both A1 and A2 strains through the experimental plot, chart regions of interaction between the two mating types and record the occurrence of oospores within the plants.

Materials and Methods

Sixty-four potato plants (cv. Home Guard) were planted in John Innes No. 1 compost, in 5L pots approximately 10cm deep and placed in a controlled environment chamber under lights (18°C; 16h photoperiod). The plants were watered daily and fertilised on a weekly basis with 1:1:1 (N:P:K) (Vitax Ltd, Skelmersdale, WN8 8EF, UK.) as required. After 8 weeks the plants were transferred to a polythene tunnel (10m x 4m), arranged in a 16 x 4 layout and grown for a further two weeks, until the canopy closed.

Before the experiment began two parental strains, 95.194 (A1) and 85.150 (A2) were passaged though leaves of potato (cv. Home Guard -commercial suppliers) to restore any vigour and pathogenicity lost in culture on agar (Chapter 2). The sporangial suspension produced after passaging was filtered through muslin and adjusted to 2 x 10^3 sporangia per mL ready to be used in the experiments.

When the plants were 8-weeks-old, two plants, one from each end of the trial were removed and sprayed with a suspension of either 95.194 (A1) (plant 58) or 85.150 (A2) (plant 7) (2 x 10^3 sporangia per mL) of sporangia and zoospores using a hand-held sprayer. These plants were then placed in a metal tray containing approximately 3cm of water and a plastic hood placed over the tray to maintain high humidity. The plants were then incubated in a controlled environment chamber (18°C; 16h photoperiod) until approximately 50% of leaflets exhibited sporulating lesions (approximately 7 days). The infected plants were then returned to their allocated position within the trial on 20 September 1997. Overhead misting was used, controlled by a leaf-wetness indicator to ensure that free water remained on the foliage throughout the experiment. The movement of disease through the crop was recorded daily; the percentage of total foliage diseased of each plant scored using a plant disease assessment key (Anon, 1990). Periodically, throughout the experiment, the door of the polythene tunnel was opened to ensure overheating did not occur. This was done when the temperature inside the

polythene tunnel reached approximately 25°C. The door was situated at the A2-end of the trial, behind pot number four.

Samples of leaf material were taken as the disease progressed throughout the experiment until complete necrosis of the haulm occurred through the whole plot. The number of samples taken was dependant on the severity of disease. Samples were not taken from plants with less than 1% of the total foliage diseased. Samples could not be taken from necrotic plant material. Samples were transferred to rye agar (see Appendix 1) as quickly as possible after collection by taking small fragments of diseased leaf (approximately $2mm^2$) from the edge of the lesion with flame-sterilised forceps. This was then placed onto antibiotic-amended rye A agar (see Appendix 1) and incubated at 18°C in the dark. When contaminant-free colonisation of the agar occurred the strain was transferred onto a fresh plate of antibiotic-amended rye A agar and stored at 18°C in the dark until mating type could be determined. In addition, 24 of these strains were selected from throughout the plot for DNA fingerprinting at a later date. This was to ensure contaminant strains of *P. infestans* had not infected experimental plants.

Occurrence and infectivity of oospores

Leaflets from selected plants were collected to detect any production of oospores within plant material. This was done by choosing leaflets, where two or more lesions appeared to be intermixed, as they developed. A total of 62 leaflets were collected and tested. The leaflets were then liquefied in a glass homogeniser (BDH Merk Ltd., Lutterworth, LE17, 4XL, UK.) with 2-3mL of deionized water, depending on the size of the leaflet. The liquefied product was then examined with a microscope for the presence of any oospores.

After 18 days, following introduction of infected plants and when all the plants were fully necrotic, the pots were removed from the polythene tunnel and placed

outside, placed within individual black plastic bags and left to over-winter. Soil samples containing plant detritus were taken from the pots 26 weeks later in April 1998. Soil from the surface of the pot was selected to increase the chance of collecting oospores.

The presence of viable oospores was assayed using a modified method of Drenth *et al.* (1995). Soil (100g) was collected from each of the pots. Deionized water ($250cm^3$) was added to 100g of the collected soil in a plastic salad box and leaflets of potato (cv. Home Guard) were floated on the surface of the soil slurry (abaxial side touching the slurry). This was incubated at 10°C in the dark to encourage zoospore release from any sporangia that may have formed either from germination of oospores or that remained in the soil from parental sporangia that survived the winter period.

After 24h the leaflets were removed and incubated at 18°C under lights to facilitate development of blight lesions. This was repeated every two days for 4 weeks. Any ensuing lesions were isolated on rye agar as described above. The soil sample was incubated at 18°C under lights when not being baited with leaflets, to assist oospore germination (Whittaker *et al.*, 1991; Pittis & Shattock, 1994). DNA fingerprints were produced for each strain to determine if they were parental types or recombinants.

Data analysis

Data obtained from the experiment was analysed in the form of single pots, individual rows or by distance from the disease sources. Disease progress curves were produced with the data organised in rows. The logit mean level of disease at a particular distance was used to show the disease gradients obtained during the experiment as described by Minogue and Fry (1983a). A distance of 0.35m in the figures is approximately equivalent to 1 pot. The disease gradient was plotted for

each day that late blight was observed in the crop until day 19 when the crop was almost entirely necrotic. Pots of equal distance from the focus were grouped and the mean calculated. Data from eight pots was used to calculate the mean level of disease at a distance of 0.35m; seven pots were used to calculate the mean level of disease at a distance of 0.70m. The mean for all other distances was calculated using data from four pots (1 row). Disease levels of 0 or 1.0 could not be plotted as the logit of these values is irrational.

Regression analysis for the disease progress curves (logit level of disease against distance) was used to calculate the gradient of the curves at different distances from the source and at different times. This allowed the effect of distance from source on the curve to be observed and thus detect any secondary foci that developed or any interference from the opposite mating type in the experiment. The data for distances between 0.35 and 2.10m from the origin were used. Data outside of this range were either impossible to use (when proportion of plant diseased was 0 or 1.0) or offered no additional information.

Results

The incidence of late blight 10 days after the introduction of late-blight-inoculated potato plants into the polythene tunnel is shown in fig. 3.1. This was the first day that leaf tissue was sampled and mating type determined due to the lack of disease on previous days. In pots 26 and 32 disease was estimated at 1%, but mating type was not determined because any sampling may have affected the natural development of disease. At this point the two test strains had spread out from the original infector plants, but disease symptoms were mainly restricted to plants lying close to the infector plants.

Two days later (day 12) extensive sampling was undertaken. The mating type of strains from all diseased plants was determined. Fig. 3.2 shows that the areas immediately surrounding the original foci were dominated by the same mating types but in the centre of the plot, both A1 and A2 strains were detected.

On day 17 of the experiment, blight had reached all of the viable plants within the experiment (Fig. 3.3). The A2 strain was recorded on most of the plants of rows 1 to 8. The A2 strain did not reach beyond plant 45 whereas the A1 strain had spread to almost all of the rows.

The plants from which A1 and A2 strains were identified in a mixed culture *in vitro*, i.e. portions of leaflets with sporulating lesions that were transferred to rye agar produced oospores in the agar is shown in fig. 3.4. Of the 19 plants from which mixed-culture strains were collected, oospores were also detected in the leaves and petioles of 9 plants. From the soil of 3 of these pots (21, 22 and 23) recombinant strains (strains 1-6, shown in Table 3.5) were detected after the winter period using the soil assay method.

The progress of disease through the pot-grown potatoes is illustrated in Fig. 3.5. The data is organised in rows, with row 1 corresponding to plants 1, 2, 3 and 4; row 2 is equivalent to plants 5, 6, 7 and 8 and so on. The incidence of disease was

recorded as the proportion of the plants diseased, the mean of which is plotted against time (days).

In rows 5 and 6 the time taken for a disease level above 0.2 to be reached was the longest observed in the experiment, with disease only surpassing this level at day 16. As well as rows 5 and 6, in rows 1, 4 and rows 7 to 12 inclusive, disease appears reach this level after a much longer period (15 days) than in other rows in the plot. In contrast there was a much flatter disease progress curve in rows 2 and 3 and in rows 13 to 16 with disease reaching this level and then increasing rapidly much earlier than in other rows.

Figs. 3.6 and 3.7. illustrate disease gradients (i.e. the logit level of disease against distance (m)) originating from the A1 and A2 sources respectively. Two main peaks occurred in the curves, both corresponding to points where diseased plants were introduced. In each figure the first peak was at 0m and the second at 4.55m. The region between these two points was the area of interaction between the two foci. The figures show that as time progressed the curves become flatter. At day 15 there was a sharp increase in disease level in this zone between 1.0m and 3.8m from the A1 source (between 1.75 and 3.15 from the A2 source).

The mean level of disease at successive distances from source, with the standard deviation of this mean shown in parenthesis is given in tables 3.1 and 3.2.

Regression analysis for the disease progress curves (logit level of disease against distance plots) is shown in Tables 3.3 and 3.4. In Table 3.3 (day 15) there was a marked change in the gradient of the disease progress curve, with the curve becoming less defined and flatter. A similar effect was seen at day 15 and even more so at day 18 (Table 3.4).

The 24 samples that were taken from the foliage during the experiment were found to have mating type and DNA fingerprints identical to one of the parental strains. Table 3.5 shows the DNA fingerprints for the parental strains and the 6 strains recovered from soil. The fingerprints for the parental strains were identical for 21 out of the 25 bands, only differing at bands 6, 8, 9 and 16. These bands were absent in the A1 strain (95.194) but present in the A2 strain (85.150). The recovered strains were identical to the parental strains for all of the bands that both parents shared with the exception of strains 2 and 6. Band 2 was present in both of the parents, but absent in these two strains. All of the recovered strains have different fingerprint patterns from the those of the parents, with the exception of strain 3, which had the same fingerprint as the A1 parent but was of A2 mating type and sensitive to metalaxyl. Isolates 1 and 4 had identical banding patterns, but differed in their sensitivity to metalaxyl. Only the strains shown in Table 3.5 were recovered suggesting that no sporangia or mycelial fragments survived the winter.

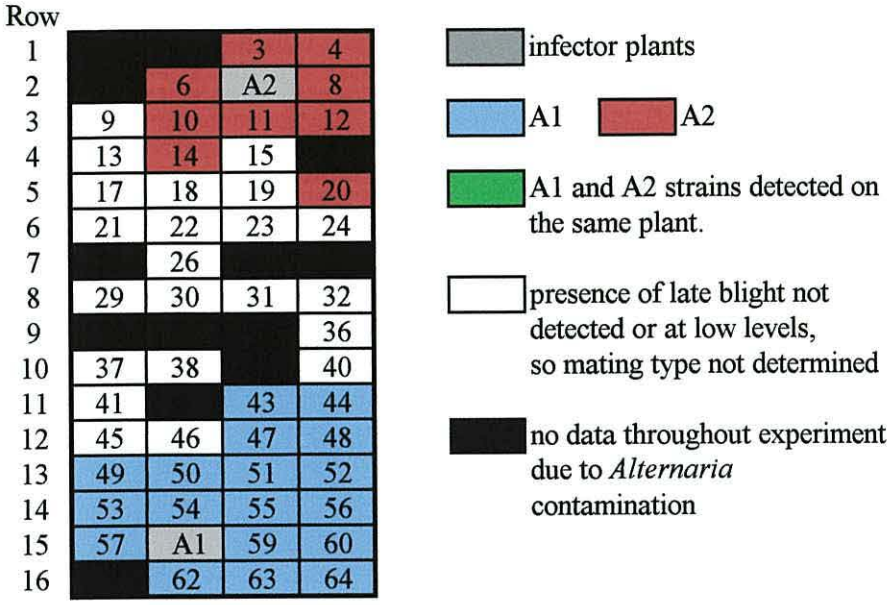


Figure 3.1 Plan of polythene tunnel showing the extent of late-blight through the pot-grown potato plants (cv. Home Guard) 10 days after the introduction of diseased plants.

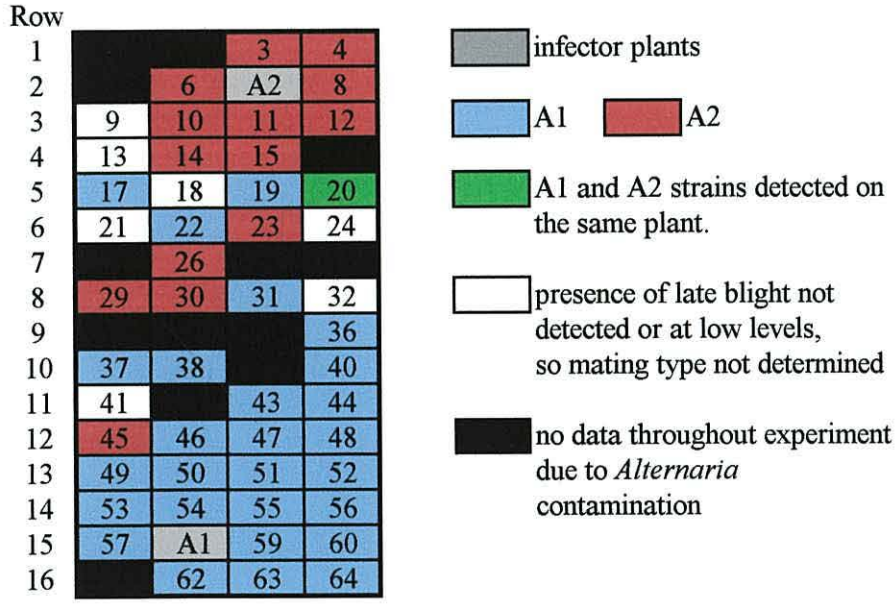


Figure 3.2 Plan of polythene tunnel showing the extent of late-blight through the pot-grown potato plants (cv. Home Guard) 12 days after the introduction of diseased plants.

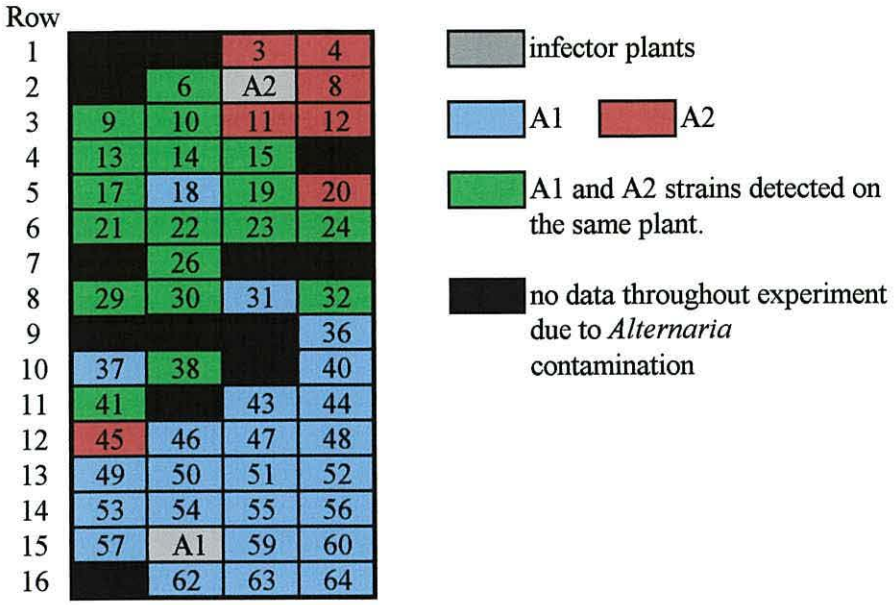


Figure 3.3 Plan of polythene tunnel showing the extent of late-blight through the pot-grown potato plants (cv. Home Guard) 17 days after the introduction of diseased plants.

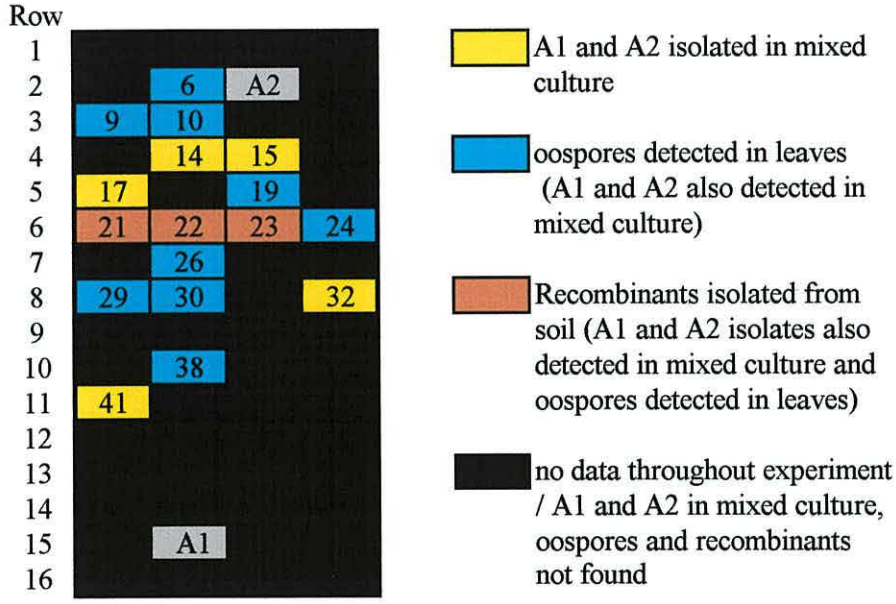


Figure 3.4 Plan of polythene tunnel showing the plants where mixed-culture self-fertile isolates were found, on which of these plants oospores were detected and from which of these pots recombinants were isolated from soil. Data collected throughtout the experiment.

Figure 3.5 Disease progress curve showing the progress of late-blight in each row over time.

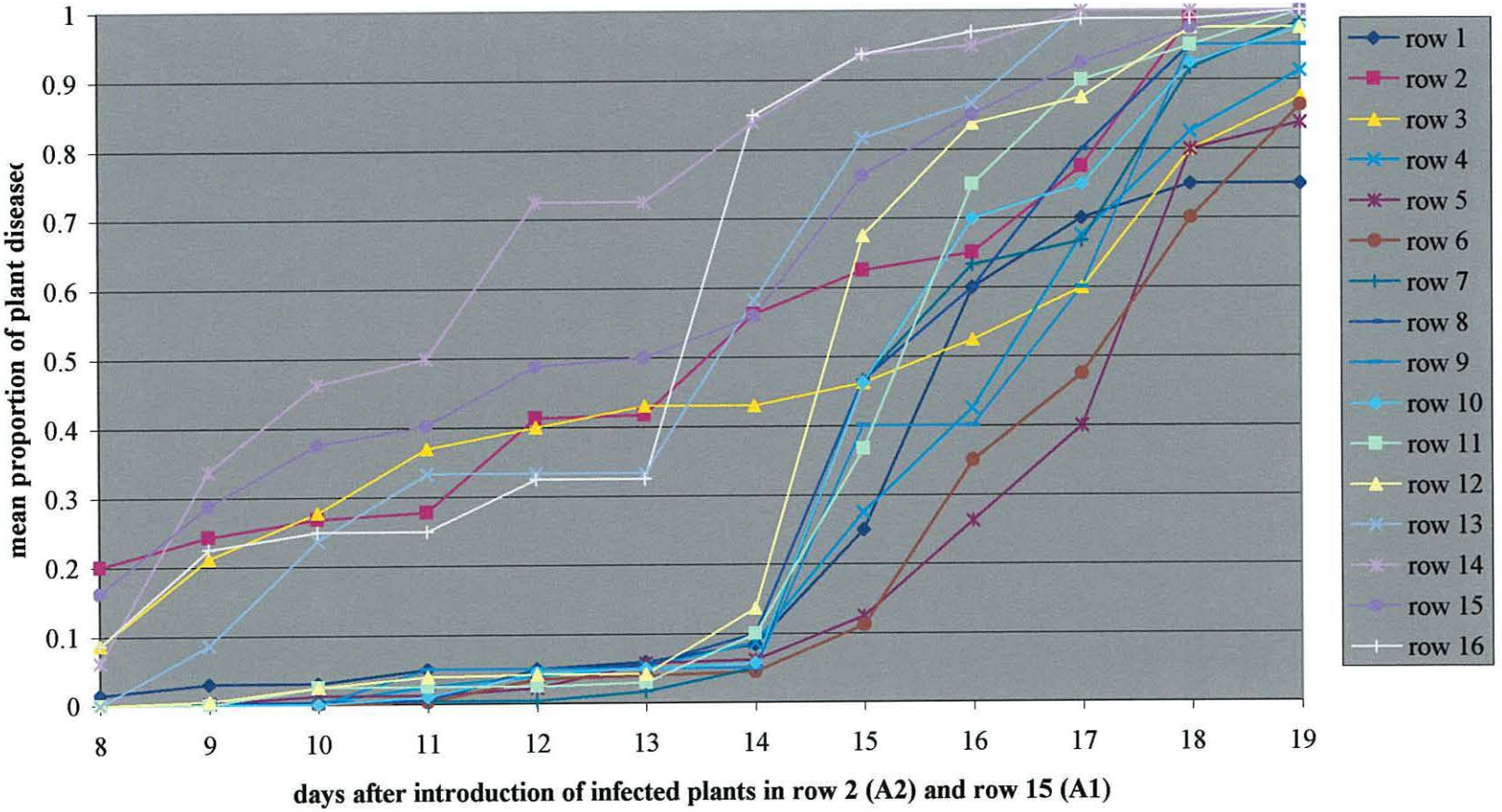


Figure 3.6 Disease level (logit) for days 8 to 19 of the experiment at increasing distance (m) from A1 focus.

