On the evolution of pesticide resistance in *Phytophthora infestans*

an experimental evolution approach

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Contents

Thesis abstract

Resistance to antimicrobial agents is a serious problem for both medicine and agriculture. The initial success of such toxins is due to absence of resistant genotypes in pathogen populations before treatment. The initial low frequency of resistance may be explained by negative pleiotropic effects of the resistance mutations on fitness in the absence of the toxin. However, when resistant and sensitive natural isolates are compared, no cost of resistance may often be seen. This high fitness of resistant genotypes is explained by the existence of compensatory mutations, i.e. mutations that ameliorate the negative effect of the initial resistance mutation. The accumulation of these compensatory mutations can only occur at a sufficient rate if the population of resistant genotypes is large, which in part depends on the pesticide use. As a result, resistance may be stably maintained in a population after pesticide treatment, whereas it was absent before pesticide treatment.

Resistance to the phenylamide pesticide metalaxyl is common in populations of *Phytophthora infestans*, the oomycete that causes the late blight disease in potato. This resistance evolved within a few years after metalaxyl became commercially available in 1977. Such fast evolution of pesticide resistance may present a serious threat for potato production. However, fast evolution of resistance to other pesticides has not been described in *P. infestans*, whereas resistance to metalaxyl and other pesticides are common in other oomycetes.

In the absence of metalaxyl, resistant isolates of *P. infestans* have a higher fitness during epidemics compared to sensitive isolates. This observation has led to the assumption that metalaxyl resistance has a direct positive effect on epidemic fitness. A reduction of the survival during the winter has been put forward as an explanation for the absence of resistance before the commercial release of metalaxyl. However, given a high mutation rate towards metalaxyl resistance, a direct positive effect of metalaxyl resistance would always lead to a high frequency of resistance, even in the absence of metalaxyl.

In this thesis, we report on an experimental evolution approach to investigate the fast evolution and apparent stability of metalaxyl resistance in *P. infestans*. We used an experimental evolution procedure because it has several advantages. First, it enables a detailed inspection of the trajectory of evolutionary changes (e.g. in resistance phenotype). Second, it enables comparisons of fitness components between near-isogenic genotypes that only differ in their sensitivity to metalaxyl, whereas conclusions based on comparisons of field isolates and offspring from sexual crosses are hampered by differences in the genetic

background.

Two experiments were performed to investigate the rate of evolution of resistance to a number of pesticides. First, we used a fluctuation test to estimate the mutation rate towards resistance against metalaxyl, fluazinam and cyazofamid. This fluctuation test, however, was not suitable to estimate the mutation rate in *P. infestans*, due to background growth of sensitive genotypes for metalaxyl and due to the low amount of zoospores that can be obtained from a single colony for the other two pesticides. Second, we analyzed the response of selection on low pesticide concentrations. Serial transfer of zoospores on a constant low concentration of metalaxyl resulted in full resistance. The same procedure on a low concentration of cyazofamid with a similar effect on growth rate, however, did not result in full resistance.

The genotypes that emerged after the selection procedure were compared with the sensitive ancestor with respect to a number of fitness components in the absence of metalaxyl. This approach did not reveal a cost of resistance. On the contrary, resistant genotypes showed a higher *in vitro* fitness compared to their sensitive ancestor. However, the results from a population survey during a nation-wide ban of metalaxyl containing pesticides in the Netherlands were suggestive of a negative effect of resistance on winter survival and on epidemic fitness. These results suggest that the relative high frequency of metalaxyl resistant isolates on untreated fields can only be explained by assuming that metalaxyl resistance has an initial cost that is followed by compensatory mutations that only restores epidemic fitness. A new selection procedure that included serial transfer of zoospores, revealed a small initial cost on *in vitro* performance by increasing the lag phase before the start of growth. Subsequent compensatory evolution under the selection conditions could then explain the absence of a cost of resistance in previous experiments. However, no such compensatory evolution was directly observed in this latter selection procedure.

The results presented in this thesis suggest that metalaxyl resistance is selected on a wide range of concentrations, whereas this is not the case for (some) other pesticides. This may explain the relative fast evolution of resistance to metalaxyl relative to that for other pesticides. Additionally, the absence of resistance before the commercial release of metalaxyl can be explained by a negative effect due to delaying growth. Finally, the methodology of experimental evolution may be a valuable addition to the multitude of methods used to study the population dynamics of this important pathogen.

Chapter 1

General Introduction

Stefan Bosmans

Historical account on potato late blight

One of the most devastating plant pathogens is *Phytophthora infestans*, the causal agent of the late blight disease in potato and tomato. This pathogen became instantaneously famous when it ran havoc among potato fields in Ireland between 1840 and 1850. Partially because potato was the prime food source for poor Irish people, this outbreak resulted in a famine that killed over one million people and caused a mass migration of people from Ireland to other parts of the world (Scholthof, 2007). Nowadays, this pathogen is still a threat to world-wide potato production and can only be controlled by the largest amounts of chemical pesticides used in agriculture to protect crops (Niederhauser, 1993),

The first identification of *P. infestans* as a pathogen dates back to the 1860's when Anthony de Bary showed that it was the cause of the disease affecting potato and not a secondary infection (Kelman and Peterson, 2002). Although the knowledge on its biology increased gradually in those early studies, *P. infestans* received less attention from mycologists than model ascomycetes and basidiomycetes, such as *Neurospora crassa* and *Schizophylum commune* (Judelson, 1997b). This lack of attention was primarily caused by the difficulties to work with this biotrophic organism in the laboratory. However, the recent advances in molecular biology have sparked new research, which radically changed our view on the biology of this organism. A brief overview of these old and new insights in the biology of *P. infestans* will be presented in this chapter.

Basic biology of *Phytophthora infestans*

The success of a pathogen is largely determined by its ability to spread from host to host. *P. infestans* is able to do this by producing vast numbers of asexual spores that are dispersed by wind or water. The spread of the pathogen by means of these asexual spores is especially efficient on humid days (Porter and Johnson, 2004) when the temperature is not too high (Mizubuti and Fry, 1998) and the sun does not shine too vigorously (Mizubuti et al., 2000). Under these circumstances, a single infection can kill a field of previously healthy potato plants within a few weeks (Fry, 2008).

Asexual reproduction

Asexual reproduction starts when sporangiophores emerge through the stomata of infected tissue. These sporagiophores carry lemon shaped sporangia (Figure 1). Each sporangium contains several nuclei and can detach from the rest of the mycelium, after which it can be carried by wind or water for several kilometers (Aylor et al., 2001). Given the large number of sporangia that are produced by a single infection, i.e. in the order of 10^5 (Flier et al., 2007), some of them will likely land on new host tissue where they can germinate.

Sporangia are unable to germinate in the absence of water. If immersed in water, however, they can germinate in two ways: either directly by producing a germ tube or indirectly by releasing zoospores (Judelson and Blanco, 2005). These zoospores are flagellated cells that lack a wall and contain a single nucleus. They are able to swim a short distance until they will encyst, i.e. eject their flagella and make a sticky cell wall that allows adherence to the host's cuticula. Each encysted zoospore can then germinate by producing a germ tube (Latijnhouwers et al., 2003). The shift between the two types of germination is influenced by temperature: direct germination of the sporangia is more frequent at temperatures above 15 °C, whereas the release of zoospores is more common at temperatures below 15 °C (Judelson and Blanco, 2005; Tani and Judelson, 2006). Despite these differences, both types of germination can result in a successful infection of host tissue.

After germination, the host tissue is entered by penetration of the host epidermis (Latijnhouwers et al., 2003). After entering the host, mycelium grows between the cells and forms haustoria, i.e. structures that penetrate the cell walls to enable extraction of nutrients from the cells. Successful infection also requires the production and excretion of large numbers of gene products to manipulate the host's response. These products include protease inhibitors (Birch et al., 2006), cell wall degrading enzymes (Latijnhouwers et al., 2003) and effectors that enter the host's cytoplasm using a conserved motif (Ellis et al., 2006; Haldar et al., 2006; Win et al., 2007). After a successful infection, the pathogen will continue its growth within the host and will eventually start producing new sporangia.

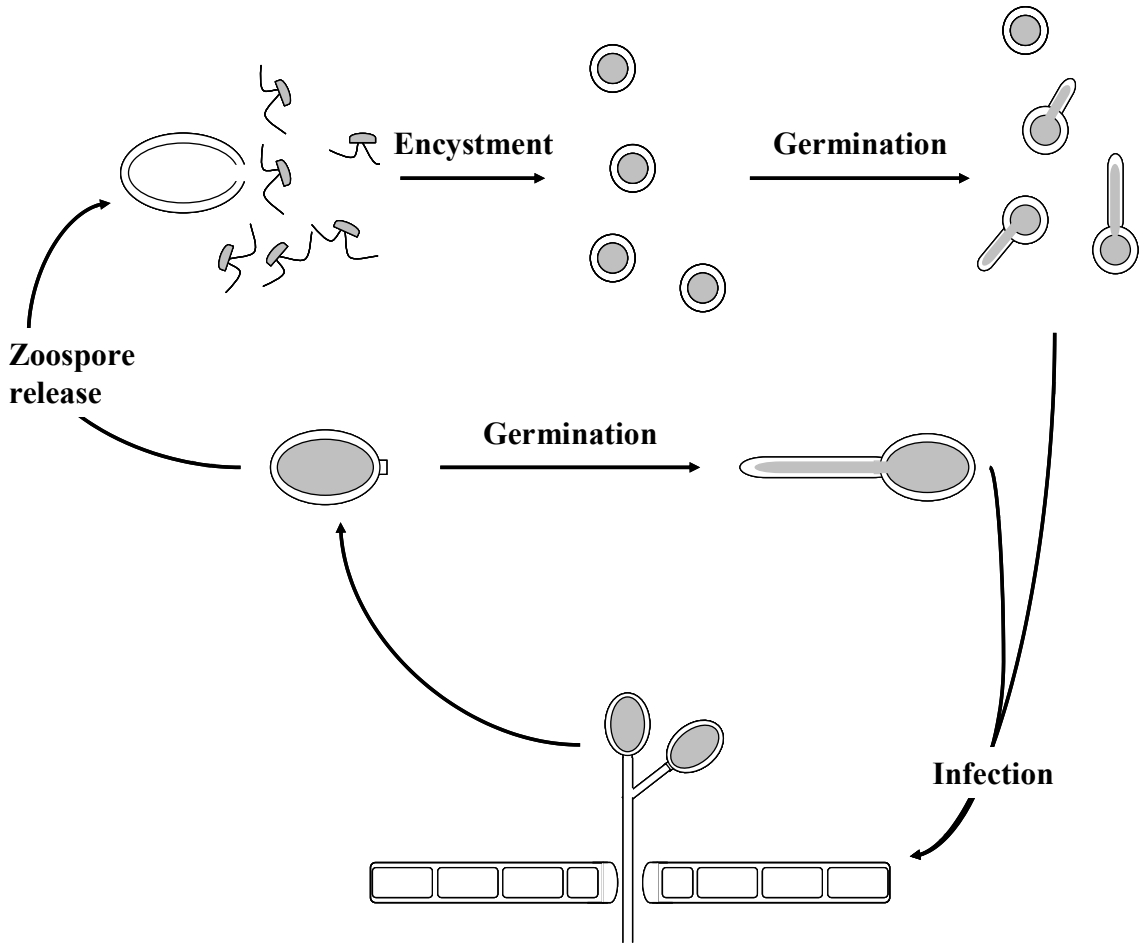

Figure 1. A schematic representation of the asexual life cycle of *P. infestans*.

The sexual cycle and genetic recombination

The asexual cycle is the main mode of reproduction during epidemics. However, *P. infestans* is also capable of sexual reproduction. Although it is of lesser importance during epidemics, sexual reproduction may still play a crucial role in the biology of the pathogen by allowing genetic recombination that may lead to a higher fitness during epidemics and thus to more severe epidemics (Gavino et al., 2000).

P. infestans is heterothallic with two distinct mating types, denoted A1 and A2. Sexual reproduction is initiated when two individuals of opposite mating type meet and form female and male sex organs, respectively called oogonium and antheridium (Figure 2). Each mating type can form both male and female organs, although some genotypes show preference for either a female role or a male role (Judelson, 1997a). Meiosis takes place inside the sex organs and fertilization occurs after fusion of the sex organs. After fertilization a thick-walled

oospore, i.e. the actual zygote, is produced. These oospores are capable of surviving outside the host and can remain infectious for several years (Turkensteen et al., 2000; Fernandez-Pavia et al., 2004).

The sexual cycle does not necessarily lead to outcrossing: some progeny may be products of selfing (Knapova et al., 2002) and others may be apomictic, i.e. offspring that is genetically similar to one of the two parents (Judelson and Yang, 1998; Carter et al., 1999). The existence of apomictic offspring is just one of those poorly understood phenomena of the sexual cycle. However, selfings are not unexpected given the fact that the female sex organs can also fuse with the male sex organs of the same genotype (Judelson, 1997a). Moreover, some genotypes have been shown to be self-fertile, i.e. they produce oospores in the absence of the other mating type (Judelson, 1996; Smart et al., 1998; Smart et al., 2000). Selfing increases the likelihood for homozygosity of rare recessive traits, e.g. the ability to infect a resistant cultivar (May et al., 2002).

The strong clonal structure observed in some populations of *P. infestans* (Cooke et al., 2006) and other *Phytophthora* species (Dobrowolski et al., 2003) seems consistent with the absence of sexual recombination. Despite this clonal structure, zoospores obtained from a single colony can show substantial variation in growth characteristics (Caten and Jinks, 1968), virulence (Caten, 1970; Abu-El Samen et al., 2003b) and AFLP genotype (Abu-El Samen et al., 2003a). Additionally, aneuploid (e.g. trisomic) and polyploid offspring (e.g. tetraploid) have been observed regularly among sexual offspring (Carter et al., 1999; van der Lee et al., 2004). This latter suggests that *P. infestans* is not strictly diploid. These changes in ploidy level could play a role in its evolution. Indeed, some new *Phytophthora* species appear to be allopolyploids that contain the DNA of two different species (Érsek et al., 1995; Brasier et al., 1999; Brasier, 2000). These observations show that *P. infestans* has a flexible genome that may facilitate adaptation in the absence of sexual recombination (Goodwin et al., 1995b).

Additionally, parasexual processes (Pontecorvo, 1956) may also play a role in generating variation within clonal lineages. These processes are typically initiated whenever two somatic nuclei fuse. The resulting polyploid nucleus may then revert to the normal ploidy level by the random loss of chromosomes, which may lead to new combinations of chromosomes, i.e. causing new recombinant genotypes. In addition, mitotic crossing over and gene-conversion may result in loss of heterozygosity or genome rearrangements.

P. infestans is coenocytic, which means that several nuclei reside in the same

cytoplasm without being separated by a cell membrane. This cellular organization increases the chance of fusions of nuclei and thus of parasexual processes that may result from it. Several examples of parasexual processes have indeed been observed in *Phytophthora* species. For instance, Gu and Ko (1998; 2000) observed a full parasexual cycle in *P. parasitica*. Additionally, Pipe et al. (2000) observed a potential product of a parasexual cycle in a natural occurring heterokaryon of *P. infestans*. These observations clearly show that parasexual processes can play a role. However, the magnitude of the impact of these processes on the biology of *Phytophthora* remains unknown.

Host resistance

Potato plants are not just innocent victims waiting to be killed by *P. infestans*. In fact, they possess a sophisticated resistance mechanism. The first step is to recognize the initial signals of an infection. Detection of these signals may then trigger a programmed cell death known as the hypersensitive response, which can stop the infection at an early stage and prevent it to become systemic (Kamoun et al., 1999). The signal that triggers this response is comprised of one or more of the gene products that the pathogen uses for infection. Because these gene products prevent the infection of resistant hosts, they are referred to as "avirulence" factors (Laugé and De Wit, 1998). This term may be somewhat confusing, since these factors are used in normal infection of a fully susceptible host. However, this nomenclature is chosen because the "wild-type" phenotype, involving an intact avirulence factor, prevents the pathogen to infect a resistant potato cultivar.

This resistance mechanism may pose a selection pressure on *P. infestans*. Indeed, some clonal lineages gained the ability to infect resistant potato cultivars that were initially resistant to this lineage (Goodwin et al., 1995b). Moreover, the introduction of new resistant potato cultivars resulted in adaptation in the pathogen population that broke these novel resistance mechanisms (Fry, 2008). Such sudden changes in the virulence phenotype are remarkable, but may be explained by the fact that they are caused by chromosomal deletions (Van der Lee et al., 2001) and that these genetic changes are recessive (May et al., 2002). Hence they may occur frequently and are not efficiently removed by natural selection.

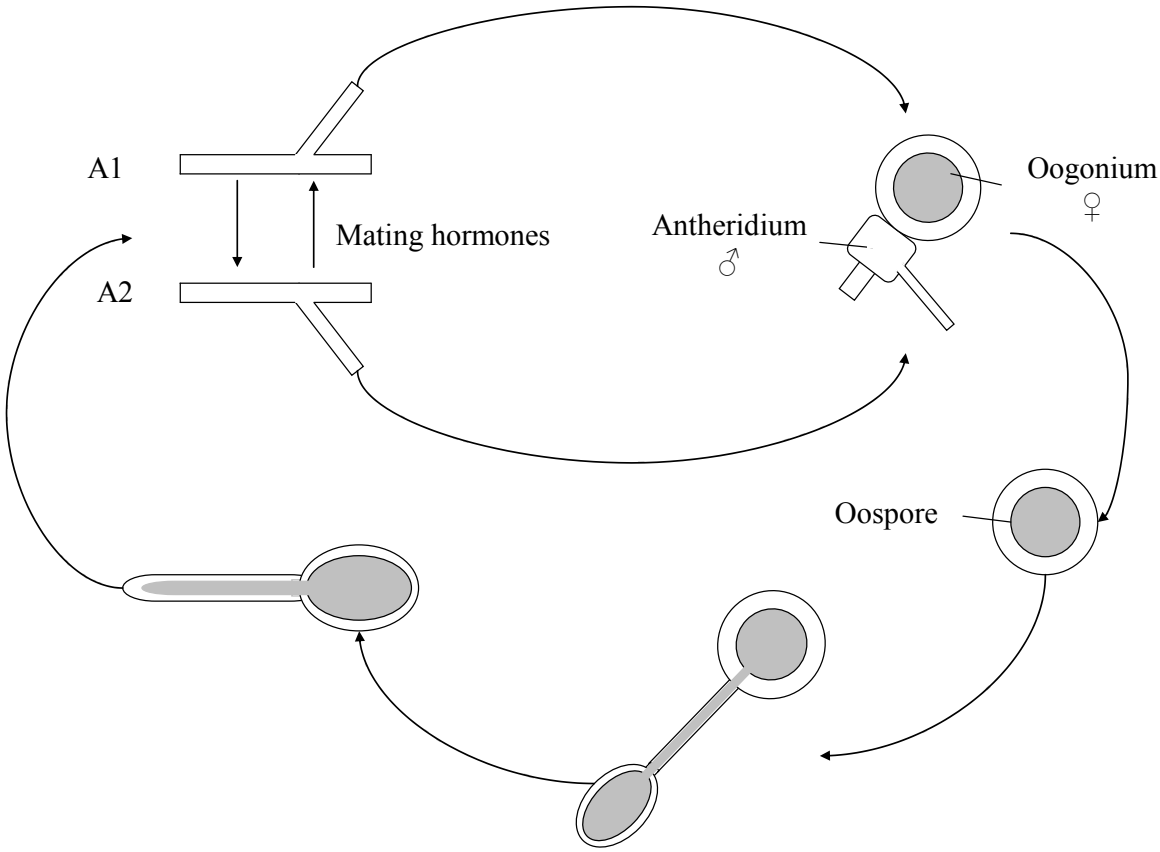

Figure 2. A schematic representation of the sexual life cycle of *P. infestans*.

The phylogenetic position of Phytophthora

The genus *Phytophthora* comprises several of the most devastating plant pathogens (Blair et al., 2008). This genus belongs to the Oomycetes: a large group of organisms that contains pathogens that parasitize a wide variety of species ranging from plants to fish (Kamoun, 2003). With its white mycelium and asexual reproduction with spores, oomycetes were traditionally regarded as a sub-group of the fungal kingdom (Caten and Day, 1977; Money, 1998). A closer look, however, reveals some striking differences with fungi: e.g., the mycelium of oomycetes is not haploid (Judelson and Blanco, 2005) and its cell wall mainly consists of cellulose-like substances (Bartnicki-Garcia, 1968) with only a small amount of chitin (Werner et al., 2002). Together with recent molecular phylogenetic data, these differences have led to the reclassification of oomycetes into a taxon that is more related to golden brown algae than to both animals and fungi (Baldauf et al., 2000; Figure 3).

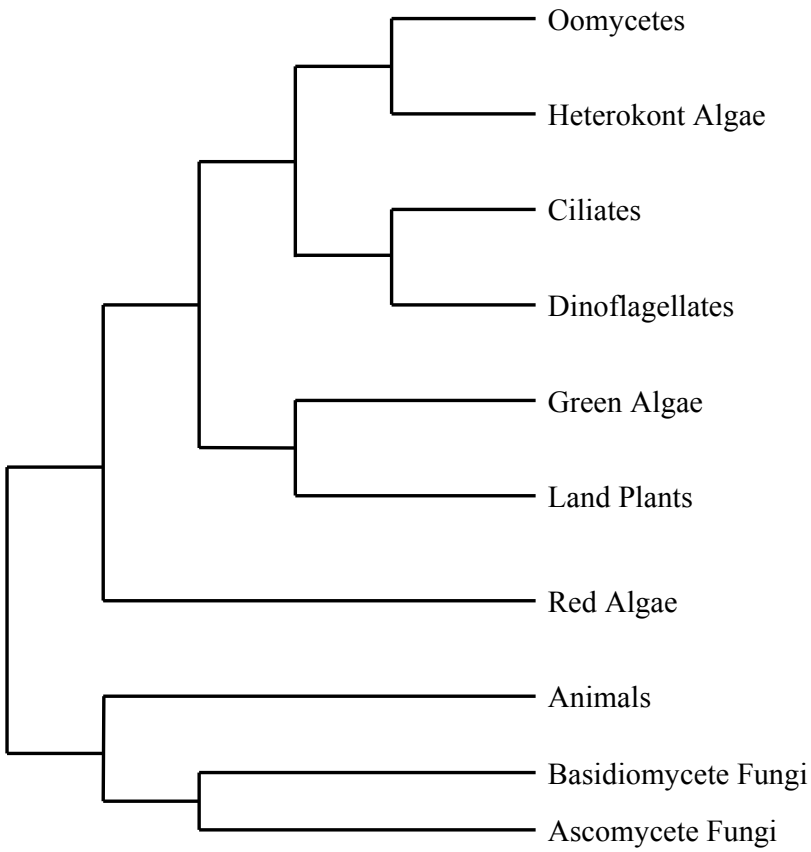

Figure 3. Phylogenetic tree showing the evolutionary relationships between major eukaryotic groups. Note the evolutionary distance between fungi and oomycetes. Adapted from Kamoun et al.(1999).

Historical migrations

Although the phylogenetic position is commonly accepted among researchers, the center of origin of the pathogen remains the subject of a lively debate (Ristaino, 2002). A large group of researchers has assumed that the Toluca Valley in Mexico is the center of origin, because the local population there shows the highest level of genetic variation (Goodwin et al., 1992; Grünwald and Flier, 2005). Furthermore, this population was the only one that reproduced sexually, since both mating types occur in equal frequencies (Goodwin et al., 1992) and molecular markers show Hardy-Weinberg equilibrium (Tooley et al., 1985). Recently, however, several investigations place the center of origin in the Andes rather than in Mexico (Gomez-Alpizar et al., 2007).

The debate on the center of origin has its consequences for our understanding of historical migrations involving *P. infestans*. For instance, the first epidemics that struck potato

fields in Europe in the 1840's were initially believed to be initiated by a single genotype that migrated together with potatoes from the Toluca Valley. Partly as a result of the low number of migrants, only one clonal pathogen lineage prevailed during the largest part of the 20th century in Europe and the U.S. (Goodwin et al., 1994). This even led to the assumption that this lineage was responsible for the very first epidemic that caused the Irish potato famine (Fry et al., 1993). However, infections on herbarium specimens revealed that the first epidemic was caused by another clonal lineage that disappeared thereafter (Ristaino et al., 2001). This latter observation indicates that at least two migration events have occurred between the first epidemic and the 1970's, suggesting that migration events were not as infrequent as originally suggested.

The re-emergence of late blight

Up until the 1970's, populations of *P. infestans* outside the Toluca valley consisted only of genotypes with the A1 mating type. Hence, reproduction was restricted to the asexual cycle, which severely reduced the genetic variability of those pathogen populations (Goodwin, 1997). During the 1980's, however, severe changes in the global population structure were observed, which were accompanied by an increase in the severity of epidemics (Fry and Goodwin, 1997). The first indication of the forthcoming changes was the first ever observation of the A2 mating type in Switzerland (Hohl and Iselin, 1984). This observation was followed by isolation of the A2 mating type in other parts of Europe (Shaw et al., 1985; Fry et al., 1991), suggesting that sexual reproduction was now possible in areas outside the Toluca Valley (Goodwin et al., 1995a). The migration of A2 genotypes was accompanied by additional genotypes of the A1 mating type (Fry et al., 1993). This new population quickly replaced the clonal lineage that dominated Europe for so long (Drenth et al., 1994).

This sexual population had several presumed advantages over the previous clonal population. First, sexual reproduction enables the production of oospores which greatly facilitates the survival of host-free periods (Turkensteen et al., 2000; Levin et al., 2001), whereas the clonal population depended on limited survival in storage potatoes or potatoes that were left behind in the soil. Second, sexual recombination could produce genotypes that are more aggressive (Gavino et al., 2000). Finally, sexual reproduction can counteract Muller's ratchet, i.e. the accumulation of deleterious mutations in asexual populations (Muller, 1964). Despite these presumed advantages, sexual reproduction still plays a minor

role (Fry et al., 1991; Cooke et al., 2003) and oospores are not a major inoculum source at the beginning of epidemics in Europe (Zwankhuizen et al., 2000).

Oomycete research

"The genomic revolution"

Traditionally, oomycetes are regarded as difficult and challenging organisms to work with in laboratory experiments (Judelson, 1997b). This is partly due to the specific characteristics of these organisms, including the absence of dominant markers, the masking of recessive markers and the biotrophic nature of these pathogens (Judelson and Blanco, 2005). Nevertheless, the biology of oomycetes has always been a challenging subject for an active community of researchers. The recent advances in genomics have opened several opportunities for this community (Kamoun and Smart, 2005). For instance, genome sequences of two *Phytophthora* species, *P. ramorum* and *P. sojae*, have been published (Tyler et al., 2006) and the genome sequence of *P. infestans* will follow soon (Govers and Gijzen, 2006). This genomic revolution is expected to shed more light on molecular mechanisms of processes such as in infection of host tissues and sporangium development (Kamoun and Smart, 2005; Birch et al., 2006). Moreover, these new approaches may also help in answering longstanding evolutionary questions.

Evolutionary biological studies of oomycetes

Although they belong primarily to the realm of plant pathologists, oomycetes may also be of interest for evolutionary biologists. For instance, the similarity between fungi and oomycetes in growth form and infection structures represents a good example of convergent evolution (Latijnhouwers et al., 2003). Furthermore, the observation that genes that are important for infection show remarkable resemblance with those of fungi is also suggestive of convergent evolution (Randall et al., 2005), although horizontal gene transfer between oomycetes and fungi cannot be ruled out (Richards et al., 2006).

Several evolutionary aspects of Oomycete biology have already been addressed by others. For instance, several papers have elucidated the evolutionary relationships between and within oomycete species (Gomez-Alpizar et al., 2007; Blair et al., 2008). Additionally, population genetic studies have been extensively monitoring populations of *Phytophthora*

infestans (Cooke et al., 2003). These studies, however, only describe evolutionary and population genetic patterns, but do not investigate the underlying mechanisms that cause these patterns. Knowledge of the underlying causes may help explain population changes and perhaps predict future changes. One example that clearly reflects the need for such research is the case of the evolution of pesticide resistance, which will be dealt with in the following case study.

A case study: pesticide resistance

The annual costs associated with potato late blight are estimated to be around 3 billion US $ annually (Flier, 2001), of which a large part is accounted for by the large amounts of pesticides used (Johnson et al., 2000). The possibility of the development of resistance to any of these chemicals is therefore a potential problem that could threaten worldwide potato production. A famous example of the danger of resistance development in *P. infestans* and other oomycetes is resistance to metalaxyl and its enantiomer mefenoxam (metalaxyl-M) (Gisi and Cohen, 1996; Figure 4).

Upon its commercial release, metalaxyl showed good curative and protective effects against *P. infestans* (Gisi and Cohen, 1996). It reduces mycelium growth and sporulation by inhibiting the activity of ribosomal RNA polymerase (Davidse et al., 1983). However, this single target makes it vulnerable to the evolution of resistance, since only a single genetic change could be sufficient to cause resistance. Indeed, resistance was already observed within a few years after the commercial release (Davidse et al., 1981; Dowley and O'Sullivan, 1981). Moreover, resistance to this pesticide has been described in a number of other oomycete plant pathogens (Bruck et al., 1982; Schettini et al., 1991; Lamour and Hausbeck, 2000; Gent et al., 2008). During subsequent years, metalaxyl resistant genotypes reached a high frequency in populations. This high frequency and the ease at which resistant and sensitive isolates can be distinguished in the laboratory (Figure 5) resulted in the use of metalaxyl resistance as one of several phenotypic and genetic markers that are commonly used in studies on the population structure (Cooke, 2004).

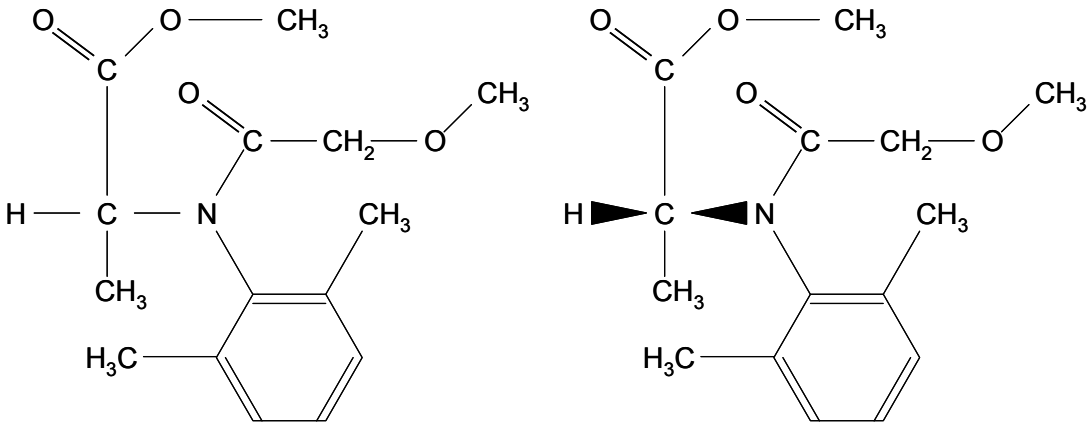

Figure 4. The chemical structure of metalaxyl (left) and its enantiomer (metalaxyl-M).

The fast evolution of metalaxyl resistance is in agreement with the general assumption that *P. infestans* has a flexible genome (van der Lee et al., 2004) and can be regarded as a pathogen with a high risk of resistance development (McDonald and Linde, 2002). Indeed, single zoospores obtained from the same *in vitro* colony show substantial variation in their sensitivity to metalaxyl (Abu-El Samen et al., 2003b). The risk of resistance development to other pesticides with a single cellular target should therefore also be high. Nevertheless, such resistances have not yet been described in field populations (Gisi and Sierotzki, 2008).

An explanation for the exclusiveness of metalaxyl resistance could lie in its genetics. For instance, it is generally accepted that metalaxyl resistance is governed by a single locus with intermediate dominant effect (Shattock, 1988; Lee et al., 1999), although additional loci with small effect, can have a substantial effect on the resistance phenotype (Fabritius et al., 1997; Judelson and Roberts, 1999; Lee et al., 1999). The action of this dominant mutation with large effect would facilitate the evolution of resistance (Jasieniuk et al., 1996). The failure of the pathogen to become resistant to other pesticides could then be explained by the recessive nature of other resistance mutations (Young et al., 2001; Stein and Kirk, 2004). However, this failure could also be the result of anti-resistance strategies taken by the agrochemical industry, such as the reduction of pesticide applications and the use of mixtures containing chemically unrelated pesticides (http://www.frac.info).

