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**Virulence and Multiple infections of *Hyaloperonospora arabidopsidis* (Gäum.) Göker, Riethm., Voglmayr, Weiss & Oberw. on *Arabidopsis thaliana* (L.) Heyhn.**

Shanerin Falab

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Virulence and Multiple infections of  
*Hyaloperonospora arabidopsidis*  
(Gäum.) Göker, Riethm., Voglmayr.  
Weiss & Oberw. on *Arabidopsis*  
*thaliana* (L.) Heynh.

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Spécialité de doctorat : Biologie

Thèse présentée et soutenue à Orsay, le 3 août 2018, par

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# I. GENERAL INTRODUCTION

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## What are parasites?

Parasite was derived from the Greek word *Parasitos* composed of *para* (near) and *sitos* (food)- meaning one who eats at the table of another. This specifies the way co-habiting organisms gain and lose resources. In a biological context, parasites are organisms living temporarily or continuously with (in/on) interspecific partners, termed hosts, from which they obtain resources for their growth and reproduction. Damage to the host, including injuries that negatively affect their morphology, physiology or reproduction, caused by parasite exploitation are common features of this extreme interspecific interaction, called parasitism (see Araújo et al., 2003).

Several different types of parasitism exist. Parasites that live inside their hosts are called *endoparasites*, those living outside or on their hosts are called *ectoparasites* and those with a distinct free-living stage off any host are called *parasitoids*. Parasites are also classified by size to distinguish the very small (up to a few hundred micrometres) with short generation times such as viruses, bacteria, unicellular fungi and protists, called *microparasites*, from large multicellular *macroparasites* that can be seen with the naked eye, for example helminths, acanthocephalans, parasitic insects, parasitic plants, etc., (Schmid-Hempel, 2011). Fungi and oomycetes that cause diseases, mainly of plants, fall someplace between micro- and macroparasites. The propagules are often microscopic, as is the case for many macroparasites, but the parasite growing on the plant is multicellular and can often be seen with the naked eye during part of its life cycle. Specifically, fungal and oomycete plant parasites are categorized according to their lifestyles among biotrophic, necrotrophic and hemibiotrophic parasites. Biotrophic pathogens grow and reproduce inside living plant tissue, but necrotrophic pathogens typically kill their infected host tissue by secreting a toxin or degrading the cell walls, then feed on these dead cells. Hemibiotrophic pathogens initially invade plants in a biotrophic way and after an establishment stage, turn to a necrotrophic lifestyle, killing the infected host cells later in their life cycles (Agrios, 2005).

## Why does parasitism exist in nature and what does it do?

It is speculated that the majority of species on earth are parasites. Furthermore, parasitism has evolved repeatedly from free-living forms (Windsor, 1998). We know this because most major groups of organisms include some parasites, meaning that the parasitic life style evolved after the origin and diversification of the groups. Also parasites are everywhere and infest all species, with even parasites

having parasites (Araújo et al., 2003; Schmid-Hempel, 2011). The transition from free-living to parasite involves some common features, such as morphological simplification and loss of functions that are no longer essential to the parasites (Janouskovec & Keeling, 2016) that are protected within their hosts from much environment variation, enjoying more stable temperature and chemical environments (Tinsley et al., 2005).

Parasitism plays important evolutionary and ecological roles. In the first place, parasitism can be seen as responsible for the evolution of eukaryotes, following the incorporation of an alpha-proteobacterium into an archaea host (Araújo et al., 2003). Parasites have also been proposed as the ecological force that compensates the costs of sex and recombination to explain the maintenance of sex (Hamilton, 1980). The long-term interaction between parasites and their hosts are responsible for a large number of associated co-adaptations involved in co-evolution. Within antagonistic interactions between host and parasite, parasite exploitation of hosts selects for increasing and novel host resistance mechanisms while these defence mechanisms in turn select for the counter-adaptations by the parasites.

In an ecological context, parasites play an important role in generating, eroding and maintaining biological diversity of their hosts. For instance, parasites are selective agents on host life-history traits, i.e., time to maturity, fecundity, body size, dispersal ability, etc., and this directly influences host population dynamics (Ladin et al., 2016). In addition, parasites also indirectly affect host population dynamics by affecting the dominance of competing host species. For example, the presence of parasites can change community structure and allow coexistence of competing species by changing their dominance hierarchies (Thomas et al., 2005). Consequently, host population changes mediated by parasites also affect their prey and predator densities thereby involving and affecting trophic interactions (Borgsteede, 1996; Thomas et al., 2005).

### **Parasite virulence – reduction in host fitness**

By definition, parasites negatively impact their host fitness and the negative effect of parasites on host growth, survival or reproduction is called virulence. There is some debate about whether virulence represents an unavoidable outcome of parasite exploitation or whether it represents an adaptive strategy of the parasites (Anderson & May, 1982; Ewald, 1983; Read, 1994; Ebert & Herre, 1996; Frank, 1996). In other words, virulence may be either an adaptation or a by-product of the parasitic lifestyle. Many parasites are pathogens that directly impact human health or cause economic losses especially to agricultural production (Murrell, 1990; Roberts & Marks, 1994; Zhou et al., 2005; Nicol et al., 2011; Otranto & Eberhard, 2011; Amenu, 2014; Rodenburg et al., 2016). Many studies link parasite virulence to

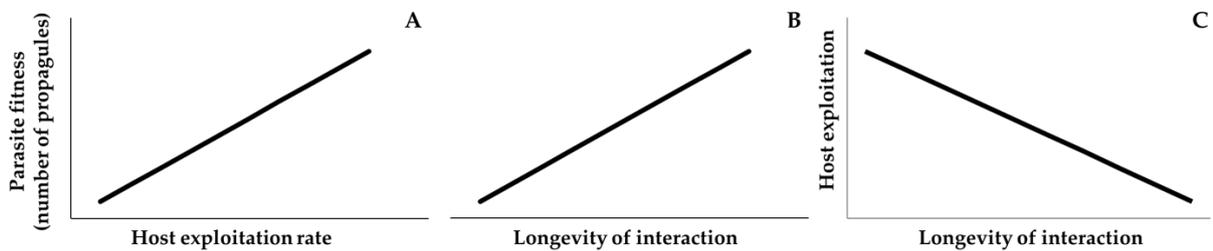
parasite growth or reproduction within the host and transmission among hosts, suggesting that higher virulence is associated with higher parasite fitness (Ewald, 1983; Bull, 1994; Levin, 1996; Poulin & Combes, 1999; Araújo et al., 2003; Mandal, 2011). This has been demonstrated in a number of cases where parasites with higher virulence transmit better (Ebert & Mangin, 1997; Ferguson et al., 2003; Salvaudon et al., 2005; de Roode et al., 2008). One mechanistic interpretation is that higher parasite virulence results from parasites taking more host resources, thereby causing more host damage, and that this higher resource level translates into enhanced parasite fitness through higher multiplication and/or transmission rates after successful infection (Read, 1994; Alizon et al., 2009).

The damage associated with virulence will depend in part on the kind of host resources that are being used by the parasites. Host resources can be divided into three broad categories – those used for growth, for maintenance and for reproduction. If parasites take resources destined only for growth, this should mostly modify age and size at maturity, host body size and host fecundity, but also, indirectly, longevity, if smaller hosts suffer higher mortality, for example, from predation. If parasites take only resources from host reproduction, called castrating parasites, these may have little or no impact on host growth or survival, and can sometimes even increase host longevity (Baudoin, 1975). Castrating parasites are expected to exhibit maximum virulence, leading to complete host castration (O’Keefe & Antonovics, 2002; Sloan, Giraud, & Hood, 2008). Parasites that take host resources away from maintenance and use these to make parasite propagules will increase host morbidity or mortality. However, the increase in parasite fitness from increased host exploitation, which causes virulence, should be limited by the negative effects of parasite exploitation on host survival (Levin & Pimentel, 1981; May & Anderson, 1983b; Knolle, 1989). This leads to a trade-off between parasite replication and the longevity of the interaction, (Figure 1.1), developed below.

### **If virulence is adaptive, how does it evolve?**

Parasites cause morbidity and mortality to their host by taking host resources away from necessary host functions for the production of parasite propagules. Parasites that have higher rates of host exploitation are expected to have higher fitness, because they take more resources from their hosts and can therefore produce more offspring (Figure 1.1A). At the same time, all other things being equal, a parasite that can exploit its host for a longer time should also have higher fitness (Figure 1.1B). However, these two effects are not independent, because host exploitation is likely to modify longevity, with parasites that take more resources damaging their hosts more and killing them more quickly (Figure 1.1C). Therefore, maximising parasite fitness represents a trade-off between maximising replication rate and maximising longevity, at

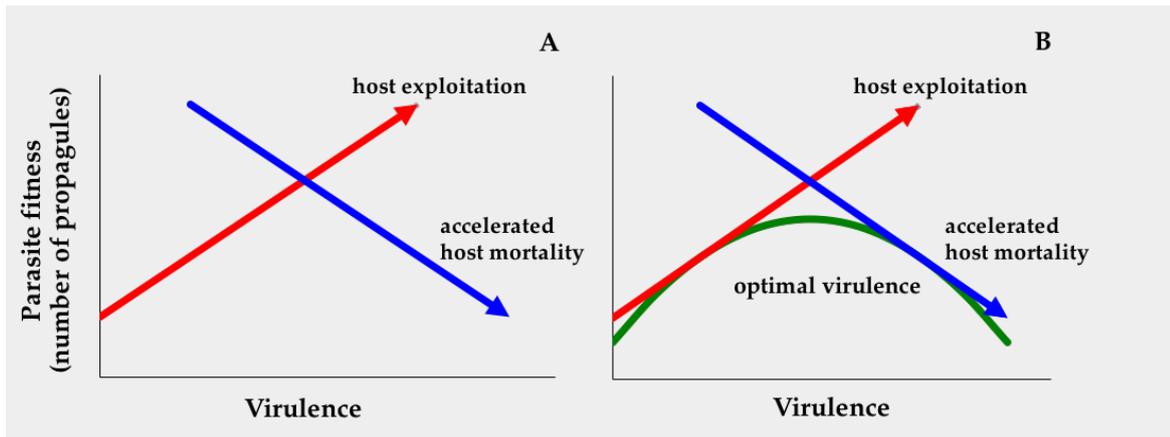
least in the case of parasites that kill their hosts by diverting important host resources away from maintenance. Here I consider a number of scenarios of how parasites and hosts interact, and the virulence consequences, when parasites are using the host maintenance budget for their own reproduction.



**Figure 1.1** Schematic expected relationships between A: parasite fitness (parasite offspring production) and host exploitation rate; B: parasite fitness (parasite offspring production) and longevity of the interaction between host and parasite. C: host exploitation and longevity of the interaction.

### **Optimal virulence: a trade-off between parasite replication and longevity of the interaction**

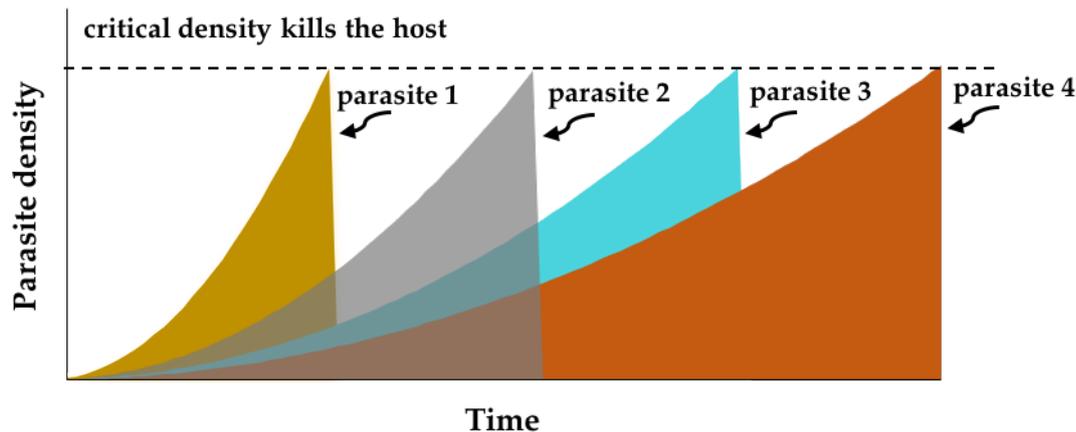
The trade-off between host exploitation rate (taking a lot of host resources and turning these into parasite propagules) and host mortality rate, which limits the amount of time that a parasite can exploit its host, generates an optimal, intermediate parasite virulence strategy that will be selected by natural selection (Figure 1.2). However, the position of the optimum virulence will vary as a function of several population parameters such as host population density and host lifespan or extrinsic mortality risk. For example, natural selection should favour higher virulence when there are many available susceptible hosts and lower virulence when there are few (Ebert & Mangin, 1997; Herre, 1993). Similarly, in environments with high extrinsic host mortality, natural selection should favour high virulence compared with environments with lower extrinsic host mortality (Stearns, 1991). Therefore, we expect that the optimal virulence will vary among populations of hosts and parasites under different ecological conditions. Similarly different parasite and host genotypes may vary in the exact nature of the relationships between virulence and both parasite reproduction and host longevity, so intrinsic features of parasite and host genetics may generate variation of optimal virulence (Alizon & van Baalen, 2005; Bull & Luring, 2014).



**Figure 1.2** A: Parasite fitness as a function of two components of virulence: rate of host exploitation and accelerated host mortality. B: illustrates the trade-off model of optimal parasite virulence that balances the gains and losses of increasing virulence.

### Variation in level of virulence among parasite strains

Figure 1.3 shows the hypothetical dynamics of several strains of parasite that differ in their host-exploitation strategies. These hypothetical parasites replicate within their host up to a critical, lethal, density, produce and transmit propagules throughout the infection. Parasite 1 has the fastest host exploitation and thus kills the host earliest. This high virulence strategy leads to earlier transmission but fewer overall progeny. Parasite 4 has the slowest host exploitation (lowest virulence) and kills the host last, and it produces the most progeny over the longest time. From this perspective, it looks as though parasite 4's strategy is superior to the others because it has the highest overall fitness. However, there are other factors that need to be considered. For example, if extrinsic mortality is very high, such that most hosts will die at an early age, the strategy of parasite 4 may have lower success under these conditions, so parasite 1 may be selected under conditions of high extrinsic mortality.