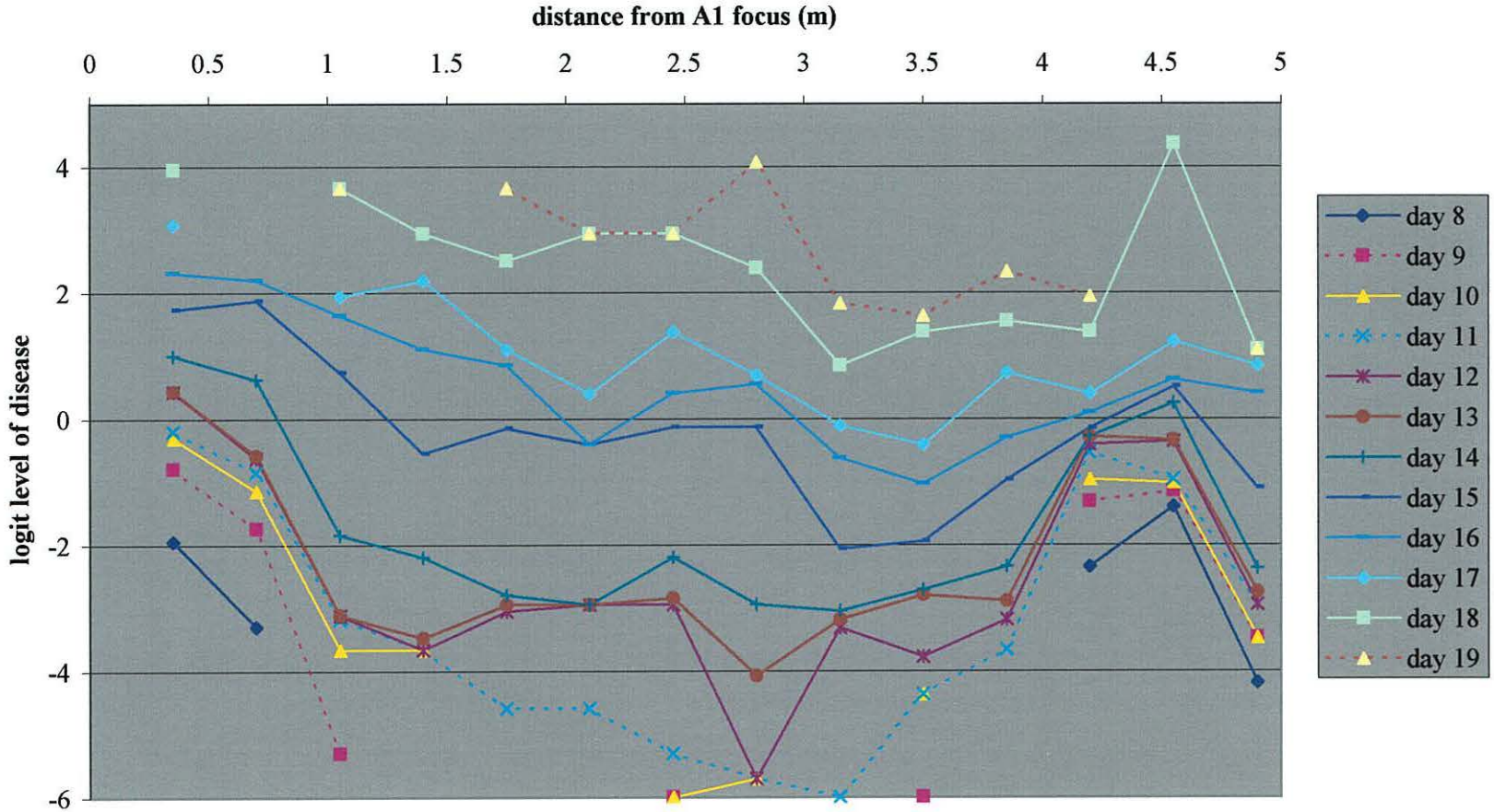


Figure 3.7 Disease level (logit) for days 8 to 19 of the experiment at increasing distance (m) from A2 focus.

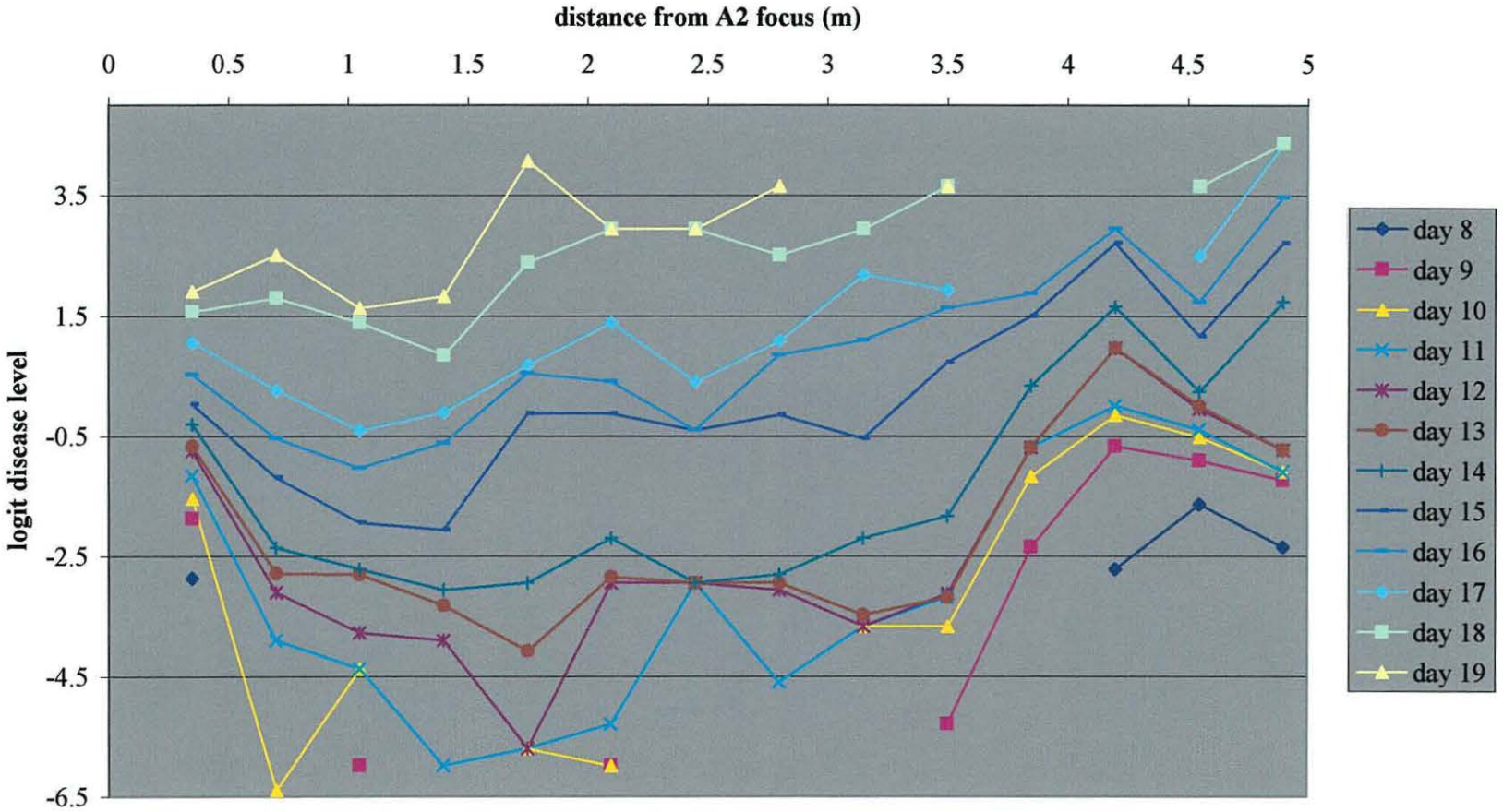


Table 3.1 Mean level of disease at sequential distances from A1 disease focus.

distance from source (m)	Mean proportion of plant diseased /($\sigma^{\dagger}$)																					
	Day 8		Day 9		Day 10		Day 11		Day 12		Day 13		Day 14		Day 15		Day 16		Day 17		Day 18		Day 19	
0.35	**0.13**	(0.13)	**0.31**	(0.26)	**0.43**	(0.29)	**0.45**	(0.28)	**0.61**	(0.32)	**0.61**	(0.32)	**0.73**	(0.33)	**0.85**	(0.31)	**0.91**	(0.21)	**0.96**	(0.11)	**0.98**	(0.04)	**1.00**	(0.00)
0.70	**0.04**	(0.07)	**0.15**	(0.13)	**0.24**	(0.12)	**0.30**	(0.18)	**0.35**	(0.21)	**0.36**	(0.22)	**0.65**	(0.30)	**0.87**	(0.19)	**0.90**	(0.17)	**1.00**	(0.00)	**1.00**	(0.00)	**1.00**	(0.00)
1.05	**0.00**	(0.00)	**0.01**	(0.01)	**0.03**	(0.03)	**0.04**	(0.05)	**0.04**	(0.04)	**0.04**	(0.04)	**0.14**	(0.12)	**0.68**	(0.28)	**0.84**	(0.14)	**0.88**	(0.15)	**0.98**	(0.03)	**0.98**	(0.03)
1.40	**0.00**	(0.00)	**0.00**	(0.00)	**0.03**	(0.03)	**0.03**	(0.03)	**0.03**	(0.03)	**0.03**	(0.04)	**0.10**	(0.10)	**0.37**	(0.15)	**0.75**	(0.23)	**0.90**	(0.10)	**0.95**	(0.05)	**1.00**	(0.00)
1.75	**0.00**	(0.00)	**0.00**	(0.00)	**0.00**	(0.00)	**0.01**	(0.00)	**0.05**	(0.03)	**0.05**	(0.02)	**0.06**	(0.03)	**0.46**	(0.37)	**0.70**	(0.35)	**0.75**	(0.29)	**0.93**	(0.10)	**0.98**	(0.03)
2.10	**0.00**	(0.00)	**0.00**	(0.00)	**0.00**	(0.00)	**0.01**	(0.03)	**0.05**	(0.04)	**0.05**	(0.00)	**0.05**	(0.00)	**0.40**	(0.00)	**0.40**	(0.00)	**0.60**	(0.00)	**0.95**	(0.00)	**0.95**	(0.00)
2.45	**0.00**	(0.00)	**0.00**	(0.01)	**0.00**	(0.01)	**0.01**	(0.01)	**0.05**	(0.00)	**0.06**	(0.01)	**0.10**	(0.00)	**0.47**	(0.46)	**0.60**	(0.35)	**0.80**	(0.20)	**0.95**	(0.05)	**0.95**	(0.05)
2.80	**0.00**	(0.00)	**0.00**	(0.00)	**0.00**	(0.01)	**0.00**	(0.01)	**0.00**	(0.01)	**0.02**	(0.03)	**0.05**	(0.00)	**0.47**	(0.47)	**0.63**	(0.32)	**0.67**	(0.31)	**0.92**	(0.10)	**0.98**	(0.03)
3.15	**0.00**	(0.00)	**0.00**	(0.00)	**0.00**	(0.00)	**0.00**	(0.01)	**0.04**	(0.05)	**0.04**	(0.05)	**0.05**	(0.04)	**0.11**	(0.06)	**0.35**	(0.13)	**0.48**	(0.17)	**0.70**	(0.29)	**0.86**	(0.14)
3.50	**0.00**	(0.00)	**0.00**	(0.01)	**0.01**	(0.03)	**0.01**	(0.03)	**0.02**	(0.03)	**0.06**	(0.03)	**0.06**	(0.03)	**0.13**	(0.06)	**0.26**	(0.11)	**0.40**	(0.14)	**0.80**	(0.14)	**0.84**	(0.11)
3.85	**0.00**	(0.00)	**0.00**	(0.00)	**0.00**	(0.00)	**0.03**	(0.02)	**0.04**	(0.02)	**0.05**	(0.04)	**0.09**	(0.05)	**0.28**	(0.10)	**0.43**	(0.10)	**0.68**	(0.13)	**0.83**	(0.22)	**0.91**	(0.09)
4.20	**0.09**	(0.18)	**0.21**	(0.39)	**0.28**	(0.43)	**0.37**	(0.45)	**0.40**	(0.45)	**0.43**	(0.46)	**0.43**	(0.46)	**0.46**	(0.42)	**0.53**	(0.36)	**0.60**	(0.36)	**0.80**	(0.27)	**0.88**	(0.19)
4.55	**0.20**	(0.40)	**0.24**	(0.47)	**0.27**	(0.49)	**0.28**	(0.48)	**0.41**	(0.44)	**0.42**	(0.44)	**0.56**	(0.51)	**0.63**	(0.43)	**0.65**	(0.41)	**0.78**	(0.29)	**0.99**	(0.02)	**1.00**	(0.00)
4.90	**0.02**	(0.01)	**0.03**	(0.03)	**0.03**	(0.03)	**0.05**	(0.00)	**0.05**	(0.00)	**0.06**	(0.01)	**0.09**	(0.02)	**0.25**	(0.07)	**0.60**	(0.28)	**0.70**	(0.42)	**0.75**	(0.35)	**0.75**	(0.35)

† Standard deviation of mean disease level.

Table 3.2 Mean level of disease at sequential distances from A2 disease focus.

distance from source (m)	Mean proportion of plant diseased /($\sigma^{\dagger}$)																					
	Day 8		Day 9		Day 10		Day 11		Day 12		Day 13		Day 14		Day 15		Day 16		Day 17		Day 18		Day 19	
0.35	**0.05**	(0.14)	**0.13**	(0.32)	**0.18**	(0.36)	**0.24**	(0.37)	**0.32**	(0.39)	**0.34**	(0.39)	**0.42**	(0.47)	**0.51**	(0.40)	**0.63**	(0.34)	**0.74**	(0.32)	**0.83**	(0.29)	**0.87**	(0.23)
0.70	**0.00**	(0.00)	**0.00**	(0.00)	**0.00**	(0.00)	**0.02**	(0.02)	**0.04**	(0.02)	**0.06**	(0.03)	**0.09**	(0.04)	**0.23**	(0.11)	**0.37**	(0.12)	**0.57**	(0.20)	**0.86**	(0.18)	**0.93**	(0.08)
1.05	**0.00**	(0.00)	**0.00**	(0.01)	**0.01**	(0.03)	**0.01**	(0.03)	**0.02**	(0.03)	**0.06**	(0.03)	**0.06**	(0.03)	**0.13**	(0.06)	**0.26**	(0.11)	**0.40**	(0.14)	**0.80**	(0.14)	**0.84**	(0.11)
1.40	**0.00**	(0.00)	**0.00**	(0.00)	**0.00**	(0.00)	**0.00**	(0.01)	**0.02**	(0.05)	**0.04**	(0.05)	**0.05**	(0.04)	**0.11**	(0.06)	**0.35**	(0.13)	**0.48**	(0.17)	**0.70**	(0.29)	**0.86**	(0.14)
1.75	**0.00**	(0.00)	**0.00**	(0.00)	**0.00**	(0.01)	**0.00**	(0.01)	**0.00**	(0.01)	**0.02**	(0.03)	**0.05**	(0.00)	**0.47**	(0.47)	**0.63**	(0.32)	**0.67**	(0.31)	**0.92**	(0.10)	**0.98**	(0.03)
2.10	**0.00**	(0.00)	**0.00**	(0.01)	**0.00**	(0.01)	**0.01**	(0.01)	**0.05**	(0.00)	**0.06**	(0.01)	**0.10**	(0.00)	**0.47**	(0.46)	**0.60**	(0.35)	**0.80**	(0.20)	**0.95**	(0.05)	**0.95**	(0.05)
2.45	**0.00**	(0.00)	**0.00**	(0.00)	**0.00**	(0.00)	**0.05**	(0.03)	**0.05**	(0.04)	**0.05**	(0.00)	**0.05**	(0.00)	**0.40**	(0.00)	**0.40**	(0.00)	**0.60**	(0.00)	**0.95**	(0.00)	**0.95**	(0.00)
2.80	**0.00**	(0.00)	**0.00**	(0.00)	**0.00**	(0.00)	**0.01**	(0.00)	**0.05**	(0.03)	**0.05**	(0.02)	**0.06**	(0.03)	**0.46**	(0.37)	**0.70**	(0.35)	**0.75**	(0.29)	**0.93**	(0.10)	**0.98**	(0.03)
3.15	**0.00**	(0.00)	**0.00**	(0.00)	**0.03**	(0.03)	**0.03**	(0.03)	**0.03**	(0.03)	**0.03**	(0.04)	**0.10**	(0.10)	**0.37**	(0.15)	**0.75**	(0.23)	**0.90**	(0.10)	**0.95**	(0.05)	**1.00**	(0.00)
3.50	**0.00**	(0.00)	**0.01**	(0.01)	**0.03**	(0.03)	**0.04**	(0.05)	**0.04**	(0.04)	**0.04**	(0.04)	**0.14**	(0.12)	**0.68**	(0.28)	**0.84**	(0.14)	**0.88**	(0.15)	**0.98**	(0.03)	**0.98**	(0.03)
3.85	**0.00**	(0.00)	**0.09**	(0.08)	**0.24**	(0.14)	**0.33**	(0.25)	**0.33**	(0.25)	**0.33**	(0.25)	**0.58**	(0.39)	**0.82**	(0.28)	**0.87**	(0.23)	**1.00**	(0.00)	**1.00**	(0.00)	**1.00**	(0.00)
4.20	**0.06**	(0.06)	**0.34**	(0.31)	**0.46**	(0.29)	**0.50**	(0.26)	**0.73**	(0.30)	**0.73**	(0.30)	**0.84**	(0.19)	**0.94**	(0.09)	**0.95**	(0.10)	**1.00**	(0.00)	**1.00**	(0.00)	**1.00**	(0.00)
4.55	**0.16**	(0.18)	**0.29**	(0.27)	**0.38**	(0.33)	**0.40**	(0.32)	**0.49**	(0.36)	**0.50**	(0.36)	**0.56**	(0.38)	**0.76**	(0.44)	**0.85**	(0.30)	**0.93**	(0.15)	**0.98**	(0.05)	**1.00**	(0.00)
4.90	**0.09**	(0.10)	**0.23**	(0.05)	**0.25**	(0.10)	**0.25**	(0.10)	**0.33**	(0.15)	**0.33**	(0.15)	**0.85**	(0.24)	**0.94**	(0.09)	**0.97**	(0.05)	**0.99**	(0.02)	**0.99**	(0.02)	**1.00**	(0.00)

† Standard deviation of mean disease level.

Table 3.3 Gradient of logit disease progress curve at increasing distance (m) from A1 focus.

	distance from A1 focus (m)					
Day	**0.35**	**0.70**	**1.05**	**1.40**	**1.75**	**2.10**
11	-4.26	-3.64	-3.31	-2.75	-2.46	-2.24
12	-5.07	-4.22	-2.86	-2.02	-1.50	-1.82
13	-5.07	-4.07	-2.75	-1.98	-1.46	-1.42
14	-4.04	-3.44	-2.97	-2.47	-1.80	-1.55
15	-1.43	-2.28	-1.77	-1.14	-1.13	-0.88
16	-0.96	-1.20	-1.15	-1.48	-1.19	-0.94

Table 3.4 Gradient of logit disease progress curve at increasing distance (m) from A2 focus.

	distance from A2 focus (m)			
Day	**0.35**	**0.70**	**1.05**	**1.40**
11	-4.58	-4.27	-3.19	-2.26
12	-4.32	-2.88	-3.05	-1.54
13	-3.03	-2.27	-2.10	-1.25
14	-3.43	-2.45	-1.71	-0.94
15	-2.82	-2.01	-0.34	0.18
16	-2.23	-1.12	-0.01	0.25
17	-2.09	-1.18	-0.32	0.26
18	-0.27	-0.74	0.19	0.66
19	-0.39	-0.31	1.04	0.82

Table 3.5. Mating type, origin, metalaxyl sensitivity and DNA fingerprints of parental isolates and isolates of *Phytophthora infestans* recovered from soil from pots left to overwinter.