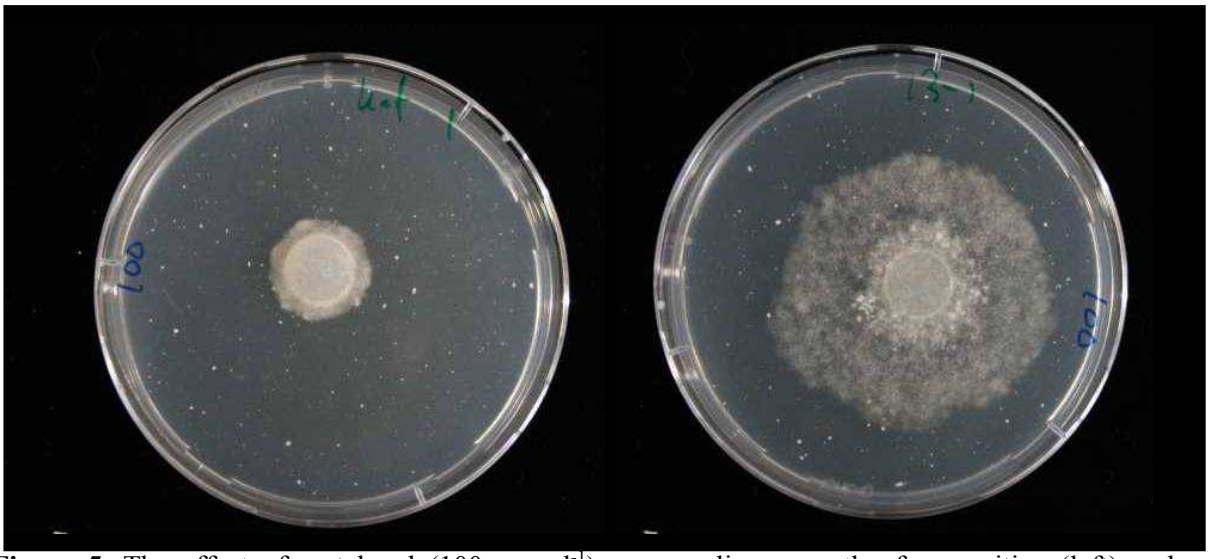

Figure 5. The effect of metalaxyl (100 µg ml^{-1}) on mycelium growth of a sensitive (left) and a resistant (right) strain of *P. infestans* after 10 days of growth. Both genotypes have a similar genetic background, but differ in the sensitivity to metalaxyl (for details see chapter 3).

The high speed of evolution of resistance to metalaxyl is not the only strange phenomenon associated with this resistance. For instance, several studies suggest that resistant genotypes have a higher fitness during epidemics than sensitive genotypes (Kadish and Cohen, 1988a; Kadish and Cohen, 1988b; Kadish and Cohen, 1989). A similar effect of metalaxyl resistance has been observed in other oomycetes (Cafe and Ristaino, 2008; Gent et al., 2008; Hu et al., 2008). The absence of resistance prior to the commercial release of metalaxyl can than be explained by a lower survival of resistant genotypes during host-free periods (Kadish and Cohen, 1992). This is in agreement with the recurrent observation that the frequency of resistant genotypes on untreated fields is low at the beginning of each growing season, but increases during epidemics (Gisi and Cohen, 1996; Gisi et al., 2000). Other studies, however, suggest either no effect (Lee et al., 1999) or a negative effect of metalaxyl resistance on components of fitness during epidemics (Dowley, 1987). Nation-wide bans of metalaxyl during the 1980's in Ireland and the Netherlands resulted in a decrease in the frequency of resistant genotypes in those countries (Dowley and O'Sullivan, 1985; Davidse et al., 1989), suggesting that the positive link between metalaxyl resistance and fitness is not as clear as suggested in some papers.

A possible lack of a large cost of resistance is rather unexpected, because resistant mutations are generally believed to infer a fitness cost in the absence of the pesticide

(Baucom and Mauricio, 2004; Gagneux et al., 2006). Although a cost of resistance is commonly accepted among evolutionary biologists (Andersson, 2006), simply cancelling the use of a particular pesticide may not result in a decrease in the frequency of resistance mutations. This apparent stability of resistance may be caused by the compensatory effects of additional mutations selected in the resistant background (Levin et al., 2000; Nagaev et al., 2001; Schoustra et al., 2006). It is not unlikely that a similar compensation may explain the fact that metalaxyl resistance has generally not been associated with a reduction in fitness.

To conclude, metalaxyl resistance has been used extensively as a genetic marker in population studies in *P. infestans*. However, several basic factors underlying the population dynamics of this resistance are poorly understood. For instance, no solid explanation can be given for the apparent exclusiveness of metalaxyl resistance and the absence of resistance to other pesticides. Additionally, the lack of negative fitness effects of metalaxyl resistance needs further explanation. Clearly, experiments on the underlying factors of metalaxyl resistance are needed to be able to explain the exclusiveness and the dynamics of this resistance in field populations with respect to resistance to other pesticides.

Experimental evolution

Traditionally, evolutionary biology has depended on indirect comparative studies to explain evolutionary patterns and understand evolutionary processes and mechanisms. The underlying mechanisms and processes that are responsible for these patterns have been outside the reach of direct observation. During the past 20 years, however, researchers have started to test hypotheses of these underlying mechanisms using microbes by allowing populations of microbes to evolve in the laboratory, a methodology called ‘experimental evolution’ (Lenski et al., 1991; Cowen, 2001; Colegrave and Collins, 2008). The use of microbes for this direct approach to study evolutionary processes is motivated by a number of advantages that they share: high reproduction rates, large population sizes and the ease to manipulate environmental parameters. The largest advantage that microbes provide for this approach is their ability to be revived after storage at -80 °C in a non-evolving state, so that population samples from different generations can be compared directly (Elena and Lenski, 2003). This approach has been successful in studying several aspects of evolutionary theory such as: the role of mutation rates (de Visser et al., 1999), the causes and consequences of

trade-offs between life-history characters (Schoustra et al., 2006) and the role of environmental heterogeneity on adaptive radiation (Habets et al., 2006).

Several questions regarding the evolution of pesticide resistance have been successfully addressed using experimental evolution. One specific topic of resistance evolution that received much attention is the initial cost of resistance and the occurrence of compensatory evolution, i.e. the emergence of additional beneficial mutations whose benefit is conditional upon the presence of the resistance mutation. Experimental evolutionary studies suggest that an initial cost followed by compensatory evolution is common in a wide range of prokaryotes (Bouma and Lenski, 1988; Schrag et al., 1997; Moore et al., 2000) and viruses (Burch and Chao, 1999). Similar approaches in a limited number of eukaryotic species also revealed the action of compensatory mutations (Cowen et al., 2001; Estes and Lynch, 2003; Schoustra et al., 2006).

The evolution of pesticide resistance and compensatory evolution have been primarily studied in model microbes and human pathogens, but not in plant pathogens. Since filamentous fungi are a major group of plant pathogens, the experimental evolutionary approach with the model ascomycete *Aspergillus nidulans* used by Schoustra et al. (2006) sheds some light on the evolution of pesticide resistance in plant pathogens. Consistent with the results on human pathogens, this approach revealed a reduction of fitness caused by the initial resistance mutation that was rapidly followed by compensatory evolution that restored the fitness of the resistant genotypes to a similar or even higher fitness level than that of the sensitive ancestor.

The biological differences between oomycetes and fungi could imply that the results obtained by Schoustra et al. (2006) are not applicable to oomycete plant pathogens. For instance, recessive mutations are immediately subjected to selection in haploid fungi, but not in oomycetes. An experimental evolutionary approach using an oomycete could therefore prove useful in helping to understand the evolution of pesticides resistance in this group of organisms. *P. infestans* is a relatively good candidate for such an approach, because it has features that makes it suitable for an experimental evolution approach: it is able to grow on artificial agar medium (Medina and Platt, 1999) and can be successfully revived after storage in liquid nitrogen (Flier et al., 2003).

Several experimental evolutionary studies had already been performed in *P. infestans* to investigate the risk of resistance against several pesticides (Young et al., 2001; Stein and

Kirk, 2004). These studies typically involved experiments in which plugs of mycelium were serially transferred on artificial medium and did not include analyses of the cost of resistance. These methods thus neglected the production and germination of sporangia and zoospores, while Schoustra et al. (2005) have shown that the outcome of selection using asexual spores can differ markedly from selection using mycelium plugs. Their results clearly showed that the speed of evolution was markedly higher when using asexual spores. Hence, evolution experiments using serial transfer of asexual spores are necessary to investigate important parameters, such as the mutation rate towards resistance that shape the evolution of pesticide resistance in *P. infestans*.

Scope and outline of this thesis

The primary scope of this thesis is to investigate the underlying factors that would facilitate the evolution of a persistent population of resistant genotypes of *P. infestans*. An experimental evolution procedure is developed and used to estimate basic genetic parameters, such as the mutation rate and the cost of resistance. The emphasis will lie on resistance to metalaxyl, since this is the only well-described case of a rather stable resistance in *P. infestans*. Two main topics will be dealt with during this thesis: factors that influence the rate of resistance evolution (**Chapter 2** and **Chapter 3**) and the effect of metalaxyl resistance on fitness in the absence of metalaxyl (**Chapter 4** and **Chapter 5**)

Chapter 2 describes attempts to estimate the mutation rate towards resistance using a fluctuation test, specifically developed for *P. infestans*. Several features of the biology of *P. infestans* severely hamper the use of this test for the estimation of the mutation rate. The suitability of this procedure and the importance for the speed of resistance evolution of this and other population genetic parameters are discussed.

Chapter 3 describes a serial-passage experiment to assess the risk of resistance development on sub-lethal concentrations. Theoretical models predict that evolution of resistance will be fastest at lower concentrations. Under these circumstances, however, fully resistant genotypes may be out-competed by less resistant genotypes. The relatively fast evolution of metalaxyl resistance could then be partially explained by the fact that low concentrations of metalaxyl will select for full resistance, whereas this is not the case for other pesticides with a single cellular target, such as cyazofamid. In order to test this hypothesis, zoospores were transferred on medium with a low concentration of metalaxyl and cyazofamid to observe whether full resistance can evolve on these sub-lethal concentrations.

Chapter 4 contains an investigation on the effect of metalaxyl resistance on the epidemic fitness, i.e. the fitness in the field during the growing season. Several papers have described a positive effect of metalaxyl resistance on epidemic fitness, despite the fact that resistance mutations are generally believed to have a negative effect on fitness. If these reports have general validity, the frequency of resistant isolates in field populations would also be high in the absence of metalaxyl. Furthermore, comparisons between near-isogenic

resistant and sensitive clones should then reveal positive fitness effects of metalaxyl resistance. In this chapter, we describe the results from two types of experiments in which we tested both these expectations. First, patterns of resistance in field populations were analyzed during a seven year period that included a period of three years in which metalaxyl was prohibited. Second, epidemic fitness components of resistant genotypes from **chapter 3** were compared with those of their ancestor.

Chapter 5 describes an experiment in which the selection procedure similar to the one used in **chapter 3** is used over relatively short time periods to investigate whether metalaxyl resistance has an initial effect on *in vitro* performance in the absence of metalaxyl that would be compensated during the selection procedure.

Chapter 6 is a discussion of the results presented in this thesis. These results will be discussed in the light of the current state of research on pesticide resistance in *P. infestans*. Additionally, exciting new directions of research on this topic are presented.

Chapter 2

Estimating the mutation rate towards pesticide resistance in *Phytophthora infestans*

Stefan Bosmans, J. Arjan G.M. de Visser, Alfons J.M. Debets and Rolf F. Hoekstra.

Abstract

Estimations of the mutation rate towards resistance in *Phytophthora infestans* are relevant for the prediction of the risk of resistance development to current and future treatment strategies. Here, we report an attempt to estimate this parameter by using a fluctuation test, a procedure that is commonly used in bacterial genetics. First, we tried to calibrate a fluctuation test using metalaxyl-M as a selective agent because resistance against this pesticide is dominant and well established. The calibration of the fluctuation test with metalaxyl-M was ineffective due to its non-lethal effect *in vitro* causing background growth of sensitive mycelium. However, the frequent occurrence of faster growing mycelial sectors on plates amended with metalaxyl-M is suggestive of a mutation rate that is appreciable. Second, we used this procedure to estimate the mutation rate towards resistance against two pesticides with a lethal effect *in vitro*: fluazinam and cyazofamid. Fluctuation tests using lethal concentrations of cyazofamid (1.0 µg ml^{-1}) and fluazinam (5.0 µg ml^{-1}) however did not yield resistant colonies, which indicates a mutant frequency that is lower than one mutant in every 10^7 zoospores. The limitations of the use of fluctuation tests as well as the relevance of estimates of the mutation rate in *P. infestans* are discussed with respect to the risk of resistance development.

Introduction

Some of the most devastating plant pathogens belong to the oomycetes; a group of organisms that was traditionally placed within the fungal kingdom due to their fungal-like appearance. More recent evidence, however, revealed that they belong to a distinct group that is more related to heterokont brown algae than to either fungi or animals (Baldauf et al., 2000). The notion of its distinct phylogenetic position reveals the need for research on this group of organisms since it is difficult, if not impossible, to understand its biology based on results obtained from fungi (Govers, 2001).

One of the focal species for oomycete research is *Phytophthora infestans*, the causal agent of the late blight disease in potato. As a result of the economic importance of this disease, pathogen populations have been monitored for many years (Cooke et al., 2003). These studies merely describe changes in population structure and do not test evolutionary predictions or estimate relevant evolutionary parameters. Knowledge on these parameters, however, would help in understanding and predicting changes in population structure.

One of these evolutionary parameters is the mutation rate, which is important for several reasons. On the one hand, a high mutation rate may be disadvantageous by increasing the frequency of deleterious alleles at mutation-selection equilibrium; hence it would increase the genetic load (Agrawal and Wang, 2008). On the other hand, a high mutation rate may be advantageous by facilitating adaptation to changing environmental conditions in populations of moderate size (Elena and Lenski, 2003; Colegrave and Collins, 2008).

Knowledge of the mutation rate in *P. infestans* is essential for predictions on how this organism will respond to current and future treatment strategies (Jasieniuk et al., 1996). For instance, current treatment strategies depend heavily on the use of chemical pesticides (Johnson et al., 2000), whereas future treatment strategies likely involve the introduction of new resistance genes into potato cultivars (Fry, 2008). The rate at which pathogen genotypes emerge that are resistant to a pesticide or that are able to infect resistant potato cultivars is an important factor in predicting the durability of these treatment strategies.

A fluctuation test (Luria and Delbrück, 1943) could be a suitable method for estimating the mutation rate in *Phytophthora infestans*. Such tests have been used extensively as a method to estimate mutation rates to resistance against pesticides in bacteria (Sniegowski et al., 1997) and is relatively easy to perform. First, a number of populations is initiated with a small inoculum and allowed to grow in the absence of a selective force. After this growth

phase, these populations are plated onto plates containing a selective ingredient, e.g. an antibiotic. The mean and variation in the number of resistant colonies between populations can then be used to estimate the mutation rate.

In principle, a fluctuation test can be used for *P. infestans*, because it reproduces asexually by producing a large number of zoospores that contain a single nucleus. This method, however, has several prerequisites to work properly: the production of sufficient spores (uninucleate zoospores) and a selectable marker. Additionally, because the asexual life cycle of oomycetes does not contain a haploid phase, a fluctuation test in *P. infestans* should involve dominant markers. Resistance against metalaxyl-M may be such a marker, because it is easily selected under laboratory conditions (Young et al., 2001) and because it is likely governed by a single dominant mutation (Lee et al., 1999). Furthermore, resistant genotypes can easily be distinguished from sensitive ones by simple growth inhibition assays (Gisi and Cohen, 1996). Resistances against other pesticides are less well understood or have not been described in field populations (Gisi and Sierotzki, 2008). Nevertheless, a fluctuation test could aid in our understanding of the potential of evolution of resistance against these chemicals.

In this chapter, we evaluated the experimental possibilities for a fluctuation-test to assess the mutation rate towards resistance to several pesticides in *P. infestans*. We first used metalaxyl-M as a selective agent, since resistance towards this pesticide is dominant and field resistance is well established. Next, we used two additional pesticides as selective agent: fluazinam, which is a general uncoupler of energy production (Brandt et al., 1992), and cyazofamid, which has a single target (Mitani et al., 2001a). The observed limitations of a fluctuation test for estimating mutation rates in *P. infestans* are discussed.

Methods

Culture conditions and collection of zoospores

Several field isolates of *P. infestans* were randomly chosen from collection in the liquid nitrogen storage facility at Plant Research International in Wageningen, the Netherlands (Flier et al., 2003) and compared for their *in vitro* production of sporangia. The density of sporangia was observed visually on three different media: V8, pea and Rye B (Caten, 1970; Medina and Platt, 1999). On average, the amount of sporangia was highest on

Rye B (data not shown). The isolate that produced the largest amount of sporangia on Rye B was chosen for the development of a fluctuation test.

Zoospore suspensions were created by flooding sporulating mycelium with 10 ml cold water (6°C) and storing it in the cold room (4°C) for 2.5 hours to induce zoosporogenesis. The resulting zoospore suspension was then transferred to sterile test tubes and put on ice for 30 minutes. During this period, zoospores swim upwards, whereas mycelial debris and sporangia settle at the bottom (Vijn and Govers, 2003). The top 1 ml of each test tube, which is enriched with zoospores, was then transferred to an Eppendorf tube and vortexed for one minute to induce encystment of the zoospores, i.e. the change from motile cells without a cell wall to immotile cells with a cell wall. The density of these suspensions was assessed using a haemocytometer and diluted if necessary.

Sensitivity tests

Stock solutions of metalaxyl-M and fluazinam (provided by Syngenta) were created by dissolving a technical grade solution in DMSO. The stoch solution of cyazofamid was created by dissolving the commercial product (providided by Plant Research International Wageningen) in water. Rye B was amended with different concentrations of the pesticides Care was taken that each medium contained a similar amount of DMSO.

Petridishes with rye B amended with pesticide at various concentrations. were inoculated with a 50 µl droplet of a zoospore suspension containing $1x10^5$ zoospores ml^{-1}.These plates were incubated at 18°C in the dark. Colony size was monitored by measuring two perpendicular diameters on day 5 and day 7 after inoculation. The relative growth rate at each concentration was then calculated by dividing the linear growth rate at that concentration, i.e. the difference between the colony size at day 5 and at day 7 after inoculation, by the growth rate on un-amended medium. The lowest concentration that reduced the relative growth rate to 0 %, i.e. the lowest lethal concentration, was then used in the fluctuation test.

Mycelium growth on artificial medium amended with different concentrations metalaxyl was further analyzed by estimating two *in vitro* growth parameters, i.e. the growth rate during colony expansion and the duration of the lag-phase before the colony size exceeded the initial inoculum size. The growth rate was estimated by linear regression of colony diameter on time. Only time-points during colony expansion (i.e. when the diameter

was between 1.6 cm and 7 cm) were used in this analysis. The duration of the lag phase was estimated using linear regression of time on diameter during colony expansion. The resulting regression model was used to calculate the time-point at which the diameter was 1.6 cm (the typical size of an inoculum droplet). Differences between these growth parameters on un-amended medium and on medium amended with metalaxyl were then analyzed using *t*-tests.

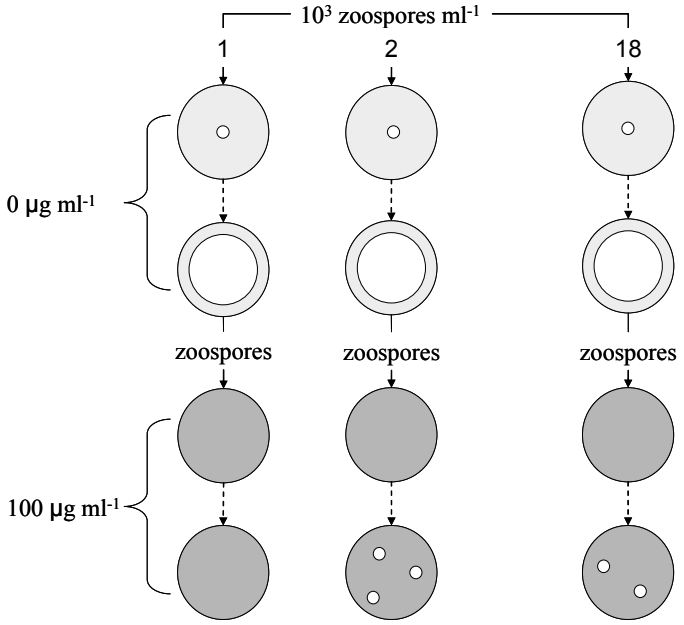

Figure 1. Overview of a fluctuation test with metalaxyl-M. At the start of the experiment, a 50 µl droplet containing 10^3 *P. infestans* zoospores ml^{-1} was pipetted onto the center of plates without any metalaxyl-M and plated out. Two weeks later, all zoospores from these plates were transferred onto plates containing a selective concentration of metalaxyl-M. The number of colonies that are able to grow on these plates can then be used to estimate the mutation rate towards metalaxyl-M resistance.

Fluctuation test

Figure 1 shows a schematic overview of the fluctuation test used in this experiment. A zoospore suspension containing 10^3 zoospores ml^{-1} was used to inoculate 18 plates containing un-amended Rye B medium. Additionally, the initial droplet was spread out using a glass rake. These plates were incubated at 18 °C in the dark for 10 days. After this period, zoospores were collected from each plate as described above with a minor modification: after the 30 minutes on ice, the top 5 ml was transferred to a new test-tube and vortexed for 1 minute after which 1 ml was used as a counting sample. The concentration of encysted

zoospores in this counting sample was estimated with a haemocytometer after which the remainder of the counting sample was mixed with the remaining 4 ml. Each suspension (5 ml) was then pipetted onto a new plate containing the lethal concentration of a pesticide. One day later, the excess of water was removed by gentle suction with a 5 ml pipette. All plates were further incubated at 18 °C in the dark and were monitored daily for the presence of mycelium. The number of colonies that emerged on medium amended with the pesticide was counted. A local computer program (P.J. Gerrish, unpublished) was then used to calculate the mutation rate.

Results

Isolate NL01096

The isolate with the largest production of sporangia on rye B amongst our random subset was NL01096. This isolate was obtained from a potato field in the Netherlands in 2001. Its mating type is A2 and its mitochondrial haplotype IIA (data not shown). Furthermore, this isolate proved to be metalaxyl sensitive according to the definition used by Cooke et al (2003).

Sensitivity tests

Mycelium growth is affected by all pesticides, but the magnitude of this effect differs between the pesticides (Figure 2). Both fluazinam and cyazofamid fully reduce the mycelium growth rate at concentrations of 5.0 μg ml^{-1} and 0.1 μg ml^{-1}, respectively (Figure 2 A & B). Metalaxyl-M, however, largely reduced the mycelium growth rate, but this reduction reached its maximum at concentrations around 0.1 μg ml^{-1} and did not increase at higher concentrations (Figure 2C). A similar incomplete reduction of growth rate was also observed in other isolates (data not shown) and appears to be a general characteristic of metalaxyl-M.

A closer look upon the inhibitory effects of metalaxyl at low dosages revealed that it causes a delay in the onset of colony growth on all concentrations tested (Table 1; Figure 3). A reduction of growth rate was observed on all but the lowest concentration (0.001 μg ml^{-1}). A similar approach using low dosages of fluazinam revealed an additional striking pattern, next to a reduced growth rate (Figure 4). Colonies that were formed on plates with a sub-lethal concentration of fluazinam did not reach a similar colony size as the control. When inoculated on larger plates (∅ 13 cm), the growth did not stop at the same colony size, but continues until it reaches its maximum size, which is again smaller than that of the ancestor.

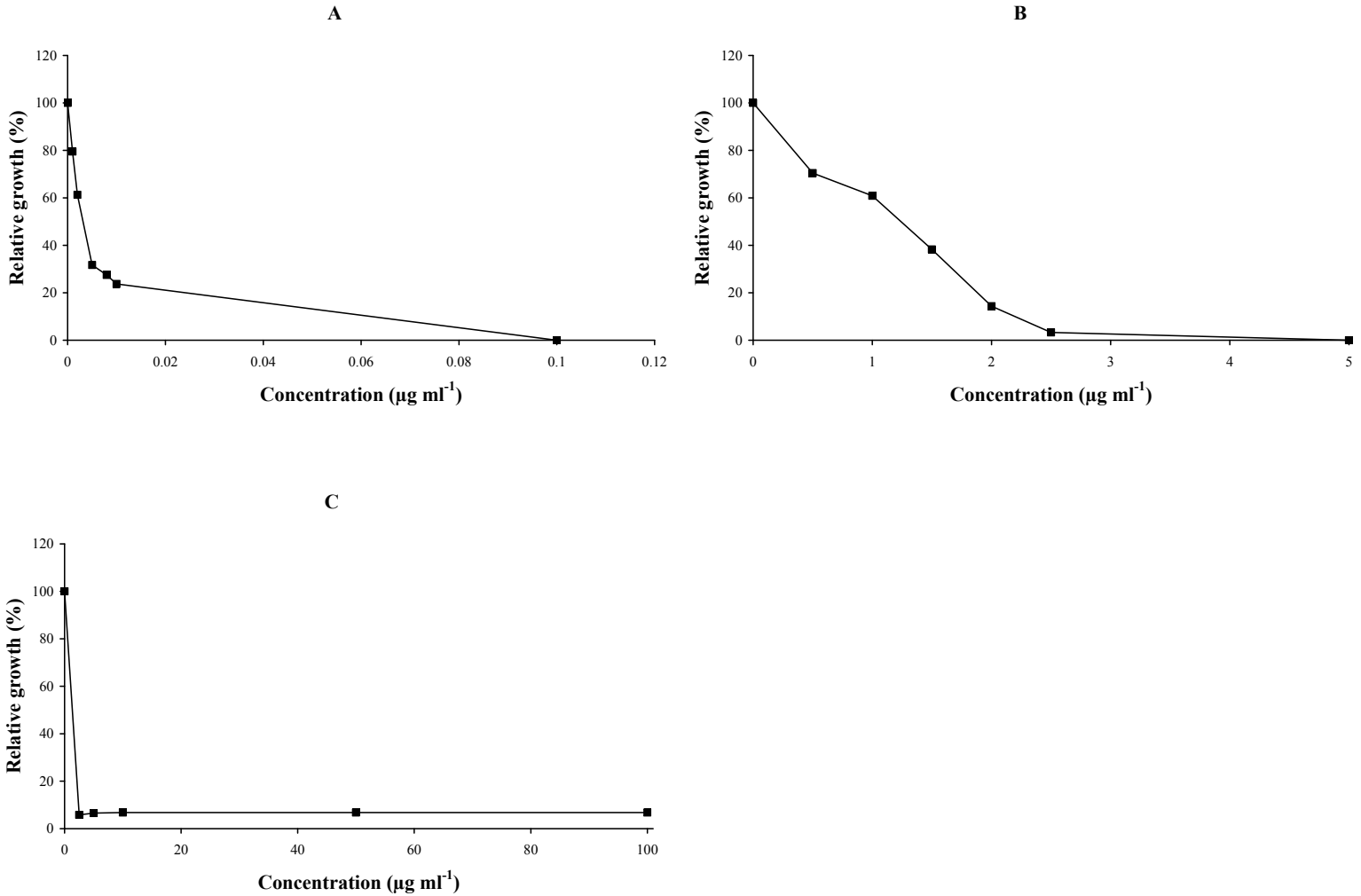

Figure 2. Dose response curves of isolate NL01096 on Rye B medium amended with either cyazofamid (panel A), fluazinam (B), or metalaxyl-M (C). The relative growth was calculated as colony diameter after 7 days of growth relative to that on medium without any pesticide.

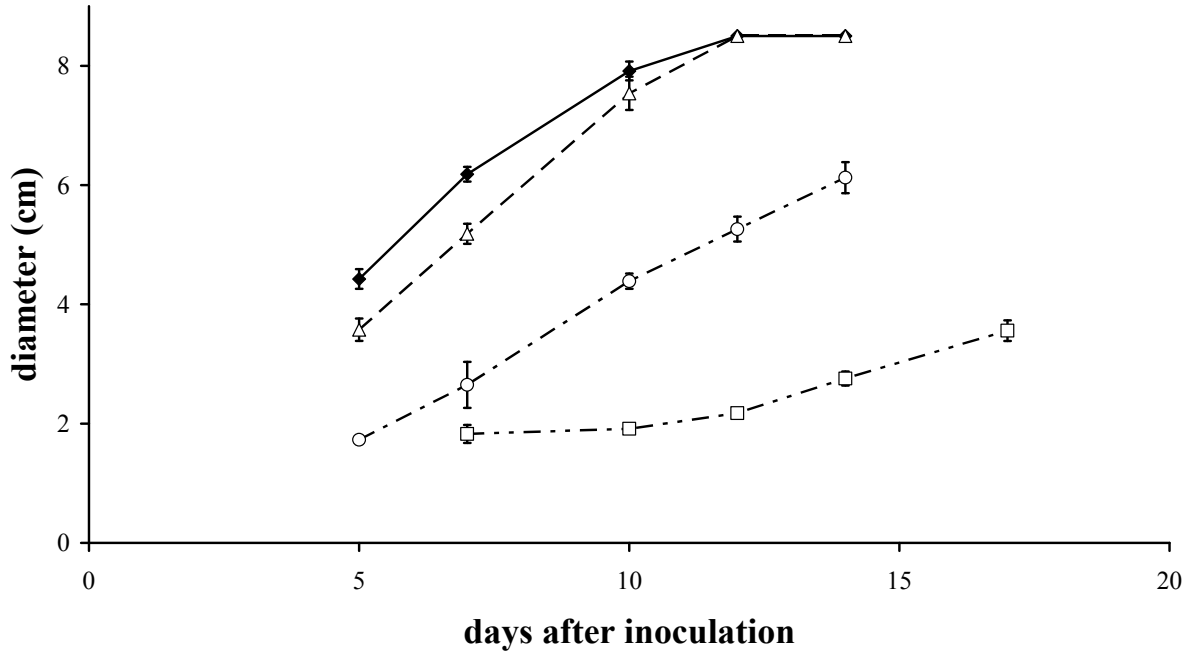

Figure 3. The colony diameter of isolate NL01096 as function of time on medium amended with different concentrations metalaxyl-M following inoculation with zoospores. The concentrations used were 0 (◆); 0.001 (△); 0.01 (○) and 0.1 µg ml^{-1} (□). Error bars represent 95% confidence limits.

Table 1. Estimations of the growth rate and duration of the lag-phase of *P. infestans* isolate NL01096 on V8 amended with various concentrations of metalaxyl (Figure 3).

Concentration (µg ml^{-1})	Growth rate cm day^{-1} (± s.e.m.)[a]		Lag phase days (± s.e.m.)[a]	
0	0.89 ± 0.03		1.71 ± 0.18	
0.001	0.90 ± 0.05	n.s.	2.62 ± 0.10	***
0.01	0.55 ± 0.03	***	4.31 ± 0.29	***
0.1	0.29 ± 0.05	***	9.33 ± 0.71	***

[a] "*"; "**" and "***" represent a significant difference (obtained from *t*-tests) with the growth rate on V8 without metalaxyl at a significance level of 0.05; 0.01 and 0.001, respectively. "n.s." represents a non-significant difference with growth rate on medium without metalaxyl.

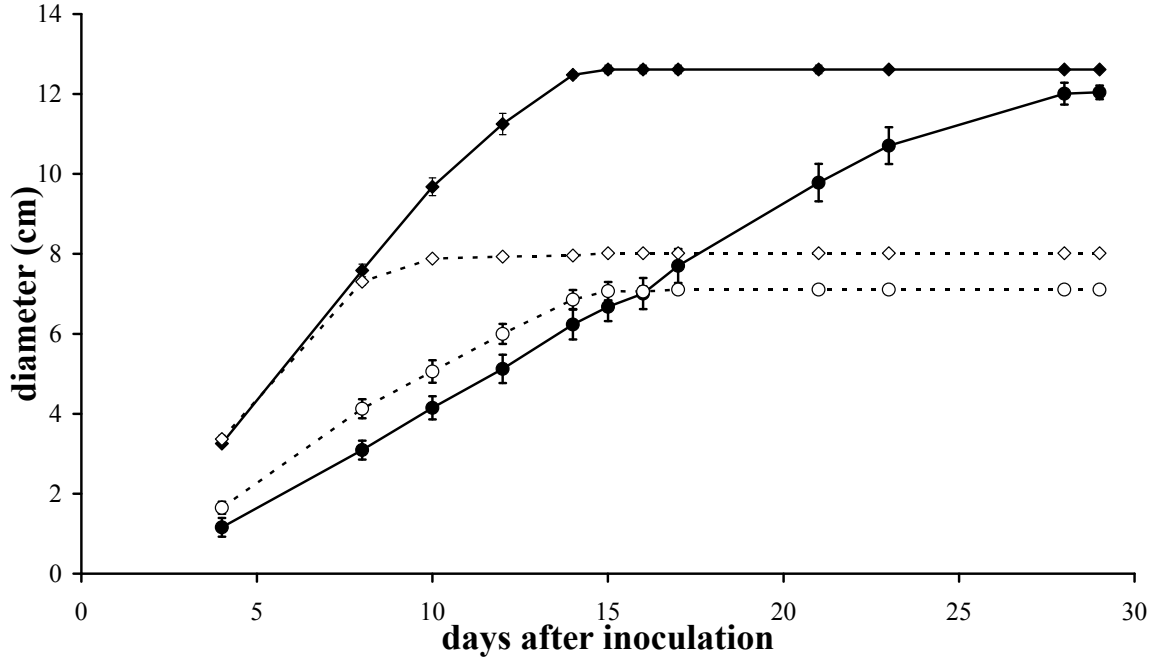

Figure 4. Colony diameter of isolate NL01096 as a function of time on un-amended medium (◇ and ◆) and medium amended with 1.0 µg ml^{-1} fluazinam (○ and ●). Open symbols represent colonies on normal sized plates (∅9 cm) and the closed symbols represent colonies on large plates (∅13 cm). Error bars represent 95 % confidence limits.