**Figure 1.3 Dynamics of parasite density within hosts.** Different parasites have different rates of replication, which are associated with their exploitation strategies. Parasite 1 has the highest exploitation rate, parasite 4 the lowest. Parasite numbers increase within hosts up to a critical density, which kills the host. The areas under the curves represent the total number of propagules produced by each different parasite strategy, which is a measure of fitness. This assumes that all propagules have similar probabilities of transmission and infection of new hosts. Fitnesses are ranked as follows:  $4 > 3 > 2 > 1$ .

### **Multiple infection- multi-genotypes of infecting parasites within-host**

However, parasites may not be alone within hosts, and the presence of other coexisting parasites within the same host can modify optimal virulence in important ways (Bremermann & Pickering, 1983; Herre, 1993; Knolle, 1989; Mosquera & Adler, 1998; Alizon, 2008; Ebert & Bull, 2008). Multiple infections can involve different species of parasites co-occurring in the same host at the same time. Infections by different pathogen species in humans cause noteworthy impacts on human health (Petney & Andrews, 1998; Balmer & Tanner, 2011; Wewalka et al., 2014), for example several pathogens co-infect with HIV and influence drug resistance (Abu-Raddad et al., 2006; Pawlowski et al., 2012; Birger et al., 2015). Ticks, fish and amphibians are often infected with different parasite species, which can interfere or enhance the effect of the different parasites (Johnson & Buller, 2012; Kotob et al., 2016; Warne et al., 2016; Raileanu et al., 2017). These interactions between different species can of course modify the selective environment for the various parasite species, leading, for example, to competitive exclusion of one or the other species (Levin & Pimentel, 1981; Bremermann & Thieme, 1989).

However, when considering how the particular virulence strategy of a species of parasites evolves, it is important to consider the range of phenotypes expressed by different genotypes within a parasite species

and how different genotypes of the same species will interact within a host (Read & Taylor, 2001; Alizon, 2013). Multiple genotypes of a parasite species within a single host have been observed in several different natural host-parasite systems including plant-viruses (Tatineni et al., 2010; Jo et al., 2017), plant-fungi (López-Villavicencio et al., 2007; Susi et al., 2015a; Susi et al., 2015b), snail-trematodes (Karvonen et al., 2012), and cotton rat-bacteria (Chan & Kosoy, 2010), indicating that multiple infections occur commonly in nature.

### **Where do multiple infections come from?**

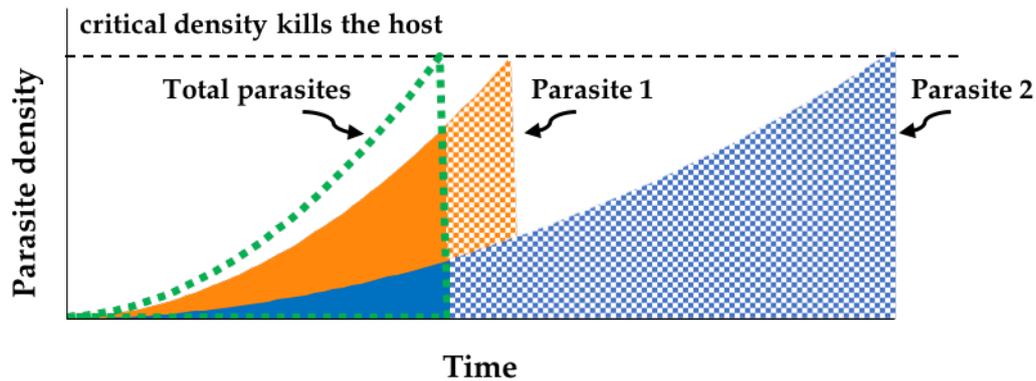
New genotypes of parasites can arise *de novo* within a host via different evolutionary forces, such as mutation and recombination, and this within-host evolution can have enormous impact on drug resistance and within-host adaptation (see Didelot et al., 2016). However, multiple infections probably often result from co-infection, with different genotypes infecting the same host (May & Nowak, 1995). Co-infection can result in long-term co-existence by more than one co-infecting pathogen strain or superinfection with only transient co-existence because one strain, usually supposed to be the more virulent one, displaces the other, less virulent one, rapidly (Nowak & May, 1994; May & Nowak, 1995; Mosquera & Adler, 1998) and hosts infected by more virulent strains cannot be infected by a less virulent genotype (Bremermann & Thieme, 1989). Regardless the origin of the genetic diversity among parasite strains within hosts, the presence of other parasite strains can modify both the level of virulence expressed by the infection, i.e., the overall virulence of the mixed infection, which may be different than the virulence of an infection with only one of the parasite strains (Alizon et al., 2013) and the selection environment for the optimal virulence strategy of all parasite strains infecting or present (Ewald, 1983; van Baalen & Sabelis, 1995). It is important to note here that a mixed infection may have a different effect on the host, expressed in the overall virulence of the infection, than would an infection by a single genotype. Independent of the effect on the host, in a mixed infection, the presence of more than one parasite genotype may alter the fitness payoffs of the infection for each genotype present. Mixed infections may lead to lower, similar or possibly even higher fitness benefits for a particular parasite strain than it would get by being the only genotype present in a single infection. Therefore we need to consider the effects of mixed infections on both the host and on the parasites. Below I discuss a number of different scenarios to expose the diversity of situations that may be encountered with mixed infections.

To return to the scheme already used to present the different virulence strategies, recall the situation of different parasite strains differing in exploitation strategies. Parasite 1 reproduced the fastest but killed its host most rapidly, producing fewer offspring overall than did the parasite strains with lower virulence. However, when two strains that differ in virulence co-infect a single host at the same time, if they

maintain the same exploitation strategy when in competition, clearly the more virulent one will produce more propagules than the less virulent one in the mixed infection, because the combined density of the parasites will cause host death when the more virulent parasite has produced relatively more propagules than the less virulent one. Similarly, in a case of superinfection, the more virulent strain will eliminate the less virulent one when this latter strain has reproduced little or not at all. Thus multiple infection will lead to the evolution of higher virulence because selection will act against the most prudent strains with the lower virulence strategies, even though this leads to overall lower production of parasite propagules (Frank, 1992; Nowak & May, 1994; van Baalen & Sabelis, 1995; May & Nowak, 1995; Mosquera & Adler, 1998; Nowak & Sigmund, 2002).

### **Virulence in multiple infections**

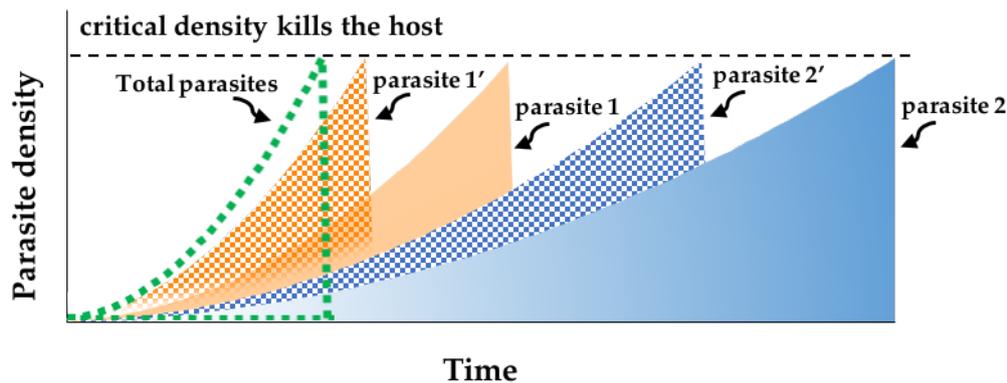
In the cases considered below, the case of co-infection with coexistence is considered. However, superinfection, with competitive exclusion of the less virulent strain will generate similar selection pressures on the virulence strategy. Figure 1.4 shows the outcome of a multiple infection by two parasite strains that differ in host exploitation and virulence strategy. If virulence is proportional to parasite density within-host then multiply-infected hosts will die sooner than those infected with a single genotype, as long as the parasites replicate at the same intrinsic rate whether alone or with a co-infecting genotype (Wargo & Kurath, 2011). Therefore, multiple infections have higher overall virulence even in the absence of any phenotypic plasticity of the parasites. Furthermore, the more virulent parasite genotype is more abundant within the host than is the less virulent one at this point, so it will have higher relative fitness. The difference in relative fitness between the different parasite genotypes increases as their difference in host-exploitation (virulence) strategy increases. Therefore selection will readily favour a much more virulent parasite but will not distinguish between parasites with similar virulence, because these would have similar relative fitness. Here, and in the figures that follow, I consider that transmission will be a function of the density of parasite propagules, i.e., that both parasite genotypes are transmitted from mixed infections in function of their relative abundance. In this case, natural selection will lead to the evolution of higher virulence when multiple infections are common, as is predicted by many theoretical models (Frank, 1992; Nowak & May, 1994; van Baalen & Sabelis, 1995; May & Nowak, 1995; Mosquera & Adler, 1998; Nowak & Sigmund, 2002).



**Figure 1.4** Dynamics of parasite density within hosts for single infection versus multiple infections when parasites have fixed exploitation and replication rates. Single infection: see Figure 4. Multiple infections: total parasite density (dotted line) represents the sum of the two genotypes within the host. The host is killed when the total parasite density reaches the critical density, which here occurs more rapidly than with a single infection of the more virulent strain. At this time, the relative fitness of the more virulent parasite 1, represented by the area under the orange curve, is far greater than the relative fitness of the less virulent parasite 2, represented by the area under the blue curve.

### **Virulence in multiple infections with plasticity**

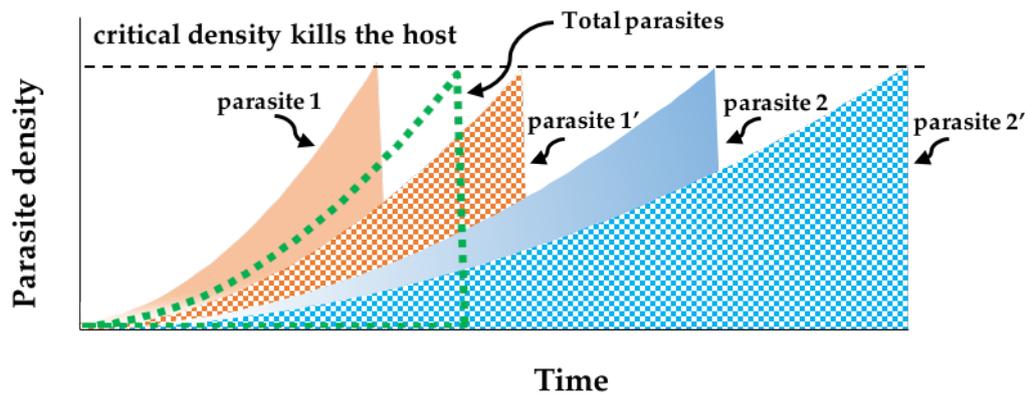
Figure 1.5 shows the outcome of a multiple infection by two parasite strains that differ in host exploitation and virulence strategy, and whose virulence strategy is phenotypically plastic, increasing in the presence of a second parasite strain. This kind of phenotypic plasticity could be a trait of the parasites themselves, but could also occur if the host defence system is less efficient at combatting mixed than single genotype infections, thereby resulting in higher parasite densities over the time course of the infection (de Roode et al., 2003). If virulence is proportional to parasite density within-host then multiply infected hosts will die sooner than those infected with a single genotype, as above. For parasites with phenotypically plastic host exploitation strategies and replication rates that increase in the presence of a second parasite strain, virulence will be even greater in multiple infections. As above, the parasite with the greater virulence strategy will have higher relative fitness. Therefore, the virulence of multiple infections is even higher when both co-infecting parasites exhibit this kind of plastic host exploitation and virulence, and natural selection will favour higher virulence.



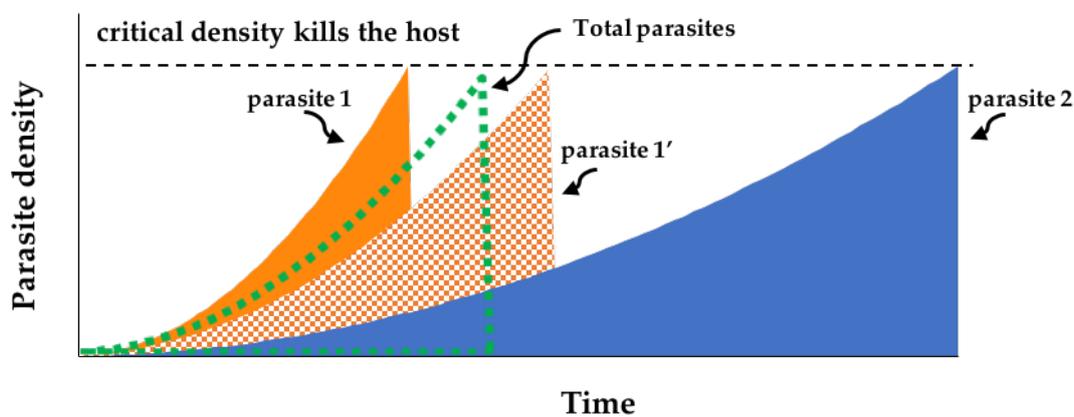
**Figure 1.5 Dynamics of parasite density within hosts when parasite exploitation strategies and virulence are plastic, increasing in response to the presence of another parasite strain.** If parasites respond to the presence of a competitor by increasing their exploitation strategies and hence their virulence, both parasite strains will replicate faster in mixed (dotted area, parasite x') than in single infections (solid area, parasite x). With this type of plastic effect, the overall virulence of multiple infections would be even greater, and selection would favour the more virulent over the less virulent genotypes when multiple infections are common even more.

Figure 1.6 shows the outcome of a multiple infections by two parasite strains that differ and are plastic in host exploitation and virulence strategy. In contrast to the scenario in Figure 1.5, their host exploitation and virulence strategies are decreased in the presence of a second strain, for example if parasites compete symmetrically and interfere with each other's ability to acquire or process resources. In this example, virulence of multiple infections is lower than that of single infection with the more virulent parasite strain but the relative fitness of the more virulent parasites is nonetheless higher than that of less virulent ones when multiple infections are common, so selection still favours higher virulence under such conditions.