Isolate	Origin	Mating type	Metalaxyl sensitivity	RG57 Fingerprint†																								
				1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
95.194 (A1)	**Dyfed**	A1	sensitive	1	1	1	0	1	0	1	0	0	1	0	0	1	1	0	0	0	0	1	1	1	1	0	1	1
85.150 (A2)	**Suffolk**	A2	resistant	1	1	1	0	1	1	1	1	1	1	0	0	1	1	0	1	0	0	1	1	1	1	0	1	1
1	**pot 21**	A1	sensitive	1	1	1	0	1	1	1	1	0	1	0	0	1	1	0	1	0	0	1	1	1	1	0	1	1
2	**pot 22**	A1	sensitive	1	0	1	0	1	1	1	0	0	1	0	0	1	1	0	0	0	0	1	1	1	1	0	1	1
3	**pot 22**	A2	sensitive	1	1	1	0	1	0	1	0	0	1	0	0	1	1	0	0	0	0	1	1	1	1	0	1	1
4	**pot 22**	A1	resistant	1	1	1	0	1	1	1	1	0	1	0	0	1	1	0	1	0	0	1	1	1	1	0	1	1
5	**pot 23**	A2	resistant	1	1	1	0	1	1	1	1	0	1	0	0	1	1	0	0	0	0	1	1	1	1	0	1	1
6	**pot 23**	A1	sensitive	1	0	1	0	1	1	1	0	1	1	0	0	1	1	0	1	0	0	1	1	1	1	0	1	1

† Variable bands are highlighted in red.

Discussion

The two strains were found together on the same plant, either as a mixed culture or on separate plant parts in 18 out of the 64 plants. Although the sampling was thorough, it is very possible, that on plants where A1 and A2 strains were found independently, the two strains were in fact growing together in several places on the plant. The fact that oospores were detected in the leaves from 12 of the test plants (6, 9, 10, 19, 21, 22, 23, 24, 26, 29, 30, and 38) suggests a high likelihood that when two mating types are present in a system and conditions for disease are favourable, oospores will form readily in the foliage.

The region of the plot where the A1 and A2 strains were found on the same plant and additionally in mixed culture, spans a total of 10 rows (rows 2 to 11), but was skewed towards the A2 origin. The A1 strain used in this experiment was shown to be more aggressive on cultivar Home Guard than the A2 strain (see Chapter 4), possibly contributing to the higher rate of progress compared with the A2 strain throughout the whole experiment. The single door of the polythene tunnel that housed the experiment was situated at the A2 end of the trial, behind pot 4, and was opened when temperatures exceeded approximately 25°C. The movement of air along the trial from row 1 to row 16 could have aided the spread of the A2 strain, accounting for the A2 strain reaching only the left side of rows 10, 11 and 12 (when looking in through the door).

The A2 strain was not detected in Rows 13 to 16, nor where oospores detected. From Fig. 3.5 it can be seen that by day 12, when the A1 strain had reached all of these rows, the mean proportion of disease was above 0.30 and by day 14 was above 0.55. It is likely that the plants in question were in a severe state of necrosis before the A2 strain reached them and so the opportunity for colonisation by the A2 strain, and subsequent oospore formation was reduced.

The latent period observed from the time of first introduction of diseased plants into the system to first symptoms on neighbouring plants was 8 days. After a further 7 days there was a sudden increase in disease levels in the middle portion of the trial, (Fig. 3.5) suggesting that secondary spread occurred from the primary source and a secondary disease focus had developed. This is supported by that fact that there is a marked change in the primary disease gradient after day 14 at the A1 end of the trial (Fig. 3.6; Table 3.3). The change is not so marked from the A2 source at this point, but is nevertheless present (Fig. 3.7; Table 3.4). A sharp change in gradient did occur at day 18, likely to have been caused by the establishment of the A1 strain (or a daughter A2 disease focus) in the region close to the A2 origin. This would flatten the secondary disease gradient further as the focus becomes less distinct, symptomatic of secondary spread or background contamination (Gregory, 1968).

Row 6 was the only row from which recombinants were recovered. Figure 3.5 shows that, along with row 5, the period before a marked increase in disease occurred was longer than in other rows in the experiment. This may have resulted in a longer period available for oospore formation before complete necrosis and so more oospores could have been produced. A higher number of oospores produced in this row than in others could account for the recovery of infectious propagules from only this row.

Although more oospores may have been produced in row 6, oospores were detected freely throughout the experiment. From the apparent ease with which oospores were formed it would have been expected to recover a higher number of recombinants. There are several possible explanations for why this did not occur:

(i) The crosses used may have carried many lethal genes and so may have a low germination rate. The same cross was used in experiments outlined in Chapter 4; no problems were found with germinating oospores of this cross *in vitro* so a genetic explanation seems unlikely.

(ii) Under the conditions experienced in the experiment oospores may have been largely malformed or aborted and therefore low percentage germination would be expected after over-wintering. Romero and Erwin (1969) showed that many immature sexual bodies of *P. infestans* failed to produce oospores, when exposed to light during the formation of the sexual stage. Instead of forming mature oospores with thick walls, these spores germinated directly forming germ-tubes and terminal sporangia. When the oospores were incubated in the dark and the sexual stage was forming, Romero and Erwin (1969) found that abundant mature oospores were formed. It is possible that the oospores formed in the pot-grown potatoes in this experiment where largely immature and germinated before the winter period, although all of the oospores that were observed appeared to be normal.

(iii) The low frequency of germinating of infectious propagules observed may also be explained by the fact that oospore germination in some species of *Phytophthora* is often asynchronous. Oospores of *Phytophthora* formed in a particular season may germinate after different periods of dormancy, thus sustaining a low but continuous population in the soil (Duncan, 1980).

(iv) The soil used in the experiment was not sterilised, as it was the aim to achieve conditions as close to those in the field as possible. Antagonism by other fungi and bacteria is a possible factor resulting in a low number of sexual progeny being detected in the soil. It has been shown that several species of bacteria and fungi are antagonistic to species of *Phytophthora* including *Pseudomonas*, *Bacillus* and *Streptomyces* species (Broadbent & Backer, 1974) and *Trichoderma* species (Brasier, 1971; Reeves & Jackson, 1972).

(v) It is feasible that the survival of oospores throughout the winter may have been hindered by the environmental conditions. As the pots were kept in black plastic bags for several months it is possible that high temperatures achieved on sunny days may have contributed to a reduced survival rate (Drenth *et al.*, 1995; Fay &

Fry, 1997). It is possible that only in the pots that contained very high numbers of oospores did enough survive to be detected.

(vi) The method by which the progeny were recovered from soil may have inherent flaws. Soil type, incubation temperature and ratio of soil to water and shape of container used may all have contributed to a low rate of retrieval. The number of strains recovered in this experiment was low, but comparable to the results achieved by Drenth *et al.* (1995), who recovered a maximum of 23 recombinants from 1Kg of soil, isolated after a 5 month exposure in soil. As the number of oospores produced in leaves could not be estimated because the overall frequency was very low compared to other soil components and their small size makes them difficult to find, it is difficult to draw conclusions from the number of progeny recovered, other than the production of viable oospores, capable of over-wintering, without artificial inoculation of plants is possible.

(vii) No parental-type strains were recovered from the soil, indicating that not enough mycelial and sporangial inoculum had survived the winter to infect the leaves in the soil assay. This is supported by the work of Zan (1962) who found that the maximum persistence of sporangia in soil, able to infect plant material was 77 days.

It can be seen that when two strains of *P. infestans* originate from two separate point sources there is a high probability, under suitable conditions for disease spread, that they will infect the same plant before complete necrosis occurs. The outcome of which is sexual reproduction.

The fact that 6 oospores from a small plot, such as in this experiment, were able to over-winter, germinate and infect a floating leaflet is important. If such a frequency were observed under field conditions then oospores would be deemed of major importance. If an individual oospore were to survive either a single winter and go on to infect a volunteer, or survive several seasons until a new crop

of potatoes were planted the resultant genotype could go on and spread through a crop.

If oospores are able to form, over-winter and germinate to infect a new crop then this could have significant consequences on disease control and crop husbandry and ultimately costs involved in producing potatoes. The occurrence of a sexually-reproducing population may result in fungicide-resistance problems, as the pathogen would be to adapt more quickly. To overcome this, tight control will have to be applied to the distribution and use of fungicides, with any that fail removed from the market quickly. Disease may be seen earlier in the season as oospores germinate and infect shoots as they emerge. It may become necessary to use fungicides in the field earlier to protect crops from this. This will obviously increase costs unless other strategies prove effective. The removal / desiccation of volunteer plants will be very important. This will minimise the formation of oospores late in the season and remove hosts early in the growing season. It may be that the end-of-season sprays that are used in the desiccation of foliage will prove effective in maintaining the current asexual population, but this is not certain.

Conclusions

- When strains of *P. infestans*, of different mating type, are present in system and conditions are conducive to disease, oospores may form readily in the foliage of a potato crop.
- A high number of oospores produced in a system does not ensure that high numbers will survive to the next growing season to germinate and cause disease.
- Only small numbers of sexual recombinants may be required to cause cultivation problems.
- Cultural and / or chemical control may need to be modified if a sexually reproducing population of *P. infestans* is established outside Mexico.

Chapter 4

The effect of propamocarbHCl on formation, germination and infection potential of oospores of *Phytophthora infestans*

Introduction

The possibility of a sexually reproducing population of *P. infestans* outside of Mexico, along with the addition of the oospore to the disease life-cycle raises concerns with regard to the control and management of potato late-blight in the field (Fry *et al.*, 1993).

The effect of different fungicides on oospore formation, viability, germination and infection potential in *P. infestans* and other members of the order Pythiales has been investigated by several workers including Duncan (1985), Bissbort *et al.* (1997) and Hanson and Shattock (1998b).

The phenylamide fungicide metalaxyl has been shown to have efficacy in inhibiting the formation of oospores in crosses in leaf disks of potato when either or both of the parental strains are phenylamide sensitive (Hanson & Shattock, 1998b). Hanson and Shattock (1998b) also demonstrated that in a cross between two phenylamide resistant strains, the results of metalaxyl treatments were not significantly different from those seen in the controls used (Hanson & Shattock, 1998b). Nevertheless, the number of oospores produced in leaf disks that were treated with metalaxyl close to the time of inoculation with *P. infestans*, was lower than in tests where the application of the fungicide was delayed. This could indicate some protective potential in such cases, but as the frequency in many regions of both A1 and A2 phenylamide resistant strains is low (Deahl, *et al.* 1995; Gisi & Cohen, 1996; Day & Shattock, 1997) the importance of this attribute may be minor.

The morpholine fungicide dimethomorph has been shown to inhibit the formation of oospores of *Plasmopara viticola* in leaf disks (Bissbort *et al.*, 1997). A 5-hour protective treatment with a concentration of 0.5µg/mL dimethomorph reduced oospore formation, but at this level the area of disease was not affected. Curative treatments (applied 48h after inoculation) of 10µg/mL and 25µg/mL reduced oospore formation by 34 and 99% respectively.

The effects of fungicides on oospore germination in *Phytophthora fragariae* have also been investigated. Duncan (1985) carried out extensive studies on the effects of four fungicides on germination of oospores of *P. fragariae* produced in the roots of strawberry plants. It was concluded that from captafol, dichlofluanid (contact fungicides) fosetyl-Al and metalaxyl (systemic fungicides), only fosetyl-Al had any effect on inactive oospores.

With resistance to the phenylamide fungicides being widespread since the 1980s (Davidse *et al.*, 1989) it is important to establish the effect of other systemic protectant fungicides, currently used, on the sexual stage of *P. infestans* in order to anticipate any future control problems.

PropamocarbHCl has been used to control a range of oomycetes including *Pseudoperonospora humuli* on hops (Hunger & Horner 1982), *Peronospora tabacina* on tobacco (Anon, 1995), Pythium blight on bean (Papavizas *et al.*, 1977) and is currently used in the control *P. infestans* on potato (Samoucha & Cohen (1990); Anon, 1995).

The mode of action of propamocarbHCl against the oomycetes is not fully understood although several hypotheses have been put forward. Reiter (1994) proposed that propamocarbHCl affects lipid synthesis. It has also been suggested that sterols have a role in the mode of action of propamocarbHCl, forming a propamocarbHCl-sterol complex, preventing the uptake of sterols into the cell (Papavizas *et al.*, 1978).

The specific activity of propamocarbHCl on oosporogenesis or oospore viability is unknown, although it is expected that any chemical that inhibits the asexual growth of an organism would have an inhibitory effect on the sexual stage. In *P. infestans* this effect may be as a result of the inhibition of mycelial growth, preventing A1 and A2 strains from coming into contact. It is possible that propamocarbHCl has a direct inhibitory effect on the sexual stage of *P. infestans* by interfering with oospore formation through the ability to prevent sterols from being absorbed into the cell.

It is the aim of this study to determine any effect of the carbamate fungicide propamocarbHCl on oospore formation *in planta* and additionally any effect that this compound has on oospores produced *in vitro* and germinated in the presence of propamocarbHCl.

Materials and Methods

The effect of propamocarbHCl on the formation of oospores of *P. infestans* in potato leaf disks (cv. Home Guard)

Six isolates (3 A1 isolates and 3 A2 isolates) were used in this study, selected because of their reliability to form oospores in adequate numbers that germinated well *in vitro*.

The method of Bardsley *et al.* (1996) was used to assess the sensitivity to propamocarbHCl of the 6 parental isolates to be used in the oospore production assay. PropamocarbHCl solution in the form of PrevicurN (72.2% a.i., AgrEvo UK Ltd., Saffron Walden, CB10 1XL, UK.) was used and diluted as required in deionized water. Six concentrations of propamocarbHCl were used in the assay: 1, 2, 5, 10, 20, 50 and 100µg/mL plus a deionized water control.

Nine crosses of the isolates listed in Table 2.1 were each made on 1.4cm diameter leaf disks of potato (cv. Home Guard). An aliquot of the propamocarbHCl test solution (2mL) was placed into each well of a 25-well repli-dish and the leaf disks floated on the test solution (abaxial side up). Five replicates were used in each test. A mixed suspension of sporangia (2 $x10^3$ per mL; A1 and A2 isolates in equal proportion) was chilled at 10°C for 3-4h to allow zoospore release. A volume of 25µL of this sporangia/zoospore suspension was then dropped onto the centre of each of the test leaf disks and the repli-dish incubated at 18°C under lights (16h photoperiod) for four weeks. An assessment of the control leaf disks and the test disks was made after 7 days to ensure that infection had occurred using the method of Bardsley *et al.* (1996).

The number of oospores per leaf disk was determined by placing each leaf disk on a glass slide and squashing it under a cover slip. This was then examined under a microscope and the number of oospores in each leaf disk determined by either a complete count, when oospores appeared to be at a low frequency (approximately 0-500 per disk), or by an estimation. This was done by counting the number of oospores in ten fields of view at x10 magnification before multiplying by 86 to

give the total for each disk [each disk was found to have the surface area equivalent to 86 fields-of-view at x10 magnification].

Table 4.1 The scoring method used to estimate the amount of sporulation of *P. infestans* on floating leaf disks of potato (cv. Home Guard).

Score	Observation
0	Sporangiophores absent
1	1-4 sporangiophores per disk
2	5-12 sporangiophores per disk
3	Moderate sporulation, but only visible under a binocular microscope
4	Sporulation visible with naked eye

The effect of propamocarbHCl on oospore production in the leaves of 8-week-old potato plants (cv. Home Guard)

Eight-week-old potato plants (cv. Home Guard; eighteen plants for each treatment) were sprayed with solutions of 0, 600, 900 and 1200µg/mL propamocarbHCl using a mechanised precision over-head sprayer at a rate of 200L/ha [equivalent to 0, 0.12, 0.18 and 0.24L/ha a.i.) propamocarbHCl in the form of PrevicurN (72.2% a.i., AgrEvo UK Ltd., Saffron Walden, CB10 1XL, UK.) was used and diluted in deionized water as required. The plants were then left to dry naturally overnight.

In advance, the 2 test isolates (selected on the basis that they produced a regularly high number of oospores *in vitro*) 95.194 (A1) and 85.150 (A2) were passaged on potato leaves and a sporangial suspension produced as described above. This suspension was then adjusted to 2 x 10^3 sporangia/mL; A1 and A2 isolates mixed in equal proportion and chilled at 10°C for 3-4h to allow zoospore release. All of the test plants were sprayed with this sporangia/zoospore suspension, over the entire surface of the plant, until run-off. Six plants were placed in each of 12 metal trays containing water. Plastic hoods were put over the plants to maintain high humidity and then incubated for 10 days at 5, 18 or 24°C under lights (16h photoperiod). Disease was assessed after one week incubation under the plastic

hoods. After two weeks the number of oospores in a leaflet was estimated by first measuring the surface area of the leaflet [using a computerised digital image analyser], and then liquefying the leaf in a glass homogeniser (BDH Merk Ltd., Lutterworth, LE17, 4XL, UK.) in a known volume of water. The number of oospores in 50µL was then counted (5 aliquots of 50µL taken for each leaflet) and the total number of oospores in the leaflet estimated (see Chapter 2). A total of ten leaflets were taken from each plant.

The effect of propamocarbHCl on oospore production in field grown potato plants

Potato plants (cv. King Edward and Maris Piper) were planted in the field at East Winch Hall, East Winch, Norfolk, UK. The plots for each cultivar consisted of 9 rows. The two cultivars were separated by 6 untreated rows [3 rows of King Edward and 3 rows of Maris piper]. Once the potato plants had begun to emerge, 20 plots were marked out for each cultivar [2 sets of 10 plots running parallel to each other] 3 rows by 3 plants, separated by a single buffer row. Plants between each plot were removed to aid walking through the plots when spraying and access to the plots for sampling.

After 9 weeks the potato plants were sprayed for the first time with propamocarbHCl in the form of PrevicurN at a rate of 0.65L/ha, applied at a concentration of 400µg/mL using a manually operated sprayer. Eight out of the 20 plots were treated (see Fig. 4.1) with propamocarbHCl with a 7 day interval between sprays for a total of 5 weeks.

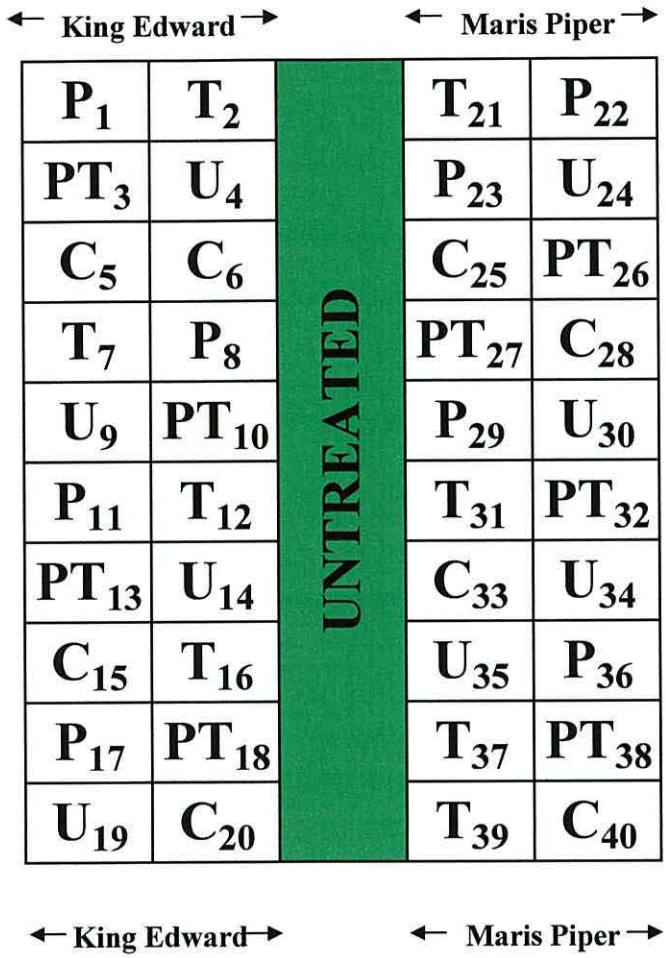
Eight days after spraying with propamocarbHCl a mixed sporangial/zoospore suspension (2 $x10^3$ sporangia per mL) of isolates 95.194 and 85.150 (see Table 2.1) in equal proportion was applied to the plants [prepared as described above] until run-off (Fig. 4.1). Fentin hydroxide was applied very late in the season after the potato crop had died and started to decay. Owing to this no further analysis of the effect of this fungicide on the survival of oospore was undertaken.

Disease was allowed to progress naturally from the last application of propamocarbHCl until 10 weeks post-inoculation when fentin hydroxide was sprayed onto the plots shown in Fig. 4.1 at 0.5L/ha applied at a concentration of 250µg/mL (Fig. 4.1).

Samples of plant material were taken throughout the season in the attempt to detect any oospores present in the haulm. Sampling was conducted most intensively 2 weeks after inoculation, when disease had become evident and enough time had lapsed for oospore formation to initiate. Plant material (4-5 leaflets) was collected from every plot daily for a further two weeks, after which sampling was conducted on a 2-weekly basis until 14 weeks post-inoculation.