Fluctuation tests

Although the effect of metalaxyl-M was not sufficient to fully reduce mycelium growth, it is still the most likely candidate to yield sufficient numbers of resistant colonies, which might appear as colonies with increased mycelium density. Therefore, we performed a fluctuation test using metalaxyl-M as the selective ingredient. We were able to collect large quantities of zoospores: each of the 18 plates yielded between 5 x 10^5 - 2 x 10^6 zoospores, which were plated out on plates containing 100µg ml^{-1} metalaxyl-M. Unfortunately, the amount of resistant colonies could not be assessed since it was impossible to distinguish individual colonies from the background growth of the majority of sensitive zoospores.

We also performed fluctuation tests with cyazofamid and fluazinam for which concentrations could be used that completely prevent background growth of sensitive colonies. After the initial growth on un-amended rye B medium, zoospores were placed onto

plates with either cyazofamid (1.0 μg ml^{-1}) or fluazinam (5.0 μg ml^{-1}). However, no colonies were observed on the selection plates. Because the minimum amount of zoospores that we tested was at least 9 x 10^6 zoospores (18 x 5 x 10^5), we come to the conservative estimation that the frequency (thus not the mutation rate) of resistant mutants to each of these pesticides does not exceed 1 mutant in every 10^7 zoospores.

Sectors emerge regularly on medium amended with metalaxyl-M

During growth experiments on various media amended with metalaxyl-M, we observed several sectors of mycelium with a markedly higher growth rate (Figure 5A). These faster growing sectors were observed in approximately one out of 20 colonies. Mycelial plugs from these sectors were transferred to new plates amended with metalaxyl-M and allowed to grow after which single zoospores were obtained from each colony. Several of these single zoospore derivatives showed a large improvement of the growth rate compared to the ancestor on medium with metalaxyl-M (Figure 5B & 5C). Unfortunately, these sectors can not be used to estimate the mutation rate since the exact time-point of the initial mutation can not be assessed.

Discussion

In this chapter, we tested the suitability of fluctuation tests for estimating the mutation rate to pesticide resistance in *P. infestans*. A fluctuation test is a suitable method if the following prerequisites are met: the ability to collect large numbers of asexual spores and the ability to distinguish mutants from wild-type individuals. For *P. infestans* this means: sufficient numbers of zoospores and dominant mutations that cause pesticide resistance. *A priori*, metalaxyl-M appeared a good candidate as a selective agent since it has a large effect on mycelium growth and dominant resistance is well established in field populations (Gisi and Cohen, 1996; Cooke et al., 2003). However, addition of metalaxyl-M to the medium only resulted in a non-lethal inhibition of mycelium growth. As a consequence, it appeared impossible to distinguish resistant colonies from mycelium that is formed by the majority of sensitive zoospores. Attempts with two other pesticides for which lethal concentrations could be applied failed to yield any resistant colonies due to insufficient zoospore production or the absence of dominant resistance mutations.

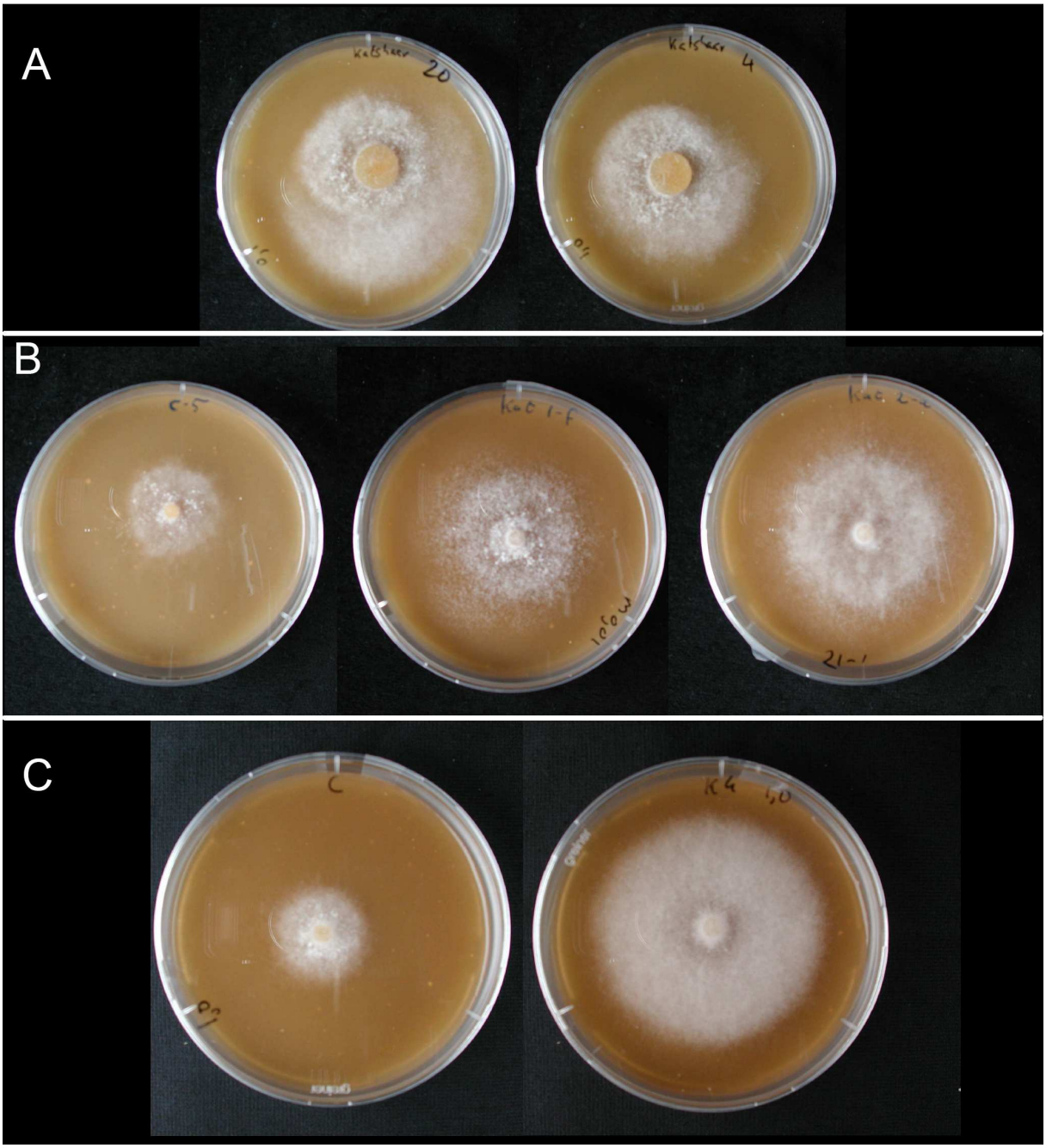

Figure 5. The occurrence of faster growing sectors with a reduced sensitivity to metalaxyl-M on V8 medium. Panel A shows sectors emerging out of a sensitive mycelium. Panels B and C show colonies from single zoospores obtained from faster growing sectors after growing 11 days on V8 medium amended with 0.01 µg ml^{-1} (B) or 1.0 µg ml^{-1} (C) metalaxyl-M. The plate on the left in panels B and C is the sensitive control growing on the same concentrations of metalaxyl-M.

The problems with the fluctuation test reported in this study may reflect yet another problematic feature of *P. infestans* as experimental system (Judelson and Blanco, 2005). Here we briefly evaluate several features of its biology that trouble the use of a fluctuation test. First, due to the diffuse growth of mycelium, it is difficult to distinguish individual colonies. Second, the number of zoospores that can be isolated from one Petri dish is in the order of 10^5-10^6 zoospores. This number is similar to that reported in other studies (Vijn and Govers, 2003), but may be insufficient for performing a proper fluctuation test when the mutation rate is low. Third, the non-haploid nature of the zoospores hampers the expression of recessive mutations. Together, these features suggest that a fluctuation test is not suitable for estimating the mutation rate in *P. infestans*.

The observation that metalaxyl-M is not lethal *in vitro* was surprising given the effectiveness of this pesticide when it became commercially available (Gisi and Cohen, 1996). However, the action of its most likely cellular target, i.e. ribosomal RNA polymerase, is also not fully stopped by metalaxyl-M (Davidse et al., 1988). Apparently, this inhibition is not sufficient to stop the production of new mycelium *in vitro*, whereas it is sufficient to stop epidemics in the field. Possibly, metalaxyl-M reduces the amount of new infections by reducing the infection efficiency (Flier and Turkensteen, 1999) or extending the latent period (Skelsey et al., 2005), as the growth rate appears to be mostly inhibited just after germination, i.e. before the colony size exceeds the initial inoculum size.

The observation that both cyazofamid and fluazinam were lethal at low doses *in vitro*, was not surprising since both pesticides were highly effective in field trials (Evenhuis et al., 2006). The relatively large effect of cyazofamid at low concentrations is not surprising as this substance primarily acts on cytochrome b (Mitani et al., 2001a), which is a crucial part of the electron transport chain in the mitochondria. Fluazinam is known to be a general uncoupler of the oxidative phosphorylation (Guo et al., 1991). This mode of action may explain the difference in the final diameter of colonies growing on sub-lethal concentrations (Figure 4), because the lower energy efficiency will reduce the amount of biomass that can be created before the medium is depleted. This reduction in efficiency may become lethal on higher concentrations of fluazinam.

Despite the apparent failure of the fluctuation tests using metalaxyl-M, the observation that faster growing sectors occur regularly, suggests a high rate of evolution towards metalaxyl-M resistance. Indeed, single zoospores from a single metalaxyl sensitive colony

can differ substantially in growth characteristics (Caten and Jinks, 1968) and AFLP genotype (Abu-El Samen et al., 2003a), but also in the sensitivity to metalaxyl-M. Some of these single zoospores had EC_{50} values (the concentration that reduces growth by 50%) exceeding 100 µg ml^{-1} (Abu-El Samen et al., 2003b), indicating an immediate shift to full resistance. These results indicate that the frequency of resistant zoospores from a sensitive colony, i.e. the mutant frequency, is high.

This relative fast rate of evolution of metalaxyl resistance can have several causes. First, because metalaxyl-M acts on a large protein complex, i.e. ribosomal RNA polymerase, it is possible that mutations in more than one sub-unit can cause resistance. Indeed, the chromosomal position of the resistance locus can differ between genotypes (Fabritius et al., 1997). Second, the gene or genes responsible for metalaxyl-M resistance may be situated in a mutation hotspot, a genomic position with a higher mutation rate relative to other genomic positions (Rogozin and Pavlov, 2003). Third, the fact that metalaxyl resistance is governed by at least one dominant mutation will greatly facilitate its evolution (Jasieniuk et al., 1996). Finally, metalaxyl resistance has never been associated with a large cost (Lee et al., 1999). The absence of such costs can lead to a high mutant frequency, even when mutation rates are low (Oliver et al., 2004).

Because we did not observe any resistant colonies on plates amended with a lethal concentration of cyazofamid or fluazinam, the mutation rate towards resistance for these two pesticides can not be assessed. However, the frequency of resistant mutants, i.e. 1 out of 10^7 zoospores, is substantially lower than that reported for metalaxyl (Abu-El Samen et al., 2003b). We may therefore conclude that resistance to these pesticides will not evolve as quickly as resistance to metalaxyl did at the beginning of the 1980's.

Several explanations can be given here for the absence of mutants resistant to cyazofamid or fluazinam. First, the resistance mutations may be recessive, which may be caused by a negative pleiotropic effect of the resistance mutation on fitness (Bourguet and Raymond, 1998). Additionally, these negative pleiotropic effects may be responsible for a low mutant frequency. For cyazofamid, such pleiotropic effects may exist given the central role of cytochrome b in cellular respiration. However, resistance against other inhibitors of the cytochrome b has been observed in other oomycetes (Gisi et al., 2000; Gisi et al., 2002) suggesting a high risk for resistance development against cyazofamid in those species. Second, full resistance may depend on more than one mutation. This may be of particular

importance for fluazinam, because it does not act on a gene product that can be altered by a simple mutation.

The rate of resistance evolution is not solely influenced by the mutation rate; other population genetic parameters, such as the population size, may also influence it. Therefore, a low mutation rate does not necessarily mean that the risk of resistance development is low. For instance, *P. infestans* is renowned for the large number of asexual spores it produces per single lesion, which lies in the order of 10^5 sporangia (Flier et al., 2007). The chance of the emergence of resistant genotypes is relatively high in such large populations of nuclei, even if mutation rates are low (Jasieniuk et al., 1996). On the other hand, a low mutation rate may be disadvantageous, since the host-free period during winter is regarded as a serious population bottleneck in *P. infestans* (Goodwin, 1997). Such bottlenecks severely decrease the effective population size for adaptation to novel conditions, which causes a high mutation rate to be beneficial for the evolution of resistance (de Visser et al., 1999; Elena and Lenski, 2003). Hence, the effect of the mutation rate on the evolution of resistance depends heavily on the effective population size.

To conclude, the only dominant selectable marker that was known beforehand, i.e. metalaxyl-M resistance, appeared not to be suitable for application in a fluctuation test, because of the non-lethality of metalaxyl-M *in vitro*. Additionally, the mutant frequencies on plates with either fluazinam or cyazofamid were too low for accurate estimation of the mutation rate. Therefore, we believe that fluctuation tests are not really feasible for estimating the mutation rate towards pesticide resistance in *P. infestans*, largely due to the lack of suitable dominant markers and the insufficient production of asexual spores within feasible culture volumes. However, differences in the rate of resistance evolution may be due to other factors besides differences in mutation rate, such as the cost of resistance. Knowledge of these factors will help in understanding the relative fast evolution of metalaxyl resistance compared to other resistances (Gisi and Sierotzki, 2008).

Chapter 3

Selection for full resistance on sub-lethal pesticide concentrations in *Phytophthora infestans*

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Abstract

One factor that affects the rate of resistance evolution is the range of concentrations on which full resistance is favored over low-level resistance. Differences in the size of these ranges may explain differences in the rate of evolution between several pesticides. Here, we use the late blight pathogen *Phytophthora infestans* to test the ability to select for full resistance at sub-lethal concentrations of two pesticides. We performed an experimental evolutionary procedure in which we serially transferred zoospores of two sensitive genotypes on sub-lethal concentrations with comparable growth-reducing effects of either metalaxyl, which rapidly generated resistance in the field, or cyazofamid, for which resistance is no problem. We found rapid heritable adaptation to the concentrations used for both isolates and both pesticides. However, the specificity of the response differed radically: selection on metalaxyl led to full resistance, whereas selection on cyazofamid was specific for the sub-lethal concentration used. Consequently, full metalaxyl resistance may be selected on a broader range of concentrations than resistance to cyazofamid. Our results do indeed suggest that rapid selection of full resistance is possible at sub-lethal concentrations for some, but not all pesticides, which may help to understand differences in the speed of evolution of pesticide resistance in natural settings.

Introduction

Agriculture and medicine depend heavily on antimicrobial compounds for pest control. However, many compounds have become ineffective due to the occurrence of resistant genotypes in pathogen populations (Palumbi, 2001). In order to prevent a similar build-up of resistance to new pesticides, it is crucial to gain more knowledge on the factors that influence selection of resistance mutations.

The result of selection for resistance depends largely on the pesticide concentration that is encountered by the pathogen. On the one hand, if pesticide concentrations are high enough to kill all sensitive genotypes, resistance will only be caused by mutations with a large effect (on resistance) that were already present in the population at a low frequency (Zhou et al., 2000; Drlica, 2003). These mutations may cause full resistance, i.e. they allow growth on concentrations that are much higher than those used to control sensitive populations. On the other hand, if pesticide concentrations are low, mutations with small effect (on resistance) may also be selected (Negri et al., 1994; Perfeito et al., 2007). Selection on a lower concentration may thus lead to a low level resistance (Baquero, 2001). Moreover, mutations with small effect may occur more frequently and large effect mutations may have large negative pleiotropic effects (Lande, 1983). As a result, small-effect mutations may be favored over large-effect mutations on a range of sub-lethal concentrations.

The pesticide concentration can also affect the rate of resistance evolution. On the one hand, sub-lethal concentrations may quickly select for full resistance, especially at intermediate concentrations due to an optimal balance between effective population size and strength of selection (Lipsitch and Levin, 1997). On the other hand, conditions favoring low-level resistance may eventually culminate into full resistance, but this evolutionary pathway is generally slower (Baquero, 2001).

Full resistance to the pesticide metalaxyl is common in present-day populations of the oomycete *Phytophthora infestans*, the causal agent of the devastating late blight disease in potato (Cooke et al., 2003; Cooke et al., 2006). The single mode of action of the pesticide, i.e. inhibition of ribosomal RNA polymerase (Davidse et al., 1988), suggests that resistance may be caused by a single mutation, which would facilitate its evolution. Indeed, resistance evolved in a relatively short period following the commercial release of metalaxyl (Davidse et al., 1981; Dowley and O'Sullivan, 1981). Genetic analyses using sexual crosses revealed the likely action of a single mutation with large effect (Shattock, 1988). However, mutations with

small effect also have a substantial effect on the level of sensitivity to metalaxyl (Fabritius et al., 1997). These small effect mutations may also be responsible for the large variation of metalaxyl sensitivity that has been observed among zoospores derived from the same sensitive colony (Abu-El Samen et al., 2003b). Even more, they may be responsible for the variation of sensitivity that is observed among sensitive genotypes in field populations (Grunwald et al., 2006).

As a result of the fast evolution of resistance to metalaxyl, several other pesticides were made available. Surprisingly, hardly any resistance has been observed for these other pesticides. For instance, cyazofamid is a relatively new pesticide that reduces electron transfer in mitochondria by specifically interacting with cytochrome b (Mitani et al., 2001a; Mitani et al., 2001b). Despite this single mode of action and the existence of resistant laboratory mutants (Ziogas et al., 2006), no resistance against cyazofamid has so far been described in field populations.

The large difference in the rate of resistance evolution for metalaxyl and for other pesticides is striking and may be explained by measures to prevent resistance build-up. However, this pattern may also be explained by differences in the range of concentrations on which full resistance can evolve. Full resistance to metalaxyl is then favored on a wider range of concentrations than full resistances to other pesticides, like cyazofamid. Indeed, selection for full resistance on inhibitory concentrations appears to be much easier for metalaxyl than for other pesticides in several studies (Davidse, 1981; Young et al., 2001; Stein and Kirk, 2004). These studies, however, were all conducted by serially transferring faster growing sectors of mycelium and did not include crucial stages of the asexual life cycle such as the production of sporangia and the germination of zoospores (Judelson and Blanco, 2005). A procedure including these stages will resemble the natural situation more closely and may yield different results (Schoustra et al., 2005).

Because metalaxyl resistance evolved relatively fast compared to resistance to cyazofamid, we hypothesize that full metalaxyl resistance is favored on a broader range of inhibitory concentrations than full resistance to cyazofamid. Hence, a concentration of metalaxyl may select for full resistance, whereas this may not be the case for a concentration of cyazofamid with a similar effect. We performed a selection experiment in which we serially transferred zoospores from two initially sensitive genotypes on artificial medium with a constant sub-lethal concentration of one of two pesticides: metalaxyl or cyazofamid.

Selection on medium with metalaxyl yielded genotypes that showed an increased growth rate on the selective concentration. This adaptation was stable, confirming that it is a heritable change, and increases growth rate on higher concentrations as well. In contrast, heritable adaptations to the selective concentration of cyazofamid were observed, but these adaptations were not beneficial at higher concentrations.

Methods

Collection of zoospores

Mycelium was grown on Petri dishes containing rye B agar (Caten and Jinks, 1968) in the dark for 10-14 days at 18 °C. Zoospore suspensions were created by flooding these cultures with 10 ml cold water (6°C) and storing them in the cold room (4°C) for 2.5 hours to induce zoosporogenesis. The zoospore suspension was then transferred to sterile test tubes and put on ice for 30 minutes. The top 1 ml of each test tube, which is enriched with zoospores (Vijn and Govers, 2003), was then transferred to an Eppendorf tube and vortexed for one minute to induce encystment of the zoospores. The concentration of these suspensions was assessed using a haemocytometer and adjusted to 2 x 10^5 cysts ml^{-1}. The suspensions were then used to inoculate new plates by placing a 50 µl drop (containing 1 x 10^4 cysts) onto the center of the plate.

Experimental evolutionary procedure

An experimental evolutionary procedure was initiated with one clone from isolate NL01096 (metalaxyl sensitive; A2 mating type) and one from isolate VK98014 (metalaxyl sensitive; A1 mating type). Both isolates were obtained from the liquid nitrogen storage facility at Plant Research International in Wageningen, The Netherlands (Flier et al., 2003). The concentrations used during the procedure were 0.01 µg ml^{-1} metalaxyl-M (an isomer of metalaxyl) and 0.002 µg ml^{-1} cyazofamid. Previous experiments showed that mycelium growth was inhibited but not stopped at these concentrations (data not shown). Furthermore, the inhibition of mycelium growth rate was similar at these concentrations.

In order to obtain a clone, i.e. mycelium derived from a single zoospore, 50 µl of a zoospore suspension containing 1x10^4 zoospores ml^{-1} was put onto the center of a plate containing Rye B agar and spread out using a glass rake. These plates were incubated

overnight at 18° C in the dark. The next day, small pieces of agar containing a single germinated zoospore cyst were cut out under a dissecting microscope and transferred to new small Petri dishes (∅ 5.5 cm) with Rye B agar.

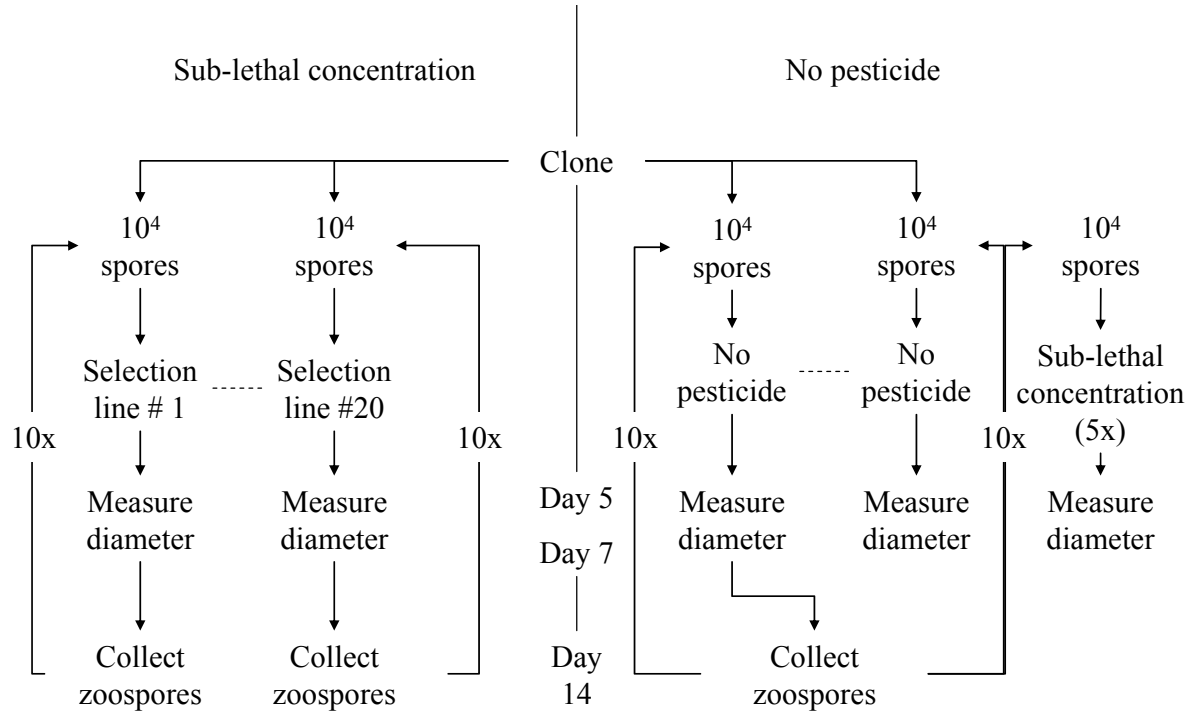

Figure 1. Schematic representation of the experimental evolutionary procedure. Selection plates contained the sub-lethal concentration of the pesticide, i.e. 0.01 μg ml^{-1} metalaxyl or 0.002 μg ml^{-1} cyazofamid, and control plates did not contain any pesticide. A clone is the mycelium derived from one individual zoospore.

A graphical representation of the procedure is given in Figure 1. At the start of the procedure, a zoospore suspension (2 x 10^5 cysts ml^{-1}) was collected from a sensitive clone, i.e. mycelium derived from a single zoospore. This suspension was used to initiate 20 parallel selection lines by inoculating 20 plates containing rye B medium amended with a sub-lethal dose of the pesticide. A parallel control line was created by inoculating five plates containing un-amended rye B medium. Fourteen days later, zoospore suspensions (2 x 10^5 cysts ml^{-1})

were obtained from each selection line and used to inoculate new plates with the same pesticide concentration. Additionally, a zoospore suspension from an un-amended plate was used to inoculate five new amended plates and five new un-amended plates. Colony growth was monitored during each cycle by measuring two perpendicular diameters on day five and day seven after inoculation.

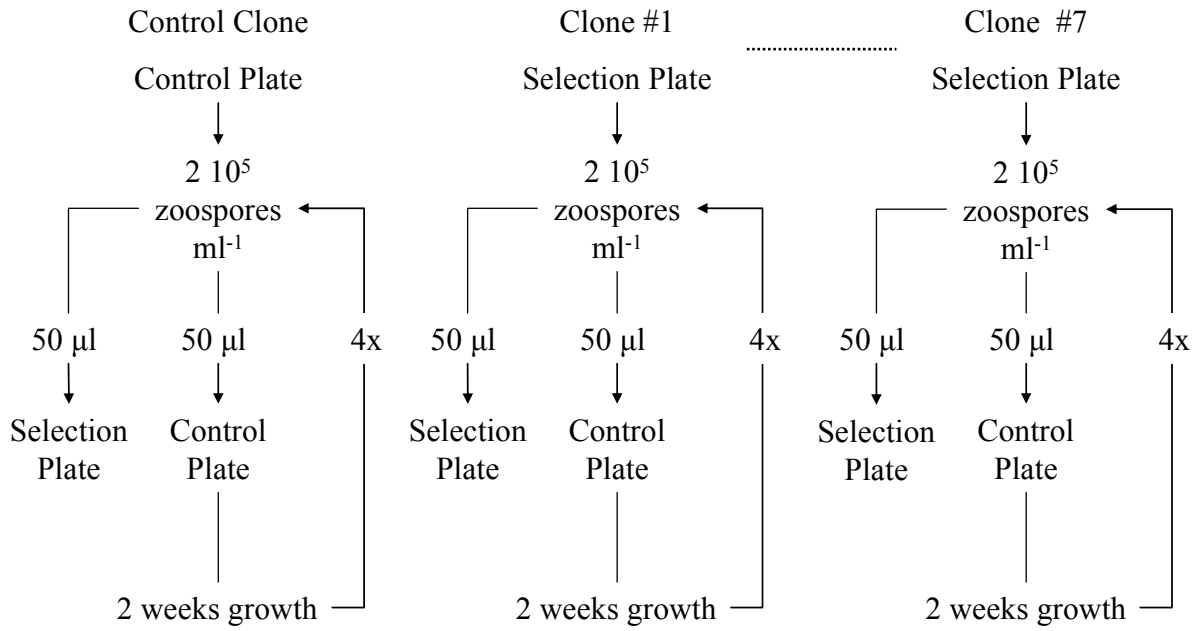

Figure 2. Schematic representation of the experiment on the stability of the adaptation. Selection plates contained the sub-lethal concentration of the pesticide, i.e. 0.01 µg ml^{-1} metalaxyl or 0.002 µg ml^{-1} cyazofamid, and control plates did not contain any pesticide. A clone is the mycelium derived from one individual zoospore.

After 10 cycles, the procedure was terminated and new clones were collected from each selection plate and from one control plate. Each clone was stored in liquid nitrogen for use in future experiments. A code was assigned to each clone. This code consists of two numbers separated by a hyphen: the first number represents the selection line (1 to 20) and the second represents the number of the zoospore from which the clone was derived.

From each procedure, two selection lines that showed an adaptation were chosen for further characterization. These characterizations were performed on two clones per selection line and involved both an analysis on the stability and the sensitivity of the adaptation. Additionally, one clone from the control plates was added to this subset to serve as a near isogenic sensitive control.

Stability of the adaptation and sensitivity to higher concentrations

Prior to the experiment, clones from the selection experiment were maintained four weeks on Rye B amended with the selective concentration (0.01 μg ml^{-1} metalaxyl or 0.002 μg ml^{-1} cyazofamid) and the clone from the sensitive control was maintained on un-amended Rye B. Zoospore suspensions were obtained and used to inoculate plates with increasing pesticide concentrations. Per clone, five plates were inoculated per concentration, for a total of six concentrations of metalaxyl-M (0; 0.001; 0.01; 0.1; 1.0 and 10 μg ml^{-1}) and five concentrations of cyazofamid (0; 0.002; 0.02; 0.1 and 1.0 μg ml^{-1}). Colony diameters were monitored daily by measuring two perpendicular diameters on each colony. Two weeks later, zoospore suspensions were obtained from one un-amended plate from each clone and used to inoculate five new un-amended plates. This cycle was repeated four times after which the growth rate was assessed one more time on plates containing the selective pesticide concentration (Figure 2) to asses the stability of the adaptation.

Data analysis

The colony growth rate during the selection procedure was calculated as the difference between the diameters on day 5 and day 7 during each cycle and expressed relative to that of the parallel control line on un-amended medium. Growth rates of individual lines were transformed by subtraction of the growth rate during the first cycle. The response to selection of individual lines was then analyzed using linear regression through the origin with the transformed growth rate as the dependent variable and number of cycles as the explanatory variable. Differences in the response between NL01096 and VK98014, i.e. the ancestors, was analyzed using a repeated measures ANOVA on the growth rate with cycle as the within subject factor and ancestor as the between subject factor.

In order to analyze differences in the sensitivity to higher concentrations, the change in diameter was calculated for each time point as the difference between the diameter at that time point and the diameter at the first measurement. The growth rate was assessed by linear regression through the origin for each concentration separately with the change in diameter as the dependent variable and the time since the first measurement as the explanatory variable. The slope of the regression line of each single zoospore was compared with that of the ancestral genotype. Time points at which the colony diameter of one colony exceeded 7 cm were excluded from the analysis to avoid possible growth inhibition by the plate's edge. Differences in the growth rate between the different genetic backgrounds were analyzed using an ANOVA with ancestor as fixed factor, selection line nested within ancestor and clone nested within selection line.

The stability of the adaptation was analyzed using *t*-tests to compare differences in growth rate on amended medium. Serial Bonferroni corrections (Rice, 1989) were used to correct for multiple comparisons. All statistical analyses were performed using SPSS 15.0.

Results

Metalaxyl

At the end of the selection procedure, 12 of the 20 NL01096 selection lines and 13 of the 20 VK98014 selection lines had survived without infection (which is a notorious problem for this slow growing organism). On average, the selection lines showed an increase in growth rate under the selective conditions when estimated with linear regression ($F_{1,133} = 26.6$, $P < 0.001$ for NL01096; $F_{1,186} = 7.30$, $P < 0.001$ for VK98014; Figure 3A and 3B), although this increase was significantly faster for NL01096 (see Table 1). The response to selection, however, varied among individual lines: four of the NL01096 lines showed no significant increase, whereas all of the VK98014 lines showed significant increases of the growth rate after sequential Bonferroni correction. Additional variation in the onset of adaptation was observed, with some lines showing an almost immediate increase in growth rate and others showing a later increase in growth rate (Figure 3A and 3B). The rapid response seen in some lines was consistent with the observation that sectors of mycelium with a higher growth rate were frequently observed during pilot experiments on medium with metalaxyl-M (Chapter 2).