Figure 1.7 shows the outcome of a multiple infection by two parasite strains that differ in host exploitation and virulence strategy and where only one, here the more virulent strain, is plastic, for example because the more virulent strain is more suppressed by the host defence system (McKenzie & Bossert, 1998) or if the less virulent strain is a better competitor that suppresses the success of the more virulent one, as has been observed for the trematode *Schistosoma mansoni* (Gower & Webster, 2005). If the more virulent genotype has decreased host exploitation and virulence strategy in the presence of a second parasite strain, this will reduce its relative fitness advantage, though selection still favours the more virulent genotype over the less virulent one.

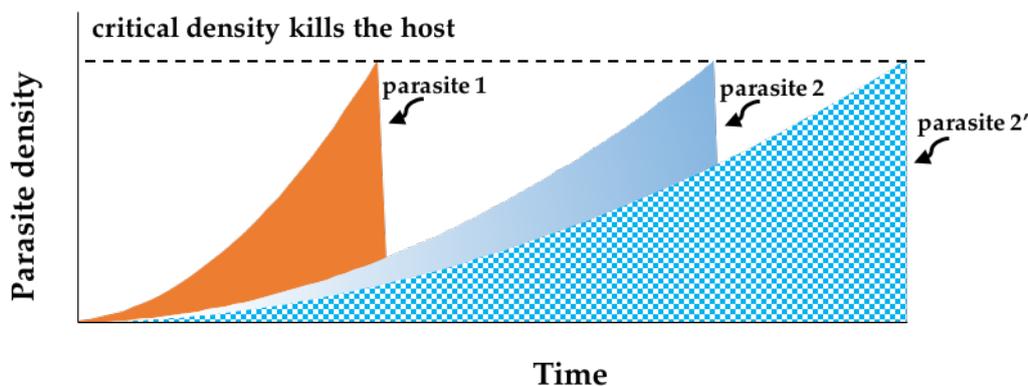


**Figure 1.6 Dynamics of parasite density within hosts when parasite exploitation strategies and virulence is plastic, decreasing in response to the presence of another parasite strain.** If competition between parasites causes a decrease in their exploitation strategies and hence their virulence, both parasite strains will replicate less well in mixed (dotted area, parasite x') than in single infections (solid area, parasite x). With this type of plastic effect, the overall virulence of multiple infections would be lower than that of a single infection with the more virulent parasites, but, if the rank of the genotypes does not change, selection would still favour the more virulent over the less virulent genotypes when multiple infections are common.



**Figure 1.7 Dynamics of parasite density within hosts when only one (the more virulent) parasite strain shows plastic exploitation strategy and virulence.** If interference competition by the less virulent parasite (parasite 2) causes a decrease in exploitation strategy of more virulent parasite (parasite 1) and hence its virulence, the more virulent parasite strain will replicate less well in mixed (dotted area, parasite 1') than in single infections (solid area, parasite 1). With this type of plastic effect, the overall virulence of multiple infections would be lower than that of single infections by the more virulent strain, but, if the rank of the genotypes does not change, selection would still favour the more virulent over the less virulent genotypes when multiple infections are common.

Figure 1.8 shows the outcome of a multiple infection by two parasite strains that differ in host exploitation and virulence strategy and where only one, here the less virulent strain, is plastic, for example because of asymmetric competition, whereby the more virulent strain is a stronger competitor and can suppress the less virulent one (Ewald, 1983; Antia et al., 1994). Several experimental studies show that the more virulent genotype is a superior competitor in mixed infections, e.g., Mice-*Plasmodium chaubadi* (Taylor et al., 1997; de Roode et al., 2005; Bell et al., 2006), PKS (post segregation killing) plasmids- *E. coli* (Cooper & Heinemann, 2005), *Daphnia magna*- *Pasteuria ramosa* (Ben-Ami et al., 2008; Ben-Ami & Routtu, 2013), fish rhabdovirus- steelhead trout (Breyta et al., 2016). Here selection favours the more virulent genotype over the less virulent one with relative fitness of the more virulent strain being even greater than in the previous examples.



**Figure 1.8 Dynamics of parasite density within hosts when only one (the less virulent) parasite strain shows plastic exploitation strategy and virulence.** If the more virulent strain (parasite 1) is more competitive and suppresses the less virulent one (parasite 2), the more virulent parasite strain will have similar fitness in mixed and in single infections. With this asymmetric competition, the overall virulence of multiple infections is determined by the more virulent strain, and selection favours the more virulent over the less virulent genotype when multiple infections are common.

## Parasite-Parasite interactions other than competition

### *Co-operation for host exploitation*

In all the scenarios described above, parasites are directly or indirectly competing with each other for a limited set of host resources. The resources that one strain uses are not available for the other, so the presence of a second strain, or a competitor, reduces the resource budget available for each. However,

there are other ways in which parasites can interact within a host, for example, as already mentioned above, via the host, if one strain activates the defence system of the host that may then suppress other strains. In addition, some parasites secrete extra-cellular compounds that are involved in resource uptake from hosts. Such extra-cellular compounds represent public goods if other individuals than the one that produces them can use them to their advantage (West et al., 2007). For example, siderophores produced by bacteria to take up soluble iron (Fe (III)) can lead to higher growth of all infecting genotypes and higher overall virulence (West & Buckling, 2003). Similarly, invertase secreted by the rice blast fungus, *Magnaporthe oryzae*, enhances overall virulence of mixed infections on rice plants even when an invertase-producer co-infects with a cheating mutant that does not produce this public good (Lindsay et al., 2016). When parasites produce public goods that are used for exploiting the host, multiple infections are expected to have higher overall virulence than single infections when multiple infections produce more of the compounds that contribute to host exploitation. However, the evolutionary trajectory resulting from the situation where multiple infections are common is less clear, because cheaters can arise, lowering the mean production of these compounds at the population level (Turner & Chao, 1999). However, it is important to note that the selective advantage of cheaters is frequency-dependent. Cheaters gain when they are rare and often share hosts with public-goods producers, but this advantage is reversed when they are common, as shown in experimental studies of co-infection in pathogenic bacteria (Harrison et al., 2006; Pollitt et al., 2014).

***Conflict and spite: Multiple infections lead to lower virulence***

Some parasites also produce costly compounds that inhibit the growth or survival of competitors, for example bacteriocins that inhibit other bacterial strains or species. This is considered a spiteful behaviour because the producer strain pays a high cost of production of these compounds to damage others. Spiteful interactions of different strains of parasites within a host are expected to reduce the overall virulence of mixed infections compared with single strain infections, but this effect should vary as a function of the degree of relatedness of the different coexisting parasite strains (see Gardner et al., 2004; Buckling & Brockhurst, 2008). Experimental work has validated these theoretical expectations. For example, when a bacteriocin-producing and a non-bacteriocin-producing strain of *Pseudomonas aeruginosa* co-infected wax moths, the bacteriocin-producer significantly suppressed the growth of the non-bacteriocin-producer when at intermediate starting densities, i.e., when the degree of relatedness of the different coexisting parasite strains was low. Under these same conditions the overall virulence, measured in host mortality, was minimised (Inglis et al., 2009). Similarly, entomopathogenic *Xenorhabdus* species vary in their ability to suppress the growth of other strains and this influences the virulence of the infection, with infections including bacteriocin-producers having lower overall virulence (Bashey et al., 2012).

These studies show that the production of bacteriocins in mixed infections of bacterial strains with low relatedness reduces the overall virulence of the infection. Furthermore, the bacteriocin-producing strains had a selective advantage in these conditions, producing relatively more propagules than the non-producing strains. This implies that multiple infections select for bacteriocin production, i.e., a spiteful strategy, which then reduces virulence in two ways. First, selection for antagonistic compounds those suppress competitors will involve physiological costs that may be paid in fitness even if bacterial strains are alone in a host. Second, these antagonistic compounds will reduce the population growth of bacteria in mixed infections with non-relatives, thereby reducing the overall virulence of mixed infections. These two effects were shown following experimental evolution over four passages of multiple genotype infections versus single genotype infections on four independent lineages in *Bacillus thuringiensis*. Multiple infections showed lower overall virulence than single infections and selection in multiple genotype infections led to an increase in competitor suppression, with associated fitness costs for three out of four of the lineages. It took longer those strains that had been selected in multiple infections to kill their hosts. Therefore multiple infections selected for lower virulence compared to single infections by selecting for an increase in the level of competitor suppression (Garbutt et al., 2011).

### **Coexistence of infecting parasite genotypes within-host**

The above introduction shows that multiple infections can have a variety of both ecological and evolutionary outcomes, increasing or decreasing overall virulence and selecting for higher or lower virulence. For multiple infections to play these important roles, however, it is necessary that different strains of parasite encounter each other and interact for access to hosts and exploitation of hosts, i.e., that the presence of one parasite genotype influences the fitness of another when they are sharing a host. This interaction can have different effects, among others, interference or scramble competition for access to hosts, scramble competition for access to resources from hosts, shared production of public goods or interference and suppression of one parasite by the other after gaining access to the host. As discussed above, these different types of interaction will have different effects on the reproductive success or fitness of the different genotypes of parasites, on the overall virulence expressed by the infection and on the evolutionary trajectory of virulence.

Even if there is little coexistence of different parasite genotypes within hosts, competition for access to hosts or competitive exclusion of one genotype by another, as in the case of superinfection, can drive the evolution of virulence. If more virulent genotypes are better competitors for access to hosts, then their presence in a population will drive the evolution of virulence by excluding the less virulent strains. If more virulent parasites outcompete less virulent ones within hosts and displace them, then even in the

absence of long-term coexistence, virulence will evolve to higher levels (May & Nowak, 1994; de Roode et al., 2005). On the other hand, without real coexistence of multiple strains within hosts, the combined virulence of a mixed infections should not be different from that of a single infection with the winning parasite genotype such as in Ben-Ami et al. (2008), unless virulence is modified by how the host mounts its defence and response to mixed infections is more or less costly than that to a single infection (Choisy & de Roode, 2010).

Whether there is coexistence between parasites depends on a number of different factors. Coexistence of different strains within hosts will be promoted if co-transmission is common, i.e., if successful transmission requires more than one genotype (Colijn et al., 2010; Alizon, 2013) and if within-genotype is greater than between-genotype competition (Colijn et al., 2010). Indeed requiring the presence of two different genotypes that differ in exploitation strategy for successful transmission prevents the more virulent one from eliminating the less virulent one. Alizon (2013) explores how co-transmission influences the evolution of virulence by aligning the evolutionary interests of the two parasites, and shows that co-transmission favors less virulent strains and lower competitiveness within-host.

Like coexistence of species within communities, it makes sense that when different parasites compete for the same resources, i.e., share the same niche, coexistence will be difficult (Seabloom et al., 2015) and one could expect genotypes that differ more in resource exploitation strategy to be more likely to coexist than those that are more similar. On the other hand, when parasite exploitation relies on the production of public goods, i.e., cooperation, more related genotypes will be more likely to cooperate, so the nature of the interaction between the parasite strains within the host will influence whether coexisting parasites are more or less related. High degrees of relatedness have been found in a number of host-parasite systems, both in natural populations (López-Villavicencio et al., 2007; Nkhoma et al., 2012; Wong et al., 2017) and following experimental inoculations, where more related strains were more likely to coinfect and coexist (Koskella et al., 2006; López-Villavicencio et al., 2010), suggesting that interactions between parasites strains may be cooperative in some systems.

### **Experimental evolution: selection within-host and between-host**

The theoretical frameworks for the effects of multiple infections and within-host competition have been outlined above. Though this framework is quite clear, there are still few experimental studies on the evolutionary outcomes of multiple infections on virulence evolution (Zhan & McDonald, 2013). These authors discuss the advantages of microbial systems and particularly plant parasites for carrying out evolution experiments to test the predictions of these theories. Indeed, there is a range of sophisticated

tools available for quantifying pathogen numbers and discriminating between strains, and this allows the study and quantification of competition between different strains of pathogens (see Zhan & McDonald, 2013). However, there are technical barriers to using experimental evolution approaches for studying the evolution of virulence under multiple infections, the most important being the difficulty of ensuring that multiple strains really interact over the long term within individual hosts. As we will see below, inoculating with a mixture of strains, or sequential inoculation with different strains, does not ensure that multiple strains will truly coexist within hosts (see also Wille et al., 2002).

## **Overall virulence**

Because of the difficulty in ensuring long term persistence of multiple infections, many studies investigating multiple infection work on a single generation of infection and compare the phenotypes of hosts exposed to multiple versus single infections of the same strains because this is technically feasible. However, as noted by Alizon (2013) the phenotype of the mixed infections, or overall virulence, cannot be used to predict the trajectory of virulence evolution.

The phenotype of multiple versus single infections by viruses (Ojosnegros et al., 2010; Gil-Salas et al., 2012; Salvaudon et al., 2013; Breyta et al., 2016; Tollenaere et al., 2017), bacteria (Inglis et al., 2009; Bashey et al., 2012; Lass et al., 2013; Pollitt et al., 2014; Stacy et al., 2016), protists (Balmer et al., 2009; Duncan et al., 2015) and fungi (López-Villavicencio et al., 2011; Buono et al., 2014; Susi et al., 2015a; Susi et al., 2015b) have been explored. Some of these studies, (for example, Balmer et al., 2009; Inglis et al., 2009; Ojosnegros et al., 2010; Bashey, et al., 2012; Gil-Salas et al., 2012; Lass et al., 2013; Salvaudon et al., 2013; Breyta et al., 2016; Tollenaere et al., 2017) quantify the different genotypes in multiply infected hosts. This provides information on the competitive effects and selection within the host, in other words which strategy or which genotype has higher success, which predicts selection on virulence at least in the short term.

Co-inoculation experiments that generate multiple infections, both from mixed species and mixtures of genotypes within species, have been explored with several host-pathogen systems. The comparisons of the virulence and parasite fitness between single- and mixed infections from empirical studies involving different parasite species are summarised in Table 1A, and different parasite genotypes of the same species in Table 1B. Where possible, the observed interaction between co-inoculated parasites/genotypes in mixed infections are described.