The plots were allowed to die and decay naturally and were left to over-winter in the field until April 1998 when soil samples (approximately 1Kg of soil) were taken from each plot. Soil was taken from the upper 3-4 centimetres and areas that appeared to contain decayed potato material were actively selected. The soil collected was placed into plastic bags and transported to the University of Wales, Bangor. The presence of viable oospores in the soil was assayed using a modified method of Drenth *et al.* (1995). Deionized water (0.25L) was added to 100g of the collected soil in a plastic salad box, leaflets of potato (cv. Home Guard) were floated on the surface of the soil slurry (abaxial side touching the slurry). This was incubated at 10°C in the dark to encourage zoospore release from any sporangia present either from germination of oospores or from parental sporangia that survived the winter period.

Figure 4.1 Plan of the field trail showing planting position of two cultivars, King Edward and Maris Piper; fungicide treatments, control plots (inoculated with *P. infestans*, but not treated with fungicide) and the untreated plots (not inoculated and not treated with fungicide).



P = propamocarbHCl treated plots

T = fentin hydroxide treated plots

C = control plots

U = untreated plots

The effect of propamocarbHCl on the germination of oospores of *P. infestans*

Oospores were produced *in vitro* by mating all combinations of A1 and A2 isolates listed in Table 2.1 using the methods described in Chapter 2. After one week the first oospore formation was observed. Oospores produced in this way were used in experiments from this point and for a further three weeks.

Pieces of agar containing oospores were removed from the agar and any excess agar removed (see Chapter 2). The retrieved oospores were resuspended in a solution of propamocarbHCl at 0, 50, 100, 200 or 500µg.mL at a concentration of approximately 2 x 10^3 oospores per mL (Whittaker *et al.*, 1991; Pittis & Shattock, 1994).

The oospore suspension was then spread on soft water agar (see Appendix 1) [approximately 1mL on each of 5 plates], sealed with Parafilm (BDH Merk Ltd., Lutterworth, LE17, 4XL, UK.) and incubated at 18°C under lights (16h photoperiod). After 21 days the cumulative mean number of germinated oospores [oospores were classified as having germinated if a germ tube of at least the diameter of the source oospore was present] on each plate was determined and the mean percentage level of germination calculated.

Results

The effect of propamocarbHCl on the formation of oospores of *P. infestans* in potato leaf disks (cv. Home Guard)

The mean number of oospores produced per cm^2 by all 9 crosses, in leaf disks was calculated. Each propamocarbHCl treatment was shown to be significantly different from all other treatments ($P < 0.01$), with the number of oospores produced decreasing with an increase in propamocarbHCl concentration. That is, each increasing propamocarbHCl concentration produced a significant decrease in the number of oospores formed (Figs. 4.4 – 4.6).

The number of oospores formed appeared to be dependant on the particular isolates used. The A2 isolate IR12.94 consistently produced the most oospores at all concentrations of propamocarbHCl with all A1 parents. Only in crosses with IR12.94 as the parental A2 isolate was oospore production observed at 50µg/mL (Fig. 4.3). When IR12.94 was individually tested for sensitivity (Fig. 4.2) the median level of sporulation was not affected by an increase in the concentration of propamocarbHCl from 0µg/mL to 100µg/mL. The other A2 isolates tested (85.105 and 87.205.4) appeared to be more sensitive to propamocarbHCl, with sporulation arrested completely at 100µg/mL (Fig. 4.2). This resulted in a reduction in the number of oospores formed in each cross involving these isolates compared to the crosses with IR12.94 as the A2 parent (Figs. 4.4 to 4.6).

The A1 isolate with the highest level of propamocarbHCl tolerance was shown to be 95.194, (Fig. 4.2) which was the only A1 isolate to sporulate at a concentration of 100µg/mL propamocarbHCl. The number of oospores produced in the cross between 95.194 (A1) and IR12.94 (A2) in the absence of propamocarbHCl was the highest over all (Fig. 4.4) but with the addition of propamocarbHCl the number of oospores produced per cm^2 was reduced at concentrations of propamocarbHCl where sporulation was not affected (Figs. 4.2, 4.4).

In addition it was seen that in several crosses [95.157.2 (A1) x IR12.94 (A2), 95.194 (A1) x IR12.94 (A2), 95.183 (A1) x IR12.94 (A2), 95.157.2 (A1) x 85.150 (A2) and 95.194 (A1) x 85.150 (A2)] the level of sporulation was not affected at 10µg/mL, but the number of oospores produced was reduced markedly (Figs. 4.3 to 4.6).

The median EC_{50} values for oospore production in the presence of propamocarbHCl were calculated for each cross, all of which fell below 5µg/mL. The cross with the highest EC_{50} value was 95.183 (A1) x IR12.94 (A2), (Table 4.3). Although oospore production on distilled water was lower than other crosses with the IR12.94 isolate, and the number of oospores produced in the presence of propamocarbHCl was also lower, the effect of propamocarbHCl on this cross was less.

The cross with the lowest EC_{50} value was 95.183 (A1) x 87.205.4 (A2) (Table 4.3). All of the crosses with the 87.205.4 isolate produced fewer oospores than any other cross in the absence of propamocarbHCl. Furthermore, all of these crosses were the most sensitive to propamocarbHCl (Fig. 4.6, Table 4.3). The A1 isolate 95.183 (Fig. 4.2) was shown to be tolerant to propamocarbHCl up to 10µg/mL, but oospore formation was reduced by over 50% and arrested completely at 10µg/mL in this cross.

The effect of propamocarbHCl on oospore production in the leaves of 8-week-old potato plants (cv. Home Guard

Oospores were only detected in plants incubated at 18°C. In the plants incubated at 5 or 24°C oospores were not detected in the leaves, petioles or stems of any plant. The effect of propamocarbHCl on oospore production in whole plants at 18°C can be seen in Table 4.4. In plants sprayed with a 600µg/mL propamocarbHCl the mean level of disease recorded on the plants was 0.82 (Table

4.3). Oospores were not detected in these plants. Similarly, oospores were not detected in the plants treated with concentrations of 900 and 1200 µg/mL propamocarbHCl, even though disease levels were consistently above 0.5. The oospores were only detected in the untreated plants, with the mean number of oospores per cm^2 over the six plants tested found to be 3615.

The effect of propamocarbHCl on oospore production in field grown potato plants

The field experiment (Fig. 4.7) oospores were detected in the leaves, petioles and stems in 14 out of 40 plots, of which only one was treated with propamocarbHCl (plot PT_3). In all but three of the plots with plants not treated with propamocarbHCl (C and T) were oospores detected. No oospores were detected in uninoculated plants (U). There was no obvious difference between the two cultivars planted (King Edward and Maris Piper). No isolates (parental types or recombinants) were recovered from soil from any of the plots even though oospores were found in many plants.

The effect of propamocarbHCl on the germination of oospores of *P. infestans*

Percentage germination of oospores (treated with 0 to 500 µg/mL propamocarbHCl) from each of the crosses was not significantly affected ($P < 0.05$) by any concentration of propamocarbHCl compared with the control (Fig. 4.8).

Figure 4.2 The effect of propamocarbHCl treatments of 0, 1, 2, 5, 10, 20, 50 and 100 µg/mL on the median level of sporulation (scored on a 1-4 scale) of *Phytophthora infestans* on floating potato leaf disks (cv. Home Guard) after a 7 day incubation.

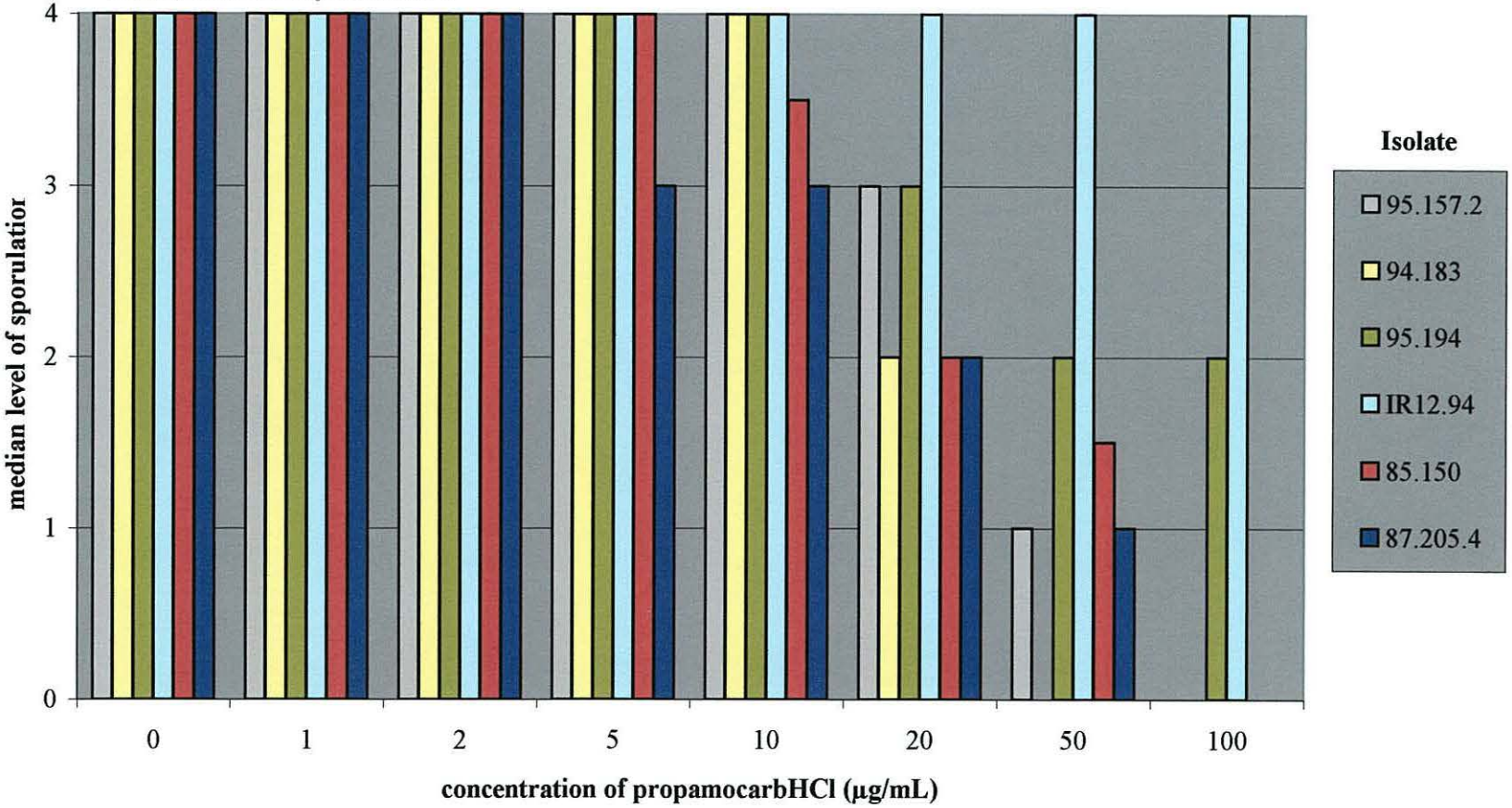
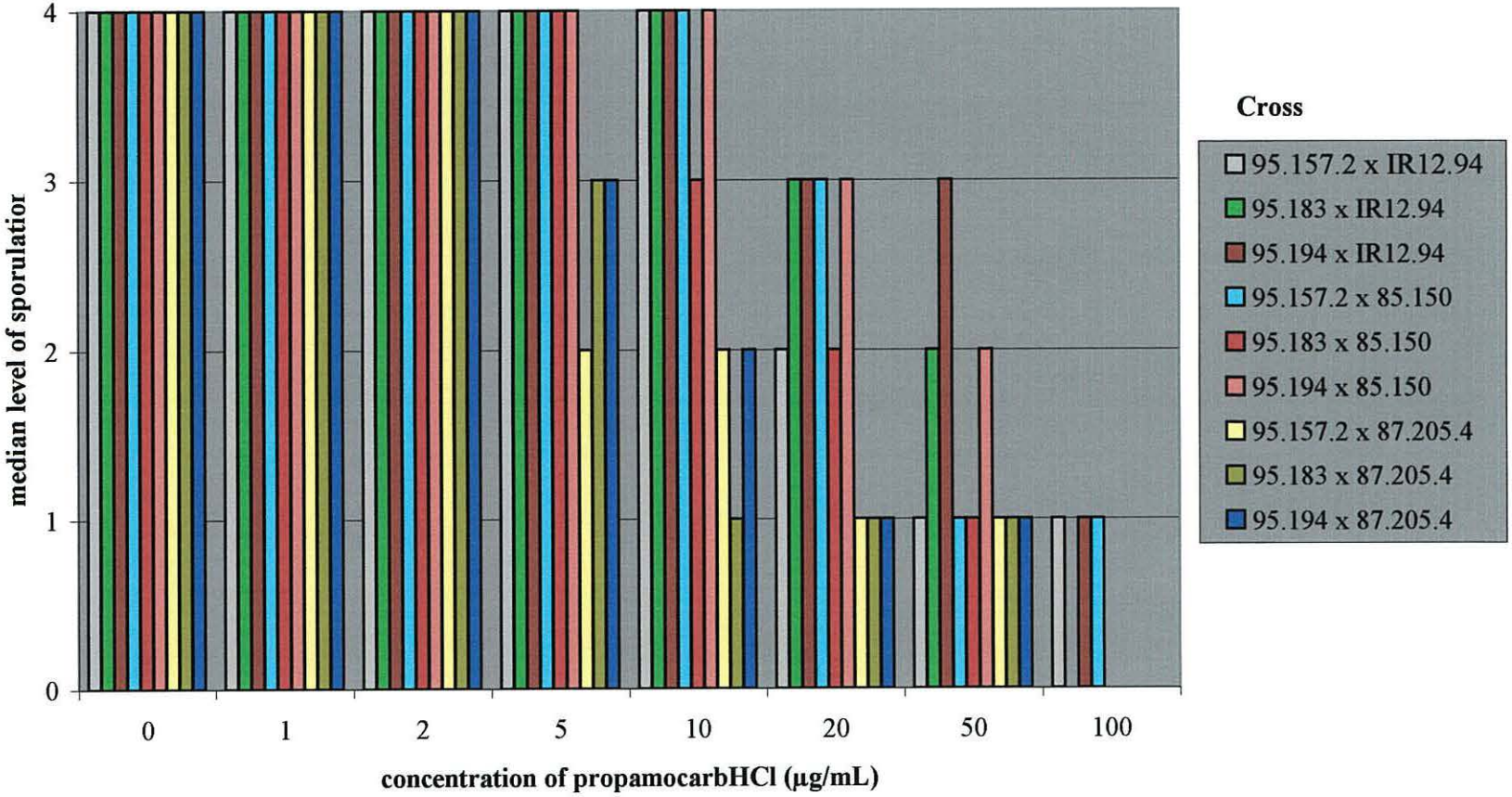


Figure 4.3 The effect of propamocarbHCl treatments of 0, 1, 2, 5, 10, 20, 50 and 100 µg/mL on the median level of sporulation (scored on a 1-4 scale) of *Phytophthora infestans* on floating potato leaf disks (cv. Home Guard) inoculated with both an A1and A2 isolate.



Figures 4.4 - 4.6 The mean effect of propamocarbHCl on the production of oospores in *Phytophthora infestans* in potato leaf disks (cv. Home Guard) in nine different crosses. Significantly different numbers are labelled a-j.

Figure 4.4

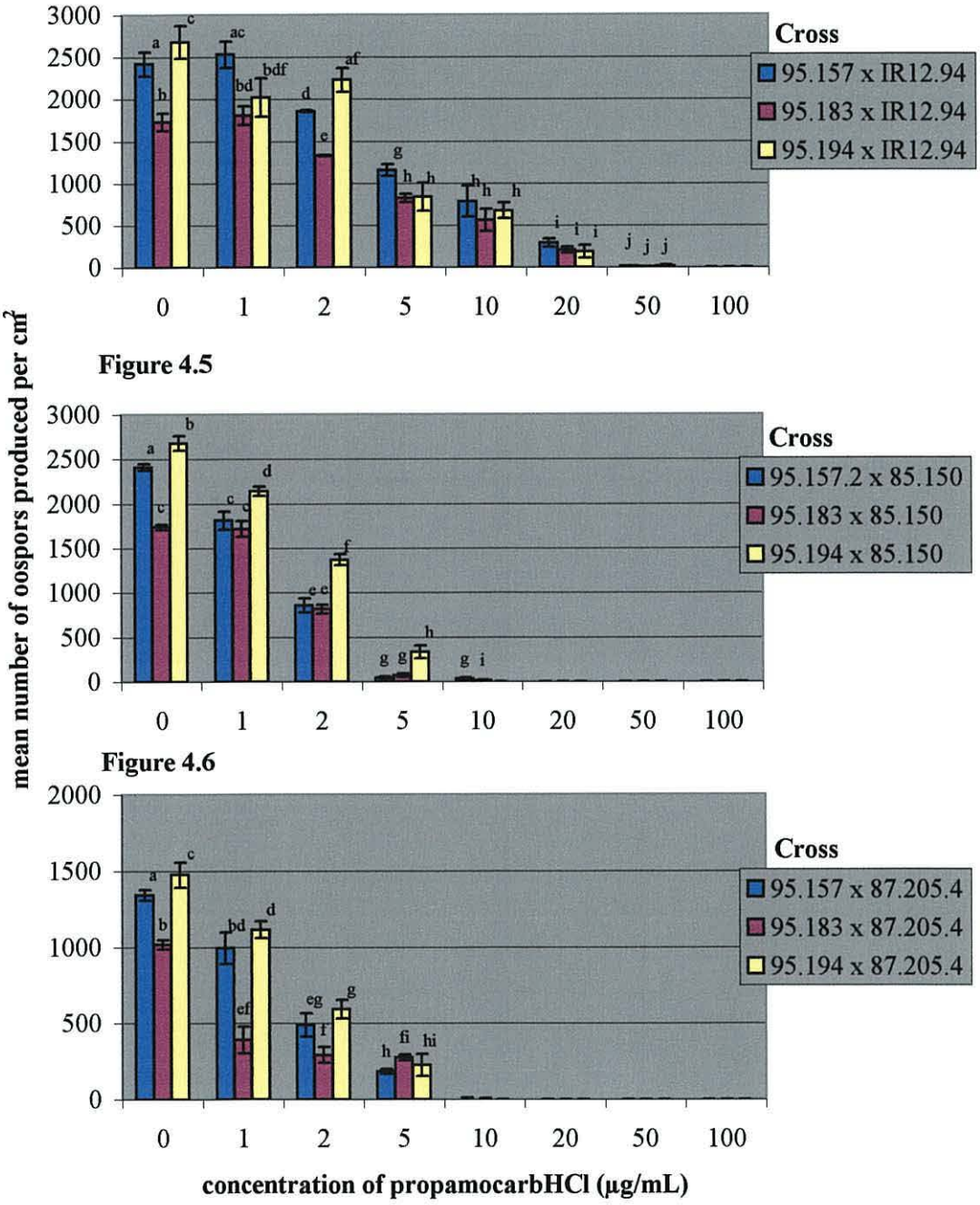
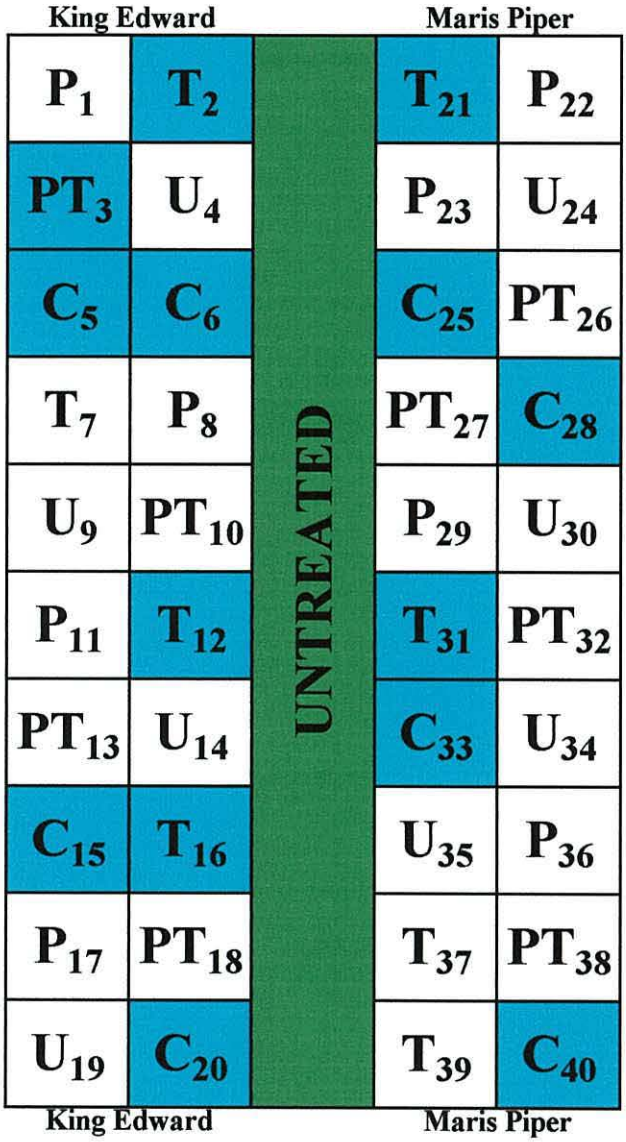


Figure 4.7 The incidence of oospores in potato plots (cv. King Edward and cv. Maris Piper) inoculated with a mixed sporangia/zoospore suspension of A1 (95.194) and A2 (85.150) isolates.