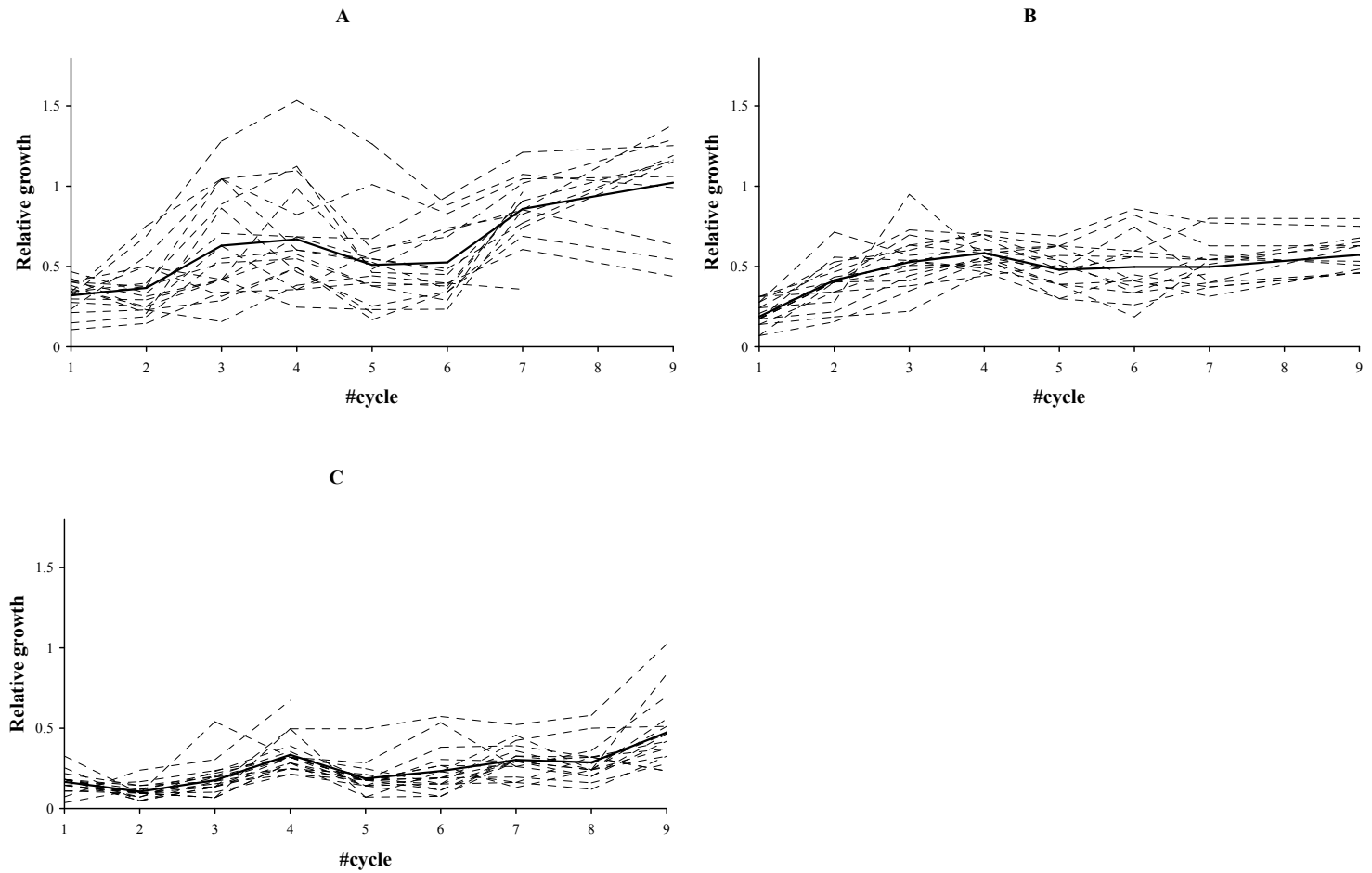

Figure 3. The relative growth rate during selection on rye B amended with 0.01 µg ml^{-1} metalaxyl using *P. infestans* isolates NL01096 (A) and VK98014 (B) and during selection with cyazofamid using isolate VK98014 (C). The relative growth was calculated as the difference in colony diameter between day five and day seven on medium with the selective agent divided by the similar difference of the sensitive control on medium without the selective agent. The dotted lines represent the individual selection lines and the solid line represents the average relative growth rate of all selection lines.

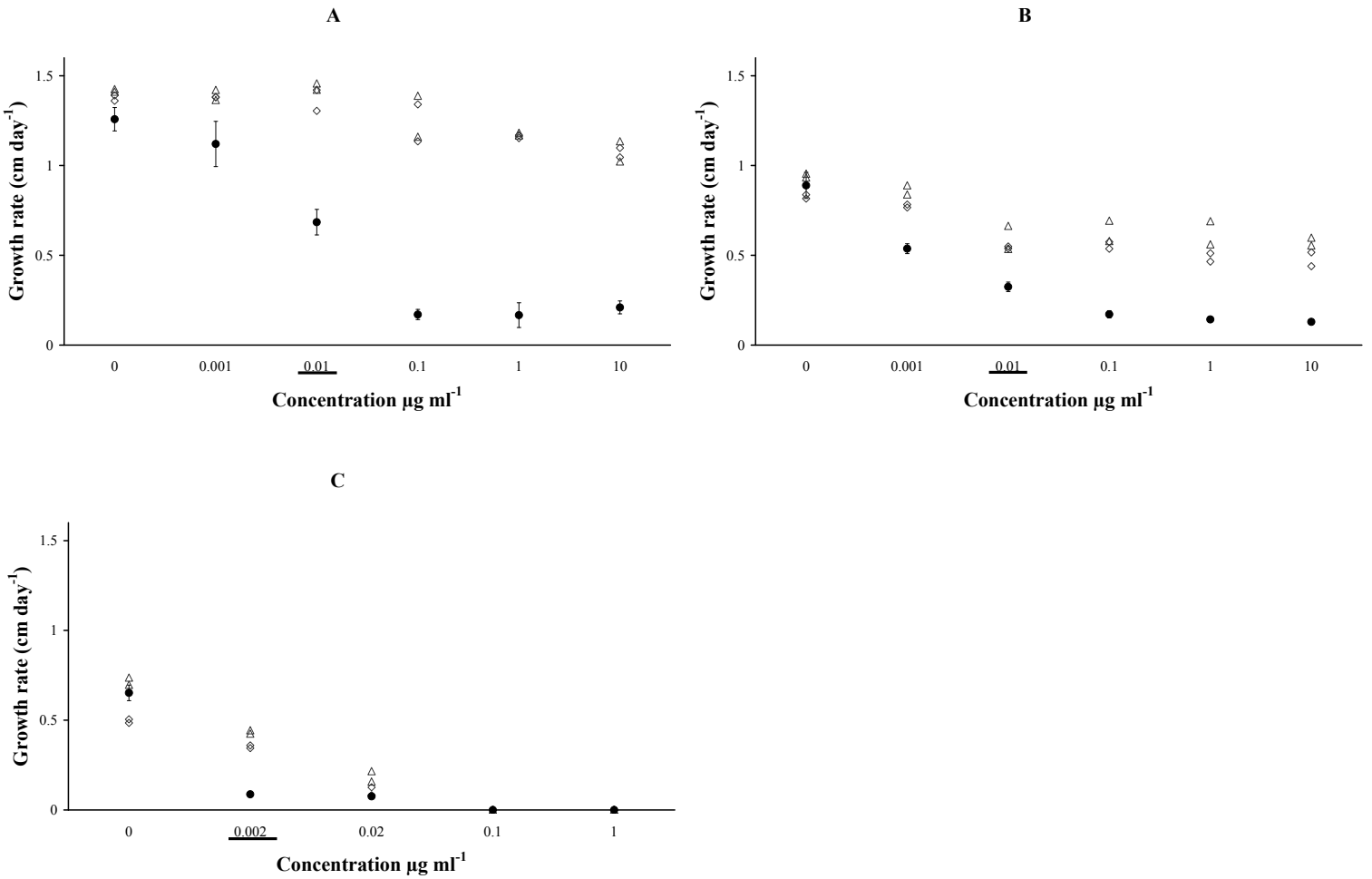

Figure 4. The growth rate (cm day^{-1}) of four clones from the selection lines (open symbols) and the control (closed symbols) as a function of the concentration (µg ml^{-1}); clones represented by the same symbol are derived from the same selection line. Each graph shows the results after a different selection procedure: NL01096 on metalaxyl (3A), VK98014 on metalaxyl (3B) and VK98014 on cyazofamid (3C). Error bars of the control represent the 95% confidence limits. The underlined concentration is used during the selection regime.

After the selection procedure was terminated, a subset of single-zoospore derivatives from the selection lines was compared with the sensitive control. The growth rate of both controls, i.e. NL01096 and VK98014, was severely reduced by metalaxyl-M but continued on concentrations up to 10 μg ml^{-1} (Figure 3A and 3B). The growth rate of the clones from the selection lines was also affected by metalaxyl-M but was significantly higher (two-sample *t*-tests, $P < 0.001$) than that of their respective control on each metalaxyl-M concentration used (Figure 4a and 4b). Moreover, several of the single-zoospore derivatives did not show a lower growth rate than that of the control in the absence of metalaxyl-M (two-sample *t*-tests, $P > 0.05$). Furthermore, after four cycles on medium without metalaxyl-M, the growth rate of the selected clones was still significantly higher on medium with 0.01 μg ml^{-1} metalaxyl-M (Table 2), suggesting that adaptations were stable over several generations. Finally, because the growth rate on medium with 10 μg ml^{-1} metalaxyl-M was between 40% and 70 % compared to that on medium without metalaxyl-M, these clones would at least be judged as intermediately resistant and some as fully resistant according to the criteria of Cooke et al. (2003).

Table 1. Repeated measures ANOVA on the mycelial growth rate during 10 cycles of selection of two ancestors of *P. infestans* (NL01096 and VK98014) on Rye B amended with 0.01 μg ml^{-1} metalaxyl.

Between subjects

Source	df	M.S.	F	p
Ancestor	1	1.23	7.53	0.012
error	23	0.16		

Within subjects

Source	df	M.S.	F	p
cycle	7	0.73	26.33	<0.0001
cycle x ancestor	7	0.15	5.56	0.0002
error	161	0.03		

Table 2. The mean growth rate (± s.e.m.) on Rye B agar amended with the concentration that was used during selection (0.01 µg ml^{-1} metalaxyl or 0.002 µg ml^{-1} cyazofamid) of clones, and a control with the same genetic background after four cycles on medium without metalaxyl. [a]: significantly different from the control after Bonferroni correction (P<0.05).

Pesticide	Ancestor	clone	Mean growth rate cm day^{-1} (± s.e.m.)	
Metalaxyl	NL01096	8-1	0.83 ± 0.04	[a]
(0.01 µg ml^{-1})		8-2	0.52 ± 0.01	[a]
		13-1	0.86 ± 0.02	[a]
		13-2	0.80 ± 0.04	[a]
		Control	0.19 ± 0.03	
	VK98014	6-4	0.94 ± 0.02	[a]
		6-5	0.96 ± 0.02	[a]
		15-1	0.89 ± 0.02	[a]
		15-5	0.91 ± 0.03	[a]
		Control	0.26 ± 0.02	
Cyazofamid	VK98014	1-1	0.58 ± 0.02	[a]
(0.002 µg ml^{-1})		1-4	0.57 ± 0.01	[a]
		7-2	0.38 ± 0.02	[a]
		7-4	0.34 ± 0.04	[a]
		Control	0.15 ± 0.03	

The effect of the adaptation depends on the genetic background as indicated by a significant genotype effect (Table 3). Indeed, the mycelium growth rates of clones from NL01096 were higher compared to the clones from VK98014 on all concentrations. This effect was particularly clear at higher concentrations, as evidenced by a significant strain x concentration interaction (Table 3). In addition, the effects of lines within strains and spores within lines were also significant, although they explain each only about 1% of the total variance, while the difference between strains explains about 75% (Table 3).

Table 3. Nested ANOVA on mycelial growth rate of two randomly chosen clones from two random evolved lines from each ancestral genotype of *P. infestans* (NL01096 and VK98014) on varying concentrations of metalaxyl.

Source	d.f.	M.S.	F	p
Concentration	5	0.0747	17.04	< 0.0001
Ancestor	1	9.0819	2070.38	< 0.0001
Concentration x Ancestor	5	0.1858	42.35	< 0.0001
Line (Ancestor)	2	0.0727	16.58	< 0.0001
Concentration x Line (Ancestor)	10	0.0133	3.02	0.0015
Clone (Line (Ancestor))	4	0.0766	17.46	< 0.0001
Concentration x Zoospore (Line (Ancestor))	20	0.0158	3.60	< 0.0001
Error	170	0.0044		

Adaptation to medium without metalaxyl-M

An additional pattern was observed during the four transfers to test the stability of the adaptation in the absence of metalaxyl-M. At the beginning of this experiment, the growth rate on medium without metalaxyl of clones from selection line 6 from VK98014 and from the control did not differ from each other (Figure 5A). After five transfers on medium without metalaxyl, however, the growth rate of the selected clones was higher than that of the ancestor on medium without metalaxyl-M. Additionally, this increase of the growth rate was accompanied by an increase of growth rate on medium amended with 0.01 μg ml^{-1} metalaxyl, which was clearly not the case for the control (Figure 5B).

Cyazofamid

Fifteen of the 20 selection lines from VK98014 survived the experiment without infection whereas none of the lines from NL01096 did. The response to selection on medium with 0.002 μg ml^{-1} cyazofamid differed from that observed in the selection experiment with metalaxyl-M. On average, colony growth rate under the selective conditions increased significantly during selection ($F_{1,173} = 25.3$, $P < 0.001$), but was typically observed later and only significant ($P < 0.05$) for six lines (Figure 2C).

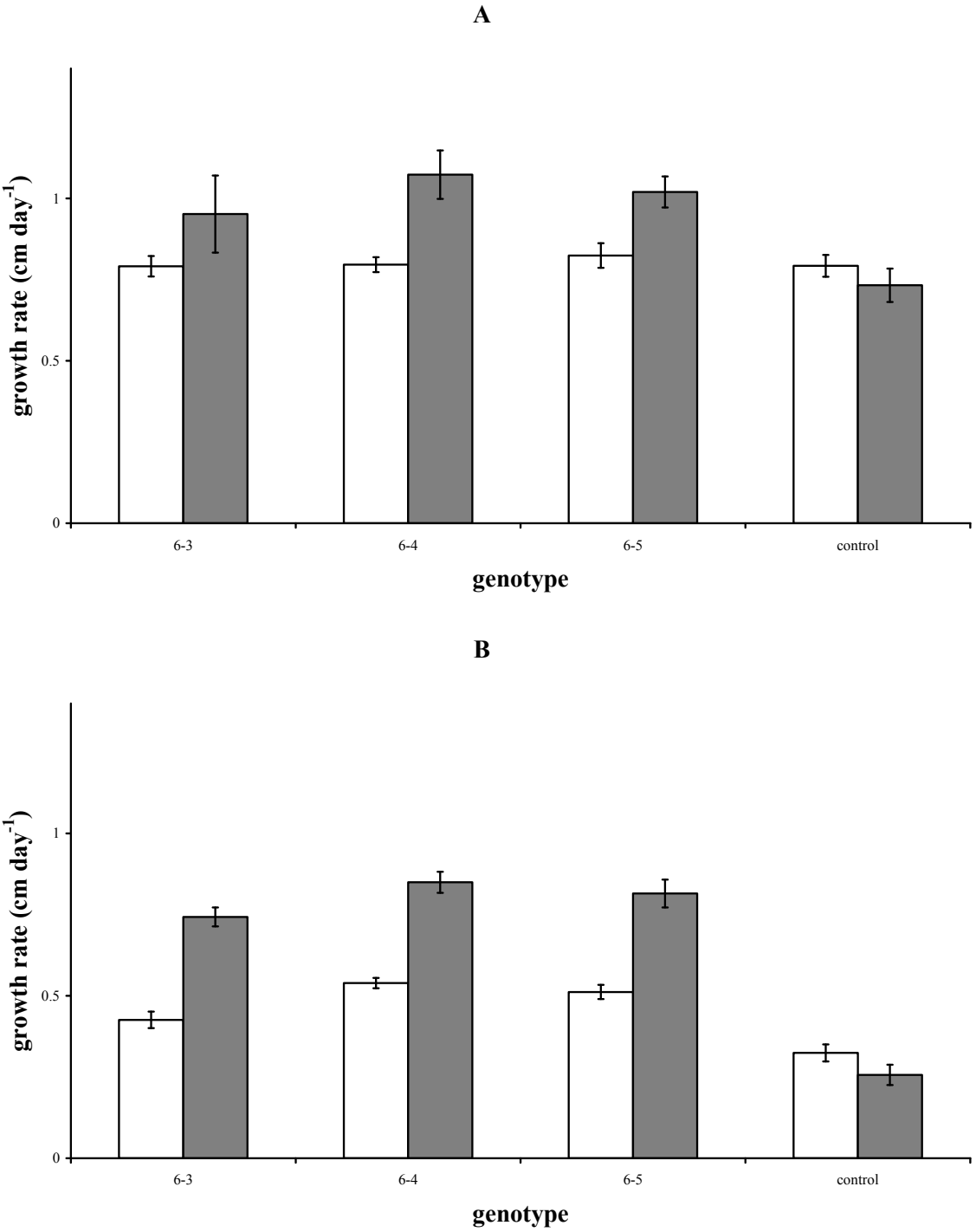

Figure 5. The growth rate before (white bars) and after (gray bars) 5 rounds of selection on Rye B medium without metalaxyl of three metalaxyl resistant clones from VK98014. The sensitive control clone is shown as a reference. Growth rates were assessed on medium without (5A) and with 0.01µg ml^{-1} metalaxyl-M (5B). Error bars represent the 95% confidence interval of the mean growth rate.

After the selection procedure was terminated, a subset of single-zoospore derivatives from the selection lines was compared with the sensitive control for the specificity of adaptation. The growth rate of the control was severely reduced by cyazofamid and growth was completely stopped on concentrations of 0.1 μg ml^{-1} and higher (Figure 4C.). Growth rates of the single-zoospore derivatives from the selection lines were also affected by cyazofamid but were only significantly higher (two-sample *t*-tests, $P < 0.001$) on a small range of concentrations. Furthermore, mycelium growth was totally absent on concentrations of 0.1 μg ml^{-1} and higher (Figure 4A and 4B).

Similar to the outcome of the procedure with metalaxyl-M, no consistent cost of resistance was observed on medium without cyazofamid, although some lines showed a reduced growth rate (Figure 4C). Similarly, this adaptation was stable after four cycles on medium without cyazofamid, (Table 2), showing that also here adaptation was stable for several generations.

Discussion

The range of concentrations on which large-effect mutations are favored over mutations with a small effect is an important factor in the evolution of resistance. By serially transferring zoospores of *P. infestans* on artificial medium with a constant sub-lethal concentration of metalaxyl, we were able to select for full resistance. However, selection on a sub-lethal concentration of cyazofamid with a similar effect on the mycelium growth rate resulted in resistance that was specific for the low concentration used. Additionally, growth of the sensitive genotypes was not stopped on metalaxyl concentrations up to 10 μg ml^{-1} whereas no growth was observed on cyazofamid concentrations exceeding 0.1 μg ml^{-1}. These results indicate that full resistance to metalaxyl can be selected on a broader range of sub-lethal concentrations (≥0.01 μg ml^{-1} to ≥10 μg ml^{-1}) than full resistance to cyazofamid (>0.002 μg ml^{-1} to <0.1μg ml^{-1}), if it exists at all. Moreover, the range of concentrations that favor full resistance to metalaxyl may include even lower concentrations. Hence, the results from this study may provide an explanation for the higher rate of evolution of metalaxyl resistance compared to cyazofamid resistance.

Because we transferred zoospores instead of mycelial plugs, our experiment differs from other selection experiments (Davidse, 1981; Young et al., 2001; Stein and Kirk, 2004).

With our approach, we better mimicked the natural situation for two reasons. First, by allowing production and germination of zoospores under selective conditions, we selected for mutant genotypes with the highest fitness during their entire *in vitro* asexual life-cycle. Serial transfer of fast growing sectors, on the other hand, would only select for mutant genotypes with increased mycelium growth rate, which may suffer from negative pleiotropic effects on other fitness components. Second, because *P. infestans* is coenocytic (its hyphae do not contain true cells), transferring mycelial plugs may keep heterokaryons intact, including those that contain combinations of sensitive and resistant nuclei (Bruin and Edgington, 1982). Zoospores generally contain one nucleus; hence the formation of zoospores will disrupt heterokaryons and partially prevent the 'hitchhiking' of sensitive nuclei. Because zoospores are believed to be the primary source of infection (Judelson and Blanco, 2005), heterokaryons are also disrupted under field conditions.

Despite the differences between our procedure and that of others, the outcome was qualitatively similar (Young et al., 2001): selection for full resistance was rapid on sub-lethal concentrations of metalaxyl, but absent on sub-lethal concentrations of other pesticides. This similarity suggests that, at least for metalaxyl, the evolution of resistance followed a similar path. Indeed, we observed faster growing sectors during the cycle that preceded a significant growth rate increase. If a sector is caused by a mutation that does not seriously impair the production and germination of zoospores, it will contribute more zoospores to the next generation. In that case, selection via zoospores will be largely equivalent to selection using mycelium.

The observation that the adaptations to cyazofamid and to metalaxyl were stable, suggests that these adaptations are not caused by a plastic response but by heritable changes (genetic or epigenetic). Because cyazofamid resistance has not yet been described, it is unknown what kind of heritable change is responsible for the adaptation, however, much more is known about the genetics of metalaxyl resistance (Gisi and Cohen, 1996). At least two known factors are involved that may positively influence the rate of resistance evolution. First, metalaxyl resistance is believed to be caused by an intermediate dominant allele with large effect, which is influenced by the action of additional loci with small effect (Shattock, 1988; Fabritius et al., 1997). This is particularly important because, unlike fungal and bacterial pathogens, *P. infestans* does not have an extended haploid phase during its life cycle. In the absence of sexual recombination, a recessive mutation will then only be selected when

it becomes homozygous due to mitotic recombination or by the rare occasion of the same mutation in the other allele. Second, substantial variation in growth characteristics (Caten and Jinks, 1968), AFLP genotype (Abu-El Samen et al., 2003a), virulence and metalaxyl resistance (Abu-El Samen et al., 2003b) have been observed among zoospores derived from the same sensitive colony. The fast increase in growth rate and the occurrence of fast growing sectors in our procedure with metalaxyl are in line with those observations, suggesting a high mutation rate towards metalaxyl resistance. Finally, the contribution of the genetic background on the sensitivity to metalaxyl suggests that the level of resistance is not solely determined by a single mutation but also by additional loci with a smaller effect.

The lack of a significant cost of resistance to metalaxyl is surprising as resistance mutations are believed to incur a fitness cost in the absence of the particular pesticide (Roux et al., 2004; Andersson, 2006; Lopez-Rodas et al., 2008). However, metalaxyl resistance has always been associated with a high epidemic fitness in several countries, which suggests a direct positive effect of resistance (Kadish and Cohen, 1988b; Gisi and Cohen, 1996). This result may also be explained by differences in genetic background between sensitive and resistant field isolates. Randomization of the genetic background by sexual recombination, however, revealed no association between resistance and aggressiveness (Lee et al., 1999). Unfortunately, sexual crosses of *P. infestans* are characterized by a low offspring survival (Knapova et al., 2002), hence small negative effects may be missed. However, the observation of an increase in growth rate during 5 transfers in the absence of metalaxyl may indicate that additional mutations may influence the effect of the resistance mutation. This would than indicate that small negative effects of the resistance mutation may be indeed be influenced by the genetic background.

Several factors may explain the lack of selection for full resistance to cyazofamid in our experiments: a mutation with a large effect on cyazofamid resistance may be recessive, may carry a large negative pleiotropic effect or may not occur frequently due to a lower mutation supply rate. Because we do not know whether large-effect mutations exist, we can not answer to what extent the effect of dominance and pleiotropy can explain the absence of such a mutation in our experiment. However, assuming a constant genome-wide mutation rate, the mutation supply rate will be directly coupled to the number of mitoses per unit of time. Because both pesticide concentrations had similar inhibitory effects on growth rate at the start of the experiment, we do not expect a large difference in the mutation supply rate

between the two experiments. However, because it is possible that more mutations can cause full resistance to metalaxyl (Judelson and Roberts, 1999) than to cyazofamid, the mutation supply rate may still be higher for metalaxyl resistance. Nevertheless, we can safely conclude that, similar to other pesticides (Young et al., 2001; Stein and Kirk, 2004; Yuan et al., 2006; Cohen et al., 2007), resistance against cyazofamid is unlikely to occur as fast as metalaxyl resistance on sub-lethal concentrations.

Our results may help to predict the risk of resistance evolution under current treatment strategies. For instance, infrequent applications of a high concentration have been advocated to prevent fast selection of new resistant genotypes in a totally sensitive population (Lipsitch and Levin, 1997). However, pesticide concentrations vary in time and space in a field situation even when sufficient amounts are used. These variations may be caused by several factors including catabolic break down and diffusion (Cremieux and Carbon, 1992; Elliot et al., 1995). Furthermore, plant growth may further dilute the concentration for pesticides, like metalaxyl, that are taken up and distributed within the plants (Evenhuis et al., 2006). The resulting heterogeneous environment could facilitate resistance evolution, because resistant genotypes that emerge on low concentrations can migrate to higher concentrations (Kepler and Perelson, 1998). Hence, metalaxyl resistance can easily develop because it is beneficial at a large range of concentrations. Resistance against cyazofamid, however, is less likely to occur under these circumstances, because it can only be selected at a narrow range of high concentrations.

To conclude, our results suggest that adaptation to sub-lethal concentrations has different outcomes for different pesticides. We believe that the fast evolution of metalaxyl resistance is an extreme case of resistance evolution in *P. infestans*. However, new pesticides may suffer from a similar evolutionary response. Therefore, we propose that the inclusion of a similar experimental evolution approach may help to asses the risk of resistance under sub-lethal concentrations in the development of new pesticides. Furthermore, our approach can be used to answer more fundamental questions on the evolution of resistance, e.g. the pleiotropic effects of resistance mutations on other fitness and virulence components (Baucom and Mauricio, 2004) and the potential for compensatory evolution (Levin et al., 2000; Schoustra et al., 2006) in this economically important pathogen.

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Chapter 4

On the relationship between metalaxyl resistance and epidemic fitness in *Phytophthora infestans*

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Abstract

In previous studies on *Phytophthora infestans*, the potato late blight pathogen, resistance to metalaxyl was usually associated with a higher epidemic fitness, i.e. the fitness during the potato growing season. This is unexpected since resistance mutations are generally believed to have a negative effect on fitness in the absence of the pesticide. In this study, we combine observations from the field and the laboratory to study this effect of metalaxyl resistance in more detail. First, the effect of metalaxyl resistance on epidemic fitness was studied by surveying the Dutch field populations during a seven year period. This revealed a negative effect of metalaxyl resistance on epidemic fitness; the frequency of resistant isolates was significantly lower during years in which metalaxyl was not used but increased when metalaxyl was used again. Furthermore, the annual dynamics of resistance development suggest that resistant isolates are selected anew each year, but only in the presence of metalaxyl. Second, an experimental evolution approach was used to estimate the effect of metalaxyl resistance on epidemic fitness components in a previously sensitive genetic background. No consistent effect of metalaxyl resistance on fitness on detached leaflets of potato cultivar Bintje was observed. However, a small positive effect on *in vitro* fitness components was shown. A new model is proposed to explain both the patterns in the field and observations in the laboratory.

Introduction

Some deleterious mutations may become beneficial when environmental parameters change. Examples of such mutations are those that cause resistance to pesticides; they increase fitness in the presence but decrease fitness in the absence of the pesticide (Andersson and Levin, 1999). This negative pleiotropic effect may explain the low frequency of resistance mutations in pathogen populations before any pesticides are used. In *Phytophthora infestans*, however, resistance to metalaxyl is typically associated with a higher fitness during the potato growing season, i.e. epidemic fitness, even in the absence of metalaxyl (Kadish and Cohen, 1989). The fact that this association is not restricted to a single genotype or country may suggest that metalaxyl resistance has a direct positive effect on epidemic fitness (Gisi and Cohen, 1996).

A direct positive effect of metalaxyl resistance on epidemic fitness is surprising, because metalaxyl was highly effective upon its commercial release. This effectiveness does suggest that the incidence of resistance was low at that time, although an occasional resistant isolate (culture obtained from a single infection) has been described in old collections (Daggett et al., 1993). Given the apparent positive effect on epidemic fitness, the absence of resistance prior to the commercial release of metalaxyl has been explained by a reduced survival of metalaxyl resistant isolates during the winter (Kadish and Cohen, 1992). However, together with the high frequency at which mutants are obtained from sensitive colonies (Abu-El Samen et al., 2003b; Chapter 2), a direct positive effect of metalaxyl resistance on epidemic fitness would also lead to a high frequency of resistant isolates in the absence of metalaxyl. Hence the absence of resistance prior to the commercial release of metalaxyl needs another explanation.

If metalaxyl resistance does indeed have a positive effect on epidemic fitness, the frequency of resistant isolates would also increase when metalaxyl is totally absent. However, this frequency went down dramatically in the Netherlands and in Ireland during periods in the 1980's when metalaxyl was completely banned in those countries (Dowley and O'Sullivan, 1985; Davidse et al., 1989). This would suggest that metalaxyl resistance has a deleterious effect on epidemic fitness in field populations. However, these results could also be explained by the large population displacement that took place around that time (Fry, 2008). An analysis of the population during a more recent nation-wide ban of metalaxyl would therefore provide additional information on this subject.

Despite having a possible deleterious effect in field populations, metalaxyl resistance is still associated with higher epidemic fitness in many studies on *P. infestans* (Kadish and Cohen, 1988a; Kadish and Cohen, 1988b). Similar positive effects of metalaxyl resistance were observed in other oomycetes (Gent et al., 2008; Hu et al., 2008), which suggests that it is a common feature of this resistance. However, these studies are performed by comparing sensitive and resistant isolates that are genetically dissimilar. Effects of the resistance mutation can therefore be masked by differences in genetic background. The confounding effects of differences in genetic background can be removed by analyzing offspring from sexual crosses between sensitive and resistant isolates (Lee et al., 1999) or by introducing the resistance mutation into a sensitive background by genetic transformation. However, the large variation of sensitivity and survival rate among offspring from a single sexual cross, which are both typical for *P. infestans* (Knapova et al., 2002), would not allow the detection of small effects. On the other hand, genetic transformation is not feasible as long as the exact genetic cause of resistance remains unknown (Shattock, 1988; Fabritius et al., 1997).

The approach of experimental evolution with microbes (Elena and Lenski, 2003) is a suitable alternative for introducing resistance into a previously sensitive background for studying the cost of resistance, especially when the mechanism of resistance is not fully understood. The strength of this method lies in the fact that evolved (resistant) individuals can be compared with a near-isogenic un-adapted (sensitive) ancestor. This method is more sensitive than analyses using sexual crosses of randomly chosen field isolates or offspring from a sexual cross, because the sensitive and resistant individual in this approach are genetically more similar than randomly chosen isolates from the field population. Similar experimental evolutionary experiments were indeed successful in revealing a significant fitness cost of resistance in a number of other organisms, such as bacteria and fungi (Gagneux et al., 2006; Schoustra et al., 2006; Lopez-Rodas et al., 2008).

In this paper, we combine the observations from two experiments to investigate the effect of metalaxyl resistance on epidemic fitness in *P. infestans*. First, if resistance has indeed a positive effect on epidemic fitness, we would expect that the frequency of resistant isolates would remain high in the absence of metalaxyl. Therefore, we analyzed the frequency of resistant isolates in Dutch field populations during a period that spanned seven years (2000-2006), including a three year period in which metalaxyl was banned (2001-2003). Second, in order to see whether the positive association between resistance and epidemic

fitness in some studies is due to differences in genetic background, we analyzed individual fitness components of clones, i.e. single zoospore derivatives that became resistant after an experimental evolutionary procedure and compared them with their sensitive ancestor. The results from these two experiments are not in favor of a direct positive effect of metalaxyl on epidemic fitness. The analysis of the field population does suggest that metalaxyl resistance causes a reduction in epidemic fitness, since it is only selected anew each year in the presence but not in the absence of metalaxyl. Additionally, no consistent effect of resistance on individual fitness components was observed with our experimental evolutionary approach. Reasons for the observed patterns from the two experiments are discussed.

Methods

Analysis of metalaxyl resistance in field populations

Field isolates were obtained by placing leaflets that showed signs of infection in a Petri dish underneath a tuber slice of cultivar Bintje and stored at 15°C. One week later, pieces of mycelium that grew through the potato slice were transferred to new Petri dishes containing pea agar amended with 100 μg ml^{-1} ampicillin.

For each field isolate, the sensitivity to metalaxyl-M was analyzed on Petri dishes containing pea agar amended with 0, 5 or 100 μg ml^{-1} metalaxyl-M. A plug of mycelium (∅ 5 mm) from the edge of an actively growing colony was put onto the center of each plate. Plates were stored at 20 °C in the dark for six days after which the colony size was assessed by measuring two perpendicular diameters. Three sensitivity classes were distinguished based on the growth rate on amended plates relative to the growth rate on un-amended plates: the sensitive class contains isolates with a relative growth rate lower than 40% on 5 and 100 μg ml^{-1}, the intermediate class contains isolates with a relative growth rate larger than 40% on 5 μg ml^{-1} but lower than 40% on 100 μg ml^{-1} and the resistant class contains isolates with a relative growth rate larger than 40% on 5 and 100 μg ml^{-1}.