## **Thesis Objectives**

In this thesis I explore the outcomes of inoculations whether with single or multiple strains of a natural pathogen *Hyaloperonospora arabidopsidis* on *Arabidopsis thaliana* plant. I compare several aspects of the phenotypes resulting from single versus multiple-strain inoculations in terms of:

1. infection success (whether each inoculated plant get infection or not)
2. infection phenotypes (latent period, sporulation intensity, transmission success and virulence on sporulating plants)
3. Infection success of individual strains

Moreover, I investigate co-occurrence of inoculated strains resulted from both co-inoculation and sequential inoculation, using the same strain combinations, in order to test the effect of time lag on co-infection success, also variation in overall infection phenotypes, and infection success of individual strains.

**Table 1A** summarised recent empirical studies on mixed infections of different parasite species.

Host	Parasites	Total “virulence” (measured phenotype)	Parasite fitness	Parasite interaction within-host
Rice ( <i>Oryza</i> spp.)  Tollenaere <i>et al.</i> , 2017	<i>Rice yellow mottle virus</i> (RYMV) and <i>Xanthomonas oryzae</i> pathovar <i>oryzicola</i> (Xoc)	<b>mixed &gt; bacteria alone</b> <b>mixed = virus alone</b>  (plant growth)	Bacteria: mixed > single  Virus: mixed ≤ single, depending on genotype	Antagonistic effect of bacteria on virus fitness Synergistic effect of virus on bacteria fitness
Human (experiments in murine)  Ramsey <i>et al.</i> , 2011 Stacy <i>et al.</i> , 2014; 2016	Commensal bacterium ( <i>Streptococcus gordonii</i> ) and Oral pathogenic bacterium ( <i>Aggregatibacter actinomycetemcomitans</i> )	<b>mixed &gt; single</b> pathogen alone  (murine abscesses weight)	<i>A. actinomycetemcomitans</i> : mixed > single  <i>S. gordonii</i> : not measured (observed present or not)	Synergistic effect of presence of <i>S. gordonii</i> on <i>A. actinomycetemcomitans</i> fitness and virulence
Mosquito ( <i>Aedes aegypti</i> )  Duncan <i>et al.</i> , 2015	Microsporidian parasites <i>Vavraia culicis</i> and more virulent <i>Edhazardia aedis</i>	<b>mixed &gt; single</b> of each parasite  (host mortality)	mixed < single for both parasites	Not observed
Rainbow trout ( <i>Oncorhynchus mykiss</i> )  Louhi <i>et al.</i> , 2015	Six bacterial strains ( <i>Flavobacterium columnare</i> ) and five trematode fluke genotypes ( <i>Diplostomum pseudospathaceum</i> )	<b>mixed &gt; single</b> of each parasite  (host mortality)	Trematode: mixed > single, depending on fluke-bacteria genotype combination  Bacteria: not measured	Synergistic effect of presence of bacteria on fluke fitness
Mice ( <i>Mus musculus</i> )  Lass <i>et al.</i> , 2013	Gastro- intestinal helminth ( <i>Heligmosomoides polygyrus</i> )  Respiratory bacterial pathogen ( <i>Bordetella bronchiseptica</i> lux <sup>+</sup> ; self-bioluminescent strain)	<b>mixed &gt; single</b> of each parasite  (host mortality)	mixed > single for both parasites	Not observed
<i>Cucurbita pepo</i> cv. ‘Dixie’  Salvaudon <i>et al.</i> , 2013	<i>watermelon mosaic virus</i> (WMV) and <i>zucchini yellow mosaic virus</i> (ZYMV)	<b>mixed &gt; single</b> of WMV  <b>mixed = single</b> of ZYMV (plant size)	WMV: mixed < single ZYMV: mixed = single	Antagonistic effect of ZYMV on WMV fitness

**Table 1A** summarised recent empirical studies on mixed infections of different parasite species (continued).

Host	Parasites	Total “virulence” (measured phenotype)	Parasite fitness	Parasite interaction within-host
Cucumber ( <i>Cucumis sativus</i> cv. Albatros)  Gil-Salas <i>et al.</i> , 2012	<i>Cucumber vein yellowing virus</i> (CVYV)  <i>Cucurbit yellow stunting disorder virus</i> (CYSDV)  <i>Zucchini yellowmosaic virus</i> (ZYMV)	Observed only in CYSDV – CVYV combination  <b>mixed</b> CYSDV + CVYV > <b>single</b> , depending on level of CVYV  (plant height, internode length, dry weight and fruit yield)	Relative within-combination for mix  <b>CVYV+ ZYMV:</b> mixed = single for both single viruses  <b>CYSDV+ CVYV:</b> CYSDV mixed > single CVYV mixed = single  <b>CYSDV+ ZYMV:</b> CYSDV mixed = single ZYMV mixed < single	Synergistic effect of CVYV on CYSDV fitness  Antagonistic effect of CYSDV on ZYMV fitness
Pacific chorus frogs ( <i>Pseudacris regilla</i> )  Johnson & Buller, 2012	Trematodes ( <i>Ribeiroia ondatrae</i> and <i>Echinostoma trivolvis</i> )	<b>mixed</b> > <b>single</b> of each parasite (delayed metamorphosis)  <b>mixed</b> = <b>single</b> of each parasite (host size and mass)	mixed < single for both parasites	deduced apparent competition (cross-immune): each parasite had a negative effect on persistence of the other within host.

**Table 1B** summarised recent empirical studies on mixed infections of different genotypes within species.

Host	Parasites	Total “virulence” (measured phenotype)	Individual genotype’s fitness in mixed infection measured?	Parasite fitness	Parasite interaction within-host
Snail ( <i>Biomphalaria glabrata</i> )  Gleichsner et al., 2018	Three strains of <i>Schistosoma mansoni</i>	<b>mixed &lt; single</b> of each parasite  (host mortality)	YES	Parasite reproduction for two genotypes mixed infection:  <b>mixed &gt; single</b> of both genotypes  Relative reproduction of both genotypes in mixed infection:  <b>More virulent &gt; less virulent genotype</b> (but varied with time after infection)	Competitive suppression by more infective strain, and with kin selection (unrelated strains showed higher total parasite production)
Crustacean ( <i>Daphnia magna</i> )  Thompson et al., 2017	Bacteria <i>Pasteuria ramosa</i>	mixed intermediate between two single strains, depending combination and host sex  (host life span)	NO	Density of the two strains together in mixed infection intermediate between two single strains	Not observed
Zebra fish ( <i>Danio rerio</i> )  Kinnula et al., 2017	Bacteria <i>Flavobacterium columnare</i>	2-strain mix intermediate between two single strains  3 strain mix > single or 2-strain mix (host mortality)	YES	No difference in fitness of any parasite genotypes among treatments	Interference competition, assumed cell-to-cell contact inhibits growth of other genotypes
Rice (cultivar CO39)  Lindsay et al., 2016	Rice blast fungus ( <i>Magnaporthe oryzae</i> ): wild-type and invertase non-producing mutant	<b>mixed &gt; single</b> at some mixtures, intermediate between two single at other concentrations  (area of disease lesion)	NO	Spore production of the two strains together in mixed infection > two single strains	Cooperative host exploitation

**Table 1B** summarised recent empirical studies on mixed infections of different genotypes within species (continued)

Host	Parasites	Total “virulence” (measured phenotype)	Individual genotype’s fitness in mixed infection measured?	Parasite fitness	Parasite interaction within-host
<i>Plantago lanceolata</i>  Susi <i>et al.</i> , 2015a; 2015b	Fungus <i>Podosphaera plantaginis</i>	<b>mixed &gt; single</b> of each parasite  (number of infected leaves)	NO	Spore production of the two strains together in mixed infection > two single strains	Not observed
<i>Silene latifolia</i>  López-Villavicencio <i>et al.</i> , 2010; Buono <i>et al.</i> , 2014	Fungus <i>Microbotryum lychnidis-dioicae</i>	<b>mixed &gt; single</b>  (degree of plant sterilization)	NO	Spore production of the two strains together in mixed infection > two single strains	Interference competition for number of infected flowers and scramble competition for spore production
Wax moth larva ( <i>Galleria mellonella</i> )  Pollitt <i>et al.</i> , 2014	Pathogenic bacteria ( <i>Staphylococcus aureus</i> ): Wild-type and <i>agr</i> mutants that do not use quorum sensing	mixed infection intermediate between two single strains, where wild-type has higher virulence than the <i>agr</i> mutant  (host mortality)	YES	In mixed infections: the <i>agr</i> mutant has higher relative fitness than the wild-type, when rare.	Cheater profits from quorum-sensing cooperation when rare
Freshwater cladoceran ( <i>Daphnia magna</i> )  Ben-Ami & Routtu, 2013	Bacterial strains ( <i>Pasteuria ramosa</i> )	<b>mixed ≈ single</b> of higher virulent genotype at some combinations, and depending on concentration of some genotypes in mixed inoculum  (host survival)	YES	<b>More virulent genotype:</b> mixed ≈ single  <b>Less virulent genotype:</b> mixed < single.  (depending on spore dose of genotype in starting inoculum)	Competitive exclusion by more virulent strain (depending on genotypes)

**Table 1B** summarised recent empirical studies on mixed infections of different genotypes within species (continued).

Host	Parasites	Total “virulence” (measured phenotype)	Individual genotype’s fitness in mixed infection measured?	Parasite fitness	Parasite interaction within-host
Wax moth larvae ( <i>Galleria mellonella</i> )  Bashey <i>et al.</i> , 2012	Insect-killing bacteria ( <i>Xenorhabdus bovienii</i> ):  Focal strain with Xenorhabdacin producer (inhibitory) or noninhibitory competitor	<b>mixed</b> (with inhibitory competitor) < <b>single</b>  <b>mixed</b> (with noninhibitory competitor) $\approx$ <b>single</b>  (rate of host mortality)	YES	Bacteriocin producing strains > bacteriocin-sensitive focal strain, non-bacteriocin producing strains $\approx$ bacteriocin-sensitive focal strain  Bacteriocin and non-bacteriocin producing strains < bacteriocin-resistant control strain	Spiteful interaction via bacteriocin
Baby hamster kidney 21 (BHK-21) cells  Ojosnegros <i>et al.</i> , 2010	Two <i>Foot-and-mouth disease virus</i> (FMDV) clones; MARLS and p200	<b>mixed</b> < <b>high virulence genotype</b> but <b>mixed</b> $\approx$ <b>low virulence genotypes</b>  (rate of cell mortality)	YES	High virulence genotype > low virulence genotype at low dose  High virulence genotype < low virulence genotype at high dose	Interference interaction
Mice ( <i>Mus musculus</i> )  Balmer <i>et al.</i> , 2009	Protozoan parasite strains ( <i>Trypanosoma brucei</i> )	mix of virulent and avirulent genotypes: mixed infection intermediate between two single strains  mix of two virulent genotypes: <b>mixed</b> $\approx$ <b>single</b>  (host survival)	YES	Virulent genotype: mixed < single  avirulent genotype: mixed < single  mixed < single for both genotypes	Competitive suppression

**Table 1B** summarised recent empirical studies on mixed infections of different genotypes within species (continued)

Host	Parasites	Total “virulence” (measured phenotype)	Individual genotype’s fitness in mixed infection measured?	Parasite fitness	Parasite interaction within-host
<p>Waxmoth larvae (<i>Galleria mellonella</i>)  Inglis <i>et al.</i>, 2009</p>	<p>Bacteria (<i>Pseudomonas aeruginosa</i>): Bacteriocin (pyocin) producer sensitive competitor</p>	<p>Virulence varies with relative frequency of a bacteriocin producer.  50% &lt; 1%, 99% of the two genotypes  No single infections tested.  No effect for genotypes that produce no bacteriocin  (host mortality)</p>	<p>YES</p>	<p>Total bacterial density lower at 50% mix than at 1 or 99%  relative fitness of bacteriocin producer is highest at 50% frequency.  For mixtures of genotypes that produce no bacteriocin, relative fitness of the strains is similar</p>	<p>Spiteful interaction via bacteriocin</p>
<p>Freshwater cladoceran (<i>Daphnia magna</i>)  Ben-Ami <i>et al.</i>, 2008</p>	<p>Bacterial strains (<i>Pasteuria ramosa</i>): Sequential and mixed infection</p>	<p><b>Mixed infections:</b>  <b>mixed <math>\approx</math> single of</b> more virulent genotypes or intermediate between the two genotypes depending on the genotypes  <b>Sequential inoculation:</b>  <b>mixed <math>\approx</math> single of</b> more virulent genotypes, of less virulent genotypes or intermediate between the two genotypes depending on the genotypes  <b>mixed = single</b> less virulent strain, when first inoculated with less virulent strain  (host longevity)</p>	<p>YES</p>	<p><b>More virulent strain:</b> mixed <math>\approx</math> single  <b>Less virulent strain:</b> mixed &lt; single</p>	<p>Competitive exclusion by more virulent strain</p>

**Table 1B** summarised recent empirical studies on mixed infections of different genotypes within species (continued)

Host	Parasites	Total “virulence” (measured phenotype)	Individual genotype’s fitness in mixed infection measured?	Parasite fitness	Parasite interaction within-host
Mice ( <i>Mus musculus</i> )  Secondary host: mosquito ( <i>Anopheles stephensi</i> )  de Roode <i>et al.</i> , 2003; Wargo <i>et al.</i> , 2007	<i>Plasmodium chabaudi</i> strains:	<b>Mixed <math>\approx</math> single</b> of virulent strain  <b>Mixed <math>&gt;</math> single</b> of avirulent strain  (host mortality, weight loss, red blood cell loss)	NO	Overall parasites $\approx$ single infection of virulent genotype	Competitive suppression by virulent genotype
Mice ( <i>Mus musculus</i> )  Secondary host: mosquito ( <i>Anopheles stephensi</i> )  Bell <i>et al.</i> , 2006; de Roode <i>et al.</i> , 2005a; de Roode <i>et al.</i> , 2005b; Taylor <i>et al.</i> , 1997)	<i>Plasmodium chabaudi</i> strains	Not observed in comparison between mixed and single infections  (weight loss and red blood cell loss)	YES	<b>More virulent genotype:</b> mixed $\approx$ single <b>Less virulent genotype:</b> mixed $<$ single. There is more competitive suppression when there is more difference in virulence  <b>Total transmission success</b> (in mosquitoes) <b>from</b> mixed $<$ single competitive suppression decreased with increasing relative virulence of two genotypes	Competitive suppression by more virulent strain