Plots in which oospores were detected

P Treated with propamocarbHCl and inoculated with *P. infestans*

T Treated with fentin hydroxide and inoculated with *P. infestans*

C Inoculated with *P. infestans* only

U Plants uninoculated

Figure 4.8 The effect of propamocarbHCl treatments of 0, 50, 100, 200 and 500 µg/mL on the germination of oospores of *Phytophthora infestans* after a 21 day incubation on water agar.

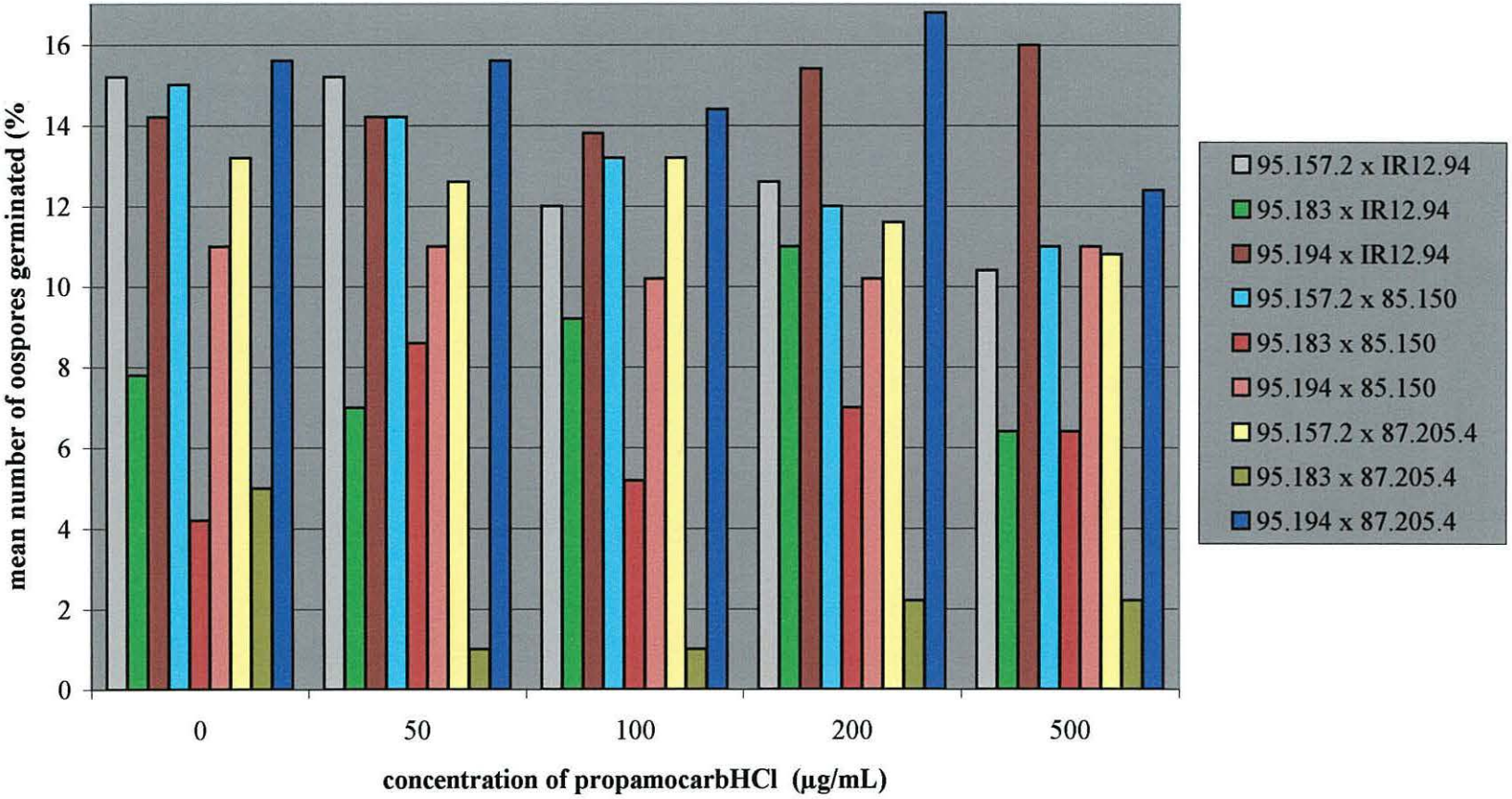


Table. 4.2 EC_{50} values of the effect of propamocarbHCl on oospore production in *Phytophthora infestans* grown on leaf disks.

Cross	EC_{50} value (µg/mL)
95.157.2 x IR12.94	4.79
95.183 x IR12.94	4.98
95.194 x IR12.94	3.63
95.157.2 x 85.150	1.54
95.183 x 85.150	1.91
95.194 x 85.150	2.08
95.157.2 x 87.205.4	1.48
95.183 x 87.205.4	<1
95.194 x 87.205.4	1.73

Table 4.3 The effect of three concentrations of propamocarbHCl on late-blight (*Phytophthora infestans*) disease levels in 8-week-old potato plants (cv. Home Guard) inoculated with a mixed sporangia/zoospore suspension of A1 (95.194) and A2 (85.150) isolates and incubated at 18°C.

concentration of	proportion of plant diseased						
propamocarb (µg/ml)	Plant 1	Plant 2	Plant 3	Plant 4	Plant 5	Plant 6	mean
0	0.95	0.95	0.95	0.95	0.95	0.95	0.95
600	0.8	0.8	0.8	0.8	0.9	0.8	0.82
900	0.95	0.95	0.6	0.9	0.85	0.85	0.85
1200	0.8	0.8	0.7	0.5	0.6	0.7	0.68

Table 4.4 The effect of three concentrations of propamocarbHCl on oospore production of *Phytophthora infestans* in the leaflets of 8-week-old potato plants (cv. Home Guard) inoculated with a mixed sporangia/zoospore suspension of A1 (95.194) and A2 (85.150) isolates and incubated at18°C.

concentration of	mean number of oospores produced per cm^2						
propamocarb (µg/ml)	Plant 1	Plant 2	Plant 3	Plant 4	Plant 5	Plant 6	mean
0	5320	0	3900	4100	2550	5820	3615
600	0	0	0	0	0	0	0
900	0	0	0	0	0	0	0
1200	0	0	0	0	0	0	0

Discussion

Since the fungisatic nature of the carbamate fungicides (Papavizas *et al.*, 1978) has an effect on the growth of *P. infestans* in potato plant material it would be expected to contribute to the inhibition of oospore production seen in this study by simply inhibiting the development of the fungus. However, it can be see from this investigation that at levels of propamocarbHCl where late blight continues to develop, the formation of oospores is inhibited. That is, oospore production is more sensitive to propamocarbHCl than either mycelial growth or sporulation. Some other mechanism must be involved in this inhibition. The mode of action of the carbamate fungicides is still not fully understood (Reiter, 1994) so it is difficult to infer a reliable explanation. Indeed it has been suggested that the mode of action of carbamate fungicides may differ between different pythiaceous fungi (Papavizas *et al.*, 1978).

Studies have been conducted which give an indication of a possible reason for the arrest of oospore production in *P. infestans* by propamocarbHCl. The effect of carbamate fungicides on pythiaceous fungi has been shown to be reversed by the addition of sterols to the growth medium (Sijpesteijn *et al.*, 1974; Papavizas *et al.*, 1978). It has been established that pythiaceous fungi lack the ability to synthesise sterols, (Hendrix, 1966) but the presence of sterols is important for the asexual and sexual reproduction in these fungi (Haskins, 1964; Hendrix, 1964; Hendrix, 1965). The exact extent of the requirement for sterols in the sexual reproduction of pythiaceous fungi has been questioned (Kerwin & Duddles, 1989). By increasing the level of unsaturated fatty acids in a system artificially it was found that oospores could be formed. However, it is accepted that sterols are required for normal oospore production (Elliott, 1994).

Papavizas *et al.*, (1978) found that propamocarbHCl impaired membrane permeability and caused leakage of cellular constituents from the mycelium of eight species of *Pythium*, but could be reversed by the addition of sterols to the growth medium. Furthermore, Papavizas *et al.* (1978) proposed that the reversal of the effects of propamocarbHCl by the addition of sterols may be as a result of the formation of a propamocarbHCl-sterol complex, so essentially blocking the effect of propamocarbHCl treatments. The formation of a sterol complex has also been suggested as the mode of action of the antibiotic nystatin in inhibiting the growth of *Pythium aphanidermatum* (Fowlks *et al.*, 1967). It is suggested that nystatin causes disruption by binding to sterols in the plasma membrane.

Langcake (1974) identified three sterols from the leaves of potato as cholesterol, stigmasterol and β-sitosterol. The amount of sterol present in potato leaves was not found to be related to the level of resistance to infection by *P. infestans*. Similar results have been demonstrated in tomato cultivars of differing susceptibility to *P. infestans* (Bradford *et al.*, 1982). Nonetheless the sterols in the potato leaves are important in both sexual and asexual reproduction and a compound with the ability to prevent uptake of these sterols may affect normal development.

If such a mechanism was proven to exist between propamocarbHCl and sterols it is feasible that propamocarbHCl may interfere with the uptake of sterols required for sexual reproduction at low concentration, and so impede oospore formation whilst not affecting mycelial growth. As species of *Phytophthora* are able to grow vegetatively in the absence of sterols (Elliott, 1994), it likely that propamocarbHCl acts in another way at higher concentrations to prevent mycelial infection of the host (possibly by the accumulation of Δ^7-hexadecenoic acid in the plasma membrane (Burden *et al.*, 1988)). PropamocarbHCL has a strong effect both on sporulation and the formation of motile zoospores (Reiter, 1994), suggesting that the blocking of sterol uptake may also be of importance in asexual reproduction.

In the experiment investigating the effect of propamocarbHCl on oospore production in leaf disks, the level of sporulation in parental isolates at 10μg/ml was not inhibited in five crosses whilst oospore production was reduced significantly (Figs. 4.3 to 4.6). This suggests that oospore production is more sensitive to propamocarbHCl than asexual sporulation in *P. infestans*.

The effect of low concentrations of propamocarbHCl on the formation of oospores, but not mycelial growth would account for the results obtained in the experiment on 8-week-old potato plants sprayed with propamocarbHCl (Tables 4.3 and 4.4) and also in the field trial where oospores were detected in only one of the plots treated with propamocarbHCl (Fig. 4.7). The inhibition of oospore production by propamocarbHCl in the field was not complete and it is possible that oospores did form in the haulm of sprayed plants but remained undetected. The dose applied (0.65L/ha) in the experiment was below the recommend field rate for potatoes (1.5 to 5L/ha) and individual plants may have been accidentally missed so accounting for this anomalous result.

The failure of any isolates (parental types or recombinants) to be recovered from the soil of the field trial could be because of numerous factors including shortcomings in the recovery procedure. One other possibility lies in the fact that the site used for the experiment (East Winch Hall, Norfolk, UK) has for many years been a site for development trials on both registered and unregistered compounds including fungicides, insecticides and herbicides. Such a long-term use of this site for this purpose may have altered the nature of the soil, both chemically and biologically. Indeed, herbicides as well as fungicides have been shown to have efficacy against species of *Phytophthora* at non-phytotoxic rates (Wilcox, 1996). It is possible that any residual compound may have contributed to a reduced recovery rate.

With the advent of genetically modified crops resistant to herbicides, the importance of herbicides in oomycete disease control should not be overlooked. The distinction between the oomycetes and other higher fungi and their close-relatedness to the chrysophytes (Gunderson *et al.*, 1987) sets a sound basis for investigations into such an area.

PropamocarbHCL was shown to have no effect on the germination of *in vitro*-produced oospores (Fig. 4.8) This would be expected as the nature of propamocarbHCl is fungistatic and is less effective in controlling mycelial growth than other parts of the fungal life-cycle (Reiter, 1994). An oospore was recorded as having germinated when the germ tube reached a length equal to that of the diameter of the oospore from which it originated. Experiments using the formation of a sporangium or further growth to asses the effect of propamocarbHCl may have yielded more conclusive results.

Conclusions

- Oospore formation was found to be more sensitive to propamocarbHCl than sporulation the occurrence of disease symptoms.. This effect was seen in leaf disks and in whole plants.
- No effect of propamocarbHCl on the germination of in vitro-produced oospores was observed.
- The mode of action of propamocarbHCl may be due to the inhibition of the uptake of sterols required for sexual; reproduction.

Chapter 5

The inheritance of sensitivity to the carbamate fungicide propamocarbHCl in *Phytophthora infestans*

Introduction

The inheritance of many phenotypic and genotypic characteristics in *P. infestans* has been investigated in depth including: mating type (Romero & Erwin, 1969; Judelson *et al.*, 1995; Judelson, 1996; Judelson, 1997), antibiotic resistance (Whittaker *et al.*, 1995), RFLP bands (Goodwin *et al.*, 1992), allozymes (Spielman *et al.*, 1990; Chang & Ko, 1992; Mosa *et al.*, 1993), mitochondrial DNA (Whittaker *et al.*, 1994) and DNA contents (Whittaker *et al.*, 1991).

The inheritance of resistance in *P. infestans* to the phenylamide fungicide metalaxyl has been the subject of extensive study (Shattock, 1988; Vorobyeva *et al.*, 1992; Fabritius *et al.*, 1997). Shattock (1988) produced single oospore progeny from matings between isolates of *P. infestans* that were either highly resistant or highly sensitive to metalaxyl. In each cross it was found that in the F_1 generation the isolates were mainly of an intermediate sensitivity to metalaxyl. The frequency distribution of these crosses was found to be skewed towards a sensitive phenotype, with a few isolates either fully sensitive or resistant. Shattock (1988) hypothesised that an incompletely dominant major gene controls resistance to metalaxyl. There is evidence in support of this from other studies undertaken in *P. infestans*. Fabritius *et al.* (1997) found that one gene conferring metalaxyl resistance was responsible for the majority of the phenotype, but additional loci of minor effect also played a significant role. It has been demonstrated that the same gene confers insensitivity to metalaxyl in both Mexican and Dutch isolates, but unusually in British *P. infestans* isolates, a gene at a different locus has been implicated (Fabritius *et al.*, 1997).

In contrast to the work of Shattock (1988) and Fabritius *et al.* (1997), Lee *et al.* (1999) suggest that metalaxyl resistance in *P. infestans* is conferred by a single dominant gene rather than an incompletely dominant major gene and that any variation in sensitivity is brought about by minor genes. These findings are in line with work undertaken in *Phytophthora sojae*, where only major genes have been found to confer insensitivity to metalaxyl (Bhat *et al.*, 1993).

The understanding of inheritance of phenotypic characteristics is important to the understanding of the population dynamics of *P. infestans* as a whole. Such phenotypic characteristics as fungicide sensitivity affect the fitness of *P. infestans* in commercial fields, but other characteristics such as virulence and aggressiveness also have a significant bearing on fitness.

The inheritance of virulence in *P. infestans* has been investigated by several workers (Romero & Erwin, 1969; Laviola & Gallegly, 1983; Tooley *et al.*, 1985a, 1986; Sweigard, *et al.*, 1987; Spielman *et al.*, 1989; Spielman *et al.*, 1990; Al-Kherb *et al.*, 1995). Amongst this work Tooley *et al.*, (1985a, 1986) found that isolates from a sexual population (Mexico) attacked more R-gene differentials than isolates from asexual populations (samples collected from the United States and Wales) but with no consistent difference in aggressiveness or fitness between isolates from the two populations.

Aggressiveness, distinct from virulence, may be defined as the degree of pathogenicity of a pathogen or race that differs in severity of pathological effects, but does not interact differentially with host varieties or cultivars (van der Plank, 1968). Aggressiveness differs from virulence, in that virulence is the degree of pathogenicity of a pathogen, where particular races interact differently with different host cultivars (Manners, 1993).

Day and Shattock (1998) studied the role of several fitness components in the population dynamics of *P. infestans*. It was suggested that a lower level of aggressiveness in the old, pre-1970s population of *P. infestans* in England and Wales, along with a lack of insensitivity to metalaxyl may have contributed to the displacement of this population by the new population. Day and Shattock detected differences in the fitness of isolates on different cultivars of potato and between isolates of different metalaxyl sensitivities. On cultivar Cara metalaxyl sensitive isolates were more aggressive than metalaxyl resistant isolates, but this difference was not observed on cultivar Maris Piper.

Flier and Turkensteen (1999) conducted similar investigations into variation of aggressiveness of *P. infestans* in Dutch field isolates. A large level of variation in aggressiveness was found from isolates within three geographic regions. Whilst latent period was found to differ significantly between isolates from the three regions, maximal growth rate and infection efficiency did not. No correlation was detected between the three components of aggressiveness tested and association between mating type and aggressiveness was not found. Due to the high level of variation seen in the isolates collected it was suggested that oospores play an important role in late blight epidemics in the Netherlands.

Sexual reproduction, it is hypothesised, serves as the major mechanism in maintaining variation and the existence of highly aggressive strains of *P. infestans* in the Netherlands. Lee *et al.* (1999) conducted a correlation study to determine any link between metalaxyl resistance and the fitness components of lesion size and sporulation capacity in progeny from two crosses of US isolates of *P. infestans*. No association between these phenotypic characteristics was observed.

Although sexual reproduction has been linked with generating variation in aggressiveness in *P. infestans*, variation not derived in this way has also been detected in other species. Kennedy *et al.*, (1986) found that 32 single-zoospore isolates of *Phytophthora fragariae* from a single field could be grouped into four

sub-clusters based on their strawberry root-rot scores. Variation in virulence between sub-clusters was observed, but additionally, differences in aggressiveness between isolates within sub-clusters were found.

Unlike the phenylamide fungicide metalaxyl, the inheritance of sensitivity to the carbamate fungicide propamocarbHCl has not been investigated. The ability of sexual reproduction to generate variation in sensitivity to this fungicide and any capacity for higher levels of insensitivity to develop through sex is unknown. From other work conducted on propamocarbHCl it seems that the mode of action of this fungicide is of a multi-site nature (Papavizas *et al.*, 1978; Reiter, 1994). If this is the case then a number of genes would be acting cumulatively and it would be expected that a cross between a highly insensitive isolate and a sensitive isolate would yield progeny with a continuous distribution of sensitivities (Georgopoulos, 1987). Isolates highly resistant to propamocarbHCl have not been detected, but variation in the level of sensitivity to this fungicide is documented (Bardsley *et al.*, 1996; J. P. Day, pers. com.)

It is the aim of this study to discover if natural variation in propamocarbHCl sensitivity is due to variation in aggressiveness or variation in responses to propamocarbHCl. Additionally, it is the aim to determine whether natural variation in propamocarbHCl sensitivity can result in an increase in insensitivity through sexual recombination.

Materials and Methods

Variation in sensitivity of progeny to propamocarbHCl

Characterisation and choice of parental isolates

Fifteen single-oospore isolates were selected from each of two crosses made between isolates 95.194 (A1) and 85.150 (A2) (cross 1) and between 95.157.2 (A1) and 87.205.4 (A2) cross 2) as listed in Table 5.2. This combination of parental isolates was chosen because of suitable genotypic and phenotypic variation, allowing recombination in the resultant progeny to be confirmed. Genetic variation was detected by using the multilocus fingerprinting probe RG57 (Goodwin *et al.*, 1992; Drenth *et al.*, 1993). The variations in phenotype used to detect recombination were mating type and metalaxyl sensitivity. As well as distinctions in the genotype and phenotype of parental isolates, the capability and reliability of the crosses to form adequate numbers of oospores that germinated well *in vitro* was fundamental in the final selection. The progeny from each cross were also characterised for mating type, metalaxyl sensitivity and RG57 fingerprint.

Determination of latent period and level of sensitivity of isolates to propamocarbHCl

Five different cultivars were used in the assay as listed in Table 5.1. These were selected to represent a wide range of foliar resistance to *P. infestans*, based on the National Institute of Agricultural Botany's Potato Variety Handbook (Anon, 1997).

The method of Bardsley *et al.* (1996) (see Chapter 4) was used to assess the sensitivity of each of the progeny produced from each of the crosses to propamocarbHCl. PropamocarbHCl solution in the form of PrevicurN (72.2% a.i.,

AgrEvo UK Ltd., Saffron Walden, CB10 1XL, UK.) was used and diluted as required in deionized water. Six concentrations of propamocarbHCl were used in the assay: 1, 5, 10, 50, 100 and 500µg/mL plus a deionized water control. Leaves from 8-week-old potato plants of each cultivar were selected and leaf disks prepared as detailed in Chapter 4. Each isolate was tested on each cultivar at each concentration of propamocarbHCl with five replicates for each isolate/cultivar/concentration of propamocarbHCl.