Mating type was determined by placing a plug of mycelium of a field isolate of unknown mating type onto a Petri dish together with a plug of mycelium from a tester isolate of which the mating type was known. Each field isolate was tested against an A1 isolate (VK98014) on one plate and against an A2 isolate (IPO-c) on the other plate. After eight days of growth at 20 °C in the dark, both plates were screened visually for oospores. Presence of

oospores on a plate indicates that the mating type of the field isolate was opposite to that of the tester isolate (Cooke, 2004).

Experimental evolutionary approach

To assess the fitness consequences of metalaxyl resistance in near-isogenic clones, we used clones of *P. infestans* isolated from a previous experimental evolutionary study (Chapter 3), in which we performed two selection procedures by serially transferring zoospores on Rye B medium (Caten and Jinks, 1968) with a sub-lethal concentration metalaxyl-M, (0.01 μg ml^{-1}). One procedure was started with a single zoospore isolate obtained from field isolate NL01096 (A2) whereas the other was started with a single zoospore isolate from field isolate VK98014 (A1). Both ancestors (NL01096 and VK98014) were sensitive to metalaxyl.

After 10 zoospore transfers, we obtained clones that showed a reduced sensitivity to metalaxyl. Each clone was isolated as a single zoospore and given a number that consisted of the number of the independent selection line and the number of the zoospore from which it was derived, e.g. VK98014 6-4 is the fourth clone that was obtained from selection line 6 in the procedure with VK98014. Additionally, we isolated control clones that had gone through the same procedure in the absence of metalaxyl. Agar plugs with mycelium from the adapted, control and ancestral clones were put into vials with 15% DMSO and were stored in the liquid nitrogen facility at Plant Research International, Wageningen, the Netherlands, for use in future experiments.

Prior to the fitness assays, several clones obtained from the selection procedure and the ancestor were revived from the liquid nitrogen (Table 1). Agar plugs with mycelium were put on rye B agar with either no metalaxyl, i.e. for the ancestral clone and other control clones, or with 0.01 μg ml^{-1} metalaxyl, i.e. for the adapted clones. Additionally, the remaining 15% DMSO solution that contains sporangia was used to inoculate tuber slices of cultivar Bintje. The mycelium that grew on rye B medium was used in the *in vitro* fitness assays, whereas the mycelium that grew on the tuber slices was used for the fitness assays on the leaflets, i.e. for the measurement of the *in vivo* fitness components.

In vitro fitness components

Two *in vitro* components were estimated for each clone: mycelium growth rate (MGR) and the germination frequency of zoospores (GF). A zoospore suspension was isolated from

each clone and diluted to $2x10^5$ zoospores ml^{-1} as described in Chapter 3. A droplet of 50 µl of this suspension was placed onto the center of a plate containing rye B medium without metalaxyl and left intact (MGR) or spread out with a glass rack (GF). Per clone, five plates were inoculated for each treatment. Petri dishes were stored at 18 °C in the dark for 24 hours after which they were sealed with parafilm and further incubated (MGR) or visually scored (GF).

Table 1. *P. infestans* clones used in the fitness assays. All clones were obtained as single zoospore derivatives from their sensitive ancestor by prolonged exposure to a sub-lethal concentration of metalaxyl-M (0.01 µg ml^{-1}).

Ancestor	Mating type	Mitochondrial haplotype	Clone	Sensitivity to metalaxyl
NL01096	A2	IIa	8-1	Intermediate
			8-2	Resistant
			13-1	Intermediate
			13-2	Resistant
			16-1	Resistant
			ancestor	Sensitive
VK98014	A1	Ia	6-3	Intermediate
			6-4	Intermediate
			6-5	Intermediate
			15-1	Resistant
			15-5	Intermediate
			ancestor	Sensitive

Mycelium growth rate was assessed daily by marking the edges of the colony on the bottom of the dish. The diameter at each time-point was then assessed at the end of the experiment as the mean of two perpendicular diameters. Because mycelium growth rate is reduced when mycelium reaches the plate's edge, measurements were continued until at least

one diameter exceeded 7 cm. The frequency of germinated zoospores (GF) was assessed visually under a dissecting microscope for at least 100 zoospores. A zoospore was considered to be germinated when the germination tube was at least twice as long as the diameter of the zoospore cyst.

In vivo fitness components

Small plugs of mycelium that emerged on the potato slices of cultivar Bintje were transferred to droplets tap-water (10 µl) on leaflets of cultivar Bintje, which were put with the abaxial side up on a 1.5 % agar plate (∅ 13 cm). One week later, a piece of a leaflet with a sporulating lesion was put underneath a new tuber slice from cultivar Bintje. Again another week later, mycelium from the tuber slices was transferred to droplets tap-water (10 µl) on leaflets of cultivar Bintje. These leaflets would provide the inoculum source to assess the lesion growth rate (LGR) and the infection efficiency (IE).

Leaflets of cultivar Bintje, with the exception of terminal leaflets, were harvested and put, with the abaxial side up, in random groups (n=5) on large Petri dishes (∅ 15 cm) containing 1.5% water agar. Sporangial suspensions were created by rinsing sporulating lesions in 10 ml tap-water. The concentration was assessed with a Coulter counter and diluted to approximately $5.0x10^4$ sporangia ml^{-1} (LGR) or to $1.0x10^4$ sporangia ml^{-1} (IE). The leaflets were inoculated by placing either one (LGR) or ten (IE) droplets of 10 µl of the sporangia suspension onto each individual leaflet. Per clone, a total of 10 leaflets were inoculated for both the LGR and IE assay. The Petri dishes were put in plastic trays that were covered with a transparent plastic bag. These trays were put at 15 °C in the dark for one day after which they were transferred to 15 °C under TL illumination with a constant day-night rhythm (16 hours light per day).

Lesion growth rate was assessed daily by measuring the largest length and the largest width along the axis perpendicular to the largest length of the lesion. The resulting area of the ellipse, (¼ π) x length x width, was calculated, square-root transformed, and the average LGR was estimated with linear regression of lesion area versus time (Colon et al., 1995).

The infection efficiency was assessed by counting the number of inoculum droplets that did not result in a successful infection. A maximum likelihood estimation of IE was calculated using the following function, which estimates the chance of obtaining the observed number of unsuccessful infections (H) given a certain infection efficiency (Sokal and Rohlf,

1995).

(1) $$L(IE|H) = P(H|IE) = \binom{100}{H}((1-IE)^k)^H((1-(1-IE)^k)^{(100-H)}.$$

In which H is the number of unsuccessful infections and where k represents the number of sporangia in each inoculum droplet. These maximum likelihood estimations were similar to the ones calculated with the traditional formula $IE=1-(H')^{1/k}$ (Colon et al., 1995), where H' represents the fraction of unsuccessful inoculations and k the number of sporangia per droplet.

Data analysis

Frequencies of the sensitivity classes over the years in the field population were analyzed using Fisher's exact tests due to low observed counts. Differences in the date of isolation of the different sensitivity classes were analyzed using a Mann-Whitney U test with sensitivity class (sensitive, intermediate or resistant) as the grouping variable and date of isolation (in number of days after January 1st) as the dependent variable.

Differences in MGR and LGR between each individual clone and the ancestor were analyzed using regression analyses with the diameter or the lesion size, respectively, as dependent variable and time after inoculation as the independent variable. Because the amounts of non-germinated zoospores were low, the differences in GF between clones and the ancestral clone were analyzed using Fisher's exact test. All statistical procedures were performed in SPSS 15.0. Sequential Bonferroni corrections were used to compensate for multiple comparisons.

Differences in IE could, in principle, be compared using a χ^2 test. However, this test does not account for differences in number of sporangia in each inoculum droplet (k). Therefore, each evolved clone was compared with its ancestor using a likelihood ratio test (Sokal and Rohlf, 1995). For each evolved clone, two different models were compared: the first model was based on the assumption that both the ancestor and the adapted clone have the same infection efficiency (null hypothesis) whereas the second model contains both individual estimations of the IE. The maximum likelihood estimation of the infection efficiency of the first model (IE') was calculated using the likelihood function

(2) $$L(H^0) = P(H_{evolved}|IE') \times P(H_{ancestor}|IE').$$

The likelihood of the alternative model is given by

(3) $$L(H^1) = P(H_{evolved}|IE_{genotype}) \times P(H_{ancestor}|IE_{ancestor})$$

and the likelihood ratio is calculated as

(3) $$\Lambda = \frac{L(H^0)}{L(H^1)}.$$

This value can than be used as a test statistic because $2\ln(\Lambda)$ follows a χ^2 distribution (Sokal and Rohlf, 1995). Both the likelihood calculations and the χ^2 test were performed using Microsoft Excel.

Results

Field data

We obtained a total of 434 Dutch isolates during the period 2000-2006, and tested them for metalaxyl resistance. Metalaxyl containing fungicides were prohibited in the Netherlands during the period 2001-2003; from 2004 and onward metalaxyl was allowed and used again. Overall, sensitive isolates were the most abundant (n = 249), followed by resistant (n = 130) and intermediate resistant isolates (n = 55). This pattern, however, differed markedly from year to year (Figure 1). The frequency of resistant isolates was low during the metalaxyl-free period (2001-2003), with only one resistant isolate obtained in 2001 (Figure 1). Subsequently, the frequency of resistant isolates increased markedly during the years in which metalaxyl was reintroduced (2004-2006). These resistant isolates were, however, isolated both on fields that were treated with metalaxyl and on fields that were not treated with metalaxyl (data not shown). Additionally, these resistant isolates were isolated at later dates compared to the sensitive ones in 2004 (Mann-Whitney *U* test, $P < 0.05$) and 2005 (Mann-Whitney *U* test, $P < 0.001$) but not in 2000 and 2006 (Figure 2). The absence of this pattern in 2000 and 2006 may, in part, be due to a low variation in sampling dates. Particularly in 2006, this low variation is caused by the late onset of epidemics in that year. Finally, metalaxyl resistance seems to be associated with the A2 mating type (Table 2; Fisher's exact test $P<0.05$).

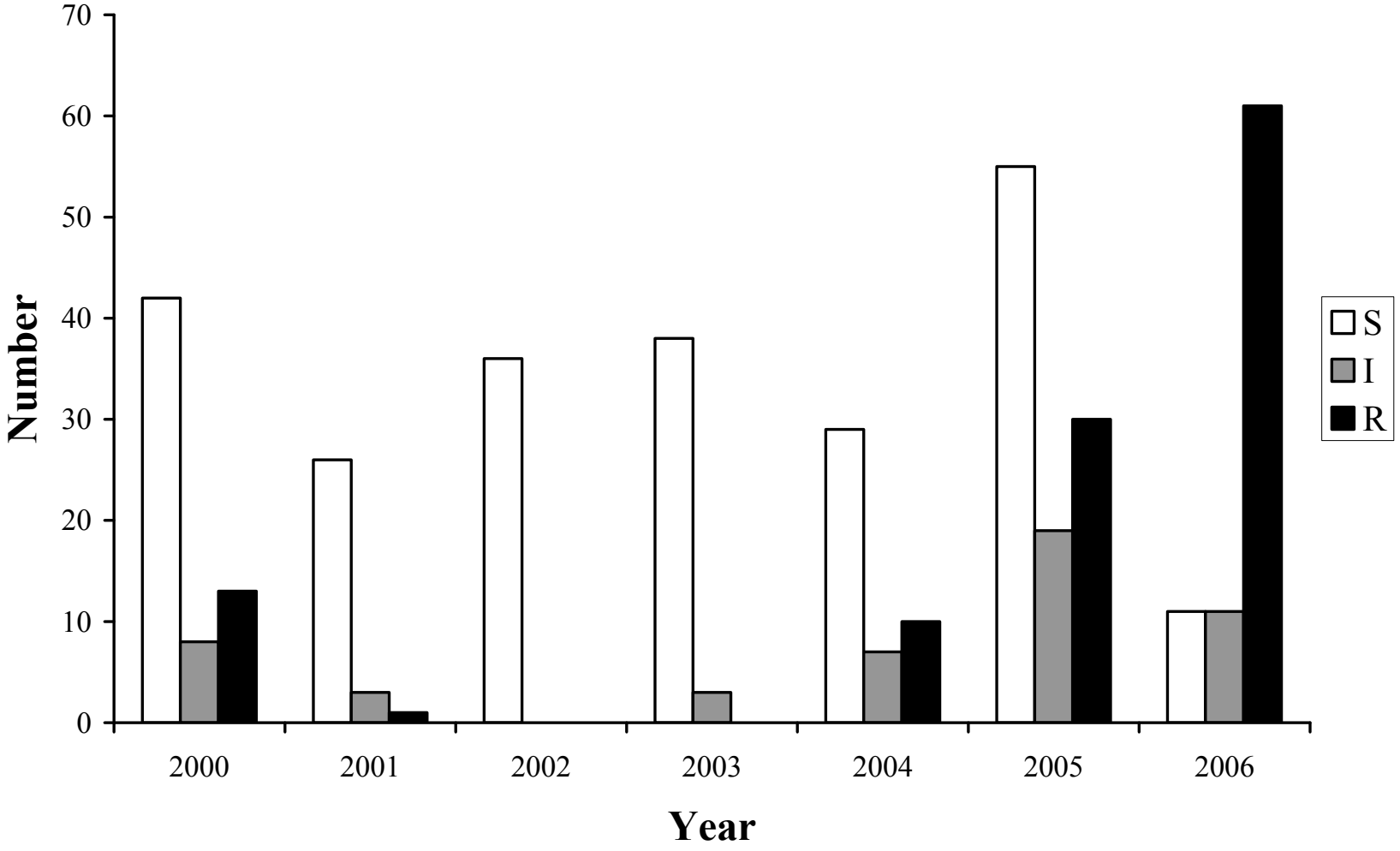

Figure 1. Distirbution of the three classes of metalaxyl sensitivity during the years 2000-2006 in the Dutch field population of *P. infestans*. The population is divided into three sensitivity classes: Sensitive (S), Intermediate (I) and Resistant (R). Metalaxyl was banned in the period 2001-2003 in the Netherlands.

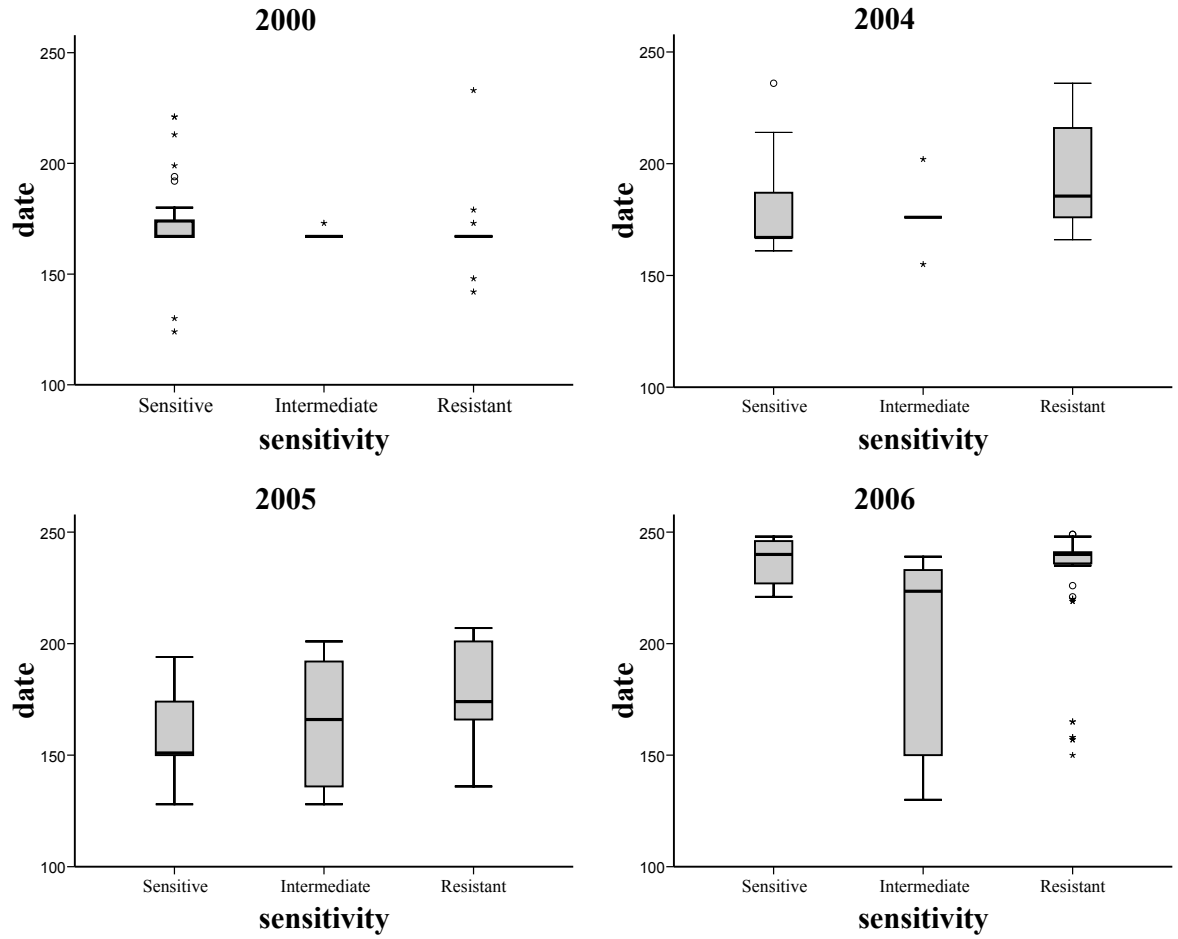

Figure 2. Box plots representing the dates of isolation (in number of days after January 1st of that particular year) of *P. infestans* isolates that were either sensitive, intermediate resistant and resistant to metalaxyl in 2000 (A), 2004 (B), 2005 (C) and 2006 (D).

Table 2. Numbers of each mating type (A1 or A2) among Dutch field isolates of *P. infestans* that belong to one of the three sensitivity classes in 2004 and 2005.

Year	Sensitivity class	Mating type	
		A1	A2
2004	Sensitive	77	56
	Intermediate	23	13
	Resistant	0	31
2005	Sensitive	84	20
	Intermediate	9	23
	Resistant	7	57

Fitness components in vitro

We analyzed epidemic fitness components for 10 clones that were obtained as single zoospore derivatives from a sensitive ancestor after prolonged and independent exposure to a low dosage of metalaxyl (Chapter 3): five from NL01096 and five from VK98014 (Table 1). Each of these clones showed a reduced sensitivity to metalaxyl and was categorized as intermediate or fully resistant according to the definition used on field isolates and according to the definition of Cooke et al. (2003)(Table 1).

Table 3. *In vitro* mycelium growth rate (MGR) and germination frequency (GF) of *P. infestans* clones (single zoospore derivatives) and their ancestor on plates containing rye B without metalaxyl. Clones were obtained after a selection procedure with a low concentration of metalaxyl-M (0.01 μg ml^{-1}). Suffices "a" and "b" represent a significant difference with the ancestor for respectively MGR and GF.

		Mycelium Growth Rate	Germination Frequency		
Ancestor	Clone	cm day^{-1} ± s.e.m.	Germinated	Not Germinated	%
NL01096	8-1	1.38 ± 0.07 [a]	123	12	91
	8-2	1.39 ± 0.04 [a]		n.d.	
	13-1	1.26 ± 0.13 [a]	162	3	98
	13-2	1.31 ± 0.03 [a]	111	3	97
	16-1	n.d.	107	7	94
	Ancestor	1.22 ± 0.03	114	4	97
VK98014	6-3	0.80 ± 0.02	53	31	63
	6-4	0.82 ± 0.01	130	70	65
	6-5	0.82 ± 0.02	96	71	57 [b]
	15-1	0.93 ± 0.02 [a]	193	49	80
	15-5	0.93 ± 0.02 [a]	97	60	62
	ancestor	0.80 ± 0.03	103	34	75

Mycelium growth rate (MGR) in the absence of metalaxyl differed between the two ancestral clones; NL01096 grew much faster than VK98014 (Table 3). However, MGR was not negatively affected by the adaptation to metalaxyl-M (Table 3). On the contrary, the adapted clones showed a higher mycelium growth rate compared to their ancestor, with the

exception of those that were derived from selection line 6 from the procedure with VK98014. These latter clones showed a similar growth rate as the sensitive ancestor although their colony size was always smaller than that of the ancestor, suggesting that mycelium growth started later (data not shown). Unfortunately, colonies of clone NL01096 16-1 were lost in the course of the experiment (suffered from fungal infections); hence it was not included in this analysis. However, subsequent experiments showed a significantly lower mycelium growth rate of this clone compared with the ancestor (data not shown).

The frequency of germinated zoospores differed markedly between the ancestors; the germination frequency was particularly high for NL01096 (97%) compared to VK98014 (75%). Metalaxyl resistance, however, did not show a consistent negative or positive effect on zoospore germination frequency. Although the germination frequency of one single zoospore derivative from VK98014 was significantly lower than that of the ancestor, other clones from this procedure as well as all clones from the procedure with NL01096 had a similar germination frequency as their ancestor (Table 3).

Fitness components in vivo

Lesion growth rate on detached leaflets appeared to be unaffected by metalaxyl resistance, because no adapted clone of NL01096 showed a significant change in this parameter compared to the sensitive ancestral clone (Table 4). However, infection efficiency appeared to be affected by *in vitro* adaptation to metalaxyl. Several clones of NL01096 were significantly less successful in infecting leaflets than the ancestor (Table 4). However, clone NL01096 16-1, which was subjected to the same experimental evolution procedure on medium with metalaxyl, showed no significant reduction in the infection efficiency compared to the ancestor. This suggests that the reduction of infection efficiency may not be due to the acquired metalaxyl resistance. Furthermore, the evolved clones and the additional control clone from VK98014 showed an even more extreme reduction in the ability to infect leaf tissue. This reduction was so severe that most evolved clones did not infect any leaflet; hence any comparison of *in vivo* fitness components was impossible to make for this isolate.

Table 4. Lesion growth rate (LGR) and infection efficiency (IE) of the *P. infestans* clones, i.e. single zoospore derivatives, on detached leaflets of potato cultivar Bintje. The clones were derived from the selection procedure with ancestor NL01096 on Rye B medium amended with 0.01 μg ml^{-1} metalaxyl-M (Chapter 3).

Ancestor	Clone	LGR mm day^{-1} (± s.e.m.) [b]		IE [a] H (%)	k	p [b]	sign [c]
NL01096	8-1	5.5 ± 0.5	n.s.	60	127	0.0040	**
	13-1	5.3 ± 1.3	n.s.	66	123	0.0034	**
	13-2	7.2 ± 1.1	n.s.	79	97	0.0024	**
	16-1	6.0 ± 0.9	n.s.	23	100	0.015	n.s.
	control [d]	5.3 ± 1.0	n.s.	40	97	0.0094	*
	ancestor	4.9 ± 0.9		22	111	0.014	

[a] The chance of infection by an individual sporangium (p) is calculated using the fraction of unsuccessful infections (H) and the average number of sporangia in an inoculum droplet (k).

[b] The chance of infection of an individual sporangium

[c] "*" and "**" represent a significant difference with the ancestor at a significance level of 0.05 and 0.01, respectively. "n.s." represents a non significant difference with the ancestor.

[d] Represents a clone that evolved *in vitro* in the absence of metalaxyl.

Discussion

Mutations that cause resistance to antimicrobial agents are expected to incur a fitness cost in the absence of the chemical (Bergelson and Purrington, 1996; Andersson and Levin, 1999; Sander et al., 2002; Baucom and Mauricio, 2004; Gagneux et al., 2006). This relation between fitness and resistance has been debated in the case of metalaxyl resistance in *P. infestans*. On the one hand, several observations have led to the idea that metalaxyl resistance in *P. infestans* has a direct positive effect on epidemic fitness, i.e. the number of infections of metalaxyl resistant genotypes increases faster than the number of infections of metalaxyl sensitive isolates (Kadish and Cohen, 1988b; Gisi and Cohen, 1996). On the other hand, the absence of resistance prior to the commercial release is suggestive of a negative effect of resistance. If this latter is true, the frequency of resistant isolates should decrease in the absence of metalaxyl. Additionally, the positive association between metalaxyl resistance and

epidemic fitness in some studies may then be explained by differences in genetic background rather than an association between metalaxyl resistance and epidemic fitness. In this paper, we investigated whether metalaxyl resistance does affect epidemic fitness of *P. infestans* by performing two experiments. A comparison of Dutch *P. infestans* field population during years in which metalaxyl was (2000, 2004-2006) and was not used (2001-2003). Secondly, the effect of metalaxyl resistance on individual fitness components was assessed by comparing near isogenic clones that differ in the sensitivity to metalaxyl.

Studies on field isolates

Effects of resistance on epidemic fitness can be studied indirectly by surveying changes in the frequency of resistant isolates following changes in metalaxyl use. In this study, the frequency of resistant isolates declined rapidly in Dutch field populations during a nation-wide ban of metalaxyl (2001-2003). Similar results were observed in earlier studies in Ireland (Dowley and O'Sullivan, 1985) and the Netherlands (Davidse et al., 1989). Such rapid declines suggest a negative effect of metalaxyl resistance on epidemic fitness, because a positive effect of metalaxyl resistance on epidemic fitness would cause this frequency to rise during the potato growing seasons.

The overall decline in the frequency of resistant isolates during a nation-wide ban of metalaxyl containing pesticides seems to differ from the observations made by others on the change in the frequency of resistance within a growing season (Williams and Gisi, 1992; Gisi and Cohen, 1996; Gisi et al., 2000). In those studies, the frequency of metalaxyl resistance is low at the start of the year but increases during the epidemic, even on fields that are not treated with metalaxyl. The fact that this pattern on untreated fields is observed each year is suggestive of a direct positive effect of resistance on epidemic fitness. A similar difference in timing between resistant and sensitive isolates was observed in the total Dutch population during the years in which metalaxyl was not prohibited, and sampling was possible throughout the season (i.e. 2004 and 2005). Apparently, metalaxyl resistance has a negative effect on the survival of the host-free period during the winter (Kadish and Cohen, 1992) and is only positively selected when metalaxyl is present, despite the fact that several resistant isolates were obtained from fields that were not treated with metalaxyl. This positive selection, however, does not occur when metalaxyl is totally absent. The rise in frequency of resistant isolates on untreated fields during years in which metalaxyl is not banned requires

another explanation such as migration of resistant isolates from adjacent potato crops that are treated with metalaxyl.

Historically, metalaxyl resistance has been associated with the A1 mating type. This association has been used to explain the prevalence of this mating type in the European populations during the invasion of new populations between 1980 and 1990 (Gisi and Cohen, 1996; Cooke et al., 2003). In the Dutch population, the A2 mating type and not A1 seems to be associated with metalaxyl resistance during 2004 and 2005 (Table 2). These data suggest that resistance is not restricted to one mating type and may evolve irrespective of the mating type.

Effect of the genetic background

Analyzing resistant and sensitive clones that are nearly isogenic is probably more appropriate to investigate the direct effect of resistance on fitness components than via an analysis of sexual crosses, because the genetic background may have profound effects on the effect of the mutation (Bergelson and Purrington, 1996). Using an experimental evolutionary approach, we observed a small but positive *in vitro* effect but no consistent effect of metalaxyl resistance on epidemic fitness components on detached leaflets. The absence of an effect on detached leaflets may also be caused by a general adaptation to the laboratory environment. However, if the results from the *in vitro* experiments could be extrapolated to a field situation, metalaxyl resistance does not have a large negative or positive effect on individual fitness components.

The reduced ability to infect leaf tissue that was observed in the clones obtained from VK98104 was not totally unexpected. For instance, zoospores that are collected from an individual colony can show substantial variation in growth characteristics (Caten and Jinks, 1968), molecular markers (Abu-El Samen et al., 2003a) and virulence (Caten, 1970; Abu-El Samen et al., 2003b). Because the ability to infect host tissue involves many genes (Birch et al., 2006), this variation can explain the quick loss of this ability after a period of *in vitro* growth. In the absence of selection for the ability to infect, i.e. due to growth on agar medium, mutations that reduce it will become neutral or even beneficial. As a result, these mutations may accumulate during serial transfer of *P. infestans* on artificial medium, which may lead to the lower infection efficiency observed in our *in vivo* study.

Epidemic fitness is not only determined by the ability to infect host tissue. It is

influenced by many traits including lesion growth rate and sporangia production (Flier and Turkensteen, 1999). If we could extrapolate the effects of metalaxyl resistance *in vitro* to a field situation, metalaxyl resistance would have a slight positive effect on fitness due to a higher mycelium growth rate. These slightly positive effects may become important in field situations due to the polycyclic nature of *P.* epidemics (Fry, 2008). However, the magnitude of these effects of resistance are not even close to the positive effects observed by others (Kadish and Cohen, 1988b).

Explaining the observed patterns

The positive effects of acquired metalaxyl resistance in our *in vitro* experimental evolutionary approach and other studies on *P. infestans* (Kadish and Cohen, 1988a; Kadish and Cohen, 1988b; Kadish and Cohen, 1989; Day and Shattock, 1997) are in agreement with the annual increase of the frequency of resistant isolates on fields that were not treated with metalaxyl containing pesticides. This gradual increase during growing seasons, however, is not in agreement with the total absence of metalaxyl resistance in field populations during years in which metalaxyl was banned. Therefore, we propose an alternative model for the evolution of metalaxyl resistance that may explain both observations.

The first part of this model assumes an initial fitness cost of any mutation that causes metalaxyl resistance. Furthermore, metalaxyl resistant genotypes are less able to survive the host-free period, which leads to a primarily sensitive population at the start of each growing season. Resistance mutations do occur in the surviving sensitive populations at a rate equal to the mutation rate. Any negative effect of an initial resistance mutation on epidemic fitness would then prevent a resistant clone from reaching a high frequency, since it is surrounded by a majority of sensitive clones that have a higher fitness. If metalaxyl is applied, however, these mutant clones will dominate the population. This resistant population may spread to adjacent fields, including those that did not receive any treatment with metalaxyl. The initial cost of resistance may then explain the absence of resistance development when metalaxyl is not available.

An initial cost of metalaxyl resistance seems feasible given its molecular mechanism. The most likely cause of metalaxyl resistance is a structural change in the ribosomal RNA polymerase, which is the target of metalaxyl (Davidse et al., 1983). Such a change will likely reduce the effectiveness of this protein complex and consequently the efficiency of production

of ribosomes. Given the fact that ribosomes are a key element in the production of proteins, it is likely that resistance has a negative effect on essential components in the life cycle like the production of new mycelium and germination; thereby reducing fitness. In an additional experiment, we observed an initial cost of metalaxyl resistance in terms of delayed *in vitro* mycelium growth that could indicate that the observed reduction in infection efficiency is indeed a direct effect of metalaxyl resistance (chapter 5).

The second part of this model contains two additional phenomena that are not mutually exclusive: compensatory mutations that restore the epidemic fitness of resistant genotypes and a higher likelihood of genotypes with a higher epidemic fitness to acquire resistance. Compensatory mutations have been responsible for the apparent stability of resistance in the absence of other pesticides (Andersson, 2003; Maisnier-Patina and Andersson, 2004). However, these compensatory mutations can only occur at a sufficient rate when the population size of resistant genotypes is sufficiently large (Levin et al., 2000; McCutchan et al., 2004). Such large populations can only occur as a result of the positive selective force caused by pesticide use; hence stable resistance will not evolve in the absence of the pesticide.

Compensatory evolution may also occur under the same conditions that selected for the resistance mutation (Cowen et al., 2001; MacLean et al., 2004). Some of these compensatory mutations may even increase fitness above that of the sensitive ancestor (Schoustra et al., 2006). Hence, the occurrence of compensatory evolution during our selection procedure may explain the slightly positive effect of metalaxyl resistance on the *in vitro* fitness components.

Next to compensatory mutations that can boost fitness, resistance mutations may occur more frequently in genotypes with a higher epidemic fitness. Hence, the mean epidemic fitness of resistant genotypes is higher than that of sensitive genotypes. This may be true for several reasons. First, genotypes with a higher fitness during epidemics are typically characterized by a high growth rate or by a high production of sporangia (Day and Shattock, 1997) and thus with a high rate of mitosis. Because at each mitosis there is a minute chance of acquiring a certain mutation, the mutation supply rate is higher in genotypes with a high epidemic fitness (Lipsitch and Levin, 1997). Second, genotypes with a high epidemic fitness may be capable of infecting host tissue on a wider range of sub-lethal pesticide concentrations, which may be selective for resistance (Baquero and Blázquez, 1997). On the

other hand, genotypes with a high epidemic fitness may be more sensitive to a given pesticide (Baquero, 2001). As a result, these genotypes may experience higher levels of stress, which may increase the mutation rate by reducing the effectiveness of DNA repair mechanisms (Agrawal and Wang, 2008). Finally, genotypes with a high fitness that acquired a resistance mutation may outcompete less fit genotypes with a resistance mutation.

To conclude, we showed that metalaxyl resistance has no direct positive effect on epidemic fitness (field survey 2000-2006) and that it does not directly cause a large increase in several individual epidemic fitness components (*in vitro* experiments). Additionally, we propose a model that could explain differences we observed between the laboratory and field observations. According to this model, the initial resistance mutation has a negative effect on epidemic fitness that may be compensated for by additional mutations. Additionally, genotypes with a higher epidemic fitness may have a higher chance of acquiring this initial resistance mutation. The assumptions of this model, i.e. the initial cost of resistance and compensatory evolution, could be tested in subsequent analyses.