**Table 1B** summarised recent empirical studies on mixed infections of different genotypes within species (continued)

Host	Parasites	Total “virulence” (measured phenotype)	Individual genotype’s fitness in mixed infection measured?	Parasite fitness	Parasite interaction within-host
Mice ( <i>Mus musculus</i> )  Secondary host: mosquito ( <i>Anopheles stephensi</i> )  Taylor <i>et al.</i> , 1998	<i>Plasmodium chabaudi</i> strains	<b>mixed &gt; single</b> of both strains  (weight loss and red blood cell loss)	NO		competition explained above.  <b>Total asexual replication:</b> mixed < or > single, with block effect  <b>Total sexual reproduction:</b> mixed > single infection of each genotype
Wax moth larvae ( <i>Galleria mellonella</i> )  Harrison <i>et al.</i> , 2006	Pathogenic bacteria ( <i>Pseudomonas aeruginosa</i> ): siderophore-producer and cheater	mixed intermediate between two single genotypes  (host longevity)	YES	<b>Producer growth:</b> mixed $\approx$ single  <b>cheater growth:</b> mixed > single, when cheater is rare in mix  Density of the two genotypes together in mixed infection intermediate between two single genotypes.	Cheater profits from siderophore cooperation when rare
Snail ( <i>Biomphalaria glabrata</i> )  (Gower & Webster, 2005)	Two strains of <i>Schistosoma mansoni</i> (high and low virulent)	Not observed in comparison between mixed and single infections  (host survival)	YES	<b>Less virulent genotype:</b> mixed $\approx$ single  <b>More virulent genotype:</b> mixed < single  For both mixed and sequential inoculation	Less virulent strain suppressed more virulent one

**Table 1B** summarised recent empirical studies on mixed infections of different genotypes within species (continued)

Host	Parasites	Total “virulence” (measured phenotype)	Individual genotype’s fitness in mixed infection measured?	Parasite fitness	Parasite interaction within-host
Three wheat cultivars ( <i>Triticum aestivum</i> )  Schurch & Roy, 2004	Three fungal strains ( <i>Mycosphaerella graminicola</i> ): three genotypes (1, 2, 3)	<b>mixed &lt; single</b> of genotype 3, in every combination composed of genotype 3 (1+3, 2+3, 1+2+3)  <b>mixed ≈ single</b> of genotype 1 or 2, in every combination  (seed: number and weight)	NO		Competitive interaction between genotypes with observed lower virulence and disease severity than single genotype infection.
Snail ( <i>Biomphalaria glabrata</i> )  secondary host: Mice ( <i>Mus musculus</i> )  Davies <i>et al.</i> , 2002	Two strains of <i>Schistosoma mansoni</i>	Mixed with different proportion of hatched miracidia from two genotypes:  <b>4:6 mix:</b> <b>mixed &gt; single</b> of both genotypes  <b>6:4 mix:</b> <b>mixed &gt; single</b> of lower virulent genotype  <b>mixed ≈ single</b> of higher virulent genotype  (host survival)	NO	Parasite reproductive rate for two genotypes mixed infection:  <b>mixed &gt; single</b> of both genotypes	Not observed

## II. THE PATHOGEN AND THE HOST

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### Pathogen profile

*Hyaloperonospora arabidopsidis* (Gäum.) Göker, Riethm., Voglmayr, Weiss & Oberw.  
(formerly *Hyaloperonospora parasitica*, formerly *Peronospora parasitica*)

Kingdom	Stramenopiles
Phylum	Oomycota
Order	Peronosporales
Genus	<i>Hyaloperonospora</i>
Species	<i>Hyaloperonospora arabidopsidis</i>

*H. arabidopsidis* belongs to the phylum Oomycota, now classified together with brown algae and diatoms. Previously, oomycetes were placed with the fungi because they and true fungi share several superficial morphological and physiological characters, i.e. reproduction of spores, hyphal living form, mode of nutrition and infection structure like appressoria and haustoria. However, the oomycete cell wall is mainly composed of cellulose with little or no chitin and their hyphae are always coenocytic, while true fungi have non-septate (=coenocytic) (Zygomycetes) or septate hyphae. Vegetative parts of oomycetes are diploid whereas these are haploid or dikaryotic in true fungi.

*H. arabidopsidis* is a natural obligate biotrophic pathogen of *Arabidopsis*, which means it needs to live in the host plants to gain nutrients and complete its life cycle. *H. arabidopsidis* has asexual and sexual phases in its life cycle. Asexual spores called conidia or conidiospores are produced on conidiophores. They are hyaline and around 15-30 µm diameter in size. These asexual spores are responsible for the symptoms known as downy mildew, forming a white carpet on the leaf surface, particularly on the underside (Figure 2.1). They provide rapid disease dispersal, with cycles as short as 3 days between spore deposition and new disease symptoms in nature (Clark & Spencer-Philips, 2016). Infected leaves of the host plants might show the early senescence after the asexual sporulation is performed (Figure 2.1). Conidiophores disperse following twisting of conidiophores due to changes in humidity. Sexual spores called oospores are produced inside infected plant tissues. These sexual spores are thick-walled and 25-50 µm diameter. They are resting spores that persist outside the host plant and can resist harsh environmental conditions. Oospores result from fertilization between male gametes (antheridia) and female gametes (oogonia). *H. arabidopsidis* is homothallic, with the two different gamete types found in the same thalli (Slusarenko & Schlaich, 2003) (Figure 2.2).

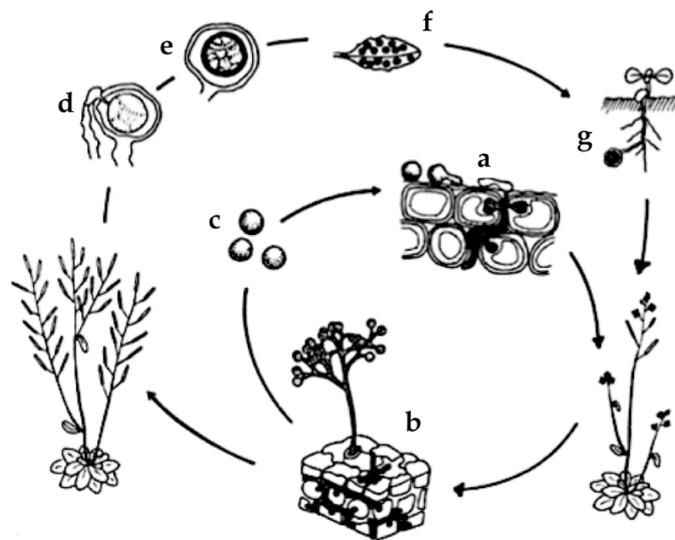
## Life cycle of the pathogen and the development of infection on host's leaf

During summer, *Arabidopsis* seeds and *H. arabidopsidis* oospores are found in resting stage in the soil. When *Arabidopsis* seeds germinate, some seedlings will become infected via their roots by germinating *H. arabidopsidis* oospores. The resulting hyphae can grow and penetrate through the intercellular spaces in a systemic manner, infecting all the plant. Hyphae inside the leaves develop haustoria that penetrate epidermal and mesophyll cell walls apoplastically and continue growth. In the leaf, some hyphae grow out via stomatal pores and produce conidiophores bearing hyaline conidiospores on the underside of the leaf. Under high humidity conditions hyphae can also pierce and produce conidiophores with conidiospores on the upper leaf surface. The conidiospores are dispersed by wind, water splash or twist of conidiophores due to changes in humidity. If they land on an appropriate host plant, these conidiospores germinate and form appressoria that pierce the leaf surface, granting the hyphae entry to the leaf and starting a new, asexual infection cycle. The asexual cycle can be rapid, from infection to sporulation in only a few days, so many cycles can occur in a single season. At the same time, sexual reproduction occurs inside the leaf when antheridia that produce male gametes fuse with oogonia filled with oospheres. Fertilized oospheres develop into oospores that remain in the leaves until they decompose. Oospores persist in leaf debris in the soil. They start new life cycle by infecting root of *Arabidopsis* seedling via oospore germination (Figures 2.2-2.3).



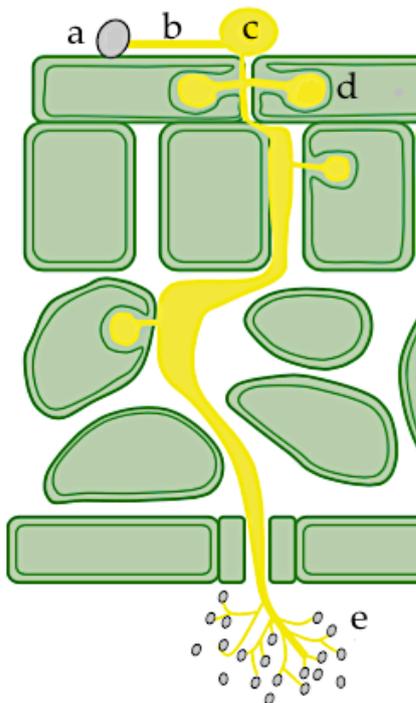
**Figure 2.1 Downy mildew symptom on *A. thaliana* caused by *H. arabidopsidis***

A. early senescence of infected leaves after pathogen sporulation; B. sporulation of pathogen on lower surface of an infected leaf; C. infected leaves showing the coniciophores baring conidiospores on both upper and lower sides namely downy mildew.

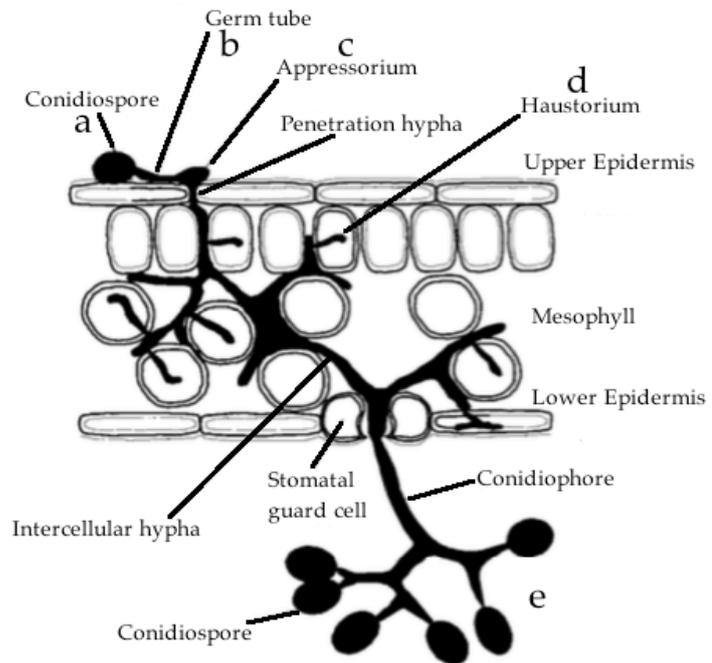


**Figure 2.2** Life cycle of *Hyaloperonospora arabidopsidis* (Slusarenko & Schlaich, 2003).

a) conidiospores germinate and start invading plant host on leaf surface to enter through intercellular space between epidermal cells. b) hyphae spread apoplastically inside an infected leaf and grow conidiophore bearing conidiospores on the leaf surface. c) conidiospores disperse to new host plants or leaves by wind or water splash. d) they form antheridia and oogonia inside infected leaves. e) fertilized oospores develops into oospores. f) oospores in an infected leaf, which will decompose on the ground. g) an oospore germinates and infects a root of a host plant seedling.



(Coates & Beynon, 2010)



(Clark & Spencer-Philips, 2016)

**Figure 2.3** Scheme of *H. arabidopsidis* infection on a leaf of a host plant

a) a conidiospore lands on the leaf surface, b) germinates and c) forms an appressorium near an epidermal cell junction or stoma, which pierces the leaf epidermis. d) The hyphae penetrate leaf cell walls, developing haustoria into epidermal and mesophyll cells, giving direct access to the plant plasma membranes. e) a hypha grows through intracellular spaces, exits via a stoma and produces conidiophores containing conidiospores on the leaf surface.

## ***H. arabidopsidis* strains used in experiments**

In this experiment I used both laboratory strains and wild strains, following Salvaudon et al. (2007). The former are strains that had been isolated, maintained by asexual re-inoculation in the laboratory for several years and characterized. I chose the parasite strains EMCO, EMWA and WACO, which I had already used in our previous experiment for exploring the dynamics of processes occurring during co-infection on one *A. thaliana* ecotype, Gb, known to be highly susceptible to infection by several strains (Salvaudon et al., 2007). Both EMCO and EMWA were provided by the Sainsbury Laboratory (John Innes Centre, Norwich, U.K.) where they had been isolated and maintained by asexual culture on specific *A. thaliana* ecotypes for more than 20 years. WACO was provided by Harald Keller, from Sophia Agrobiotech, France, where it had been cultivated by asexual reinoculation for many years. For all strains, I reanimated spores frozen at -80°C and inoculated them onto specific *A. thaliana* ecotypes: Eiffel-2 for EMCO, Cape Verde Islands [Cvi] for EMWA and Columbia [Col-0] for WACO, on which I cultivated them for at least five cycles before harvesting conidia for inoculation. Wild strains had been isolated in spring of 2004, cultivated for several asexual cycles in the greenhouse and had been stored in the -80°C freezer since 2007. I chose ORS3, collected from the campus of Université Paris-Sud, in Orsay, France and Fri5, collected from the campus of University of Fribourg, in Fribourg, Switzerland because both of these strains infected the ecotype Gb with high success (Salvaudon et al., 2005, 2007; Héraudet et al., 2008). Before starting the experiment, I cultivated all five strains on Gb for at least 5 asexual inoculation cycles in order to standardise the host environmental condition and multiply spores to have an adequate supply of fresh spores on fresh plant material. For asexual cycles of infection I collected sporulating leaves from each strain when they were sporulating heavily (between seven and ten days post-inoculation) into individual sterile 15 ml Falcon tubes. When leaves were covered with a dense mat of conidiophores, I collected only ten leaves per strain. When sporulation was patchier I collected about 20 leaves. These leaves were then immediately vortexed with 5 ml of sterile tap water for about ten seconds and the result was filtered through sterile surgical gauze. The water with spores was returned to the same Falcon tube and sprayed onto plants of Gb in the four-leaf stage, with several plants in each of ten pots, using a glass atomiser fitted with an electric pump. The glass tubes and spray head were sterilised after each use by washing them first with hot soapy water and then with 70% ethanol. The spray apparatus was sterilised by soaking in bleach solution after use with each strain.