The time from inoculation to the time that sporulation was first observed on individual leaf disks was recorded. This latent period of the isolates was recorded up to 14 days after which the leaf disks became necrotic so were scored as having zero sporulation. Additionally, the sporulation on each of the disks was scored after 7 days on a 0-4 scale (Table 4.1) by estimating the number of sporangiophores on each disk (Bardsley *et al.*, 1996).

Table 5.1. National Institute of Agricultural Botany foliar rating for resistance to late-blight of the five potato cultivars used in this study (Anon, 1997).

Cultivar	NIAB foliar resistance rating
Home Guard	**4**
King Edward	**4**
Maris Piper	**5**
Tina	**6**
Stormont Enterprise	**7**

Data Analysis

The mean lowest concentration of propamocarbHCl that gave a score of 2 (5-12 sporangiophores produced per leaf disk) from the 5 replicates was plotted against isolate (Figures 5.1 and 5.2).

Interpolated latent periods (days) were used to represent all the data generated form this experiment in order to allow the large amount of data to be represented in a concise way. The statistical program SigmaStat was used to calculate the least squares regression line (Figures 5.3 and 5.4).

R^2 values for the correlation between latent period and propamocarbHCl insensitivity were calculated by using Pearson's product moment correlation co-efficient (Tables 5.6 and 5.7).

Results

The fifteen progeny selected from each cross were initially identified as being sexual recombinants (Tables 5.4 and 5.5). Isolate 1 from cross 2 was later found to be of parental genotype (based on the limited number of markers used in this study). Variable bands were detected in the parental isolates (bands 6, 8, 9 and 16 in cross 1; and 3, 7 and 19 in cross 2). In addition to the variation seen in these bands, band 2 was found to be variable in the progeny of both crosses even though it was present in all four parental isolates. Band 16 was found to be variable in the progeny of cross 2, but was present in both parental isolates of this cross. In cross 1 isolate 2 (A1) was found to have the same DNA fingerprint as the parental isolate 94.194 (A1), but it was determined that this isolate was a recombinant because of a difference in metalaxyl sensitivity from the A1 parental isolate. Isolate 1 of cross 2 was identical to the A2 parental isolate (87.205.4).

The lowest concentration of propamocarbHCl (mean of 5 replications) required to give a score of 2 (5-12 sporangiophores seen per disk) in each of the five cultivars is shown in Figures 5.1 and 5.2. On cultivar Home Guard a concentration of 220 µg/mL was required to restrict sporulation of the parental isolate 95.194 (A1) to a score of 2. It can be seen that the concentration required giving this same score decreases as the level of horizontal resistance increases (from cultivars King Edward through to Stormont Enterprise), with only a concentration of 10µg/mL required to give a score of 2 on Stormont Enterprise. A similar response was seen with parental isolate 85.150 (A2), but with lower concentrations of propamocarbHCl required to give a score of 2 on all cultivars except King Edward, where 100 µg/mL was required as in 95.194 (A1). All F_1 isolates fell within the range of the two parental isolates on cultivar Home Guard, with the exception of isolates 2 and 15 that were both less sensitive to propamocarbHCl than the parental isolates. On other cultivars the concentration required to give a score of 2 in isolates 2 and 15 was consistently equal to or higher than the dose required by the parental isolates. On other cultivars isolate 5 exhibited less

sensitivity to propamocarbHCl than the parental isolates, but on Home Guard was as sensitive to propamocarbHCl as the parental isolate 85.150 (A2) is. On cultivar Tina isolate 5 exhibited a high level of insensitivity to propamocarbHCl, with 500 µg/mL required to give a score of 2.

In cross 2, a similar response to propamocarbHCl concentration and cultivar was seen, with the concentration of propamocarbHCl required to give a score of 2 decreasing as the level of horizontal resistance in the potato cultivar increases. Overall both parental isolates in cross 2 showed insensitivity to propamocarbHCl equal or above that that of the parental isolates of cross 1. The only isolate generated from cross 2 to show more insensitivity to propamocarbHCl than the parental isolates was isolate 13 which, on cultivars Home Guard, King Edward, Maris Piper and Tina, a concentration of 500, 500, 200 and 100 µg/mL respectfully was required to give a score of 2 (a concentration between 160% and 200% of that required in the parental isolates). The level of control in isolate 13 exhibited by propamocarbHCl was equal to that of the parental isolates on cultivar Stormont Enterprise.

No correlation was found between propamocarbHCl sensitivity and metalaxyl sensitivity, with both metalaxyl resistant and sensitive isolates exhibiting sensitivity to propamocarbHCl and a level of insensitivity higher than that seen in the parental isolates (Figures 5.1 - 5.2 and Tables 5.4 – 5.5).

The interpolated latent periods for the parental isolates and progeny of crosses 1 and 2 when treated with 0, 250 and 500 µg/mL propamocarbHCl (with standard error bars) are shown in Figures 5.3 and 5.4. Each cultivar was plotted separately with isolate plotted against latent period (a suitable scale was used for each plot). In both crosses, and on all cultivars the latent periods of none of the progeny differed significantly from the parental isolates when not treated with propamocarbHCl. It can be seen that the parental isolates 95.194 (A1) and 85.150 (A2) had a latent period approximately equal to that of the 15 progeny at a

concentration of 0 μg/mL propamocarbHCl when grown on cultivar Home Guard (Fig. 5.3). As the concentration of propamocarbHCl increased on this cultivar, so the latent period for each isolate increased. The maximum interpolated latent period was 20 days, seen in isolate 1 at a concentration of 500 μg/mL propamocarbHCl. In cross 1 none of the isolates tested exhibited a latent period significantly lower than that of the parental isolates on cultivar Home Guard, although the interpolated latent period for isolate 1 was significantly longer than other isolates at a concentration of 500 μg/mL propamocarbHCl. On cultivar King Edward latent periods significantly lower than the parental isolates are seen. Isolates 1, 2, 6 and 15 all showed latent periods shorter than either parental isolate when treated with propamocarbHCl. No significant variation from the latent periods seen in the parental isolates at different concentrations of propamocarbHCl was observed in cross 1 when grown on cultivar Maris Piper. On cultivar Tina isolates 5, 6, 13 and 15 show latent periods significantly shorter than the parental isolates when treated with propamocarbHCl. None of the progeny were found to be significantly different from the parental isolates when grown on cultivar Stormont Enterprise at 0, 250 or 500 μg/mL propamocarbHCl. Although a trend towards an increase in latent period can be seen from Figure 5.3, the only significant difference was between 0 and 500 μg/mL propamocarbHCl.

Similar results are seen in cross 2 (Fig. 5.4), with latent period increasing with concentration of propamocarbHCl. The variation in this cross is less than that seen in cross 1, with little statistical difference seen in the progeny compared with the latent periods seen in the parental isolates; none of the progeny were significantly different from both parental isolates on any of the cultivars at any propamocarbHCl concentration. On all cultivars and at concentrations of propamocarbHCl of 250 and 500 μg/mL isolate 2 consistently had a latent period longer than that exhibited by the parental isolates, although this was not statistically significant.

Some correlation can be seen between latent period and propamocarbHCl sensitivity in both of the crosses tested in these experiments (Figures 5.5 – 5.6 and Tables 5.6 – 5.7). R^2 values show that the maximum amount of correlation between these two factors that can be explained by a direct relationship is 45.97% (correlation of latent period and propamocarbHCl insensitivity on Stormont Enterprise in cross 2). This value is misleading as very little sporulation was seen in this experiment, with a score of 0 µg/mL propamocarbHCl on the whole being able to restrict the level of sporulation to a score of 2. The lowest R^2 value observed was 0.0016 (0.16% can be explained by correlation), seen in cross 1 on cultivar Maris Piper. The level of correlation that can be explained in cross 1 by an interaction between latent period and propamocarbHCl sensitivity was lower than in cross 2. In cross 2 a concentration of 0 µg/mL propamocarbHCl was able to restrict the level of sporulation to a score of 2 for several isolates on cultivars King Edward's, Maris Piper, Tina and Stormont Enterprise. The result of this would be to give the appearance that there was more correlation due to a direct interaction between the two factors tested than was actually the case. It can therefore be concluded that the level of correlation between these two factors is low.

Fig. 5.1 The lowest concentration of propamocarbHCl required to give a score of 2 in leaf-disk-sensitivity tests on isolates of *P. infestans* generated from cross 1, all isolates tested on five cultivars of potato.

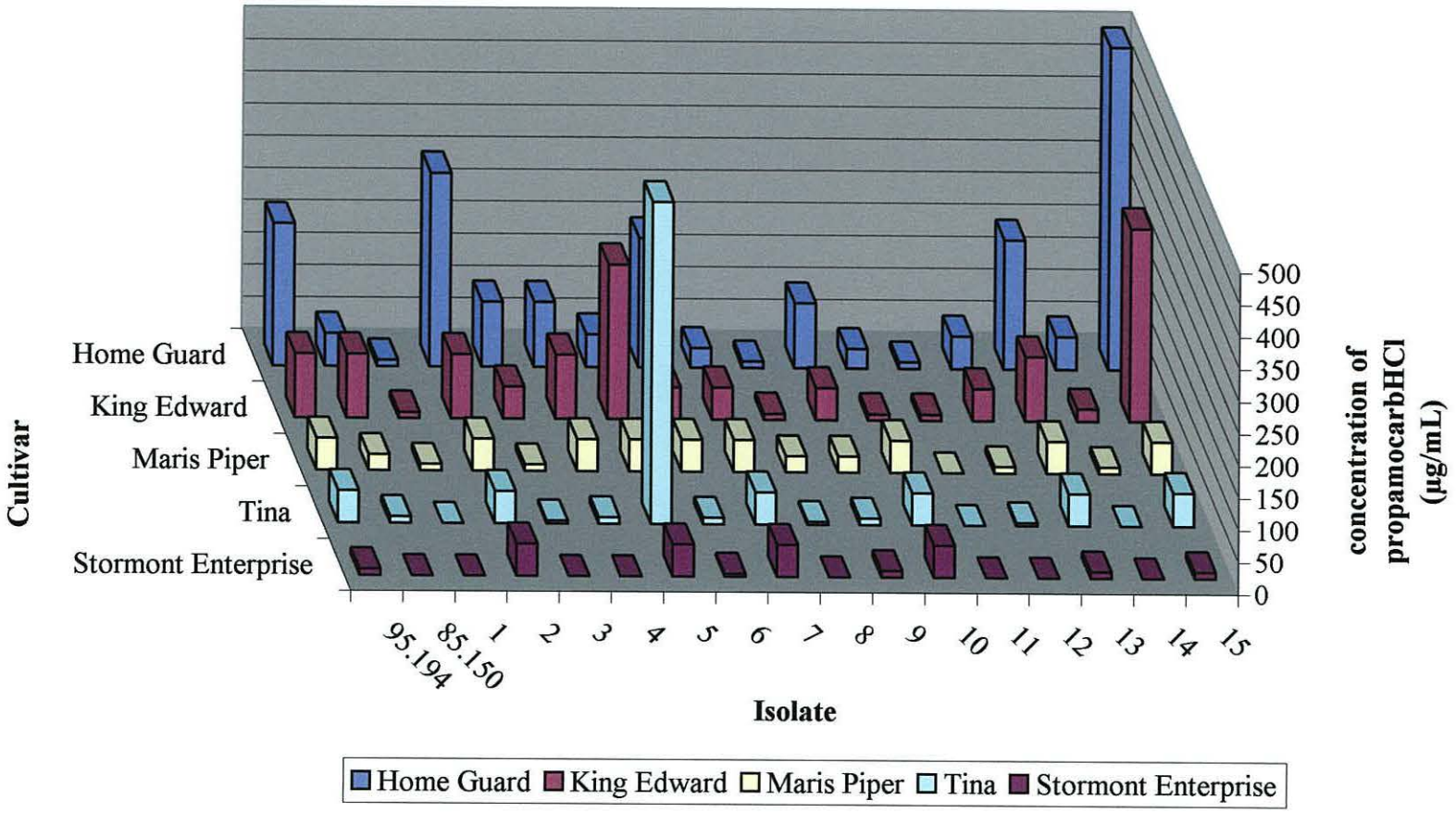


Fig. 5.2 The lowest concentration of propamocarbHCl required to give a score of 2 in leaf-disk-sensitivity tests on isolates of *P. infestans* generated from cross 2, all isolates tested on five cultivars of potato.

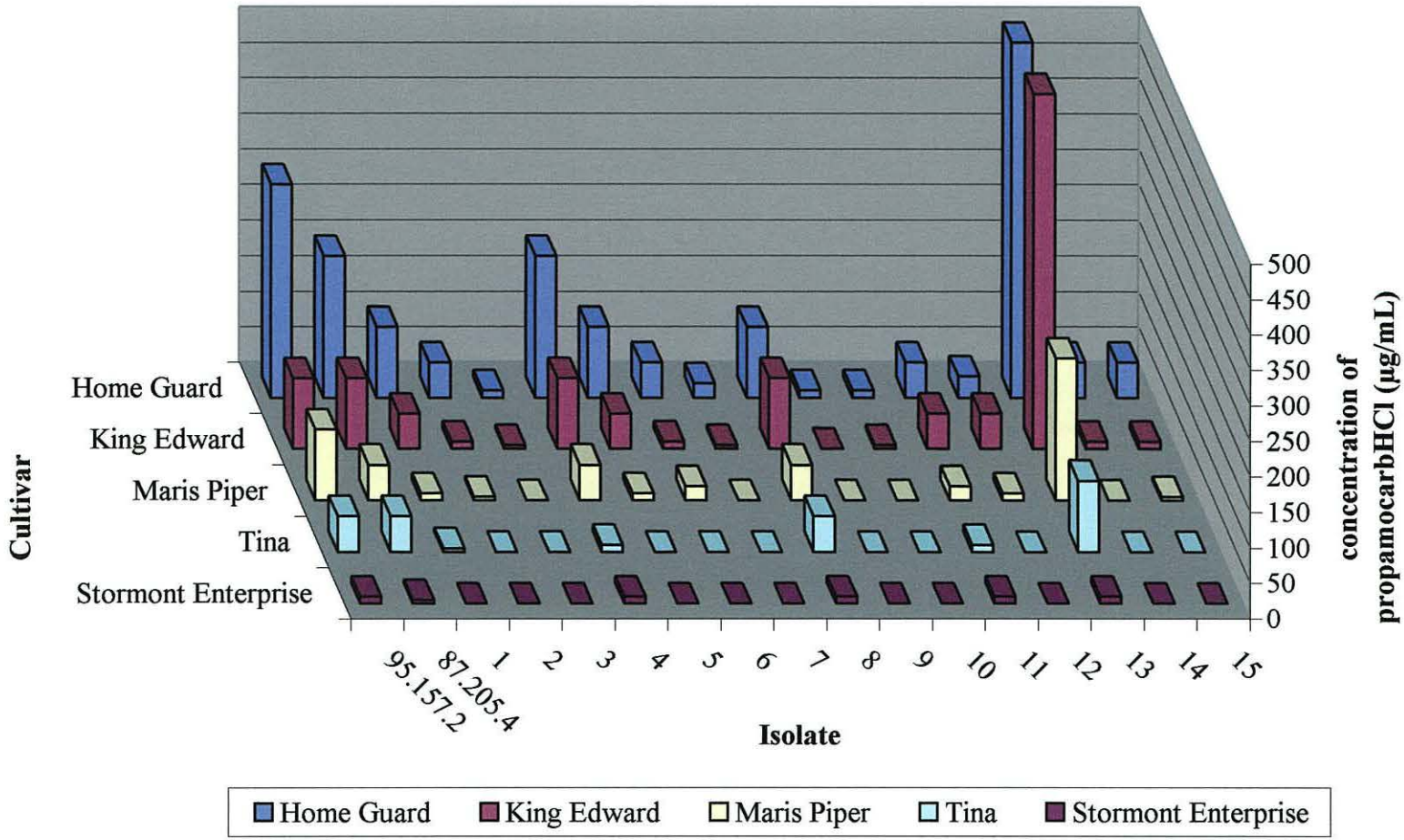


Figure 5.3 Interpolated latent period (days) of parental isolates and sexual progeny from *P. infestans* cross 1 grown on leaf disks of five different cultivars of potato floating on propamocarbHCl at three different concentrations.

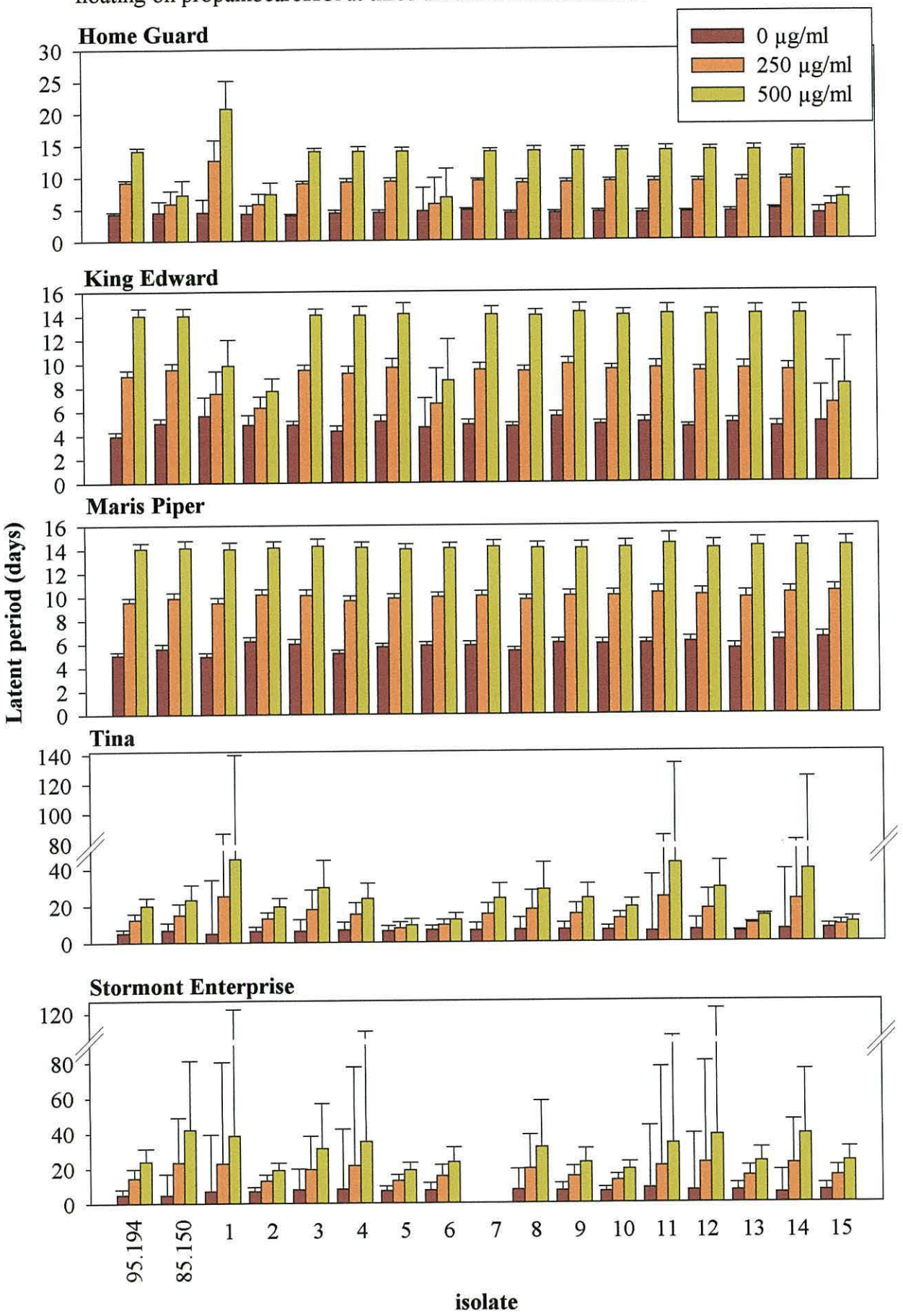


Figure 5.4 Interpolated latent period (days) of parental isolates and sexual progeny from *P. infestans* cross 2 grown on leaf disks of five different cultivars of potato floating on propamocarbHCl at three different concentrations.