Acknowledgements

This project is partly funded by the Dutch ministry of Agriculture, Nature Management and Fisheries through the Umbrella Plan Phytophthora (DWK 427). We thank Petra van Bekkum and Trudy van den Bosch for isolation of field isolates from the liquid nitrogen storage facility and for assistance during the fitness measurements.

Chapter 5

In vitro analysis of the direct fitness cost of metalaxyl resistance in *Phytophthora infestans*

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Abstract

Resistance against pesticides usually incurs a fitness cost in the absence of the pesticide. In *Phytophthora infestans*, however, resistance against metalaxyl is usually associated with a high epidemic fitness in the absence of metalaxyl. Similarly, no significant reduction in fitness was observed in genotypes of *P. infestans* after *in vitro* adaptation to metalaxyl. An experiment involving two steps was performed to assess whether the absence of a cost is due to the absence of pleiotropic effects of the resistance mutations themselves or due to compensatory effects of additional mutations. In the first step, eight clones were isolated that had just acquired an adaptation to metalaxyl-M. A comparison of *in vitro* growth parameters (duration of lag phase before the start of growth and mycelium growth rate) of these initial mutant clones with the ancestor revealed that the adaptation has a negative effect on fitness by increasing the lag phase in the absence of metalaxyl-M. In a second step, three mutant clones were used to generate selection lines that were allowed to adapt to metalaxyl-M during five more transfers. These lines showed a further improvement in the presence of metalaxyl, realized by an increase of the growth rate, but no compensation of the cost of resistance occurred. Rather, this adaptation to metalaxyl appeared to be associated with increased fitness costs due to further increases of the lag phase in the absence of metalaxyl. The results, therefore, show fitness costs associated with metalaxyl resistance caused by a delay of growth. This cost may offer an explanation for the absence of metalaxyl resistance prior to metalaxyl use despite a higher epidemic fitness that is typically associated with this resistance.

Introduction

Mutations that cause resistance against chemical pesticides hamper our ongoing fight against pathogens(Palumbi, 2001). Generally, these mutations have a low frequency in a pathogen population before a pesticide is used. This initially low frequency has been explained by a fitness cost of the resistance mutation when the pesticide is absent (Andersson and Levin, 1999). The existence of such a cost justifies the expectation that the frequency of resistant genotypes will go down after a pesticide is no longer used.

Despite this negative effect of resistance mutations, the frequency of resistant genotypes sometimes appears to be more or less stable in the absence of the pesticide (Andersson, 2003). This stability has been ascribed to compensation of the negative effect by additional mutations (Levin et al., 2000; Schoustra et al., 2006) or by hitch-hiking of the resistance mutation with a large beneficial mutation (Enne et al., 2004). Recently, however, evidence is emerging that some resistance mutations may be more or less neutral, whereas others even increase fitness in the absence of the toxin (Andersson, 2006). Clearly, knowledge of the effects of a resistance mutation on fitness and of the potential of compensatory mutations is essential for designing durable treatment strategies that prevent evolution of stable resistance in pathogen populations.

Resistance to the phenylamide pesticide metalaxyl (and its enantiomer metalaxyl-M) is well established among several species of oomycete plant pathogens (Gisi and Cohen, 1996). In *Phytophthora infestans*, the potato late blight pathogen, the frequency of resistant genotypes is relatively high in present day potato fields (Hermansen et al., 2000; Cooke et al., 2003), including fields that were not treated with metalaxyl (Gisi et al., 2000). This relatively high frequency on untreated fields has led to the general idea that metalaxyl resistance is beneficial during epidemics by directly increasing the fitness during epidemics (Kadish and Cohen, 1989), despite being largely absent prior to the commercial release of metalaxyl (Daggett et al., 1993). This absence has been explained by the lower survival of resistant individuals during host-free periods (Kadish and Cohen, 1992). However, it remains unclear whether the high epidemic fitness is caused by a direct positive pleiotropic effect of the resistance mutation or by additional compensatory mutations that may have increased the fitness of resistant genotypes.

A first attempt to study the direct effects of metalaxyl resistance has been made by Lee et al. (1999). In that study, the effect of resistance on aggressiveness components was

analyzed in a randomized genetic background by analyzing sexual crosses between sensitive and resistant genotypes. With this approach, the linkage between resistance mutations and possible compensatory or other beneficial mutations is disrupted. However, no significant effects of metalaxyl resistance, either positive or negative, were observed in that study. This may be explained by the fact that small effects are obscured by the large variation in fitness that is generated in such a cross. Such small effects of a resistance mutation may, however, become important due to the polycyclic nature of epidemics (Fry, 2008). Therefore, a more powerful method that enables detection of small effects should be used.

In a previous study, we selected for metalaxyl resistance in a sensitive background by allowing a sensitive genotype to adapt to a low concentration of metalaxyl-M (Chapter 3). We expected that this approach was more sensitive than the analysis of sexual crosses and would allow for the detection of smaller effects of metalaxyl resistance. A small positive effect of resistance on *in vitro* growth was observed in the absence of metalaxyl-M (Chapter 4), which might indicate that metalaxyl resistance is beneficial rather than costly. However, we could not rule out the possibility that compensatory mutations were selected later during the selection procedure.

In this paper, a new experimental procedure was used to assess the *in vitro* fitness cost of adaptation to metalaxyl-M. We isolated mutant clones, i.e. mycelium derived from single zoospores, immediately after the appearance of an adaptation to metalaxyl and compared them with the ancestral clone. Subsequently, selection experiments using these new mutant clones were initiated to observe the possibility of compensation later during selection on metalaxyl-M. The results show that metalaxyl resistance caused a delay of growth but no decrease in growth rate in the absence of the toxin. However, no evidence for compensation later during the selection regime was observed. This direct fitness cost may explain the absence of metalaxyl resistant mutants of *P. infestans* prior to metalaxyl use.

Methods

Ancestral genotype

A clone from the metalaxyl sensitive *P. infestans* isolate NL01096 was chosen as the ancestral genotype because this isolate has several characteristics that facilitate *in vitro* evolution: a relatively high growth rate, production of sufficient numbers of zoospores and the ability to obtain resistant clones. Furthermore, resistant clones that were obtained from this

isolate in a previous study (Chapter 3) showed a higher *in vitro* growth rate in the absence of metalaxyl-M than the sensitive ancestral clone.

Collection of zoospores and single zoospore derivatives

Zoospores were collected as described in Chapter 3. To recapitulate, a 14 day old sporulating colony on rye B agar (Caten and Jinks, 1968) was flooded with 10 ml cold water (6° C) and put in a cold room (4° C). After two-and-a-half hours, the water was poured into sterile test tubes and put on ice for 30 minutes after which the top 1 ml was transferred to an Eppendorf tube. This tube was then vortexed at maximum speed to induce encystment of the zoospores. The concentration of the resulting zoospore suspension was assessed using a haemocytometer and diluted if necessary. In general, inoculations of new plates were performed by placing a 50 µl droplet of a suspension containing $2x10^5$ zoospores ml^{-1} onto the center of the plates.

In order to obtain clones, i.e. mycelium derived from a single zoospore, a zoospore suspension was diluted to $1x10^4$ zoospores ml^{-1}. A 50 µl droplet of this suspension was put onto the center of a plate containing Rye B agar and spread out using a glass rake. These plates were incubated overnight at 18° C in the dark. The next day, small pieces of agar containing a single germinated zoospore cyst were cut out under a dissecting microscope and transferred to new small Petri dishes (∅ 5.5 cm) with Rye B agar. The mycelium that grew on these plates was used in subsequent analyses as clones.

Selection of resistant clones

Two different procedures were used to isolate initial mutant clones. These procedures are graphically represented in Figure 1. The first method was initiated by inoculation of a zoospore suspension on plates containing rye B amended with 0.01 µg ml^{-1} metalaxyl-M. Mycelium was allowed to grow at 18 ºC for 14 days after which zoospores were collected again and used to inoculate new plates containing rye B amended with 0.01 µg ml^{-1} metalaxyl-M. Again, zoospores were collected after 14 days. At this stage, however, single zoospores were collected from any colony that showed a large increase in growth rate compared to the preceding cycle.

The second method was also initiated with the inoculation of plates containing 0.01 µg ml^{-1} metalaxyl-M. However, instead of transferring zoospores after 14 days, pieces of faster growing sectors of mycelium were transferred to new plates containing rye B amended with

0.01 µg ml^{-1} metalaxyl-M. Fourteen days later, single zoospores were isolated from the mycelium that emerged from these pieces. Because the single zoospore derivatives from each method are expected to contain only the initial resistance mutation, they are used as initial mutant clones in the subsequent experiments.

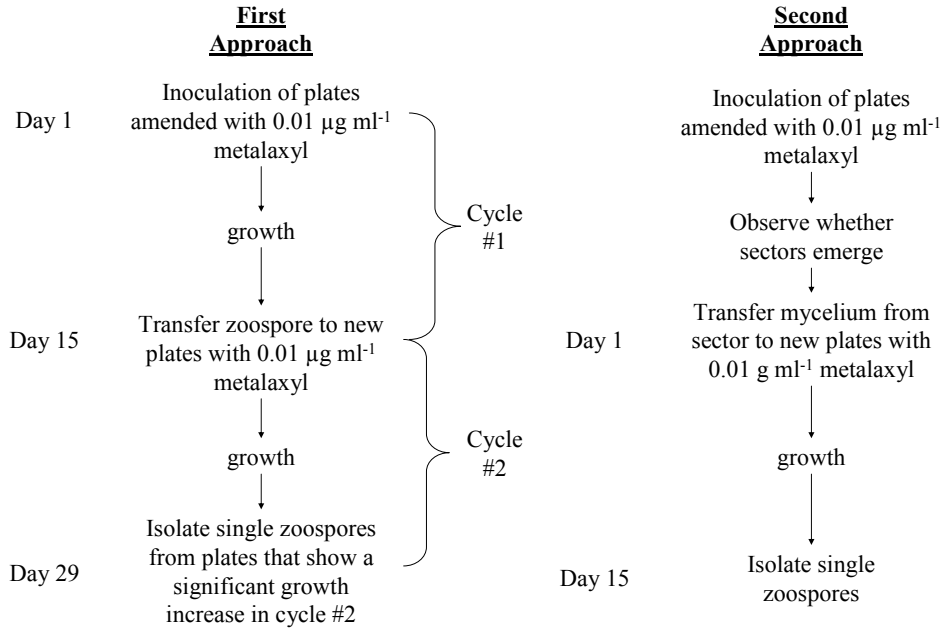

Figure 1. Overview of the two approaches used to select *P. infestans* clones with a reduced sensitivity to the pesticide metalaxyl-M.

Each initial mutant clone was given a code for easy reference. This code consists of a letter followed by two numbers separated by a hyphen. The letter refers to the procedure that was used to obtain the mutant: "Z" for zoospore transfer and "S" for isolation via sectors. The two numbers refer to the particular mutant clone within this procedure: the first number refers to the number of the selection line or number of the sector and the second number refers to the number of the clone. For instance, $S_{2\text{-}1}$ would represent the first clone obtained from the second sector and $Z_{3\text{-}2}$ would represent the second clone obtained from the third plate in the procedure using zoospore transfer. Next to the isolation of mutant clones that showed adaptation to metalaxyl-M, clones were obtained from mycelium that grew on plates that contained rye B agar without metalaxyl-M. These clones are referred to as ancestral clones.

In vitro growth of initial mutant and ancestor

In order to analyze *in vitro* growth, Petri dishes were filled with rye B with the appropriate concentration metalaxyl-M and allowed to dry overnight with the lid in place. Per clone (ancestor or mutant), Petri-dishes without metalaxyl-M (n=5) and Petri-dishes with 0.01 μg ml^{-1} (n=5) metalaxyl-M were inoculated and incubated at 18 °C in the dark. One day later, these plates were sealed with parafilm and put back at 18 °C in the dark for the remainder of the experiment. During the experiment, colony size was monitored daily by marking the edges of the colony on the bottom of the dish. At the end of the experiment, the colony size at each time-point was assessed by measuring two perpendicular diameters. The mean of these two diameters was used as an estimate for the colony size.

Selection experiment to observe compensation

Three clones obtained by zoospore transfer were subjected to a similar selection experiment that was used to create it. From each of these clones, 10 selection lines were created by inoculation of 10 plates containing 0.01 μg ml^{-1} metalaxyl-M with a zoospore suspension. Each selection line was given a code that consists of the code of the initial mutant clone followed by the number of the selection line, e.g. $Z_{1\text{-}3}$ 7 is the code for the seventh selection line obtained from mutant clone $Z_{1\text{-}3}$. Every 14 days, zoospores from each selection line were transferred to new plates containing 0.01 μg ml^{-1} metalaxyl-M. After 5 transfers, selection lines that had survived without fungal infections were analyzed for *in vitro* growth. For each selection line, plates with rye B amended with either 0 μg ml^{-1} (n=5); 0.01 μg ml^{-1} (n=5) or 10 μg ml^{-1} (n=5) metalaxyl-M were inoculated with a zoospore suspension after which growth was assessed similar to the *in vitro* growth experiment described above.

Data analysis

After inoculation of agar medium with a single droplet of zoospores on the center, *in vitro* growth is characterized by three phases: an initial lag phase before mycelium growth is observed, a linear radial growth phase, and a phase during which growth decreases in the vicinity of the plate's edge (Figure 2). During the lag phase, mycelium growth is restricted within the boundaries of the initial inoculum droplet (∅ 1.6 cm). During the linear growth phase, mycelium growth occurs outside the boundaries of the inoculum droplet and the growth rate is approximately linear. This growth phase finishes when the diameter exceeds 7

cm due to the vicinity of the plate's edge (∅ 9 cm). As a result of these different phases, analysis of *in vitro* growth is performed on two growth parameters: the duration of the lag phase and the growth rate during the linear growth phase.

The growth rate was estimated by linear regression of colony diameter on time. Only time-points during the growth-phase (i.e. when the diameter was between 1.6 cm and 7 cm) were used in this analysis. The duration of the lag phase was estimated using linear regression of time on diameter during the linear growth phase. The resulting regression model was used to calculate the time-point at which the diameter was 1.6 cm (the typical size of an inoculum droplet). This analysis was performed for each individual plate. Hence, five estimates of the growth rate and five estimates of the duration of the lag phase were obtained for each clone or selection line on each concentration.

The effect of the initial adaptation was analyzed by comparing the growth parameters of the initial mutant clones with those of the ancestral clone. The effect of subsequent selection on medium with 0.01 μg ml^{-1} metalaxyl-M was analyzed in a second analysis by comparing the growth parameters of each selection line with those of the initial mutant clone. However, the measurements on the initial mutant clones were, out of necessity, performed in a different experiment than the measurements on the selection lines. Therefore, all estimations of the two growth parameters were expressed relative to those of the ancestral clone, which was taken along in both blocks.

Statistical analyses were performed separately for each concentration of metalaxyl-M. In order to analyze the effect of the initial adaptation, one-sample *t*-tests with two degrees of freedom were performed to compare the single ancestral value for each growth parameter with the three mean values of the mutant clones obtained by zoospore transfers (by averaging replicates per spore) or with the mean values of the three sectors (by averaging the mean value for both single-spore derivatives per sector). In order to analyze the effect of the five subsequent transfers, the mean values of initial mutant clones after two cycles (n=3) were compared with the mean values of the clones after seven cycles (n=3) using a paired sample *t*-test. All estimations of the *in vitro* growth parameters and *t*-tests were performed in Microsoft Excel.

Results

Generation of resistance

Our first approach to isolate clones with a reduced sensitivity to metalaxyl, i.e. by serially transferring zoospores, yielded one colony that showed a significant increase in growth rate after the second zoospore transfer. Single zoospores were collected and three clones ($Z_{1\text{-}1}$, $Z_{1\text{-}2}$ and $Z_{1\text{-}3}$) were chosen for the characterization of *in vitro* growth and for the analysis of compensatory evolution.

Our second approach, i.e. by isolating faster growing mycelial sectors, was more successful in isolating mutant clones with a reduced sensitivity to metalaxyl-M. A total of five fast growing sectors were isolated from which clones were isolated. From these five sectors, only three yielded clones that showed a substantial reduction in the sensitivity to metalaxyl-M. Five of these clones were used in subsequent experiments: two from the first ($S_{1\text{-}1}$ and $S_{1\text{-}2}$); two from the second ($S_{2\text{-}1}$ and $S_{2\text{-}2}$) and one from the third sector ($S_{3\text{-}1}$).

Growth parameters differ between the initial mutant clones and the ancestor

The growth parameters of all clones (mutant and ancestral) are negatively influenced by metalaxyl-M as indicated by a reduction of the growth rate and a prolongation of the lag phase on medium with 0.01 μg ml^{-1} metalaxyl-M. Nevertheless, each mutant clone showed an adaptation to metalaxyl-M, because they reach a larger diameter on medium amended with metalaxyl-M (Figure 2B and 2D). In the case of the mutant clones obtained by zoospore transfer, this adaptation seems to be caused by an increased growth rate (one-sample *t*-test: t_2 = 14.28, P = 0.0049) and by a shorter lag phase (t_2 = -13.78, P = 0.0052) relative to the ancestor (Figure 3A and 3C). In the case of the mutant clones obtained from sectors, however, the adaptation is only caused by a higher growth rate (one-sample *t*-test using the average values per sector: t_2 = 61.20, P = 0.00027), because the duration of the lag phase did not change significantly (t_2 = 4.04, P = 0.056) from that of the ancestor on medium amended with metalaxyl-M (Figure 3 B and 3D).

The adaptations to metalaxyl-M appear to come with a cost: the diameter of the ancestor is larger than the diameter of each mutant clone during the linear growth phase on medium lacking metalaxyl-M (Figure 2A and 2C). The growth rate of the mutant clones obtained by zoospore transfer did not differ from the ancestor (t_2 = -2.35, P = 0.14; Figure 3A), but these clones had a significantly longer lag phase than the ancestor (t_2 = 6.85, P =

0.021; Figure 3C). Similarly, the mutant clones obtained from sectors did not differ from the ancestor with respect to the growth rate (t_2 = -0.36, P = 0.75; Figure 3B), but have a longer lag phase than the ancestor (t_2 = 24.18, P = 0.0017; Figure 3D).

The increased lag phase of the mutant clones on medium without metalaxyl-M could formally be caused by differences in the amount of zoospores in the inoculum. However, care was taken to inoculate similar amounts of zoospores by adjusting the concentration of zoospores in the suspensions. Moreover, a 10x dilution of the ancestral zoospore suspension did not reduce the growth rate and the initial diameter compared to a 3x dilution in this experiment and undiluted suspension in pilot experiments (data not shown).

Fitness consequences of five further transfers on metalaxyl-M amended medium

To see how continued selection on medium with metalaxyl-M would affect the observed cost of resistance, the three mutant clones from the procedure with zoospores were subjected to five additional transfers on metalaxyl-M containing medium with 10-fold replication. Figure 4 shows the effects of continued selection on metalaxyl-M on the two growth parameters for both medium with and without metalaxyl-M. Clearly, further adaptation to metalaxyl-M containing medium is only due to a further increase of the growth rate (t_4 = 5.93, P = 0.0041), because the lag phase does not change relative to the mutants that were isolated after two transfers (t_4 = 0.69, P = 0.53). However, the data provide no support for compensation of the cost of resistance during continued growth on medium with metalaxyl-M; after seven transfers, both the growth rate (t_4 = 2.26, P = 0.086) nor the lag phase (t_4 = 1.65, P = 0.17) differed from the mutant clones isolated after two transfers.

In order to see whether the adaptation to 0.01 μg ml^{-1} metalaxyl-M led to resistance, we analyzed the growth of the selection lines on medium amended with 10 μg ml^{-1} metalaxyl-M (Figure 5). All selection lines performed better than the ancestral genotype and are resistant according to the definition of Cooke et al. (1999). However, two lines obtained from initial mutant $Z_{1\text{-}3}$ ($Z_{1\text{-}3}$ 2 and $Z_{1\text{-}3}$ 7) showed a strikingly larger diameter throughout the assay; their diameter already exceeded five cm after nine days. These two lines also showed a decrease in the duration of the initial growth phase on medium amended with metalaxyl-M.

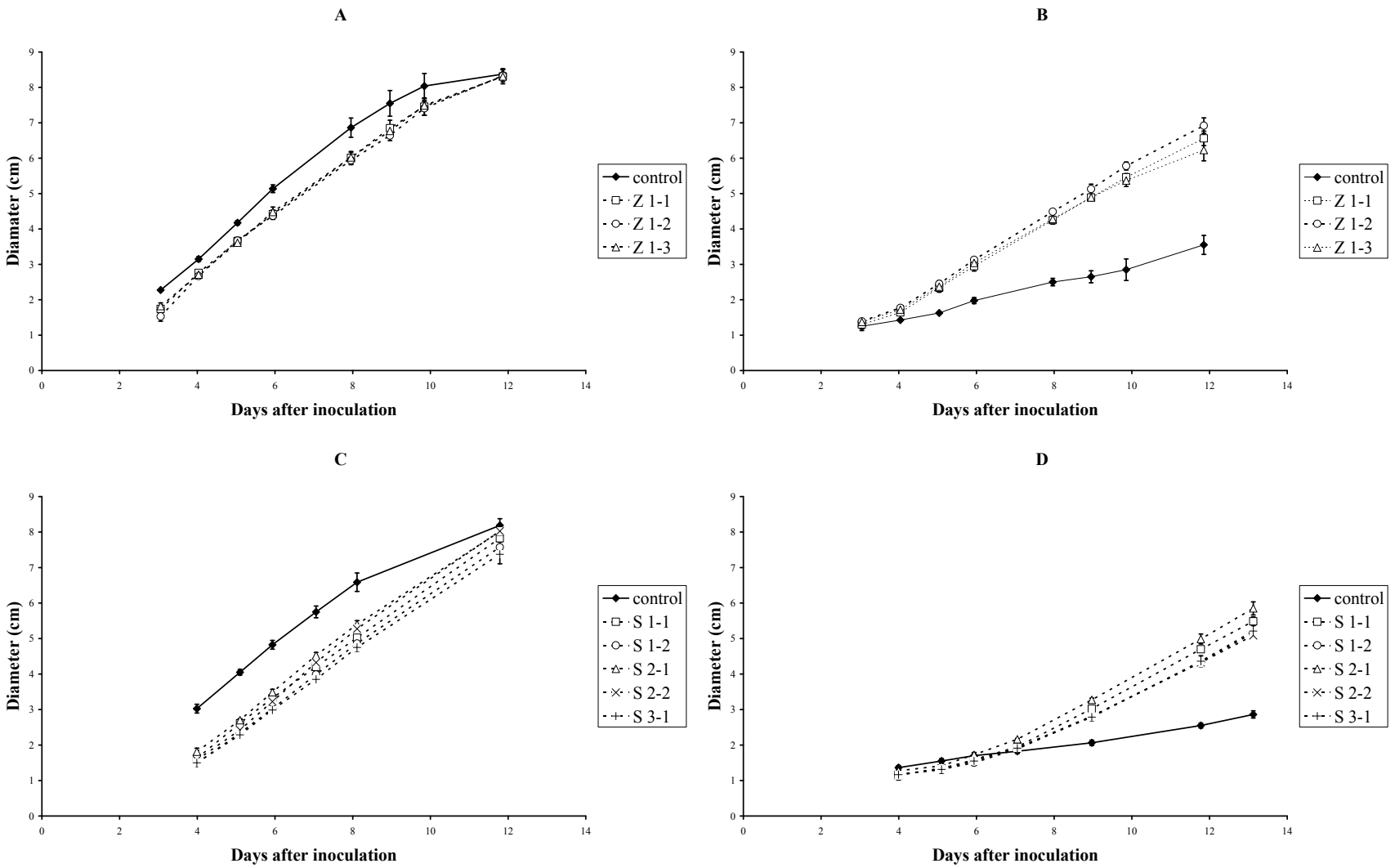

Figure 2. Diameter as a function of time of the mutant *P. infestans* clones obtained by zoospore transfer (A+B) and those obtained by isolation of sectors (C+D). Figures A and C represent growth on medium without metalaxyl-M and figures B and D represent growth on medium amended with 0.01 μg ml^{-1} metalaxyl-M. The solid line represents a sensitive near-isogenic clone. Error bars represent 95% confidence limits.

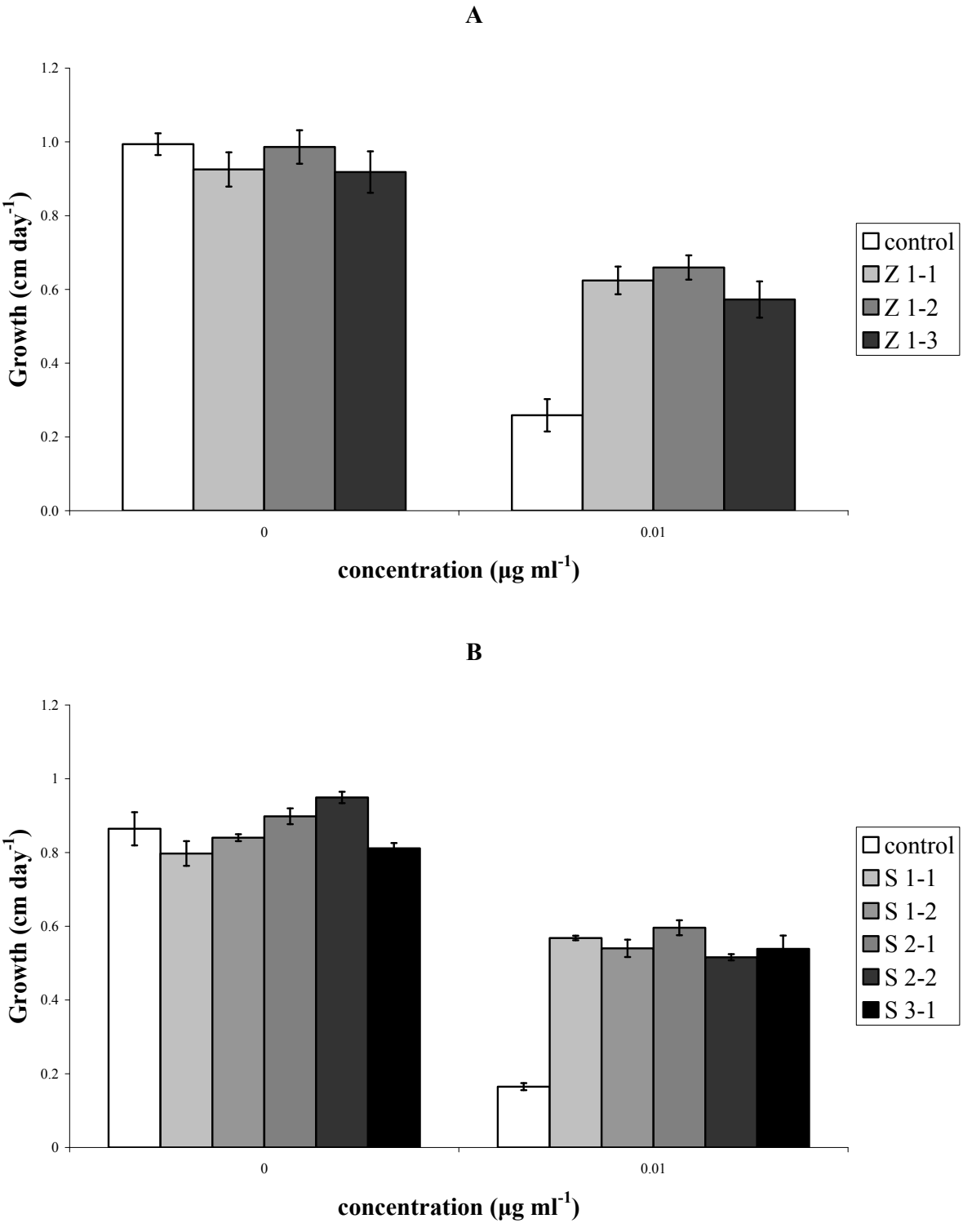

Figure 3. Growth rate (A+B) and the duration of the lag phase (C+D) of mutant *P. infestans* clones and a sensitive ancestral clone (control). Figures A and C represent the growth parameters of clones obtained by zoospore transfer and figures B and D represent the growth parameters of clones obtained by isolation of sectors on medium amended with 0.01 µg ml^{-1} metalaxyl-M.. Error bars represent 95% confidence limits.

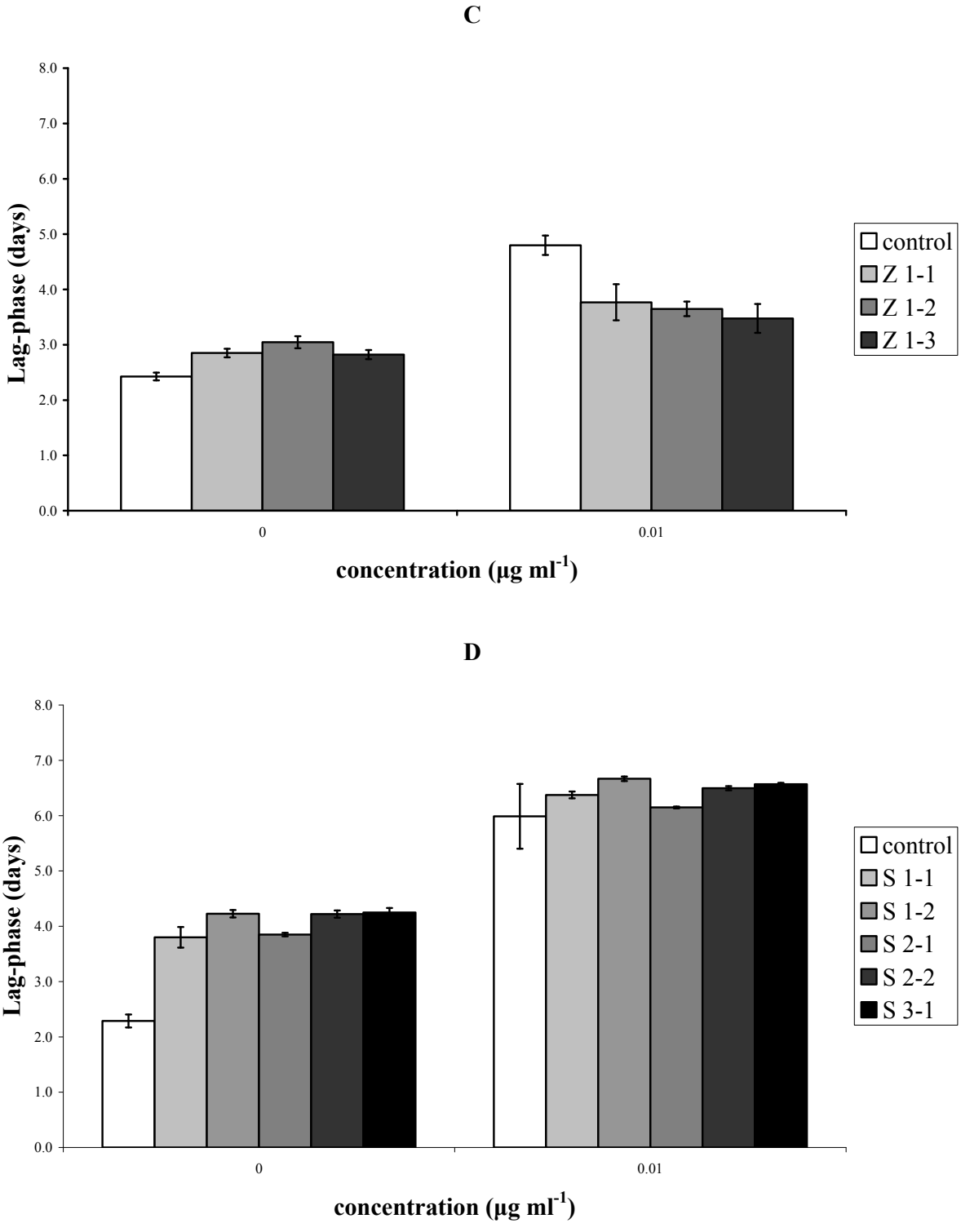

Figure 3 (continued). Growth rate (A+B) and the duration of the lag phase (C+D) of mutant *P. infestans* clones and a sensitive ancestral clone (control). Figures A and C represent the growth parameters of clones obtained by zoospore transfer and figures B and D represent the growth parameters of clones obtained by isolation of sectors on medium amended with 0.01 µg ml^{-1} metalaxyl-M. Error bars represent 95% confidence limits.