## ***A. thaliana* ecotypes used in experiments**

*A. thaliana* ecotype 'Gb' represents the selfed offspring from a single plant grown from seed originally collected from Great Britain. This ecotype has subsequently been multiplied by several additional generations of selfing and seeds had been stored in the greenhouse seed room since 2007. 'Gb' is susceptible to a large range of pathogen strains (Salvaudon et al., 2005, 2007; Héraudet et al.,

2008) and can be infected with more than one *H. arabidopsidis* genotypes at the same time from my preliminary results. I generated fresh seeds by planting out seeds produced by a single mother plant and harvesting their selfed seeds at maturity, before using them in the experiment. For the inoculation experiments, seeds were sown on soil in 5 cm diameter pots and placed in a cold chamber (5-6 °C) for ten days to synchronize germination. Then they were transferred to a growth chamber (10:14 light-dark photoperiod, 18°C ± 3 °C average temperature and 98-100% hygrometry) and left to germinate and grow until the four-leaf stage (15 days after germination). Every two days I turned the trays and changed their position in the growth chamber to homogenize growth conditions.

I used this set of isolates of *H. arabidopsidis* and ecotypes of *A. thaliana* to carry out a series of different experiments that allowed me to explore the conditions that led to multiple infections and to compare the phenotypes of infections resulting from inoculation with single isolates or mixtures of isolates. Because my aim was to investigate co-infections, it was necessary to have a molecular marker that distinguished these different strains. I developed this marker as follows.

### **III. DEVELOPMENT AND TESTING OF MARKERS (PROCEDURES AND RESULTS)**

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#### **Development and testing of microsatellite markers**

Since no sequenced genome of these particular *H. arabidopsidis* strains was available, I developed micro- and minisatellites starting from the *Hpa* EMOY2 genome sequence, which has not yet been assembled. The sequence of version 7.0.1 is available on NCBI's Genome Project website, <http://www.ncbi.nlm.nih.gov/bioproject/?term=30969>, with the Genome Project ID 30969, WGS accession numbers [ABWE01000001-ABWE01005422] and project accession number [ABWE00000000]. There are several separate contigs and a lot of ambiguous bases "N" in the sequences. These available genome data were obtained from Baxter et al. (2010). I searched for repetitive sequences using a tandem repeat detection tool, Phobos Version 3.3.12 program (Mayer, 2006-2010) beginning with contig 0 and then 1, 2, etc. I assigned Phobos to find tandem repeats by default values that used 1, -5, -5 for a match, mismatch or gap position respectively. I indicated a range of repetitive sequence length from 2 to 20 base pairs accepting imperfect repeats in order to obtain longer units. Small motif lengths (2-10 bp) give microsatellite markers, larger motif lengths give minisatellite markers. The approximately 20 bp sequences at the 3' and 5' flanking region of each repeat were designed as primers for amplifying the product by PCR. I tested the designed primers for amplification ability and length polymorphisms in EMCO, EMWA and WACO by PCR amplification. All markers were tested with at least four independent PCRs. Those showing length polymorphisms were tested with 6-12 independent PCRs to verify the consistency of amplification and fragment length (Table 3.1).

#### **DNA extraction and adjustment of concentration for testing markers**

All spore samples that were used for testing of microsatellite markers and for examining the individual strain identification from known mixtures were extracted using the NucleoSpin<sup>®</sup> Plant II (LOT 1506/005) developed by the MACHEREY-NAGEL GmbH & Co. KG. This extraction kit, based on CTAB (PL 1 lysis buffer) and SDS (PL 2 lysis buffer) methods with RNase A, is designed for plant genomic DNA isolation, of which PL1 buffer was successfully tested with fungal mycelium. Hence, I modify this kit to spore samples. In addition, the kit contains the filter that excludes the crude lysates and the spin column containing silica membrane to bind the genomic DNA that can be eluted with the

elution buffer PE (5 mM Tris/HCl, pH 8.5). This leads to high purification method for DNA extraction.

Five sporulating leaves of each tested strain were collected in individual 2 µl tube and vortexed in 500 µl lysis buffer PL1. Then, add 10 µl RNase A, mix thoroughly and incubate at 65 °C for 15-20 minutes. The lysate was loaded onto filter column and centrifuged at 11,000 x g for 2 minutes. The flow-through lysate was collected and added the binding buffer PC to generate optimal condition that DNA could bind the silica membrane on binding column. After mixing thoroughly, all mixture was loaded on the binding column and centrifuged at 11,000 x g for 1 minute. The binding column was washed twice, each followed by 11,000 x g centrifugation for 1 minute, with 400 µl buffer PW1 and 700 µl PW2 respectively to remove the contaminants, i.e., proteins, RNA, metabolites and the PCR inhibitors. Finally, the binding column was eluted with the 30 µl prepared elution buffer (65 °C), incubated at 65 °C for 10 minutes and centrifuged at 11,000 x g for 1 minute to obtain the purified DNA.

The extracted DNA was measured the concentration (ng/µl) using the NanoDrop2000<sup>®</sup> spectrophotometer launched by Thermo Fisher Scientific Inc. I used 1 µl of sample for each measurement specified to nucleic acid with elution buffer PE as a blank. I determined the quality ratio for DNA (260/280) ≥ 1.8, which is expected value of accepted purity.

PCR was performed in The Applied Biosystems<sup>®</sup> Veri<sup>®</sup> 96-Well Thermal Cycler using total volume mixture of 20 µl modified from QIAGEN<sup>®</sup>. This consists of 2 µl of 10x PCR buffer (Tris·Cl, KCl, (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 15 mM MgCl<sub>2</sub>, pH 8.7), 0.4 µl dNTPs mix (10 mM each of dATP, dCTP, dGTP, and dTTP), 0.13 µl forward primer, 0.13 µl reverse primer, 0.15 µl *Taq* DNA Polymerase (a recombinant 94 kDa DNA polymerase (deoxynucleoside-triphosphate: DNA deoxynucleotidyltransferase, EC 2.7.7.7), originally isolated from *Thermus aquaticus*, and expressed in *E. coli*), 15.19 µl nuclease free deionized water and 2.0 µl of spore suspension or extracted DNA (see also Table 3.2). Each reaction mixture was loaded into the 96-well PCR plate and amplified in a Thermal Cycler under these condition: 1 minute of initial denaturation at 94 °C, 40 cycles of the following three steps: 1) 25 seconds of denaturation at 94 °C, 2) 1 minute of annealing at 60 °C and 3) 30 seconds of Extension at 72 °C. Then, final extension was performed for 3 minutes at 72 °C. I optimized the PCR conditions by reducing the concentration of MgCl<sub>2</sub>, which allowed us to eliminate unspecific PCR bands.

Amplified PCR products were separated via gel electrophoresis of 10 µl samples run on 3 % MetaPhor<sup>™</sup> Agarose and visualized under ultraviolet (UV) light. I added Ethidium bromide to the gel directly to stain the amplified fragments. To determine the size of PCR products, I used GelPilot<sup>®</sup> 50 bp Ladder (100) (cat. no. 239025) containing different sizes of base pairs: 500, 400, 350, 300, 250,

200, 150, 100 and 50. This allowed me to distinguish different *H. arabidopsidis* strains by their different PCR product sizes.

I found polymorphisms in the size of the amplified PCR products among three strains, EMCO-EMWA-WACO, from four primer pairs of the 16 that were tested (Table 3.1). The Co 2-2 microsatellite marker showed the most distinct size polymorphic products among these three strains (Figure 3.1), while the other three markers either failed to detect the strain WACO (Co 2-1) or produced some double bands on the gels (Co 5-2 and MnSHP6) (Table 3.3). The wild strains ORS3 and FRI5 did not produce unique distinguishable bands with the Co 2-2 marker: ORS3 was not distinguishable from EMWA and FRI5 was not distinguishable from EMCO. Nonetheless, this single marker allowed me to distinguish among the three laboratory strains and between the wild strains and some of these laboratory strains in multiple inoculation experiments.

**Table 3.1** Microsatellite and minisatellite markers for distinguishing EMCO, EMWA and WACO. The amplified fragment size (bp) in the table represents the sequence from the EMOY.

Name	Sequence (5' to 3')	Repetitive unit	Fragment size (bp)	Length Polymorphism	Annealing Temperature (°C)	Amplification success per all PCR
Co0-1-F	CACAAACACTGGGAAGAAGAG	AGG	200	NO	57.9	3/4
Co0-1-R	GTTTAACCTCCTCCTCCTTC				57.3	
Co0-2-F	CTCTCATCCTAGAGATATATGTG	ATC	160	NO	57.1	3/4
Co0-2-R	GAAGCCACTATCGTCTTCAG				57.3	
Co0-3-F	GAAATAGATGTGCCCTCAATTAC	ACAG	200	NO	57.1	
Co0-3-R	GGCACTGTGTATACGAGATG				57.3	3/4
Co1-1-F	GGACCACGTACTIONTACATAG	AT	180	NO	57.3	4/4
Co1-1-R	GGACACGTGTAAGAGTTGATG				57.9	
Co1-2-F	CGCAAGGTTGAATGCCGAAC	ACAGATAG	450	NO	59.4	4/4
Co1-2-R	CTAACCGCGTTATTGACACAG				58.4	
Co2-1-F	CTTTCAAACCTACCTTATGTC	ACAG	200	<b>YES</b>	56.5	7/8
Co2-1-R	CTGCTTCGCATTCAGTGAGAC				59.8	
Co2-2-F	CTCGGTCATCGCAATTGGATG	AAAGTAGAG	210	<b>YES</b>	59.8	10/12
Co2-2-R	GCTGCTGGCAATTTCTATCC				57.9	
Co3-1-F	GTCTCCTCGGACGCTTTAC	GCGTAATGCATT	100	NO	61.4	4/4
Co3-1-R	GCGTAATGCATTCATTTGTCTC	CATTTGTCTC			56.5	
Co3-2-F	GACTGTGCGCAAGGTTGAATG	CGGATATGCCTA	200	NO	56.5	2/4
Co3-2-R	CGGATATGCCTATATACCTATG	TATACCTATG			56.5	
Co4-1-F	CTTTTAGTAAGTGTGCGCAAG	GTATAGTACGGG	350	NO	55.9	3/4
Co4-1-R	GTATAGTACGGGTAAGGGATAC	TAAGGGATAC			58.4	
Co4-2-F	GAATCATCGCGATGCTACTG	CAGCTCATCACG	130	NO	57.3	3/4
Co4-2-R	CAGCTCATCACGTACTTTGC	TACTTTGC			57.3	
Co-5-1-F	GTACATATAAGGTACACCTAAC	GAGTATGCGCAA	120	NO	54.7	4/4
Co-5-1-R	GAGTATGCGCAAGGTTGTATG	GGTTGTATG			57.9	
Co-5-2-F	GCTATTCGACATTACAGTATGC	GTTCAGCAAAGA	350	<b>YES</b>	56.5	4/6
Co-5-2-R	GTTCAGCAAAGAGTGCTAGG	GTGCTAGG			57.3	
Co-6-1-F	GACAGGCTAGATCGCACTTAG	GGTGAAGCTATT	210	NO	59.8	3/4
Co-6-1-R	GGTGAAGCTATTAGTGAGTTTG	AGTGAGTTTG			56.5	
Co-6-2-F	CATCTGCGTCTGCAAGTTCTG	GAAACAATAGAA	130	NO	59.8	4/6
Co-6-2-R	GAAACAATAGAATCCGATACTAC	TCCGATACTAC			55.3	
MnSHP6-F	GTAGGGACCGCAACCTG	GTAGGGACCGCC	191	<b>YES</b>	61.4	8/10
MnSHP6-R	GACCGACTGGCTAGCTGACT	AACCTG			59.6	

Because the goal was to identify individual strains in co-infections, I tested whether I could detect individual strains from known strain mixtures using the Co 2-2 marker (Table 3.4). I used two different approaches, one generated by mixing extracted DNA and the other generated by mixing spore suspensions of known concentration. This demonstrated whether it would be possible to detect the individual strains when both are present in co-infections, as well as testing the effectiveness of performing PCR on spore suspensions directly without a DNA extraction step. Since individual sporulating leaves sometimes contains few spores, and I feared that these could be lost during a DNA extraction process, using spore suspension immediately in PCR might avoid this problem and could be more practical with a large number of sporulating leaves from experiments. The DNA concentration of each strain was measured with a spectrophotometer and adjusted to be nearly the same before mixing. The spore suspension of each strain was prepared by adding 30  $\mu$ l of deionized water to a tube containing five sporulating leaves, vortexing without crushing the leaves, and adjusting the concentration to  $5.0 \times 10^4$  spores per ml after counting spores with a haemocytometer. I performed two independent PCRs on each mixture of extracted DNA and two independent PCRs on each spore suspension mixture. Furthermore, because infections within plants may occur at different proportions of the different strains, I tested whether it was possible to detect individual strains from known mixtures, even when they were at very different proportions (Table 3.5). I used extracted DNA of EMCO and ORS3 and varied their representation from 9% to 91% of the mixture.

Specific PCR products were successfully amplified for both or all three strains with Co 2-2 marker in all 1:1 ratio mixtures, whether testing 38 independent PCR with extracted DNA mixtures or 38 independent PCR with spore suspension mixtures (Table 3.4). For the mixtures of EMCO and ORS3 at varying proportions of the two strains, both strains were detected in almost all tests. Only EMCO failed to be detected in two out of four PCR amplifications when it was present at only 9% (Table 3.5).