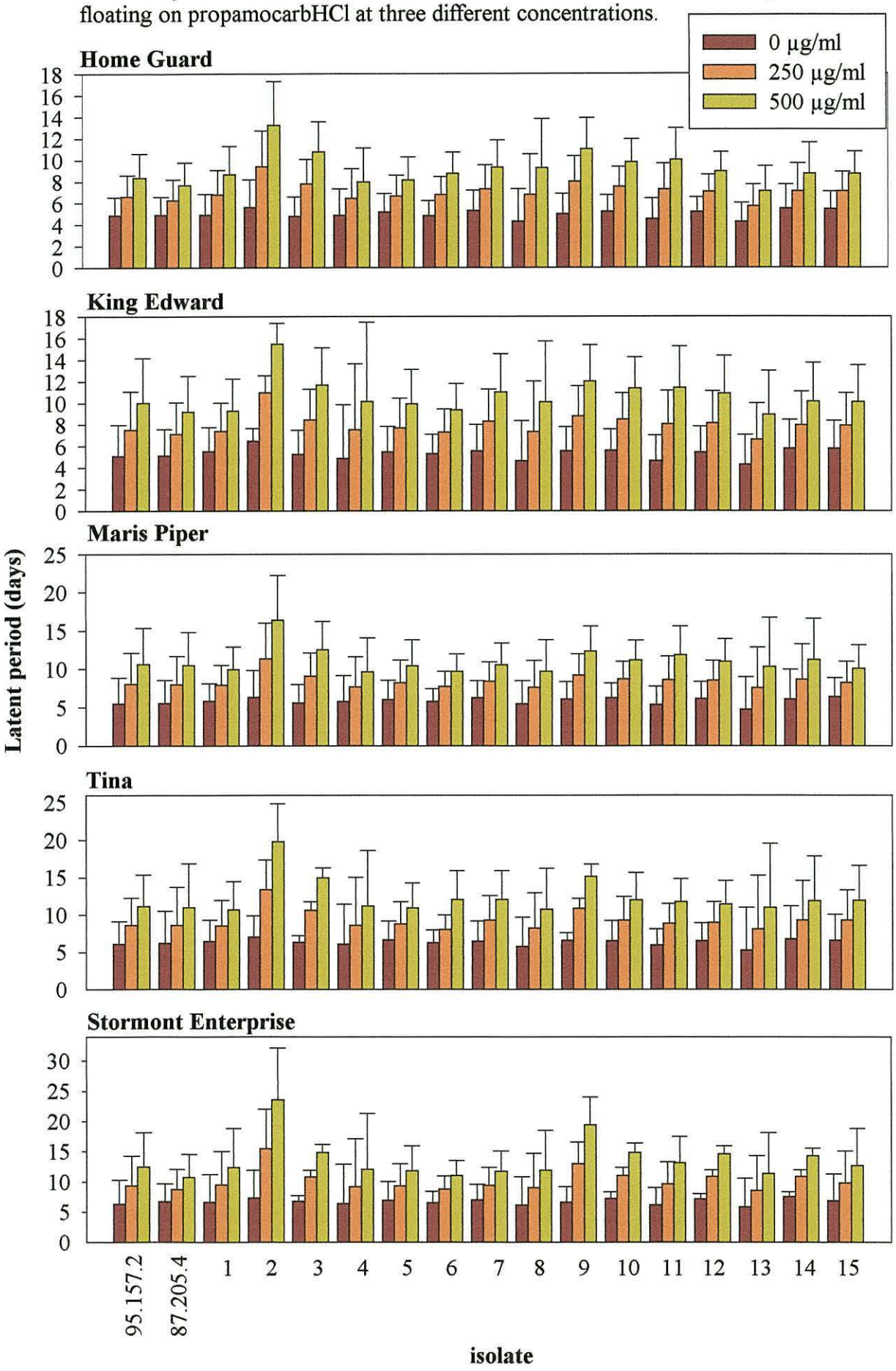


Figure 5.5 Mean lowest concentration of propamocarbHCl required to produce a score of 2 (EC_2) in *P. infestans* (cross 1) vs. mean latent period at 0 µg/ml propamocarbHCl (days), on five cultivars of potato.

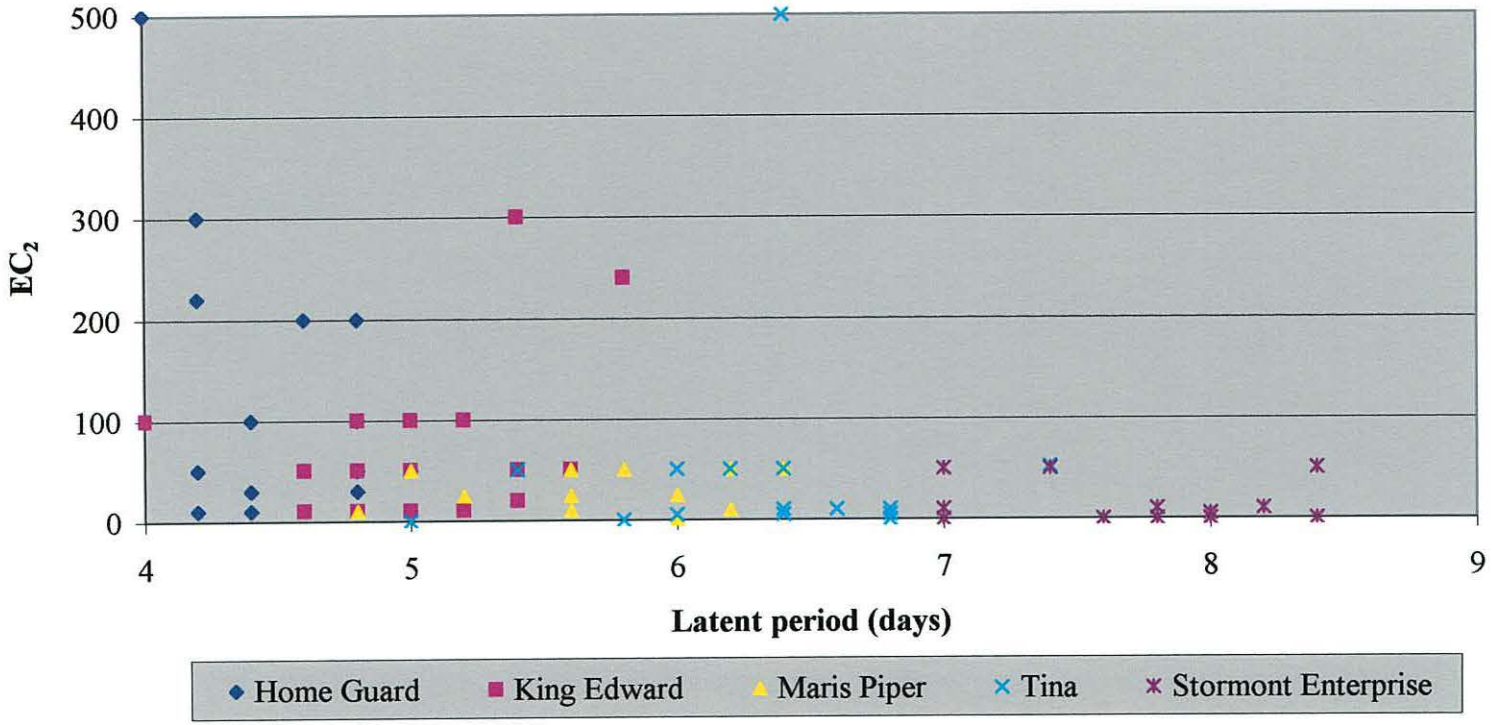


Figure 5.6 Mean lowest concentration of propamocarbHCl required to produce a score of 2 (EC_2) in *P. infestans* (cross 2) vs. mean latent period at 0 µg/ml propamocarbHCl (days), on five cultivars of potato.

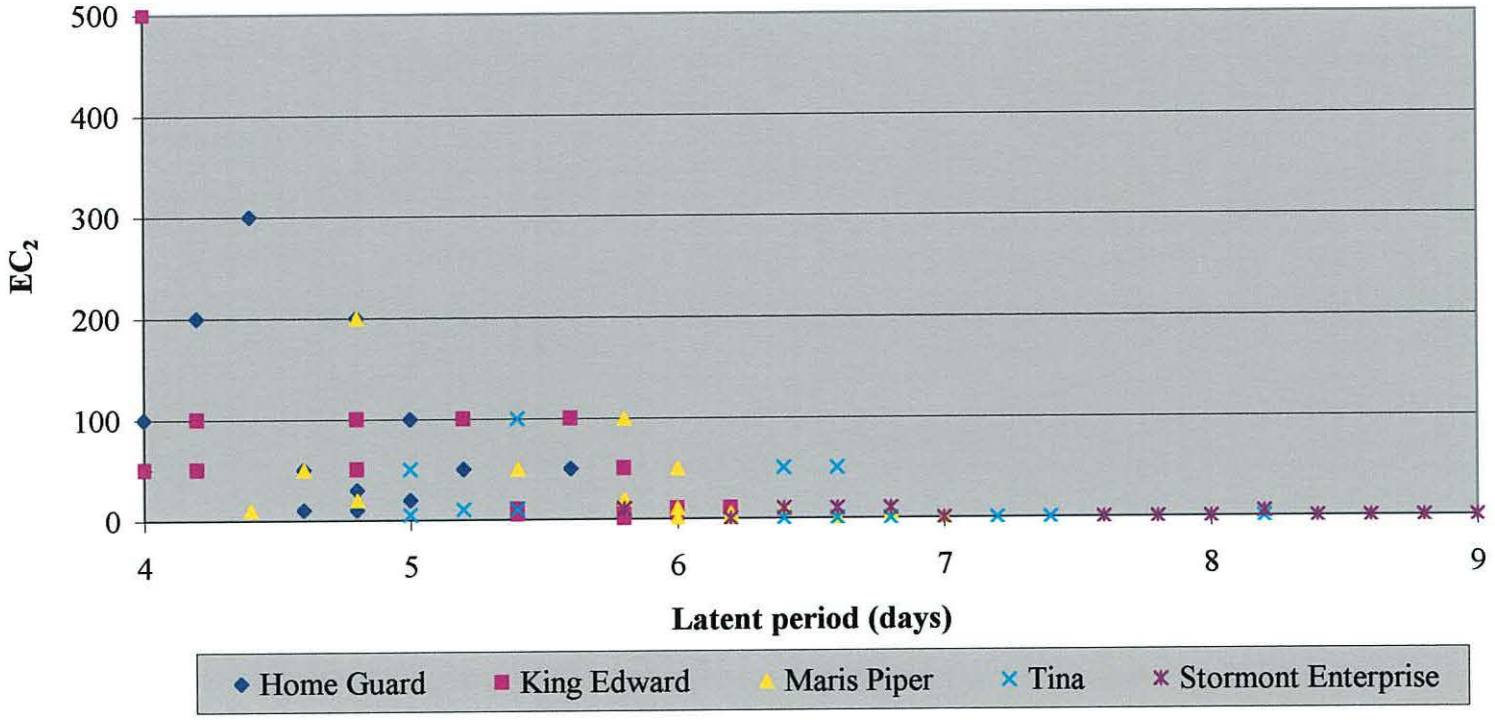


Table 5.2 Isolates used in experimentation, showing origin and metalaxyl sensitivity.

isolate	origin	mating type	metalaxyl sensitivity
95.157.2	Cambridgeshire, Maris Piper tuber	A1	resistant
95.194	Dyfed, potato leaf -cultivar unknown	A1	sensitive
85.150	Suffolk, Maris Piper tuber	A2	resistant
87.205.4	Cornwall, Pentland Hawk leaf	A2	resistant

Table 5.3 DNA fingerprints of parental isolates of *Phytophthora infestans* used to generate progeny.

Isolate	RG57 Fingerprint																							
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**	**23**	**24**	**25**
95.157.2	1	1	0	0	1	0	0	0	0	1	0	0	1	1	0	1	0	0	0	1	1	1	0	1	1
95.194	1	1	1	0	1	0	1	0	0	1	0	0	1	1	0	0	0	0	1	1	1	1	0	1	1
85.150	1	1	1	0	1	1	1	1	1	1	0	0	1	1	0	1	0	0	1	1	1	1	0	1	1
87.205.4	1	1	1	0	1	0	1	0	0	1	0	0	1	1	0	1	0	0	1	1	1	1	0	1	1

Table 5.4. Mating type, metalaxyl sensitivity and variable RG57 bands of isolates produced from cross 1 (95.194 x 85.150).

Isolate	Mating type	Mexalaxyl sensitivity	Variable bands				
			2	6	8	9	16
95.194	A1	sensitive	1	0	0	0	0
85.150	A2	resistant	1	1	1	1	1
1	A2	resistant	1	0	0	0	1
2	A1	resistant	1	0	0	0	0
3	A1	sensitive	1	1	1	0	1
4	A2	sensitive	1	1	0	0	0
5	A2	sensitive	1	1	0	1	1
6	A2	resistant	1	1	0	0	1
7	A1	resistant	0	1	0	0	0
8	A2	sensitive	1	0	0	0	1
9	A2	sensitive	1	0	0	0	1
10	A2	resistant	1	1	0	0	0
11	A1	sensitive	0	0	0	0	0
12	A1	resistant	0	0	0	0	0
13	A2	resistant	0	0	0	0	0
14	A2	resistant	1	0	1	0	0
15	A2	resistant	0	1	1	1	0

Table 5.5. Mating type, metalaxyl sensitivity and variable RG57 bands of isolates produced from cross 2 (95.157.2 x 87.205.4).

Isolate	Mating type	Mexalaxyl sensitivity	Variable bands				
			2	3	7	16	19
95.157.2	A1	resistant	1	0	0	1	0
87.205.4	A2	resistant	1	1	1	1	1
1	A2	resistant	1	1	1	1	1
2	A1	resistant	1	1	0	0	0
3	A1	sensitive	1	1	0	1	0
4	A2	sensitive	0	1	0	1	1
5	A2	sensitive	0	1	1	1	0
6	A2	resistant	0	1	1	1	0
7	A1	resistant	1	1	1	1	0
8	A2	sensitive	1	1	1	0	1
9	A2	sensitive	1	1	1	0	1
10	A1	sensitive	1	1	1	0	1
11	A1	resistant	1	1	1	1	1
12	A2	resistant	1	1	1	1	0
13	A1	sensitive	0	1	1	1	1
14	A1	resistant	0	1	1	1	1
15	A2	sensitive	1	1	1	0	1

Table 5.6 R^2 values for correlation of propamocarbHCl tolerance (EC_2 value) and latent period (days) at 0 µg/mL propamocarbHCl in cross 1 (95.194 x 85.150). Interaction between all cultivars and individual cultivars with latent period and propamocarb tolerance are shown.

Cutivar	R^2 value
All cultivars	0.1043
Home guard	0.1211
King Edward	0.119
Maris Piper	0.0016
Tina	0.0022
Stormont Enterprise	0.0167

Table 5.7 R^2 values for correlation of propamocarbHCl tolerance (EC_2 value) and latent period (days) at 0 µg/mL propamocarbHCl in cross 2 (95.157.2 x 87.205.4). Interaction between all cultivars and individual cultivars with latent period and propamocarb tolerance are shown.

Cutivar	R^2 value
All cultivars	0.2472
Home guard	0.2023
King Edward	0.3114
Maris Piper	0.225
Tina	0.1854
Stormont Enterprise	0.4597

Discussion

The latent periods observed at different concentrations of propamocarbHCl in the isolates with greater insensitivity to propamocarbHCl compared with parental isolates were not significantly different so it is unclear whether the differences were due to fitness components, specific fungicide insensitivity or a combination of these two factors. The interpolated values shown in Figures 5.3 and 5.4 were used to infer any information from the assays using the late-blight-resistant cultivars in which the level of sporulation was low. Any value for latent period over 14 days is meaningless within the experiment because the leaf disks were fully necrotic by this time.

Fry (1975) suggested that the combined effect of fungicide and late blight-resistant cultivars could be integrated so that disease might be controlled using reduced rates of fungicide. These results show that increased doses of propamocarbHCl combined with cultivars of high resistance to *P. infestans* can extend the latent period before disease symptoms become apparent. This fact is possibly more relevant to field grown potatoes where the plants are viable for much longer. These results are an indication of how doses of fungicide can be reduced when resistant cultivars are used compared with more susceptible ones.

The overall pattern in the two crosses shows that the EC_2 value decreased as latent period increased. This suggests that some variation in the level of sporulation in the presence of propamocarbHCl was due to variation in fitness and also to variation in insensitivity to the fungicide or other unrecorded factors. The degree to which these factors affected the relationship between latent period and insensitivity to propamocarbHCl varied for each of the crosses on each cultivar. Cross 2 consistently showed higher R^2 values suggesting that fitness was more important in insensitivity to propamocarbHCl in these isolates. Although care was taken to remove environmental variation from these experiments, it was possible that cross 1 was subjected to slightly different conditions than cross 2, which

could have masked the fitness and propamocarbHCl insensitivity components that were tested.

After the initial analysis of this study, Isolate 1 of cross two was found to be of identical genotype, based on the markers used, to parental isolate 87.205.4. This does not mean that isolate 1 is not a recombinant. It is possible for F_1 isolates to exhibit the same phenotype and fingerprint pattern as parental isolates. This isolate did show differences from the parental isolates when the lowest concentration of propamocarbHCl required to give a score of 2 was determined. For this reason it was decided to use this isolate in the analysis as if it was a recombinant. However, if this had been detected during the isolate selection procedure then it would have been discarded because it could not be confirmed, with the markers used here, as a recombinant. Many other isolates were discarded for this reason. It is possible that some of these were recombinants, but based on the markers used, this was not detected.

The degree of insensitivity to propamocarbHCl seen in the F_1 isolates of both crosses was variable over a range of values rather than simply exhibiting complete insensitivity or complete sensitivity. This suggested that propamocarbHCl may have a multi-site mode of action and that in order for a particular isolate to have an increased level of insensitivity compared with a parental isolate, a combination of genes is required. None of the F_1 isolates tested were fully resistant to propamocarbHCl but only had variation in the level of insensitivity observed compared to other F_1 isolates and the parental isolates. Such fully resistant isolates have not yet been detected, but it would be essential for future studies into the inheritance of insensitivity/resistance to propamocarbHCl to obtain such isolates either from the field or to produce mutants. One obstacle in producing resistant isolates through the production of mutants is the fact that *P. infestans* and propamocarbHCl make working *in vitro* difficult. *P. infestans* grows poorly in artificial, defined media and the fungicide works poorly in natural media such as

rye A agar due to its pH dependency (Guest, 1984). Attempts were made during these studies to grow *P. infestans* in defined media, but were unsuccessful.

If the environmental conditions were the same in the tests for both of the crosses it can be assumed that more than one gene is involved in the inheritance of propamocarbHCl insensitivity, although statistical error may have interfered in this experiment because of the relatively small numbers of isolates tested and the high level of variation seen in the latent periods. Nevertheless, a simple one-gene two-allele system can produce a continuous distribution of phenotypes if the genotypic means are small compared to environmental variation (Griffiths *et al.*, 1993). It is possible that this is the case here with little variation being of a genetic nature. Fitness is an important factor in the insensitivity of a particular isolate to propamocarbHCl, but also other factors not tested probably play a role. These may include such factors as insensitivity of *P. infestans* to the inhibition of phospholipid synthesis (Reiter, 1994) and the permeability of cell membranes.

In order for this method to be of use in the field the assay should be repeated with a wide range of non-related field isolates to determine the effect of reduced rates of propamocarbHCl in combination with resistant cultivars. This method may be of use in identifying useful cultivar/fungicide concentration combinations before more extensive field experiments are undertaken. The use of latent periods may be of more use to growers than other measures of disease severity such as final disease ratings. In severe blight seasons most crops will suffer from late blight infection towards the end of a season no matter what fungicide regimes are used.

It can be seen that increased level of insensitivity to propamocarbHCl can be achieved through sexual reproduction. The implications of this are important to the potato-growing industry because of the occurrence of metalaxyl resistance in the 1980s which is more dependant on fungicides such as propamocarbHCl (Dowley & O'Sullivan, 1981). It is possible that strains that are fully resistant to propamocarbHCl will eventually appear in the field, although propamocarbHCl

has long been used on several different crops to combat many different pathogens without resistance problems. One realistic problem for propamocarbHCl may be an indirect one. It has been shown that there is correlation between propamocarbHCl insensitivity and fitness. This fact may result in the fungicide adding to natural selection pressure and facilitate the development of more aggressive and fit strains.

Conclusions

- When propamocarbHCl is used together with cultivars with some resistance to *P. infestans* the latent period before disease symptoms occur can be increased.
- It is possible that propamocarbHCl has a multi-site mode of action.
- Sexual reproduction can produce increased insensitivity to propamocarbHCl.
- Complete insensitivity to propamocarbHCl was not detected in *P. infestans*.

Chapter 6

General Discussion

It has been shown in this thesis that oospores of *P. infestans* were able to form in high frequency in the leaves of pot-grown potato plants when A1 and A2 isolates originated from different foci. It is possible that the same trend might be seen in the field, but on a much larger scale with oospores forming in a field where A1 and A2 strains are present together. The frequency of oospore formation may be lower in the field because environmental conditions are not stable and so only sporadically optimal for disease proliferation, and as a result sporadically optimal for oospore formation. Whether oospores can form in the field in high numbers, under natural conditions is unknown.

It has been shown in this study that oospores were produced *in vitro* and germinated to produce colonies on an agar plates. Also it was shown that oospores were produced *in vivo*, when parental isolates were allowed to spread through a potato plot naturally. The problem lies with producing oospores *in vivo* that then germinate to produce pathogenic colonies. Until this is achieved, it is difficult to surmise that this occurs in nature outside Mexico.