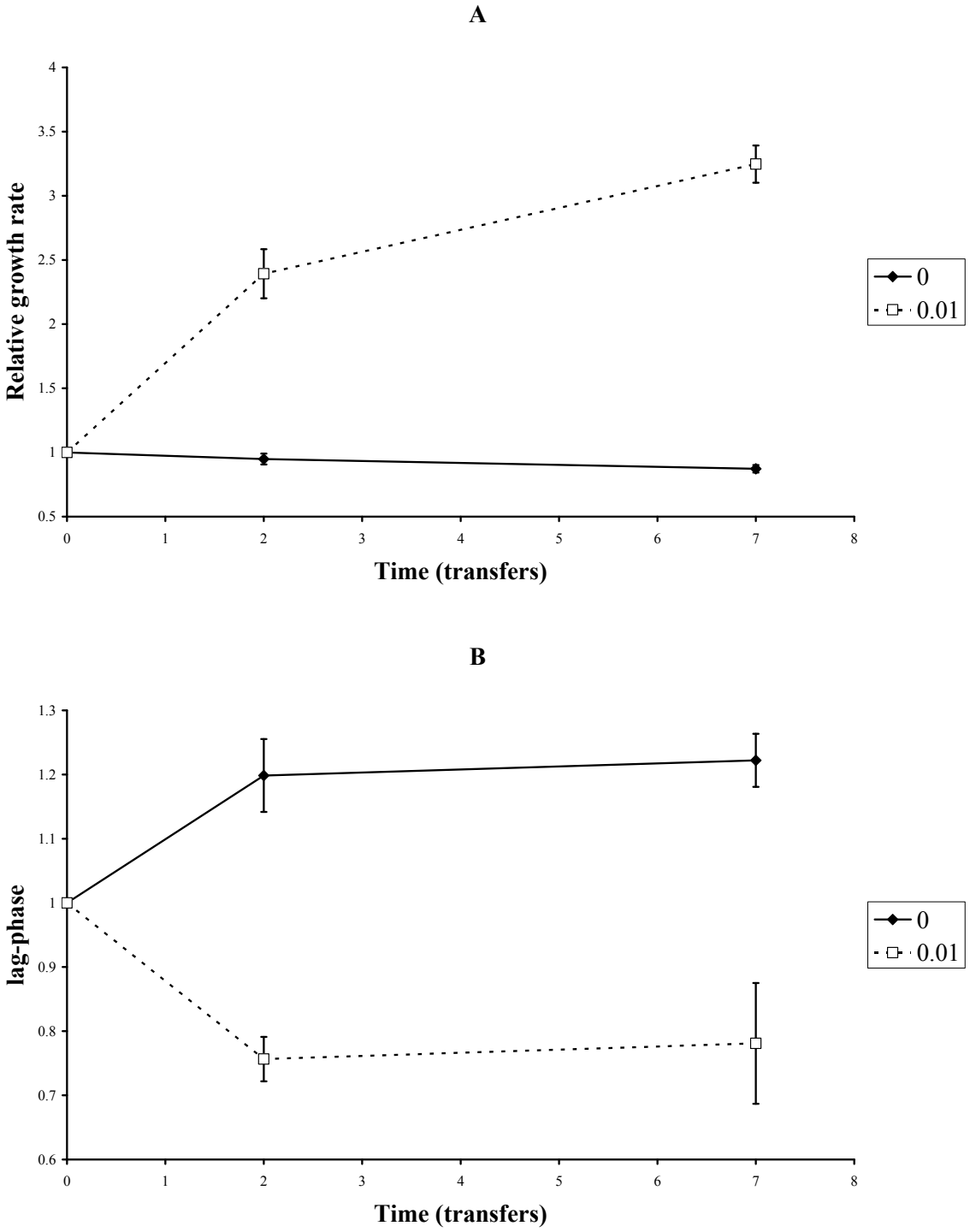

Figure 4. The growth rate (A) and the duration of the lag phase (B), on medium without (0) and medium with (0.01 μg ml^{-1}) metalaxyl-M, of the mutant *P. infestans* clones obtained by zoospore transfer. The mutant clones were analyzed after two and seven transfers on medium containing metalaxyl-M (0.01 μg ml^{-1}). Both growth rate and lag-phase are presented relative to a sensitive ancestral clone. Error bars represent the 95% confidence limits.

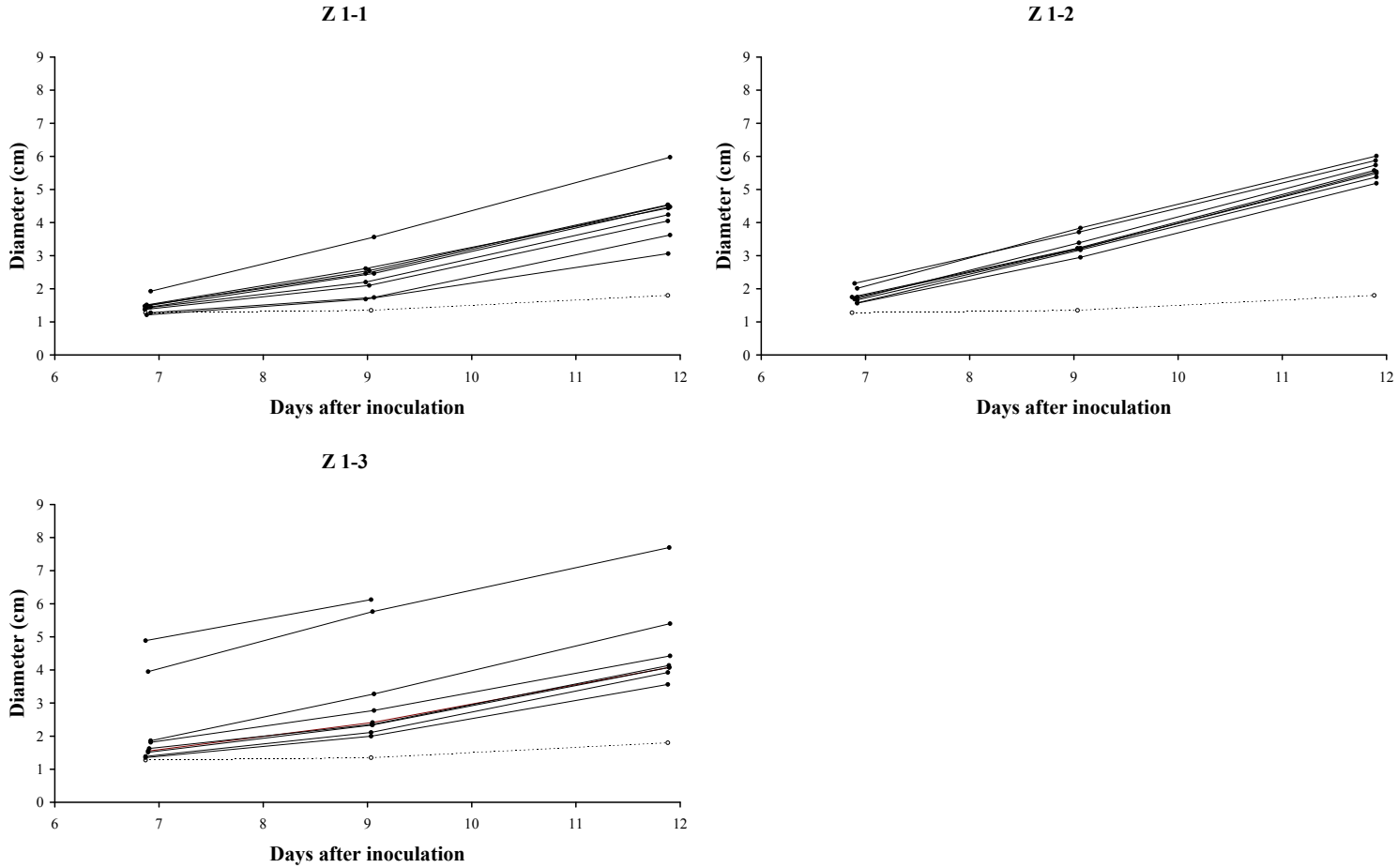

Figure 5. Colony diameter as function of time of the selection lines obtained from the three mutant *P. infestans* clones on Rye B medium amended with 10 μg ml^{-1} metalaxyl-M. Selection lines have spent five cycles on medium with 0.01 μg ml^{-1} metalaxyl-M. The growth of the sensitive ancestral clone is represented by the dotted lines, whereas the growth of the selection lines is represented by the solid lines.

Discussion

The absence of a cost of metalaxyl resistance in previous experiments with *P. infestans* (Chapter 3 and Chapter 4) can, in principle, be explained by an absence of negative pleiotropic effects of the resistance mutations or by compensation by secondary mutations during the selection procedure. The results of this study provide partial evidence in favor of the latter explanation. First, we showed that metalaxyl resistance has a negative influence on *in vitro* growth by increasing the length of the lag phase before the start of the linear growth phase. To our knowledge, this is the first report of a fitness cost in the absence of metalaxyl-M that is directly caused by the resistance mutations. Second, continued selection in the presence of metalaxyl-M led to further adaptation to metalaxyl-M via increases of the growth rate. However, we were unable to observe compensation of the initial cost during our selection regime that would explain the lack of a fitness cost in previous experiments (Kadish and Cohen, 1988b; Gisi and Cohen, 1996; Hu et al., 2008).

One may argue that a selection experiment that uses potato tissue *in vivo* would resemble the natural situation more closely, because the growth of *P. infestans* on artificial medium differs markedly from growth on potato tissues (Reis et al., 2005). However, a better understanding of the *in vitro* evolution of metalaxyl resistance could aid in our understanding of this phenomenon in field populations. Furthermore, *in vitro* selection of mutant clones has at least two clear benefits. First, random environmental factors are better controlled. Second, due to the typically low infection chance of leaflets by individual zoospores (Flier et al., 2007), an experiment involving leaflets is likely affected by the chance effects of genetic drift.

The difference in the growth parameters between the mutant clones obtained from sectors and those obtained from zoospore transfers may reflect differences in selective conditions. For instance, given that metalaxyl resistance may be caused by several mutations at different loci (Fabritius et al., 1997), mutant clones that were isolated from sectors harbor mutations that increase growth rate but that are not necessarily favored during competition with sensitive zoospores in the presence of metalaxyl-M. Mutant clones obtained by transferring zoospores will, however, harbor mutations that are beneficial throughout the entire life-cycle *in vitro*, which may differ from the former category. Alternatively, the adaptations may have started with the same mutation that is quickly followed by selection of additional mutations that improve the growth parameters during the specific procedure. The difference between the two procedures may then be explained by selection of different

additional mutations with different fitness consequences (Schoustra et al., 2005). This difference justifies the use of zoospore transfers in selection experiments, because it resembles the natural situation more closely than mycelium transfer, which has been used frequently as a method to generate resistant genotypes (Young et al., 2001; Stein and Kirk, 2004).

The fact that the prolongation of the lag phase was observed in all mutant clones suggests that this is a common effect of *in vitro* adaptation to metalaxyl-M. Here, we can only speculate on the mechanistic explanation for this pattern. For instance, the most likely cause of resistance to metalaxyl is a structural change in its target, i.e. ribosomal RNA polymerase (Davidse et al., 1983). Such a structural change will likely interfere with the normal function of this large protein complex (Shematorova and Shpakovski, 2002) and reduce the efficiency of the production of new ribosomes. Because ribosomes are a key element in protein production, a reduction in the efficiency will have its largest impact during stages in which fast protein production is important. The stages following encystment of zoospores, i.e. the loss of mobility and the production of a new cell wall (Judelson and Blanco, 2005), are known to depend particularly on the *de novo* production of proteins (Penington et al., 1989) and may therefore be more sensitive to a reduction in the efficiency of ribosome production. Indeed, the infection efficiency on leaflets of cultivar Bintje seems to be negatively influenced by metalaxyl resistance (Chapter 4).

The observed prolongation of the lag phase may explain why resistance is not selected on artificial medium without metalaxyl-M (Chapter 3). Under those circumstances, resistance mutations still occur and may end up in zoospores (Abu-El Samen et al., 2003b) as they hardly affect the growth rate. However, zoospores that contain a resistant nucleus have to compete in the expanding population with a majority of zoospores that contain a sensitive nucleus and have a shorter lag phase. The colony that results from this droplet will contain primarily sensitive nuclei and will primarily produce zoospores with sensitive nuclei. In the presence of metalaxyl-M, however, mycelium that contains resistant nuclei has a higher growth rate and, consequently, contributes more to the population of zoospores that make up the next generation. Additionally, the zoospores that contain a resistant nucleus have a shorter lag phase in the presence of metalaxyl-M and will out-compete the sensitive zoospores.

Although compensatory evolution repairing the cost of resistance in the absence of the pesticide has been reported several times (Levin et al., 2000; Schoustra et al., 2006), we did

not find evidence for it during prolonged selection on medium with metalaxyl. However, evidence for compensatory evolution during continued selection in the same environment has been reported before. In a study with the bacterium *Pseudomonas fluorescens*, MacLean *et al*. (2004) found evidence for compensatory evolution by prolonged selection under the same conditions. They found that the initial evolution of an ecotype (called *wrinkly spreader*) in their heterogeneous environment was driven by a substantial fitness gain, despite associated defects in the central carbon metabolism. Continued selection in the same environment led to further fitness increases that were caused by mutations that repaired the initial metabolic defects and compensated the fitness costs involved.

The observation that multiple mutations may increase the growth rate on medium with metalaxyl-M is in agreement with other studies that suggest a quantitative genetic nature of this resistance with the possible action of multiple loci with large and small effect (Fabritius et al., 1997; Lee et al., 1999). The observation that two selection lines showed a very large improvement of the growth parameters relative to the others suggests that more than one large-effect mutation could be responsible for the high level of resistance following adaptation on sub-lethal concentrations. This quantitative nature of this resistance may implicate that sexual recombination may break up the association between different large-effect mutations and yield intermediate resistant genotypes. Indeed, an excess of intermediate resistant isolates has been observed on field plots with both mating types (Cooke et al., 2003).

The positive association between fitness and metalaxyl resistance in field populations remains enigmatic, because the results presented in this paper indicate that resistance itself may cause a reduction in fitness. Two explanations can be given here to explain the difference between the results presented here and those in field populations. First, aggressive genotypes may be more prone to acquire resistance due to a higher rate of mitotic divisions (Lipsitch and Levin, 1997). Second, compensation may increase fitness beyond that of the sensitive ancestor (Schoustra et al., 2006). However, such secondary increases in growth rate can only occur if the population of resistant genotypes reaches a sufficient size, which is less likely in the absence of metalaxyl-M. Hence, these superior resistant genotypes will not evolve in the absence of metalaxyl-M.

To conclude, we present evidence for a direct fitness cost of metalaxyl resistance that affects the initial phase of *in vitro* growth. Although we have not found evidence for it in this study, an earlier study has shown that these costs may be compensated for by additional

mutations that can even improve the growth rate beyond that of the ancestor. The initial cost associated with resistance may explain the absence of resistance evolution on medium without metalaxyl-M, whereas compensatory evolution can explain the lack of a cost in previous experiments.

Chapter 6

General Discussion

Stefan Bosmans

Introduction

The presence of antibiotic chemicals poses a selection pressure on pathogen populations. As a result, several pathogen populations became resistant to a particular pesticide. Because medicine depends heavily on these chemicals for disease control, this may affect current and future treatment strategies. Hence, antibiotic resistance can have adverse effects on human populations. Next to this practical relevance, the emergence of resistance is an example of fast evolution and has received much attention from evolutionary biologists.

Resistance is not only a big problem in medicine but also in agriculture. For instance, pesticide resistance has been observed in a number of weed species (Baucom and Mauricio, 2004; Roux et al., 2004; Neve and Powles, 2005) and several insect species (Palumbi, 2001; Tabashnik et al., 2008). Resistance to pesticides has also been observed in numerous species that belong to the oomycetes (Gisi et al., 2002), which is a group of organisms that contains some of the most devastating plant pathogens. Knowledge of the factors that influence the evolution of resistance to pesticides in these organisms may help in designing treatment strategies that reduce the speed of resistance evolution.

In the production of potatoes, large amounts of pesticides are necessary to prevent economic losses due to *Phytophthora infestans* (Johnson et al., 2000), the oomycete that causes potato late blight. The problem of resistance development in this pathogen became apparent when resistances to the successful pesticide metalaxyl were observed a few years after its introduction (Davidse et al., 1981; Dowley and O'Sullivan, 1981). Surprisingly, resistance to other pesticides, like QoI inhibitors and CAA pesticides, has not yet been observed in field populations of this oomycete (Gisi and Sierotzki, 2008).

As a result of its fast evolution in *P. infestans*, metalaxyl resistance has been the subject of several studies (Fabritius et al., 1997; Judelson and Roberts, 1999; Lee et al., 1999). These studies have contributed to our knowledge on the genetics of this resistance. However, some aspects still have not been sufficiently explained by those studies. For instance, no satisfactory explanation has been given for the relative fast evolution of metalaxyl resistance relative to other resistances (Gisi and Sierotzki, 2008) and for the absence of this resistance prior to the commercial release of metalaxyl (Daggett et al., 1993; Gisi and Cohen, 1996) given the positive effect of metalaxyl resistance on epidemic fitness (Kadish and Cohen, 1988b).

Experimental evolution has proven a suitable method for answering fundamental evolutionary questions regarding antibiotic resistance in bacteria and other pathogens (Bouma and Lenski, 1988; Schrag et al., 1997; Burch and Chao, 1999; Moore et al., 2000; Cowen et al., 2001). A major advantage of this procedure is that genotypes from different generations during the adaptation process can be compared directly. This procedure could, in principle, be used for *P. infestans*, because this pathogen can be successfully revived after storage in liquid nitrogen (Flier et al., 2003).

In this thesis, I report results from an experimental evolutionary approach that was used in our laboratory to study the evolution and stability of pesticide resistance. In this chapter, I will discuss the results of this approach in the light of our current knowledge of metalaxyl resistance. I will present possible explanations for the fast evolution and exclusiveness of metalaxyl resistance and will discuss the absence of resistance prior to the commercial release of metalaxyl. Finally, possible future directions of research on pesticide resistance are discussed where experimental evolution could be a promising approach.

Explaining the rate of evolution of metalaxyl resistance

Genetics of metalaxyl resistance

Because *P. infestans* is diploid, differences between resistance mutations in the level of dominance may, at least in part, explain differences in rates of resistance evolution (Jasieniuk et al., 1996). The fast evolution of metalaxyl resistance may then be explained by the existence of dominant resistance mutations. Indeed, by analyzing sexual offspring of crosses between resistant and sensitive genotypes, Shattock (1988) revealed the action of at least one intermediate dominant allele that was responsible for resistance. This outcome was similar to that reported by others, although the level of dominance of the mutations differs between studies (Lee et al., 1999). Therefore, it is commonly accepted that metalaxyl resistance is governed by a partially or fully dominant allele at a single locus, which has been referred to as *MEX* (Fabritius and Judelson, 1997).

The mutation rate is another important genetic parameter in explaining the rate of evolution. On the one hand, a high mutation rate may be deleterious by increasing the genetic load (Agrawal and Wang, 2008). On the other hand, a high mutation rate may facilitate the rate of adaptation by increasing the mutation supply rate, particularly in small populations (de

Visser et al., 1999; Elena and Lenski, 2003). Estimates of the mutation rates could therefore provide important insights into the potential of *P. infestans* to adapt to new selection pressures, such as pesticides.

In **Chapter 2**, we report on attempts to estimate the mutation rate using a fluctuation test, a method that is commonly used in bacterial genetics (Luria and Delbrück, 1943; Sniegowski et al., 1997). Unfortunately, the preliminary tests using metalaxyl failed due to the non-lethal effect of metalaxyl *in vitro*. Additional fluctuation tests using the lethal pesticides fluazinam and cyazofamid also failed, because no resistant colonies were observed in those tests, despite attempts to maximize the number of zoospores tested. Based on this absence of resistant colonies, we were able to provide a conservative estimate of the frequency of mutant zoospores obtained from a sensitive colony. This frequency is likely lower than one mutant among every 10^7 zoospores for both cyazofamid and fluazinam. The mutant frequency is apparently higher for metalaxyl resistance, because numerous resistant zoospores can be obtained from a single sensitive colony (Abu-El Samen et al., 2003b). This result is backed up by our recurrent observation of faster growing mycelial sectors on medium amended with low concentrations of metalaxyl-M.

The differences in the mutant frequencies for the different pesticides observed in **Chapter 2** may be explained by differences in the mutation rates towards resistance (Rogozin and Pavlov, 2003). Similar high mutant frequencies as we observed for metalaxyl resistance were also obtained for other characters (Caten and Jinks, 1968; Caten, 1970; Abu-El Samen et al., 2003b; Abu-El Samen et al., 2003a), which could suggest a genome-wide high mutation rate. However, the mutant frequency is not only determined by the mutation rate but also by their pleiotropic effects on other traits (Oliver et al., 2004). Hence, given the large numbers of asexual spores that are produced during epidemics (about 10^5 sporangia per individual lesion (Flier et al., 2007)), resistant genotypes will always emerge in the absence of the pesticide, unless they have large negative pleiotropic effects. The absence of resistant colonies on lethal concentrations of cyazofamid and fluazinam could then be explained by a low mutation rate or by large negative pleiotropic effects on fitness of potential resistance mutations. In contrast, resistance to metalaxyl would then be caused by a mutation that occurs often or that has a small negative pleiotropic fitness effect.

Response to selection on sub-lethal concentrations

The segregation of metalaxyl sensitivity among the offspring of sexual crosses may reveal yet another explanation for the fast evolution of metalaxyl resistance. As mentioned above, it is commonly accepted that mutations at a single locus are responsible for metalaxyl resistance. Sexual offspring from crosses between sensitive and resistant parents, however, show an almost continuous spectrum of sensitivity, which makes it hard to make a clear distinction between the different sensitivity classes (Deahl et al., 1993). This variation has been explained by the action of additional loci with small effect (Grunwald et al., 2006; Judelson and Senthil, 2006). The influence of these loci with small effect can be substantial (Lee et al., 1999), indicating that metalaxyl resistance can better be regarded as a quantitative (or metric) trait that is under the influence of one or several Quantitative Trait Loci with large effect, e.g. *MEX-loci* (Fabritius et al., 1997), and other loci with small effect. These loci with small effect can have important implications for the evolution of pesticide resistance, which will be discussed below.

Theoretical studies suggest that evolution of resistance is fastest on intermediate pesticide concentrations due to an optimal balance between selection and mutation supply rate (Lipsitch and Levin, 1997; Kepler and Perelson, 1998). At intermediate concentrations, however, large effect mutations may have to compete with other mutations with small effect more often than at high concentrations where only large-effect mutants survive (Negri et al., 2000; Perfeito et al., 2007). Under conditions of relatively weak selection, these small-effect mutations may be more advantageous than large-effect mutations, especially if the large-effect mutations have a large negative pleiotropic effect on fitness (Lande, 1983). Hence, evolution on relatively low concentrations may lead to an intermediate level of resistance instead of full resistance.

In **Chapter 3**, we investigate whether full resistance to metalaxyl can be selected *in vitro* at a constant low concentration and whether this is also the case for another pesticide with a single target, cyazofamid. Other studies had suggested that resistance to metalaxyl would evolve under these conditions, whereas this is not the case for other pesticides (Young et al., 2001; Stein and Kirk, 2004). However, these studies were conducted on increasing concentrations of pesticide, which could facilitate the evolution of resistance by accumulation of small-effect mutations (Negri et al., 2000; Baquero, 2001; Perron et al., 2006). Moreover, these experiments were conducted by serially transferring plugs of mycelium, thereby

neglecting selection during crucial parts of the asexual life-cycle (see **Chapter 1** for a detailed discussion on the life-cycle). By serially transferring zoospores on a constant sub-lethal concentration we showed that full resistance to metalaxyl can evolve quickly under these conditions, whereas this is not the case for resistance to cyazofamid.

We also observed a significant better *in vitro* performance of resistant genotypes compared to their sensitive ancestor. This result may indicate that metalaxyl resistance does not have a large negative pleiotropic effect on fitness. This absence of a large cost may facilitate the evolution of metalaxyl resistance. First, it will positively influence the frequency of mutants obtained from sensitive colonies. Second, it also allows selection for high level resistance on low concentrations (**Chapter 3**).

Why resistance is absent when metalaxyl is absent?

Several studies have reported a higher fitness during epidemics of metalaxyl resistant *P. infestans* genotypes compared to sensitive ones (Kadish and Cohen, 1988a; Kadish and Cohen, 1988b). This apparent positive effect of resistance makes it difficult to explain the absence of resistance prior to the commercial release of metalaxyl. This paradox was supposed to be solved with the observation of a reduced survival of resistant genotypes during winter storage (Kadish and Cohen, 1992). However, given the high mutation rate (Abu-El Samen et al., 2003b; **Chapter 2**), a positive effect of the mutation would cause metalaxyl resistance to be selected anew each year. A lower survival of resistant genotypes during the winter would then be insufficient to prevent the increase of resistance in the absence of metalaxyl. Three additional explanations can be given here for the positive link between resistance and fitness: (i) the resistance mutation may be beneficial by itself (Luo et al., 2005), (ii) its effect may be masked by differences in the genetic background (Enne et al., 2004), or (iii) the initial cost may be compensated by additional mutations (Levin et al., 2000; Schoustra et al., 2006). In this part, I will try to unravel the mystery of the higher fitness of metalaxyl resistant genotypes by evaluating each of these possible explanations.

The cost of metalaxyl resistance

The lack of even a small negative pleiotropic effect of metalaxyl resistance would be surprising since resistance mutations are generally believed to incur a fitness cost in the

absence of the pesticide (Bergelson and Purrington, 1996; Andersson and Levin, 1999; Sander et al., 2002; Baucom and Mauricio, 2004; Gagneux et al., 2006). In **Chapter 4**, we analyzed the Dutch population of *P. infestans* during the period 2000-2006, which includes a three year period (2001-2003) during which metalaxyl was banned from the Dutch market. Metalaxyl resistance disappeared almost instantaneously at the start of the metalaxyl-free period and came back when metalaxyl returned on the market. These results indicate that metalaxyl resistance is not selected in the absence of metalaxyl. Similar effects of nation-wide bans have been observed before (Dowley and O'Sullivan, 1985; Davidse et al., 1989). Hence, these data suggest that metalaxyl resistance has no direct beneficial effect on epidemic fitness.

Genetic background

Studies on the cost of resistance to metalaxyl typically compare randomly chosen field isolates that differ in their sensitivity to metalaxyl (Kadish and Cohen, 1988b; Day and Shattock, 1997) and do not take differences in genetic background into account (Lee et al., 1999). Hence, it remains unknown whether the higher fitness of resistant genotypes is caused by a direct effect of the resistance mutation or by differences in the genetic background. Experiments that investigate the direct effect of resistance should therefore either randomize the genetic background by sexual recombination (Roux et al., 2004) or introduce the resistance mutation in a known sensitive genetic background.

Using an experimental evolutionary procedure, we were able to analyze the effect of metalaxyl resistance between near-isogenic individuals that differ in their sensitivity to metalaxyl (**Chapter 4**). This approach is believed to be more powerful than an analysis of sexual offspring, because it produces less variation than sexual crosses. However, we were also unable to observe a consistently negative effect of metalaxyl resistance on aggressiveness components on leaflets of potato cultivar Bintje, although a small positive effect was observed *in vitro*.

In **Chapter 4**, we also discussed the hypothesis that the likelihood of obtaining a resistance mutation is high in genotypes with a high epidemic fitness. This hypothesis is backed up by the observation that the increase in frequency of resistant genotypes coincides with the increase of aggressiveness during each epidemic (Gisi et al., 2000). Although this hypothesis may explain the higher aggressiveness of resistant genotypes, it does not explain the absence of resistance prior to metalaxyl use. This absence can possibly be explained by

assuming that metalaxyl resistance causes an initial cost, which is later compensated during further adaptation.

Compensation of an initial cost

Compensatory mutations are mutations that restore the negative fitness effects of resistance mutations (Levin et al., 2000; Andersson, 2003). In some cases, these compensatory mutations may even increase fitness of the resistant mutant above that of the sensitive ancestor (Schoustra et al., 2006). Hence, they could well be responsible for the higher aggressiveness of resistant individuals. In that case, the initial resistance mutation may harbor a cost that is compensated for during subsequent growth and adaptation. This may explain the apparent lack of a large fitness cost observed in **Chapter 4**.

In **Chapter 5**, we investigated whether metalaxyl resistance involves an initial fitness cost that is compensated later during the selection procedure. We observed an initial fitness cost of metalaxyl resistance that was apparent from a delayed onset of *in vitro* growth rather than from a reduced mycelium growth rate. However, we did not observe compensatory evolution under these same conditions (i.e. in the presence of metalaxyl). Nevertheless, this initial cost may explain the absence of metalaxyl resistance prior to the commercial release of metalaxyl and during years in which metalaxyl was banned from the market.

Conclusions

The results presented in this thesis provide new insights into the evolution of metalaxyl resistance. The small negative pleiotropic effect of resistance measured *in vitro* may both explain the fast evolution of resistance in the presence of metalaxyl and the lack of resistance evolution in the absence of metalaxyl. Based on these results, we are able to present a model of the evolution of metalaxyl resistance. This model involves two levels of selection: the level of single colonies and the level of sensitive and resistant sub-populations.

In a sensitive colony, resistant nuclei emerge at a rate proportional to the mutation rate. These resistant nuclei are subjected to weak negative selection in the absence of metalaxyl (**Chapter 5**), and hence may end up in some sporangia (Abu-El Samen et al., 2003b) and be transported to new plants. Low concentrations of metalaxyl are likely to occur at some places when metalaxyl is applied in the field (Cremieux and Carbon, 1992; Elliot et

al., 1995; Evenhuis et al., 2006). Growth of sensitive colonies continues under these conditions; hence mutations may occur in these colonies. Nuclei that just acquired a resistance mutation with large effect will directly outcompete the sensitive nuclei (**Chapter 2**) and the nuclei that acquired a resistance mutation with small effect in the same colony. These resistant nuclei will then end up in the majority of sporangia from this colony and are therefore more likely to infect new hosts.

Because a small number of sporangia may already contain resistant nuclei in the absence of metalaxyl, a small number of resistant genotypes may infect new hosts. This would explain the occasional isolation of metalaxyl resistant genotypes before the commercial release of metalaxyl (Daggett et al., 1993). However, these resistant genotypes are unlikely to reach a sufficiently large population size due to the fitness costs like the delay of *in vitro* growth that we observed in **Chapter 5**. This delay may reduce the efficiency of infecting new hosts (**Chapter 4**) and may also increase the latent period, i.e. the period between infection and the production of the first sporangia. Since both infection efficiency and latent period are important factors influencing the speed at which polycyclic epidemics progress (Skelsey et al., 2005), a small resistant sub-population will be quickly outcompeted by the larger population of sensitive genotypes from which it emerged. In the presence of metalaxyl, however, a resistant sub-population will likely outcompete the population of sensitive genotypes from which it emerged. Once this resistant population has reached a sufficient population size it is more likely to acquire additional mutations that compensate the cost of resistance and increase fitness during epidemics (McCutchan et al., 2004).

Implications

The results in this thesis provide explanations for the relatively fast evolution of metalaxyl resistance and for the paradox between the apparent positive effect of this resistance and the absence of resistance prior to the commercial release of metalaxyl. They also have additional implications for our view on metalaxyl resistance. These implications will be presented here, together with some future challenges in oomycete research in which experimental evolution could prove useful.

The fast evolution of metalaxyl resistance has led some scientists to believe that resistance evolution is a serious threat in this pathogen (e.g. Fry and Goodwin, 1997). Results from other studies (Young et al., 2001; Stein and Kirk, 2004; Yuan et al., 2006; Gisi and Sierotzki, 2008; Rubin et al., 2008) and from this thesis, however, suggest that the risk of development of resistance to other pesticides is not as high as for metalaxyl. In other words, metalaxyl resistance appears to be a special case that evolves fast and is common among several oomycete species. This special position of metalaxyl resistance may be caused by several features: it is likely caused by a dominant genetic change with a relatively small negative pleiotropic effect (**Chapter 5**), that is selectively favored on a wide range of (low) concentrations (**Chapter 3**). Additionally, because several mutations may cause full resistance (Fabritius et al., 1997), the mutation rate towards metalaxyl resistance may be high compared to other resistances.

Reducing the application rate of pesticides has been used as a method to reduce the selection pressure for resistance. Metalaxyl resistance, however, may benefit from this approach due to its small negative pleiotropic effect in the absence of the toxin. Because the effect of low concentrations on resistance evolution differs between pesticides, a similar approach as presented in **Chapter 3** should be included when new pesticides are introduced. Although evolution of resistance to other pesticides does not have to occur on low concentrations, one should still be careful not to increase the application rate since the evolution of full resistance is likely stimulated under those conditions.

Future challenges

The experimental evolution approach that was used throughout this thesis did help in testing several hypotheses on the evolution of metalaxyl resistance. This method can also be used to test other hypotheses on evolutionary problems in this pathogen. The strength of this approach clearly lies in the ability to compare near isogenic genotypes during evolutionary processes in this flexible genetic system. Here, I will present some evolutionary hypotheses on the evolution that can be investigated using an experimental evolutionary approach.

Explaining the link between epidemic fitness and metalaxyl resistance

In **Chapter 4**, we proposed a model to explain the association between epidemic fitness and metalaxyl resistance. This model contains two mutually non-exclusive factors that can explain this link: compensatory evolution and an increased supply of resistance mutations in genotypes with a high epidemic fitness. Based on studies from field populations, however, it is hard to distinguish between these two factors. Controlled laboratory experiments are therefore needed to assess how each of these factors is influencing this unexpected link.