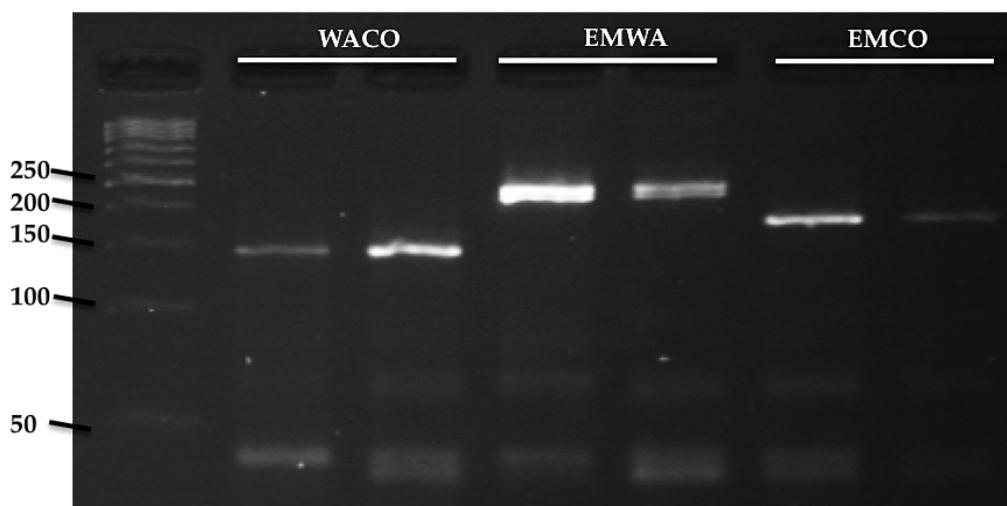
This validates that it is possible to detect individual strains in co-infections. I found no evidence for interference between amplified PCR products of the two or three tested strains when using mixtures of extracted DNA or spore suspensions. Hence, I can have confidence in the detection of individual strains from multiple infections that may differ in DNA quantity.

**Table 3.2** Optimized PCR conditions according to QIAGEN for *H. arabidopsidis* detection

Step	Time	Temperature (°C)
Initial denaturation	3 minutes	94
<b>3-step cycling:</b>		
Denaturation	25 seconds	94
Annealing	1 minute	60
Extension	30 seconds	72
Final extension	3 minutes	72
Number of cycles	40	

**Table 3.3** Fragment lengths of PCR products from primer pairs that showed length polymorphism. Those marked this an asterisk (\*) showed double bands.

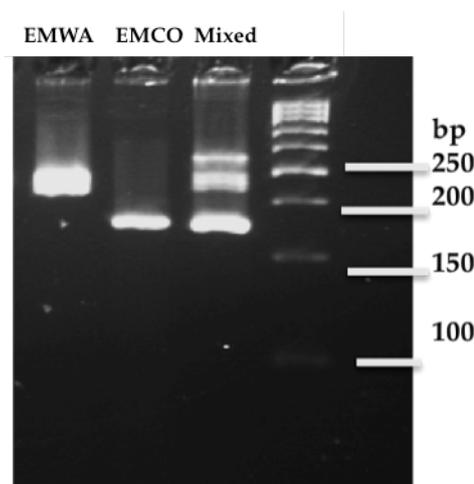
Markers	Strains	Amplified fragment size (bp)
Co 2-1	EMCO	300
	EMWA	180
	WACO	-
Co 2-2	EMCO	200
	EMWA	250
	WACO	150
Co 5-2	EMCO	300*
	EMWA	330*
	WACO	280*
MnSHP6	EMCO	150
	EMWA	200
	WACO	130*



**Figure 3.1** Gel showing length polymorphism of PCR products from primer pair Co 2-2.

**Table 3.4** Testing the detection of strains using the Co 2-2 marker on mixed spore suspensions and mixed extracted DNA of different strains, with both or all strains at equal proportions (1:1 or 1:1:1). Two independent PCRs were performed for spore suspensions and two for extracted DNA. The spore concentration of each strain was adjusted to  $5.0 \times 10^4$  spores per ml by counting with a haemocytometer before mixing and DNA concentrations were tested with a photospectrometer and adjusted before mixing. Individual PCR products for all strains were detected from all tests, whether on extracted DNA mixtures or spore suspensions.

Strain in spore mixture (1 $\mu$ l of each)		Distinction between strains from mixture	Successful amplification in PCR				
			EMCO	EMWA	WACO	FRI5	ORS3
EMCO	EMWA	YES	+	+			
EMCO	WACO	YES	+		+		
EMCO	ORS3	YES	+				+
EMWA	WACO	YES		+	+		
EMWA	FRI5	YES		+		+	
WACO	FRI5	YES			+	+	
WACO	ORS3	YES			+		+
FRI5	ORS3	YES				+	+
EMCO-EMWA-WACO		YES	+	+	+		



**Figure 3.2** Gel of PCR products amplified with primer pair Co 2-2 showing the successful detection of the strains EMWA and EMCO from pure samples and from a 1:1 mixture of extracted DNA sample.

**Table 3.5** Detection of individual strains from mixtures containing different proportions of DNA of the EMCO and ORS3 using the Co 2-2 marker. The DNA concentration of the two strains was adjusted to be approximately the same after estimating with a spectrophotometer, then mixed in different proportions to generate the strain mixtures. Nuclease free deionized water was added to obtain a total volume of 2  $\mu$ l for each PCR. Each mixture was tested in four independent PCRs.

Amount in $\mu$ l (and proportion) of each strain in mixture		Number of successful amplifications	
EMCO	ORS3	EMCO	ORS3
1.0 $\mu$ l (0.50)	1.0 $\mu$ l (0.50)	4	4
1.0 $\mu$ l (0.57)	0.75 $\mu$ l (0.43)	4	4
1.0 $\mu$ l (0.67)	0.50 $\mu$ l (0.33)	4	4
1.0 $\mu$ l (0.77)	0.30 $\mu$ l (0.23)	4	4
1.0 $\mu$ l (0.91)	0.10 $\mu$ l (0.09)	4	4
1.0 $\mu$ l	0	4	-
1.5 $\mu$ l	0	4	-
0.75 $\mu$ l (0.43)	1.0 $\mu$ l (0.57)	4	4
0.50 $\mu$ l (0.33)	1.0 $\mu$ l (0.67)	4	4
0.30 $\mu$ l (0.23)	1.0 $\mu$ l (0.77)	4	4
0.10 $\mu$ l (0.09)	1.0 $\mu$ l (0.91)	2	4
0	1.0 $\mu$ l	-	4
0	1.5 $\mu$ l	-	4
0	0	-	-

### Collection of sporulating leaves for strain identification and leaf sample preparation

For the two-strain combination experiments I collected each sporulating leaf from all recipient plants from all transmission treatments. I did not collect sporulating leaves from the primary inoculated plants for two reasons. First, for those plants used for the transmission experiment, because I genotyped all successful transmission events, this provides information about the primary infection, since we can conclude that all genotypes that transmitted had successfully infected. Second, I used these same plants for estimating plant fitness, so it was necessary to leave the rosettes intact. For the three-strain combination experiment, I collected all sporulating leaves from primary inoculated plants on day 14 post-inoculation as well as two sporulating leaves per pot of all plants inoculated with single strains and all sporulating leaves of those plants inoculated with mixtures from the additional plants used to check inoculum viability.

The individual sporulating leaf samples were collected into an individual 2.0 µl tube for parasite genotyping, placed on ice during the collecting procedure and then frozen at -20 °C until analysis. I added 30 µl of deionized water to each tube and vortexed each without crushing the leaves. I used a 2.0 µl sample of these spore suspensions directly in each PCR. For each spore sample I performed at least two independent PCRs whose products were loaded separately onto the MetaPhor™ Agarose gels. Duplicate reaction products were run on either separate or the same gels, but in the latter case, in distant lanes.

## IV. CO-INOCULATION PROCEDURE

### TRANSMISSION PROCEDURE

### PLANT FITNESS AND VIRULENCE ESTIMATION

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#### 4.1 Inoculation procedure of co-inoculation experiments

I established inoculation experiments two or three strains that could be distinguished by the microsatellite marker. For the three experiments with different pairs of strains, i.e., EMCO-ORS3, EMWA- FRI5 and EMWA-WACO, seedlings at the four-leaf stage were inoculated by pipetting a 10  $\mu$ l droplet of spore suspension onto each of the four leaves. For the three-strain mixed experiment trays of 20 pots, each containing three seedlings at the four-leaf stage were inoculated by spraying using a glass atomiser fitted with an electric pump as described above in the methods for maintaining the *H. arabidopsidis* cultures. Control plants in all experiments were inoculated with sterile water by the same method as used for spore inoculated plants, i.e., pipetting or spraying.

#### *Inoculation procedure for EMCO-ORS3, EMWA- FRI5 and EMWA-WACO experiment*

I set up four treatments (single strain-1, single strain-2, standard mixed strains, half standard mixed strains) and a control inoculated with sterile tap water for each combination of strains. It is noted that the three experiments here were independent, carried out at a different time, but each was done in the same growth chamber under the same conditions. I prepared inoculum by collecting sporulating leaves into individual sterile 15 ml Falcon tubes, vortexing them with sterile tap water and straining this through sterile gauze. I counted spores using a haemocytometer and adjusted their concentration to  $5.0 \times 10^4$  spores per ml for every strain to prepare standard inocula. I used these standard inocula to generate four inoculation treatments. I used standard inocula for the two single strain treatments. For co-inoculation, I mixed equal volumes of the two standard inocula to produce a standard mixed inoculum and also generated half standard mixed inoculum by diluting each standard inoculum to a half concentration with  $2.5 \times 10^4$  spores per ml before mixing them together. This generates different kinds of inoculum. Two pure strain inocula with equal number of spores, one mixed inoculum with the same total number of spores but half as many as each strain, and a lower dose inoculum with half the total number of spores and only one quarter the number of each strain in the mix. Trays, each containing twenty pots, each with a single four-leaf stage plant, were prepared for inoculation. For the EMWA- FRI5 combination I used a single tray for each of the 5 inoculation conditions (four

treatments and one control). For the EMCO-ORS3 and EMWA-WACO combinations I used two trays of twenty plants each per inoculation condition. As mentioned above, I inoculated these plants by pipetting a 10.0 µl-drop of inoculum onto each leaf, thus inoculating all plants within a treatment with about the same number of spores of *H. arabidopsidis*, namely about 500 spores for the standard and standard mixed inoculum and about 250 spores for the half standard mixed inoculum (Table 4.1). Control plants were inoculated with 10.0 µl-drops of sterile tap water onto each leaf.

### ***Inoculation procedure for three-strain mixed experiment***

I conducted the seven inoculation treatments with three independent single strain inoculations (EMCO, EMWA, WACO), all pairwise strain co-inoculations (EMCO-EMWA, EMCO-WACO, EMWA-WACO) and one three mixed strain co-inoculation (EMCO-EMWA-WACO) plus a control with sterile tap water. I prepared inoculum as above, collecting sporulating leaves into individual sterile 15 ml Falcon tubes, vortexing them with sterile tap water and straining this through sterile gauze. I counted spores using a haemocytometer and adjusted their concentration to  $5.0 \times 10^4$  spores per ml for every strain to prepare inocula for the single strain inoculations, to  $1.0 \times 10^5$  spores per ml for every strain for the double strain inoculations and to  $1.5 \times 10^5$  spores per ml for every strain for the three-strain inoculation. For mixed inoculations equal volumes of the different strain suspensions were mixed to provide a 6 ml spore suspension (Figure 4.1). This generated inocula with the same number of spores of each strain in each inoculum but a different total spore dose among the single, two- and three-strain mixtures. The 20 pots, each containing three seedlings at the four-leaf stage for each inoculation treatment were placed uniformly in a tray and the whole tray was sprayed with 6 ml of spore suspension inoculum using a glass atomiser fitted with an electric pump. I made sure to spray all leaves of each plant in each pot, spraying each pot for approximately 2 seconds, to ensure that all plants should have received an equivalent amount of inoculum (Table 4.1). After each spraying I washed the glass atomiser with bleach solution and ethanol to sterilise it and I cleaned the experimental area with ethanol. I first sprayed the control plants with sterile tap water. Then I carried out the single, then the double- and last, the three-strain inoculation treatment. Therefore the three-strain inoculation treatment was carried out almost one hour after the control. Five additional pots of Gb and five pots of each habitual ecotype used for spore multiplication (Cvi for EMWA, Ei-2 for EMCO, and Col-0 for WACO), each with three seedlings, were inoculated with its appropriate single strain inoculum by spraying, as above. This was to check the viability and the infection ability of each single strain inoculum. All mixed strain inocula, were similarly tested on five pots of each of the habitual ecotypes for each strain in the inoculum, for example, five pots containing three plants each or Cvi, Ei-2 and Col-0 for the three-strain mix.

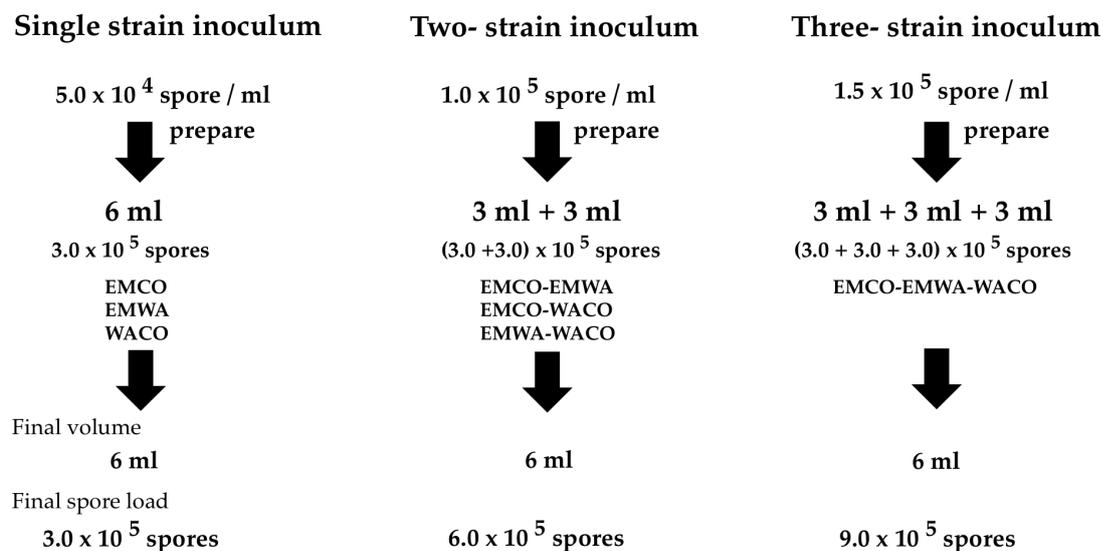
### Growth condition

The inoculated plants were grown in a growth chamber at 10:14 light-dark photoperiod,  $18^{\circ}\text{C} \pm 3^{\circ}\text{C}$  average temperature and 98-100% hygrometry. Each plant was isolated in a transparent plastic column that was placed around each pot immediately after inoculation to prevent spore dispersal and contamination. The positions of trays and plants within trays were changed every two days. Twice per day I misted with water above the plastic columns to maintain high humidity.