The oospore is important in the survival and proliferation of disease in several other phytopathogenic oomycetes including *Plasmopara viticola* (oospores overwinter in the soil in grape vine leaves), *Phytophthora megasperma* (long-term survival is facilitated by oospores), plus many *Pythium* species including *Pythium ultimum* (the oospore is the major mode of survival in most species of *Pythium*) (Smith *et al.*, 1988).

The oospore is important in other species of phytopathogenic oomycetes and oospores of *P. infestans* may form and over-winter in the field outside of Mexico. The question why oospores are scarce in European potato crops arises. The effects

of sexual reproduction on the nature of late-blight in the field have not been determined (Drenth *et al.*, 1993; Flier & Turkensteen, 1999). Many factors, such as the use of fungicides and end-of-season herbicides, are present in the commercial fields of Europe, and indeed the rest of the world that stand in the way of the oospore of *P. infestans* becoming a viable survival propagule and a threat to modern agriculture, sufficiently for financial losses to be so great that action is needed.

It has already been stated above that the oospore may be deficient in some way, or the right conditions may be lacking, making formation, survival and then symptoms of disease unlikely. Diamond (1999) calls this the Anna Karenina principal, where he quotes Tolstoy's novel, "Happy families are all alike; every unhappy family is unhappy in its own way." This principal is applied to animal domestication, but can also be applied to pathogenic propagules. In order for a plant pathogenic propagule to be successful it must fulfil the requirements that are seen in all other successful pathogens. It must be able to infect the host, survive a dormant period where the host is not available (whether this be through an alternative host, volunteers or by being soil-borne) and produce viable and pathogenic propagules in sufficient number to maintain the life-cycle. With a failure in any one of these stages the pathogen will not persist. Is this the case with the oospore of *P. infestans*? With this question in mind it is easier to identify some possible reasons for oospores of *P. infestans* not proliferating in much of the world, but being successful in others.

It is possible that there has not been enough time since the introduction of the A2 mating type into Europe for large numbers of oospores to build up in the soil. If the A2 did arrive in Western Europe in the late 1970s as suggested (Niederhauser, 1991), then the total number of seasons that A1 and A2 mating types have had to combine sexually in the field is limited due to the use of crop rotation in most regions. Seed potatoes are grown under a 7-year regime in the UK, whilst it is common to grow ware potatoes in a 4 or 5 year cycle in order to reduce the risk of

build-up of potato pests (Lutman, 1992). Under these circumstances a maximum of between 3 and 5 seasons over the last 20 years in a particular commercial field would have been available for oospores to form. It is likely that this time scale was not sufficient for oospores to reach a high enough frequency in the soil for their effects to be detected. If the low frequency of the A2 mating type in the UK (Day & Shattock, 1997) was also taken into consideration then the chance of oospores forming, surviving a winter period and then infecting a potato plant is even more remote.

There has been a recent trend to shorten crop rotations and to grow potatoes more often in favoured fields. In some areas, particularly in poorer countries, such short rotations can involve potatoes being planted in the same field on an annual basis, it is here where the effects of oospores may become apparent first (Franc *et al.*, 1994; Moodie, 1999). One such place is Jersey, where potatoes have been grown in the same field for many years, and where the A2 mating type has recently been detected, but so far only on tomato plants (J. P. Day, pers. comm.). Short crop rotations, and indeed the trend to grow potatoes in consecutive years in the same field, are well established in Holland where the A2 mating type has been present for many years (Drenth *et al.*, 1994; Goodwin & Drenth, 1997). It should therefore be of no surprise that it is in this region that the highest level of diversity outside of Mexico has been detected (Zwankhuizen *et al.*, 2000). The level of diversity and the population structure in neighbouring France is very different from The Netherlands despite the close proximity of the two countries (Lebreton & Andrivon, 1998; Flier & Turkensteen, 1999).

Understanding the reasons for the success of *P. infestans* in the Toluca Valley region of Mexico in sustaining a population that has A1 and A2 isolates in equal ratio, as well as producing viable oospores that are able to affect the population diversity, is important in understanding whether oospores will become economically important in the rest of the world (Jaime-Garcia *et al.*, 2001; Flier *et al.*, 2001).

The Toluca Valley area Mexico remains the region of the world with highest level of diversity in *P. infestans,* determined by both phenotypic and genotypic markers. Obviously there must be a reason for this - sexual reproduction occurs and oospores are viable and are able to, either directly or indirectly, cause lesions in host plants and ultimately cause disease.

The most obvious difference between the Toluca Valley and most other potato-growing regions is geographic. The Toluca Valley is at high altitude, 2600m, with potatoes grown up to 3500m (Grünwald *et al.*, 2000), but this alone cannot explain why sexual reproduction is more successful there as potatoes are grow in other regions at high altitude and there is no known correlation with altitude and oospore formation.

The other geographic feature that sets the Toluca Valley apart is latitude. The Toluca Valley lies at 19°N, a latitude only shared by North Africa, Arabia, Northern India, Burma, Thailand, Laos, Vietnam and Hawaii. Latitude has two characteristics that may have a bearing on the life cycle of *P. infestans*.

At latitude of 19°N the intensity of light will be much higher than in more northerly regions. Low latitude also has the effect of reducing the differences in day length caused by the seasons. The summers will have shorter days than more northerly regions and the winters will have longer days. This could have a significant bearing on the life cycle of *P. infestans*, but the effect of photoperiod on the overall life cycle has not yet been satisfactorily determined.

Germination of oospores of *P. infestans* has been shown to be increased by the presence of light. Chang and Ko (1991) achieved a 70% germination rate after a 20-day incubation under lights. Light has been shown to inhibit the formation of oospores in species of *Phytophthora*, whilst inducing sporangial formation (Harnish, 1965; Brasier, 1969). However, Cohen *et al.* (1997) found that light had

little effect on formation of oospores of *P. infestans* in leaves of potato and tomato when initial lesions were already established.

The importance of light in the life cycle of *P. infestans* has been studied, but these studies are disparate, often using different photoperiods, intensities and wavelengths. In experiments, such as those in this thesis, the significance of light is often considered only as a means to keep plant material alive long enough to complete the assay and the exact effect on the pathogen, independent of the plant is overlooked. This lack of consideration of light in experiments may account for differences observed in formation, survival and germination rates of oospores between and within species of *Phytophthora*. Studies in *P. infestans* comparing the effects of light intensity and photoperiod directly are needed, and indeed comparisons between *P. infestans* and other species are needed.

It is insufficient to state that light stimulates oospore germination in one species whilst inhibiting it in another when the experiments have not been carried out concurrently. Without specialized equipment, light intensity is difficult to determine. Fluorescent tubes and light bulbs do not produce the same level of light throughout their lifetime; output drops off significantly after a short time. What effect does this have on *Phytophthora* grown *in vitro*? If the studies are to determine the effect that light has on sexual and asexual reproduction in species of *Phytophthora* it is also insufficient to rely on information supplied by the vendor because there is no way to check that their quality control is adequate.

If it is determined that photoperiod and light intensity are important in the life cycle of *P. infestans* and other species of *Phytophthora,* then what might the effect on the epidemiology of late-blight around the world be?

At latitudes close to that of the Toluca Valley it would be expected that *P. infestans* would respond in a similar way, providing that all other variables are equal. For instance, in Israel, where much work has been done on *P. infestans* it

would be expected that oospores would form (given the opportunity) and germinate generating genetic diversity at a higher frequency than seen in northern Europe. Cohen *et al.* (2000) had success in producing oospores in the field in Israel (approximately 32°N) during two winter seasons. It is possible that the success of this study is partly due to geography. A short day length (or even a long period of darkness) may have contributed to the success in producing oospores in the field. Additionally this same factor may have contributed to the success of the experiment seen in Chapter 3 of this thesis where abundant oospores were produced.

The experiment seen in Chapter 3 was conducted in September and October (around equinox), when day length is approximately 12 hours (comparable to day length in Mexico), a time when potatoes are not usually grown in the UK. Normal main-crop (those likely to be infected by late-blight) potatoes are planted in April and the crop canopy closes some time in June. It is only after this that late-blight becomes a risk and fungicides are applied. This coincides with the longest day of the year, with the possibility of oospore formation and germination occurring in the following 8-10 weeks.

This may be the pattern seen at high latitudes, but other factors including fungicide treatments and the use of late-blight-resistant cultivars are likely to be more important in facilitating or inhibiting the formation and germination of oospores within the conditions and regimes that potatoes are grown in and cultivated under.

Another difference between the regime in Mexico and the rest of the world is that different cultivars are grown. This is the case between other regions, such as between the US and UK (there are few cultivars grown in both of these two countries). Mexico operates a national breeding program that produces cultivars suitable for the local conditions (Grünwald *et al.*, 2000).

Studies in the past have concentrated on race non-specific resistance to late-blight as a means to compare oospore formation in different cultivars (Chapter 5, this thesis; Hanson & Shattock, 1998a). These studies are valid in determining the likelihood of oospores forming in field grown potatoes of a particular variety, but general conclusions cannot be reliably drawn from the results to predict how other cultivars may affect oospore formation. These studies cannot be used to infer an explanation for the apparent success of *P. infestans* in producing oospores in Mexico, because many other factors, measurable and non-measurable have a role in determining the likelihood of the host facilitating oospore formation. In short, no two cultivars can be compared by any means other than by direct, simultaneous assay. This is problematic because experience of cultivars grown in Mexico is primarily found in Mexico, the same applying to each country; the availability of cultivars from other countries is also limited. How a given cultivar will precisely respond to new conditions is unknown, thus work obtained by moving a cultivar to a different geographic region may be unreliable.

The role of fungicides in reducing the likelihood of oospore build up in the soil is paramount. As fungicides have the ability to reduce the amount of disease in a potato crop, so they are able to restrict the opportunities for A1 and A2 mating types to come into contact and therefore reduce the number of oospores formed in the field. It has been demonstrated that propamocarbHCl and other fungicides including metalaxyl and dimethomorph have the ability to interfere with oospore formation at concentrations where asexual sporulation is possible (Chapter 3; Bissbort *et al.*, 1997; Hansen & Shattock 1998b). It is likely that other fungicides that have not been tested also have the ability to affect oosporogenesis at low doses where asexual sporulation is seen.

The effect on the development of *P. infestans* and on the formation of oospores by other chemicals used in routine potato crop production, and indeed used in fields where potatoes are not being grown, but do form part of the crop rotation should not be overlooked. It has been found (Wilcox, 1996) that the dinitroanaline

herbicides oryzalin and pendimethalin can protect against crown rot of Mahaleb cherry seedlings caused by *Phytophthora cryptogea*, *P. cambivora* and *P. megasperma* when applied at non-phytotoxic rates. When tested *in vitro* at a concentration of 0.25µg/ml oryzalin (equivalent to the concentration used in the field) sporangia formation was inhibited by up to 97%. Similar results were found with pendimethalin.

It is not surprising that herbicides should have an effect on species of *Phytophthora* as they are related to the chrysophytes (golden-brown algae) (Gunderson *et al.*, 1987), which are in turn related to plants. It would be of great interest to ascertain whether such herbicides and indeed other chemicals used in potato husbandry have an inhibitory effect on the development *P. infestans*, in particular on the formation of the sexual stage and survival and germination of oospores. It is common for potato crops to be treated at the end of season with either a broad-spectrum herbicide or sulphuric acid to desiccate the crop before harvest. If oospores are contained in leaves that are treated with these chemicals it seems likely that their survival would be compromised because of the toxic and caustic effects of these compounds.

With pressures against the formation and survival of oospores in commercial fields any development of new genotypes appears unlikely over short time scales. Possibly the best place to look for any population changes is in areas where fungicides are not used and potatoes are grown in the same plot every year. The main candidate for this is garden allotments where there is little use of chemicals and non-certified seed is often planted. A relatively new source of sexual inoculum could be brought about by the cultivation of increasingly popular organic crops. Organic crops account for 3-4% of all food in British supermarkets (Anon, 2000a). Although some chemicals such as Bordeaux mixture are permitted for use in organic crops (Anon, 2000b), the overall control of late-blight is less than in conventional crops, so the chance of sexual reproduction would be expected to be higher.

If the sexual stage of *P. infestans* does become established in the UK and in other countries outside of Mexico there would be a gradual build up in genetic diversity that would not necessarily be exhibited in current populations. This can be compared to seed banks, where diversity is stored in seeds in the ground over long periods, taking advantage of, and maximising, variation when conditions change (Salisbury & Ross, 1992). The age and history component of large numbers of oospores in the soil may influence the regenerative ability of late-blight. If such a mechanism had been in place before the migration of the new population into Europe in the 1970s then old, displaced populations may have survived the invasion of new genotypes and may have re-occurred at a detectable frequency at a later date. The diversity stored in such an oospore-bank would favour the development of resistance to any new chemicals and late-blight resistant varieties of potato by allowing the *P. infestans* population to respond quickly to new selection pressures.

If an oospore bank does become established then this could lead to problems with early disease being difficult to control. The effect of most fungicides currently available to farmers is preventative, rather than curative, so once late-blight symptoms are observed in a crop, it is very difficult to eradicate (Erwin & Ribeiro, 1996). Disease outbreaks before first spray, when the crop canopy begins to close, cannot be predicted easily. Current models such as blitecast (Karause, *et al.* 1975; Fry *et al.* 1983) do not account for oospores causing early outbreaks of disease and therefore would need to be modified.

Longevity of individual oospores will ultimately determine the level of oospores in the soil. It is important to determine the longevity of oospores in different soil types and in different geographic locations in order to be able to control blight comprehensively. It may become more important, than is currently the case, to control volunteer plants in order to prevent disease spreading from a field where oospores are present, to a young crop in a neighbouring field.

The role of the oospore in the population dynamics of *P. infestans* may rely on selection pressure reducing the efficacy of asexual reproduction, resulting in isolates capable of reproducing sexually. This could be brought about by late-blight becoming marginalized, with few sporadic outbreaks and the removal of all volunteers. The pathogen would be forced into a situation where the only means of over-wintering was through the oospore. This is unlikely to ever occur. However, as fungicides improve in their efficacy against late-blight and crop husbandry improves, it may result in unwanted side effects.

For a population of *P. infestans* to be capable of reproducing sexually both A1 and A2 strains must be present. This is an obvious statement, but nevertheless important. Once sexual reproduction occurs and pathogenic progeny are produced, there is the opportunity for more A2 phenotypes (along with more A1 phenotypes) to survive. Whether or not the A2 phenotypes can out-compete or co-exist with the dominant A1 phenotypes is a different question entirely. What is clear is that, with every generation, new A2 genotypes will be produced, so increasing the probability of more oospores being formed and yet more A2 phenotypes generated. In suitable conditions this accumulation may take many seasons, with an initial lag phase as seen in all populations. If A2 genotypes are to become established outside of Mexico then many factors, as outlined above, will be important in determining this outcome.

Exactly how oospores will affect the production of potatoes in this country will not be known until fields are found with a well-established oospore population. Any long-term or seasonal effects on oospore germination cannot be readily investigated. Any studies on commercial fields to look for the impact of oospores should be undertaken in regions where short rotations are used. This may prove to be one of the most important factors along with variety of potato, geographic location, soil type and fungicide treatments in governing of the nature of the sexual stage of *P. infestans*.

Appendix 1

Growth media

Rye A agar (Caten & Jinks, 1968)

Rye grains (60 g) were soaked in deionized for 36 hours at 18°C. The grains were then removed from the supernatant (retained) and macerated in a Wareing blender until 90% damaged. The resulting pulp was then incubated at 50°C for 3h and then filtered through muslin. This filtrate was then added to the supernatant and made up to 1 L with deionized water. Sucrose (20 g) and 1.5% agar was added and this was autoclaved for 15 mins at 1.08 kg cm^{-2}.

Antibiotic amendment (Day & Shattock, 1997)

Stock antibiotic solution (RAN) was made as follows: 250 mg rifamycin, 200 mg ampicillin and 500 mg nystatin were dissolved in 10 mL dimethyl sulphoxide (DMSO). 2 mL of this were then added to 1L molten agar.

Pea broth (Pipe *et al.*, 2000)

300g of frozen peas were boiled in distilled water for 10min. and autoclaved for 15 mins at 1.08 kg cm^{-2}. Two hundred and fifty µL RAN antibiotic stock solution was then added to each 500 mL of broth.

Soft water agar

Plain agar (Sigma-Aldrich Company Ltd., Gillingham, Dorset, SP8 4XT, UK.) (7.5 g) was added to 500 mL deionized water and autoclaved for 15 mins at 1.08 kg cm^{-2}.

Appendix 2

Molecular biology reagents (for further details see Sambrook and Russell, 2001)

Promega 10 x amplification buffer (supplied with Taq DNA polymerase)

500 mM KCl; 100 mM Tris HCl, pH 9.0; 1% Triton X-100.

dNTP dilutions

dNTPs supplied at 100 mM and diluted to 25 mM for each dNTP by mixing equal volumes of dNTPs together. For the PCR reaction, 480 µL SDW was added to 20 µl of dNTPs to give a final concentration of 1 mM for each dNTP.

DNA extraction buffer (200 mM Tris HCl, 250 mM NaCl, 25 mM EDTA, 0.5% SDS)

50 mL 1M Tris HCl, pH 8.5
12.5 mL 5M NaCl
12.5 mL 0.5M EDTA, pH8.0
12.5 mL 10% SDS
Made up to 250 mL with deionized water and autoclaved 15 mins at 1.08 kg cm^{-2}.

Phenol

Equilibrated phenol used, containing 0.1% hydroxyquinoline and stored under 10 mM Tris HCl, pH 8.0.

RNAase A (10 mg/mL)

10mg RNAase A prepared in 1 mL of 10 mM Tris HCl, 15 mM NaCl; pH 7.5, boiled for 10 min., dispensed into 500 µL aliquots and stored at -20°C.

TE buffer (10mM Tris HCl, 1 mM EDTA; pH 8.0)

1 mL 1 M Tris HCl, pH 8.0
200µL 0.5 M EDTA, pH 8.0
Made up to 100 mL with deionized water and sterilised by autoclaving.

50 x TAE (2 M Tris HCl, 50 mM EDTA, pH 8.0)

121.1 g Tris
28.55 mL Glacial acetic acid
50 mL 0.5 M EDTA, pH 8.0
Made up to 500 mL with deionized water.

Bromophenol blue gel loading buffer (0.25% bromophenol blue, 40% sucrose)

0.125 g bromophenol blue
20 g Sucrose
50 mL Deionized water
Filter sterilised through a sterile 0.22 µM filter unit and stored at 4°C.

Blotting buffer 1 (0.25 M HCl)

3.86 mL HCl in 500 mL deionized water.

Blotting buffer 2 (1.5 M NaCl, 0.5 M NaOH)

43.8 g NaCl
10.0 g NaOH

Made up to 500 mL with deionized water.

Blotting buffer 3 (1 M Tris HCl, 2 M NaCl; pH 7.2)

60.5 g Tris
58.0 g NaCl
450 mL deionized water added and the pH adjusted 7.2 with concentrated HCl, and made up to 500 mL with deionized water.

20 x SSC. (3M NaCl, 0.3M NaCitrate; pH 7.0)

87.6 g NaCl
44.1 g Sodium Citrate
Made up to 500 mL with deionized water and adjusted to pH 7.0 with concentrated HCl.

Antibody wash buffer 1

5.8 g Maleic acid
4.4 g NaCl
4.0 g NaOH
450 mL deionized water
Adjusted to pH 7.5 with concentrated HCl, made up to 500 mL with deionized water and 1.5 g Tween 20 added.

Antibody incubation buffer

5.8 g Maleic acid
4.4 g NaCl
4.0 g NaOH
450 mL Deionized water

Adjusted to pH 7.5 with concentrated. HCl and made up to 500 mL with deionized water. Prior to use 2.0 g Blocking reagent was added to 100 mL Antibody incubation buffer and dissolved by microwaving gently for 5-10 min.

Detection buffer

6.1 g Tris HCl

2.9 g NaCl

5.1 g $MgCl_2.6H_20$

450 mL deionized water

Adjusted to pH 9.5 with concentrated HCl and made up to 500 mL with deionized water.

Pre-hybridisation solution

25.0 mL 20 x SSC

2.0 g Blocking reagent

1.0 mL 10% N-lauroylsarcosine

2.0 mL10% SDS

75.0 mL Deionized water.

Heated gently in microwave at low power for 5-10 min.

Hybridisation solution.

50 mL pre-hybridising solution prepared and freshly denatured labelled probe DNA added.

Hybridisation wash buffer 1

50 mL 20 x SSC

5 mL 10% SDS

Made up to 500 mL with deionized water.

Hybridisation wash buffer 2

2.5 mL 20 x SSC

5 mL 10% SDS

Made up to 500 mL with deionized water.

Developer solution

100 mL developer

400 mL tap water

Stop solution

A few drops of concentrated. acetic acid placed into fixer tray, topped up with tap water.

Fixer solution

100 mL fixer

400 mL tap water

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