Although experimental evolutionary studies on other organisms were successful in showing the significance of compensatory mutations (Estes and Lynch, 2003; Schoustra et al., 2006), the experiments with *P. infestans* presented in this thesis did not show clear evidence for compensatory evolution. Results obtained from the experiments on the stability of the adaptations in **Chapter 3**, however, revealed that additional selection on medium without metalaxyl may result in an additional adaptation in some resistant clones. This adaptation resulted into an increase of the growth rate of the resistant clones above that of their sensitive ancestor on medium without metalaxyl and on medium with a low concentration metalaxyl. These results could indicate a potential role of compensatory evolution in explaining the absence of a large cost of metalaxyl resistance in our *in vitro* fitness assays. However, since this was the only observation of a possible compensatory mutation, we clearly need to study this in more detail.

The upcoming publication of the genome sequence of *P. infestans* may provide us with new opportunities to study compensatory evolution. This sequence may help us in finding the exact mutations that are responsible for metalaxyl resistance in field populations. This will make it much easier to see whether the genetic changes in our *in vitro* experiments are the same as those observed in the field population. Combining new genomic tools with an experimental evolutionary procedure would then allow us to study the effect of the resistance mutation in different backgrounds and the potential of compensatory evolution in more detail.

Present-day use of metalaxyl in potato production

Despite the problems with resistance, metalaxyl is still used in present day agriculture. In order to prevent resistance evolution, it may be used in mixtures with chemically unrelated pesticides (www.frac.info). This does not imply that resistance cannot evolve if metalaxyl is used in a mixture. The addition of metalaxyl to the mixture will, however, likely influence the speed of evolution of resistance to the other pesticides. On the one hand, some studies suggest

that metalaxyl stimulates the growth of some genotypes (Zhang et al., 1997). Metalaxyl may then increase the supply rate of mutations that cause resistance to other pesticides. On the other hand, metalaxyl may reduce the evolution of resistance to other pesticides, because it reduces the growth rate in other genotypes (**Chapter 2**) and thus the mutation supply rate (**Chapter 3**). An experimental evolution approach could show which effect metalaxyl may have on the evolution of resistance to other pesticides. Such an experiment would include selection of resistance to an antibiotic, e.g. streptomycin (Shaw, 1996), in the presence and absence of metalaxyl.

The consequences of coenocytic hyphae

Hyphae of *P. infestans* do not contain septa, but consist of several nuclei in the same cytoplasm. In fact, they can be regarded as a single cell, i.e. they are coenocytic. This may have several important implications for the evolution of metalaxyl resistance. For instance, the deleterious effect of a resistance mutation in one nucleus may be supplemented by the other nuclei in the same cytoplasm that do not bear the mutation. As a result, nuclei that contain a resistance mutation may migrate into sporangia together with non-mutated nuclei (Pipe et al., 2000; Judelson and Blanco, 2005); hence, they may migrate to new hosts despite their reduction in fitness. If zoospores (with single nuclei) are produced, however, a resistant nucleus may no longer benefit from the other nuclei, because zoospores hardly fuse after germination (Judelson and Yang, 1998). Hence, the spread of resistant genotypes in the absence of metalaxyl may be hampered by the production of zoospores. Because zoospores are primarily produced when temperatures are low, this may partially explain the low frequency of resistant genotypes at the beginning of each growing season. An experimental evolutionary procedure in which both zoospores and sporangia are serially transferred may be used to test this hypothesis.

Evolutionary potential

Traditionally, *P. infestans* is regarded as a "high risk" pathogen with a large evolutionary potential (McDonald and Linde, 2002). However, the difficulties with isolating mutants that are resistant to other pesticides in this thesis and other studies (Young et al., 2001; Stein and Kirk, 2004) may indicate that the evolutionary potential of this pathogen is low. On the other hand, in several oomycete species such as *Plasmopara viticola*, the causal agent of downy mildew on grape, resistance to other pesticides is more common (Gisi and

Sierotzki, 2008; Rubin et al., 2008). This latter suggests that resistance evolution is still possible within oomycetes, but that it does, for some reason, not occur in *P. infestans*.

At this stage, we can only guess at the reasons for the differences in resistance evolution between *P. infestans* and other oomycetes such as *P. viticola*. One remarkable difference between these two species, however, is the contribution of sexual offspring to epidemics. Populations of *P. viticola*, seems to be random mating (Gobbin et al., 2006), whereas *P. infestans* shows primarily clonal reproduction (Fry et al., 1991; Cooke et al., 2003; Cooke et al., 2006). One large benefit of sexual recombination over clonal propagation may lie in the fact that sexual recombination facilitates the production of genotypes that are homozygous for a recessive resistance mutation, particularly if selfing is also possible. This suggests that sexual reproduction would facilitate the evolution of resistance to other pesticides. However, an analysis of resistance evolution in the sexual reproducing population of *P. infestans* in the Toluca Valley did not reveal fast evolution to other pesticides than metalaxyl (Grunwald et al., 2006). Unfortunately, this experiment was only performed during one growing season; hence, the effect of sexual reproduction on the population in the following years could not be assessed. Nevertheless, these results reveal the need for a thorough analysis of the consequences of increased sexual reproduction in potato production areas.

The apparent low rate of evolution towards pesticide resistance does not imply that evolution of other characters is also slow. For instance, *P. infestans* is renowned for its ability to adapt to resistant potato cultivars (Fry, 2008). Such changes are typically the result of the loss of function of avirulence factors (Birch et al., 2006; Ellis et al., 2006). Therefore, adaptation is not necessarily caused by single point mutations that specifically alter the gene product, but may also be caused by loss of function mutations such as frame shift mutations or even large chromosomal deletions (Van der Lee et al., 2001). Such loss of function changes are likely to occur more frequently than specific point mutations. The frequency of these mutations in the absence of selection, however, will largely be influenced by their negative pleiotropic effects (**Chapter 2** & **Chapter 3**). Hence, the cost of virulence may be an important topic for understanding virulence evolution.

Non-meiotic recombination

The absence of sexual reproduction in *P. infestans* populations outside the Toluca Valley may indeed slow down the evolution of recessive traits. However, adaptations that allow infection of previously resistant potato cultivars are also generally recessive (May et al., 2002). Therefore, another explanation is needed to explain the fast accumulation of these recessive mutations. Additional phenomena may explain this quick loss of heterozygosity; mitotic recombination by crossing over, gene conversion and non-disjunction also referred to as parasexual recombination (Pontecorvo, 1956).

Because *P. infestans* is coenocytic, parasexual processes may occur regularly, since nuclei could, in principle, be able to fuse. Indeed, similar parasexual processes have been proposed to explain sudden host-shifts in some species of *Phytophthora* (Brasier et al., 1999; Brasier, 2000) or other patterns in asexual offspring (Pipe et al., 2000). The incidence of parasexual processes may be assessed by using an experimental evolutionary approach together with the availability of new molecular markers (Cooke, 2004).

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Nederlandse Samenvatting

-Dutch Summary-

Antibioticumresistentie in bacteriële ziekteverwekkers

Ziektes hebben een grote rol gespeeld in de geschiedenis van de mensheid. Het was dan ook geen wonder dat de ontdekking van antibiotica als een overwinning werd gevierd. Deze euforie werd echter minder toen de eerste berichten over resistente bacteriën binnen kwamen. Dit probleem van resistentieontwikkeling is nog steeds actueel, gezien de toename van het aantal krantenkoppen over MRSA in ziekenhuizen en multiresistente TBC in sommige delen van Europa. De evolutie van antibioticum resistentie is, ironisch genoeg, ook een mooi voorbeeld van snelle biologische evolutie en heeft dan ook in toenemende mate aandacht gekregen van evolutiebiologen.

Wetenschappelijk onderzoek heeft enkele belangrijke inzichten opgeleverd over de factoren die de evolutie van antibioticum resistentie in bacteriën beïnvloeden. Zo hebben mutaties die resistentie veroorzaken doorgaans een negatief effect op de fitness in de afwezigheid van het antibioticum. Dit negatieve effect kan verklaren waarom resistentie vrijwel afwezig is voordat een antibioticum voor het eerst wordt gebruikt. Dit negatieve effect leidt er echter niet toe dat resistenties verdwijnen wanneer het gebruik van een antibioticum wordt gestaakt. De stabiliteit van resistentie wordt verklaard door de waarneming dat andere mutaties het negatieve effect kunnen compenseren. Aangezien de kans dat een resistentie mutatie en een compenserende mutatie simultaan optreden in een sensitief genotype klein is, zal een stabiele resistente populatie zich niet ontwikkelen vóór het gebruik van een antibioticum.

Een ander belangrijk inzicht is dat de ontwikkeling van resistentie vooral snel is bij relatief lage doseringen van een antibioticum. Dit komt doordat bacteriën dan tegelijk een selectiedruk ondervinden en toch in staat zijn tot deling, waardoor de kans op nieuwe resistentie mutaties wordt vergroot. Dit inzicht verklaart mede waarom het belangrijk is een antibioticumkuur altijd af te maken, omdat dat de kans verkleint dat een grote populatie bacteriën in contact komt met een lage dosis antibioticum.

Dezelfde factoren die de evolutie van antibioticum resistentie in bacteriën beïnvloeden, lijken ook de evolutie van resistentie in andere groepen van organismen te kunnen beïnvloeden. Bijvoorbeeld, in sommige schimmels blijken resistentie mutaties ook een negatief effect te hebben op fitness in de afwezigheid van een fungicide. Ook blijkt in deze groep de mogelijkheid voor compenserende evolutie te bestaan. Er zijn echter ook voorbeelden van resistenties die geen nadelig effect op fitness lijken te hebben. Dit laatste zou suggereren dat de algemene ideeën over de evolutie van resistentie in bacteriën niet altijd

simpelweg kunnen worden geëxtrapoleerd naar andere organismen.

Pesticide resistentie in de teelt van aardappels

Ziektes van landbouwgewassen kunnen ook een enorm effect hebben op menselijke populaties. Een bekend voorbeeld van de impact van plantenziekten is de eerste uitbraak van de aardappelziekte halverwege de 19^{e} eeuw in Ierland. Als gevolg van deze uitbraak ging een groot gedeelte van de aardappeloogst verloren. Omdat de aardappel de primaire voedselbron was voor de arme Ierse populatie, resulteerde deze uitbraak in een hongersnood waarbij meer dan één miljoen Ieren het leven lieten en een grote groep het land ontvluchtten. Het bleef helaas niet bij deze ene uitbraak: de ziekte zorgt ook voor problemen in de hedendaagse aardappelteelt. De meest gebruikte manier om de ziekte enigszins onder controle te houden is door de toepassing van een grote hoeveelheid chemische bestrijdingsmiddelen. Ontwikkeling van resistentie in de ziekteverwekker zou dus een enorme invloed kunnen hebben op de effectiviteit van de huidige bestrijdingsmaatregelen.

De ziekteverwekker die verantwoordelijk is voor al deze problemen is *Phytophthora infestans*. Dit organisme behoort tot de oomyceten, een groep organismen die, op het eerste gezicht, fysieke overeenkomsten vertoont met schimmels. Deze groep verschilt echter op verschillende fronten van schimmels: ze zijn niet haploid en hebben een andere chemische samenstelling van hun celwand. Recente fylogenetische reconstructies laten dan ook zien dat deze groep totaal niet verwant is aan schimmels. Door deze verschillen kunnen resultaten uit onderzoek met schimmels niet zomaar worden gebruikt om de biologie van oomyceten te begrijpen.

De noodzaak voor gericht onderzoek aan *P. infestans* en de technische ontwikkelingen in de wetenschap hebben er toe geleid dat we steeds meer te weten komen over de genetica van *P. infestans*. De meest zorgwekkende observaties die voortkomen uit de nieuwe ontwikkelingen zijn de flexibiliteit van het genoom en het aanpassingsvermogen van *P. infestans*. Dit is in overeenstemming met de observaties dat resistenties in aardappelgewassen relatief snel worden doorbroken en resistentie tegen het bestrijdingsmiddel metalaxyl snel optrad en vrij algemeen is in hedendaagse populaties.

Resistentie tegen metalaxyl in *P. infestans* is in meerdere opzichten een vreemd verhaal. Ten eerste ontstond deze resistentie vrij snel na het gebruik van metalaxyl in de aardappelteelt, terwijl resistenties tegen andere gebruikte bestrijdingsmiddelen nog niet zijn

waargenomen in natuurlijke populaties van dit organisme. Ten tweede wordt metalaxyl resistentie geassocieerd met een hogere fitness gedurende epidemieën. Deze hogere fitness lijkt in tegenspraak met de afwezigheid van resistentie vóórdat metalaxyl werd toegepast. Deze afwezigheid werd vervolgens verklaard door een negatief effect van resistentie op de overleving gedurende de winter. Echter, gegeven de hoge snelheid waarmee metalaxyl resistentie optreedt in verschillende experimenten, zou een positief fitness effect van resistentie mutaties ertoe moeten leiden dat resistentie altijd optreedt; ook in de afwezigheid van metalaxyl. Er is dus duidelijk meer onderzoek nodig naar de factoren die de evolutie van resistentie beïnvloeden in dit organisme.

Onderzoek en resultaten beschreven in dit proefschrift

Experimentele evolutie is een methode die zeer succesvol is gebleken voor het ophelderen van factoren die de evolutie van antibioticum resistentie in bacteriën beïnvloeden. De kracht van deze methode ligt in het feit dat genotypen die ontstaan gedurende een adaptatieproces kunnen worden opgeslagen in de diepvries (-80°C) om later weer tot leven te worden gewekt. Hierdoor kunnen verschillende fases van een adaptatieproces direct met elkaar worden vergeleken. Aangezien *P. infestans* ook kan worden opgeslagen in de diepvries (in dit geval in vloeibare stikstof), kan deze methode in principe worden gebruikt om enkele factoren op te helderen die de evolutie van resistentie beïnvloeden

In dit proefschrift wordt een experimenteel evolutionaire benadering beschreven waarin getracht wordt de evolutie van resistentie tegen bestrijdingsmiddelen op te helderen. Het proefschrift bestaat globaal uit twee delen. In het eerste gedeelte (hoofdstuk 2 en 3) wordt getracht een verklaring te vinden voor de relatief snelle evolutie van metalaxyl resistentie ten opzichte van mogelijke resistenties tegen andere bestrijdingsmiddelen. In het tweede gedeelte (hoofdstuk 4 en 5) wordt getracht een verklaring te vinden voor de hogere epidemische fitness van metalaxyl resistente isolaten.

De snelle evolutie van metalaxyl resistentie

Verschillende factoren kunnen de snelheid van evolutie van resistentie beïnvloeden. Een belangrijke factor is de snelheid waarmee nieuwe resistentie mutaties optreden. Verschillen in mutatiesnelheid naar resistentie tegen verschillende pesticiden zouden de

verschillen in snelheid van evolutie kunnen verklaren, zeker in (effectief) kleine populaties. Fluctuatietesten zijn zeer succesvol gebleken voor het schatten van mutatiesnelheden in bacteriën en lijken dus een logische methode om mutatiesnelheden te meten in *P. infestans*. Deze methode bleek echter om verschillende redenen niet geschikt voor *P. infestans*. In de eerste plaats bleek metalaxyl, het enige bestrijdingsmiddel waarvoor een dominante resistentiemutatie bekend was, niet lethaal te zijn *in vitro*. Dit zorgde ervoor dat sensitieve genotypen ook in staat waren om te groeien, waardoor een onderscheid tussen resistente en sensitieve genotypen onmogelijk te maken was. In de tweede plaats is het aantal zoosporen dat wordt geproduceerd door een enkele kolonie onvoldoende om de lage mutatiesnelheden te kunnen schatten. Dit zou mede kunnen verklaren waarom fluctuatietesten met twee andere bestrijdingmiddelen die wel lethaal waren, fluazinam en cyazofamid, helemaal geen groei opleverden.

De afwezigheid van groei in deze twee laatste fluctuatietesten suggereert dat de mutatiesnelheid voor resistentie niet extreem hoog is in *P. infestans*. Een extra experiment liet echter zien dat mycelium met een verminderde gevoeligheid voor metalaxyl regelmatig ontstaat op platen met een relatief lage concentratie. Dit laatste kan worden verklaard door een hoge mutatiesnelheid voor metalaxyl resistentie. De snelle evolutie kan echter ook verklaard worden door het feit dat er verscheidene mutaties een dominante resistentie kunnen veroorzaken of door het feit dat metalaxyl resistentie een relatief klein negatief fitnesseffect heeft.

De eenvoud waarmee resistente klonen konden worden geïsoleerd van platen met een lage concentratie metalaxyl leidde ons ertoe om de rol van lage pesticide concentraties op de evolutie van resistentie te onderzoeken. Verschillende genotypen van *P. infestans* werden gekweekt op agarplaten met een lage dosis metalaxyl of een lage dosis cyazofamid. Iedere twee weken werden zoosporen van deze platen geïsoleerd en gebruikt om nieuwe platen te inoculeren. Groei op platen met metalaxyl resulteerde in klonen (mycelium afkomstig van 1 zoospore) met een stabiele aanpassing aan het gif die ook voordelig was op hogere concentraties. Deze klonen zouden als resistent betiteld worden na een standaard resistentie test. Selectie op platen met cyazofamid resulteerde in een aantal klonen met een stabiele aanpassing. Deze aanpassing was echter niet voordelig op hogere concentraties cyazofamid. Deze resultaten benadrukken de mogelijke rol van lage concentraties in de evolutie van pesticidenresistentie. De mogelijkheid tot evolutie van volledige resistentie op platen met een

lage dosis metalaxyl zou kunnen verklaren waarom deze resistentie relatief snel evolueerde en waarom cyazofamid resistentie vrijwel afwezig is in hedendaagse populaties.

De afwezigheid van een negatief fitnesseffect van metalaxyl resistentie

De waarneming dat metalaxyl resistente genotypen een hogere fitness hebben dan sensitieve genotypen strookt niet met de afwezigheid van de resistentie voordat metalaxyl werd gebruikt. Twee verschillende benaderingen werden gebruikt om deze paradox op te lossen. In de eerste benadering werd de *P. infestans* populatie in Nederland bestudeerd gedurende een periode waarin producten met metalaxyl niet waren toegestaan. Gedurende jaren waarin metalaxyl wel toegestaan is, ontstaan resistente isolaten doorgaans later in het jaar. Gedurende jaren waarin metalaxyl niet toegestaan is, worden er echter geen resistente isolaten gevonden. Deze resultaten suggereren dat resistentie alleen maar wordt geselecteerd in de aanwezigheid van metalaxyl en dus geen direct positief effect heeft op epidemische fitness. In de tweede benadering werden fitnesscomponenten van klonen afkomstig van eerdere selectie-experimenten vergeleken met die van hun sensitieve voorouder. Deze benadering liet echter geen consistent negatief dan wel positief effect zien van metalaxyl resistentie op een fitnesscomponent.

De resultaten van bovenstaande studies kunnen alleen worden verklaard in het licht van een nieuw model. Dit model omvat een initieel nadelig effect van metalaxyl resistentie dat wordt opgeheven door compenserende mutaties. Tevens kan het zijn dat metalaxyl resistentie vaker optreedt in genotypen met een hogere fitness. In het laatste experiment vinden we inderdaad aanwijzingen voor een initieel nadelig effect van metalaxyl resistentie. Dit effect is met name merkbaar gedurende de initiële groeifase na kieming van zoosporen en niet gedurende myceliumgroei. Compenserende evolutie zou dan kunnen verklaren waarom dit effect niet meer merkbaar is na langdurige aanpassing aan metalaxyl.

Conclusie

Metalaxyl resistentie in *P. infestans* heeft een aantal kenmerken die het relatieve gemak waarmee het ontstaat kan verklaren. In de eerste plaats lijkt metalaxyl resistentie te worden veroorzaakt door meer dan één mutatie met een dominant effect. Mutaties die resistentie veroorzaken tegen andere bestrijdingsmiddelen zouden wel eens recessief kunnen

zijn. In de afwezigheid van seksuele reproductie ontstaan homozygoot resistenten alleen maar door een aantal zeldzame genetische processen: dubbele mutaties, mitotische recombinatie of genconversie. In de tweede plaats kan volledige resistentie tegen metalaxyl al op lage concentraties worden geselecteerd, terwijl dat niet geldt voor andere bestrijdingsmiddelen. In de laatste plaats lijken de resistentie mutaties geen groot nadelig effect te hebben op de fitness in de afwezigheid van metalaxyl. Als gevolg daarvan kan een kleine resistente sub-populatie toenemen in omvang, ook in de afwezigheid van metalaxyl. De afwezigheid van resistentie in de *P. infestans* populatie voordat metalaxyl beschikbaar kwam kan worden verklaard aan de hand van een klein nadelig effect van de resistentie mutatie(s).

Dankwoord

What is there left for me to do in this life?
Did I achieve what I had set in my sights?
Am I a happy man or is this sinking sand?
Was it all worth it? Was it all worth it?

Queen 1988

Bovenstaande tekst werd in 1988 opgetekend door de Britse rock groep Queen in het nummer "*Was it al worth it*" dat verscheen op hun LP "The Miracle". Het nummer straalt het gevoel uit dat de vier bandleden (en mijn helden) moeten hebben gehad toen ze terugkeken op hun, tot dan toe, succesvol verlopen carrière. Dit nummer krijgt extra gewicht wanneer men beseft dat Freddie Mercury op dat moment al wist dat hij besmet was met het Hiv-virus. Op de één of andere manier bekruipt mij een soortgelijk gevoel, nu ook ik op een kruispunt in mijn leven sta met het afronden van mijn proefschrift en het starten van een carrière in het onderwijs.

Het schrijven van een proefschrift is geen simpele opgave en het effecten daarvan zijn merkbaar in je privé en sociale leven. Als daarbij ook nog eens een belangrijk persoon uit je leven wegvalt, wordt het een nog zwaardere opgave. Ik heb me dan ook menigmaal afgevraagd: "*Is it all worth it*". Het is dan ook dankzij een aantal mensen dat ik heb doorgezet en dat ik kan zeggen: "*Yes, it was a worthwile experience*". Ik kan er dan ook niet aan voorbij om een aantal van deze mensen te bedanken.

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Het sociale leven van een AIO is verweven met het dagelijkse werk. Het is daarom van enorm belang om goed op te kunnen schieten met je collega's. Bij het begin van mijn project had ik het geluk om binnen te komen in een gezellig groepje collega's. Marc Maas en Siemen Schoustra waren op dat moment aan het einde van hun project. Michelle Habets was samen met Karoly Pál één van mijn kamergenoten aan het begin van mijn project. Hun rol als kamergenoot werd later opgevuld door de nieuwe garde AIO's: Anna Visser en Bart Nieuwenhuis. Ik wil jullie bij dezen bedanken voor de vele gezellige discussies en bezoekjes aan "De Vlaamsche Reus". Merijn Salverda, "mijn AIO broertje", wil ik hier even speciaal bedanken voor de vele bieravondjes en voor de onmisbare tips over het schrijven van een proefschrift.

The social life of a PhD student is tightly linked to the work situation. Hence, the relationship with your colleagues is crucial for a nice atmosphere at work. Danny, thank you and good luck with your career in Manchester. Karoly, you are one of the nicest roommates a starting PhD student could wish for. Köszönöm, for the nice conversations and the fun we had in the lab.

Naast AIO's en analisten wordt het lab ook nog bevolkt door een aantal postdocs en andere medewerkers. Duur, bedankt voor de gezellige tijd. Tania, obregada and good luck with the addition to your family. Anne, bedankt voor de vele gezellige gesprekken en voor het feit dat je Alarik af en toe meenam naar het lab zodat ik noodgedwongen even niet met werk bezig hoefde te zijn.

Gedurende de afgelopen 4 jaar ben ik betrokken geweest bij verschillende vormen van onderwijs. Zowel bij GATC en bij eerstejaars Genetica ben ik intensief opgetrokken met Klaas Swart. Klaas, bedankt dat je me de kans hebt gegeven om wat meer te ruiken aan het onderwijs; dit heeft mijn interesse in het onderwijs zeker aangewakkerd.

De belangrijkste groep voor een onderwijsinstelling als de universiteit zijn toch wel studenten. Zelf heb ik drie MLO studenten mogen begeleiden gedurende hun stage. Iris, Josien en Geart, bedankt voor jullie input in mijn onderzoek, het heeft zeer zeker bijgedragen aan het resultaat. Naast deze 3 zijn er veel andere studenten geweest waar ik het goed mee kon vinden: Erik Bastiaans (nu AIO), Daphne Huberts, Pepijn Kooij, Auke van der Weide, Laura Grauss, Eline Hartog, Daniel Goedbloed, Daniel Engelmoer en alle andere studenten, bedankt.

Werken met *Phytophthora infestans* is wederom een uitdaging gebleken. Het feit dat het me toch gelukt is, dank ik aan de onmisbare hulp van de mensen bij PRI. Wilbert, jouw hulp was cruciaal in het begin van mijn project. Geert, Bert, Marieke, Henry, jullie gaven mij altijd het gevoel welkom te zijn en waardeerde mijn alternatieve invalshoek waarop ik naar het probleem van de aardappelziekte aankijk. Petra, jouw tomeloze inzet aan het einde van mijn project heeft alsnog een leuke dataset opgeleverd. Trudy, je mailbox stond altijd open en je was altijd bereid om mij te helpen met praktische problemen en het bijhouden van mijn selectielijnen in de vloeibare stikstof. Een promotiebaan is inderdaad een roeping maar was onmogelijk zonder jullie hulp; hartelijk dank daarvoor.

Koffie is voor een AIO als olie voor een motor. Gelukkig was ons gebouw gezegend met een kantine met het mooiste uitzicht waarin zich ook een koffieapparaat bevond. Dit apparaat was een verzamelplaats voor alle dorstige medewerkers van onze groep maar ook van andere groepen. Soms kwamen daar ook de hongerige collega's bij. Hierbij wil ik een

"vaste" groepje dorstige collega's bedanken waarmee menig gezellige discussie is gevoerd. Dyaan, Mariëlle, Linus, Corrie, Joost, Jochem, Judith en diegene die ik vast ben vergeten, dank je voor de gezellige tijd.

Voor sommige mensen is de dorst nog niet gestild met de dagelijkse portie koffie; sommige hebben de neiging om op vrijdagmiddag de koffie te upgraden naar een biertje (of wijntje). Hoewel deze gewoonte pas echt in zwang kwam aan het einde van mijn project heb ik hier veel plezier aan beleefd. Wilco, Ronnie, Leo en Henk, jullie humor heeft zeer aanstekelijk gewerkt en heeft mijn vrijdagmiddagen aanzienlijk opgevrolijkt. Dank je en op het feest nemen we er nog eentje.

Hoewel het leven van een AIO zich primair moet afspelen binnen de muren van het lab en werkkamer, is er altijd wel een beperkte hoeveelheid tijd om wat nieuwe vrienden te leren kennen. Een belangrijke groep nieuwe vrienden zijn mijn voormalige huisgenoten van de Churchillweg: "the wrestling twins" Thomas en Dennis en Karin B. Ik heb veel plezier gehad tijdens onze dronken avondjes op kamer 3. Dankzij jullie kijk ik tegenwoordig met afgrijzen naar pinguïns.

Een speciale nieuwe vriend is eigenlijk één van de weinige vrienden die ik heb over gehouden aan mijn tijd in Utrecht. Ronald, als collega 020-hater hebben we menig avondje voetbal gecombineerd met bier en gesprekken over de frustraties die je als wetenschapper hebt. Ik denk dat wij het schoolvoorbeeld zijn hoe supporters van twee verschillende topclubs toch vriendschappelijk met elkaar kunnen omgaan. Helaas begint jouw leven als supporter van "die club uit Rotterdam" aardig masochistische vormen aan te nemen. Ik hoop dat jullie dit jaar eindelijk eens boven de neusjes eindigen op de tweede plaats (De eerste plaats is al gereserveerd voor PSV; de mooiste club van Nederland).

Het feit dat ik vrij weinig vrienden heb overgehouden aan mijn tijd in Utrecht is de "schuld" van een groep vrienden die ik heb leren kennen in het jongerencentrum "JOEK" in Geldrop. Hoewel ik de laatste jaren weinig tijd heb gehad voor het onderhouden van zulke vriendschappen op afstand, heb ik de verschillende feestjes ervaren als een aangename onderbreking van mijn wetenschappelijke bezigheden. Bij dezen wil ik deze groep bedanken voor deze vriendschap, die alweer bijna 10 jaar duurt.

Een van de dingen die mij bindt met veel van mijn vrienden is toch wel mijn liefde voor Heavy Metal. Hoewel het in het botanisch centrum wemelt van de plantaardige "heavy metal accumulators", moest ik toch regelmatig uitwijken naar grote grasvelden met modder (veroorzaakt door regen, bier en het afvalproduct daarvan) om al mijn negatieve energie er eens lekker uit te schreeuwen. Hierbij wil ik dan ook al mijn metal vrienden in Wageningen, Geldrop en daarbuiten bedanken. Art, jij was er eigenlijk altijd bij om ons te brengen in je auto toen wij er nog geen hadden. Het is mij nog altijd een raadsel hoe we fatsoenlijk 600 kilometer konden rijden naar het noorden van Duitsland met een beperkte kofferbak waar zich ook nog eens een gastank in bevond.

En dan is er nog die andere belangrijke vrouw in mijn leven. Karin, ik heb beloofd niet klef te worden in dit stukje dus ik houd het kort. Je houdt het toch al mooi 9 jaar vol met deze Queen-fan en PSV supporter. Ik denk dat het volgende stukje tekst genoeg zegt hoe ik over je denk:

You and me we are destined you'll agree
To spend the rest of our lives with each other
The rest of our days like two lovers
For ever, yeah, for ever
My bijou

Queen 1990

Curriculum Vitae

Stefan Bosmans is geboren op 29 augustus 1979 te Geldrop. Na het behalen van zijn VWO diploma aan het Strabrecht College te Geldrop in 1997 begon hij in hetzelfde jaar met de studie biologie aan de Universiteit Utrecht. Gedurende deze studie kwam hij in aanraking met de evolutionaire biologie en was hij, gedurende de jaren 2000-2003, betrokken als studentenassistent bij de differentiatiefase cursus "Evolutionaire Oecologie" van dr. Gerdien de Jong en bij de eerstejaars cursus "Oecologie" in 2003. In mei 2003 studeerde hij af met afstudeervakken bij de vakgroepen "evolutionaire populatiegenetica" en bij "ecofysiologie van planten". Beide afstudeeronderwerpen hadden een sterke evolutionair genetisch karakter. In februari 2004 begon hij aan zijn promotie onderzoek bij het laboratorium voor erfelijkheidsleer, waar hij aan het onderwerp van dit proefschrift heeft gewerkt. Gedurende die periode is hij ook betrokken geweest bij het onderwijs dat werd verzorgd door deze vakgroep. Hij heeft daarbij zijn steentje bijgedragen aan de basiscursus "Genetica" in 2008, "Genetic Analysis: Tools and Concepts" in 2006 en aan "Moleculaire en Evolutionaire Ecologie" in 2007. Deze interesse in het onderwijs heeft er mede toe geleid dat hij op dit moment zijn carrière voortzet aan de Fontys leraren opleiding Tilburg waar hij betrokken zal zijn bij het opleiden van de toekomstige generatie biologie docenten.

PE&RC PhD Education Certificate

With the educational activities listed below the PhD candidate has complied with the educational requirements set by the C.T. de Wit Graduate School for Production Ecology and Resource Conservation (PE&RC) which comprises of a minimum total of 32 ECTS (= 22 weeks of activities)

Review of Literature (5.6 ECTS)
- Fungicide resistance in *Phytopthra infestans* (2004)

Writing of Proposal (5 ECTS)
- Stability of fungicide resistance in *Phytophtra infestans* (2004)

Post-Graduate Courses (5.9 ECTS)
- Basic and advanced statistics; PE&RC (2005)
- Population Genetics; Functional ecology (2006)
- Multivirate analysis; PE&RC (2006)

Competence Strengthening / Skills Courses (2.1 ECTS)
- Time planning and project management; PE&RC (2005)
- Techniques for writing and presenting a scientific paper; PE&RC (2006)

Discussion Groups / Local Seminars and Other Scientific Meetings (5.6 ECTS)
- In the tracks of evolution (2004-2008)

PE&RC Annual Meetings, Seminars and the PE&RC Weekend (2.7 ECTS)
- Current themes in ecology: experimental evolution, fundamental and applied (2004)
- PE&RC day (2004-2007)
- Introduction weekend (2005)
- Current themes in ecology: ecological and evolutionary genomics (2005)
- Najaarsvergadering Ned. Ver. Voor Mycologie; CBS, Utrecht (2007)

International Symposia, Workshops and Conferences (8.1 ECTS)
- 23rd Fungal Genetics Conference (poster); Asilomar, USA (2005)
- Oomycete molecular genetics meeting (poster); Wageningen (2006)
- 11th ESFEB conference (poster); Uppsala, Sweden (2007)

Courses in Which the PhD Candidate Has Worked as a Teacher:
- Genetical Analysis, Tools and Concepts (2006); 30 days
- Molecular and evolutionary ecology (2007); 30 days
- General Genetics (2008); 30 days