**Table 4.1** summarised inoculation procedure of co-inoculation experiments; inoculum dose, plant materials (\*each pot containing three plants), and how to inoculate.

Experiment	Treatment	Inoculum dose ( $\times 10^4$ spores/ml)	Total plants per treatment	Inoculation method
EMCO-ORS3, EMWA-WACO	Single (for each strain)	5.0	40	Pipetting (10.0 $\mu\text{l}$ -drop on a leaf)
	Mixed low dose	5.0 (half of each strain)	40	
	Mixed high dose	2.5 (half of each strain)	40	
EMWA-FRI5	Single (for each strain)	5.0	20	Pipetting (10.0 $\mu\text{l}$ -drop on a leaf)
	Mixed low dose	5.0 (half of each strain)	20	
	Mixed high dose	2.5 (half of each strain)	20	
Three-strains mixed (EMCO, EMWA, WACO)	Single (for each strain)	3.0	60*	Spraying with 6 ml inoculum (2 second per pot)
	Two strains mixed (for each mixture)	6.0 (3.0 of each strain)	60*	
	Three-strains mixed	9.0 (3.0 of each strain)	60*	

### Preparation of inocula for different inoculation treatments



**Figure 4.1** Preparation of inocula for the single-, Two- and Three strain inoculation treatments. Each inoculation was designed to deliver the same number ( $3.0 \times 10^5$ ) of spores of each strain.

## 4.2 Transmission procedure

Transmission experiments were conducted using the plants inoculated with the three pairs of strains: EMCO-ORS3, EMWA-FRI5 and EMWA-WACO. For each experiment, I used inoculated plants from co-inoculation experiment above as the source plants, which will be transmitted onto new plants through sterile water drops. Trays containing 20 pots, each containing a single four-leaf stage plant of Gb were prepared to serve as recipient plants for parasite transmission of EMCO-ORS3 and EMWA-WACO strain combinations. For the EMWA-FRI5 treatment, 20 pots, each with three recipient four-leaf stage plants of Gb were prepared.

Source plants consisted of 20 of the 40 inoculated plants from the EMCO-ORS3 and EMWA-WACO treatments and all 20 inoculated plants from the EMWA-FRI5 treatment. Transmission methods followed Salvaudon *et al.* (2005). Each plant, whether it had leaves bearing visible spores or not, was held about 10 cm above a single pot containing a four-leaf stage plant or plants and sprayed with sterile tap water. Transmission was carried out on day 12 post-inoculation for the experiment EMCO-ORS3 and EMWA-FRI5, and day 10 post-inoculation for EMWA-WACO. Water droplets, possibly containing spores from source plants, rained onto the leaves of recipient plants. I used one source plant to transmit spores to one recipient plant in the EMCO-ORS3 and EMWA-WACO experiments, and to three recipient plants of EMWA-FRI5 experiment. For the control, I used the same transmission procedure with control plants that had initially been inoculated with sterile tap water as source plants. After the transmission, recipient plants were isolated in individual transparent columns and observed daily from five to eight days post-transmission. I counted the number of sporulating leaves and collected each sporulating leaf into an individual 2.0µl tube for parasite genotyping. For the EMWA-FRI5 strain combination I considered a single pot as an observation unit and for transmission success I counted the total number of sporulating leaves observed in a recipient pot, not distinguishing on which plant they grew.

## 4.3 Plant fitness and virulence estimation

Plant fitness and virulence were estimated from EMCO-ORS3, EMWA-FRI5 and three-strain mixed inoculation experiments on day 60 post-inoculation. For EMCO-ORS3 I grew the sporulating plants that had not been used for the transmission experiment until day 60 post-inoculation for fitness estimates. For EMWA-FRI5 I used the sporulating plants from the transmission experiment. For the three-strain mixed experiment, I transplanted every sporulating plant from each pot from each inoculation treatment and two plants from each of the 10 control pots into its own individual pot. Each of the pots was isolated within an individual plastic column, either immediately after inoculation (two-strain mixed inoculation treatments) or after transplanting (three-strain mixed inoculation

treatment). I did not estimate fitness of plants in the EMWA-WACO treatment further because the infection success with WACO was so low.

Of the twenty plants from each treatment of EMCO-ORS3 and EMWA-FRI5 experiment, all control plants and observed sporulating plants were grown for fitness measures. For the three-strain mixed experiment, I transplanted every sporulating plant from each pot from each inoculation treatment and two plants from each of the 10 control pots into its own individual pot, which was isolated within an individual plastic column, on day 14 post-inoculation. At day 60 post-inoculation, I estimated total plant fitness from all three experiments following Brachi *et al.* (2012) by counting the total number of siliques on the primary shoot, the primary branches and the basal branches and multiplying these numbers by the average length of the respective siliques, estimated from three, randomly selected siliques from each type of branch. In parallel I recorded total number of siliques, seed weight from all siliques of each plant, as well as plant height from the EMCO-ORS3 and the three-strain mixed experiments. For the EMCO-ORS3 experiment, I recorded number of abortive siliques and number of early ripe siliques at day 45 post-inoculation. For EMWA-FRI5 experiment, I counted the number of branches per inflorescence, but the number of inflorescences with measured rosette diameter for the three-strain mixed experiment (Table 4.2).

The fitness characters measured at day 60 post-inoculation for the three different inoculation experiments are summarised in Table 3.2. I estimated total plant fitness from all plants in all treatment groups from all three experiments following Brachi *et al.* (2012) by counting the total number of siliques on the primary shoot, the primary branches and the basal branches and multiplying these numbers by the average length of the respective siliques, estimated from three, randomly selected siliques from each type of branch.

**Table 4.2** fitness-related trait measured or not from infected plants

Experiment	Phenotypic traits (measured or not)							
	Total fitness	Seed weight	Total siliques	Plant Height	Bushiness	Rosette diameter	No. aborted siliques	Early ripe siliques
EMCO-ORS3	YES	YES	YES	YES	NO	NO	YES	YES
EMWA-FRI5	YES	NO	YES	NO	YES	NO	NO	NO
Three-strain mixed	YES	YES	YES	YES	YES	YES	NO	NO

Virulence was estimated for each of the measured fitness traits as the difference in trait value between each infected plant and the mean of all control plants from the appropriate experiment.

## V. STATISTICAL ANALYSES

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The statistical analyses were performed using the R programme (version 3.2.2, R Core Team, 2015).

### 5.1 Day until first sporulation (latent period)

I compared the time until first sporulation among inoculation treatments and among inoculum types (single, two- and/or three-strain mixtures) using one-way ANOVA followed by a Tukey multiple comparison test for pair-wise differences.

### 5.2 Infection success

I tested whether infection success, measured from whether an inoculated plant sporulated or not, was heterogeneous among the inoculation treatments with a  $\chi^2$  test.

### 5.3 Infection intensity

#### *Cumulative number of sporulating leaves*

I recorded the number of sporulating leaves per observation unit (per sporulating plant for EMCO-ORS3, EMWA-FRI5 and EMWA-WACO experiments or per pot containing at least one sporulating plant for three-strain mixed experiment) on day last observed (on day 12 post-inoculation for EMCO-ORSAY and EMWA-FRI5 experiment, on day 10 post-inoculation for EMWA-WACO, on day 14 post-inoculation for three-strain mixed experiment). The heterogeneity in sporulating leaves per observation unit among inoculation treatments was tested using one-way ANOVA followed by a Tukey multiple comparison test for pair-wise differences.

#### *Increase in number of sporulating leaves over time*

The change in number of sporulating leaves over time was analysed using repeated measures analyses of data recorded for each plant on each day after it sporulated. Individual plants were subjects and observations were repeated over time for EMCO-ORS3, EMWA-FRI5 and EMWA-WACO experiment. In case of the three-strain mixed experiment, where pots each containing three plants, individual plants were nested within their respective pots and observations were repeated over time.

I performed an analysis of covariance using a generalized linear mixed-effects model with Penalized Quasi-Likelihood, via the *glmmPQL* function with Poisson error distribution and a logarithmic link function of the standard R package *MASS* (Venables & Ripley, 2003), to test how the inoculation treatments and day post-inoculation (=time) affected the number of sporulating leaves per plant or per pot in the case of pots containing three plants. The statistical model, detailed below, considers number of sporulating leaves (unbalanced and non-normal count data) as a function of inoculation treatment, i.e., the fixed effect, day post-inoculation and all interactions. I tested each plant as a subject for all experiments; in addition, I assigned each pot as a subject for three-strain mixed experiment. Each subject was observed multiple times, so it was possible to test whether number of sporulating leaves per plant within pot or number of sporulating leaves per pot varied over time. The (Subject|Time) term in the model stood for the repeated measures at different time points within a particular subject. In the description of all models, “\*” was used to indicate that both the main effect and the interaction between two covariables were tested, in the syntax of the R programme. *Post hoc* multiple comparisons were performed using the *lsmeans* package based on Tukey-adjusted comparisons method (Lenth, 2016). Slope estimate of each treatment was obtained from the model in order to examine how the number of sporulating leaves changed over time.

For all strain combinations the following model was tested:

Model:

Number of sporulating leaves ~ Inoculation Treatments\* Day post-inoculation + (Subject | Time)

#### **5.4 Strain identification from mixed inoculation or transmission**

I compared the number of leaves from which each strain was found by PCR with a  $\chi^2$  test to test for heterogeneity among the different inoculation treatments. For the two-strain experiments this measured transmission success of the two strains. I compared the number of leaves from which each strain was found by PCR between the different single strains, between single and mixed inoculations and between low and high dose mixed inoculations of the same strain combination. For the three-strain mixture this measured the infection success following inoculation, and I analysed whether the identity of the partner strain influenced the number of leaves from which an individual strain was found by PCR. The presence or absence of a particular strain in a leaf was assigned as a binary variable. The analysis was performed using the *glm* function of the *stats* package with a binary logistic regression and logit link function. The logits from the model were compared by a Tukey multiple comparison test for pair-wise differences using the *glht* function of the *multcomp* package (Hothorn et al., 2008).

## **5.5 Transmission experiments**

### ***Relationship between transmission intensity and latent period***

For all three experiments (EMCO-ORS3, EMWA- FRI5 and EMWA-WACO), I counted cumulative sporulating leaves from all sporulating recipient plants in each pot at eight days after transmission. I tested whether this cumulative number of sporulating leaves on recipient plants was influenced by the latent period of the primary infection of the source plants with ANCOVA using the *glm* function of the *stats* package with a Poisson error distribution and logarithmic link function for count data. This allowed me to deal with data including non-normal and unbalanced response variables.

## **5.6 Plant fitness and virulence**

I analysed variation in measured fitness-related traits and some vegetative traits among the infected plants using a Multivariate Analysis of Covariance (MANOVA) to test their combined variation across inoculation treatments for all experiments.

The variation in plant fitness-related or vegetative traits on day 60 post-inoculation and virulence of infection among inoculation treatments were tested using one-way ANOVA followed by a Tukey multiple comparison test for pair-wise differences.

ANCOVA analyses were performed for virulence on total fitness and effect of infection on some vegetative traits using the *lm* function of the *stats* package with linear model method, with number of sporulating leaves and latent period as covariables among inoculation treatments.

## VI. RESULTS AND DISCUSSIONS

### CO-INOCULATION & TRANSMISSION EXPERIMENTS

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The results below showed the outcomes of co-inoculation experiments in terms of infection success and infection phenotypes, i.e., latent period, sporulation intensity, transmission success and virulence on sporulating plants on day 60 post-inoculation, comparing between single- and mixed inoculation. Co-infection success, i.e. the successful infection of more than one strain from mixed inoculations, was quantified. I addressed the following questions here:

1. Does co-inoculation lead to multiple infections?
2. Does co-inoculation of different strains influence infection success?
3. Does co-inoculation of different strains influence infection phenotype, including virulence?

#### **Variation in infection success and infection phenotypes among inoculation treatments revealed a consequence of co-inoculations of *H. arabidopsidis* strains on the Gb ecotype of *A. thaliana***

From all our experiments the control plants, inoculated with sterilized water, never sporulated. Each inoculum led to at least some sporulation except for the case of WACO in the experiment with WACO and EMWA. However, WACO was successfully transmitted from the two mixed inoculation treatments in that experiment, demonstrating that the inoculum was viable at the time of inoculation. In the three-strain experiment I additionally checked the viability of strains by inoculating additional plants of Gb and the plants used for strain multiplication and all inoculations gave rise to successful sporulation. Results are presented in Table 6.1.

**Table 6.1** Sporulation on the additional pots used to check the viability and infection ability of the inoculum for the three-strain mixed experiment, showing the number of pots containing at least one sporulating plant, in parentheses, (total sporulating plants), observed day 14 post-inoculation. On day 14 post-inoculation, all sporulating plants from all treatments were collected for strain detection, using the Co 2-2 microsatellite marker. In square brackets I show the number of plants found to harbor the infecting strain. Single strains were inoculated on the habitual plant ecotype it had been multiplied on (Ei-2 for EMCO, Cvi for EMWA, Col-0 for WACO), while mixed inocula were used only on the habitual plant ecotypes. Five pots each containing three plants were used for every treatment.

Treatment	Number of pots containing at least one sporulating plant (sporulating plants) [plants revealing the specific Co 2-2 marker phenotype]		
	Ei-2	Cvi	Col-0
EMCO	5 (9) [8]		
EMWA		5 (12) [10]	
WACO			5 (8) [5]
EMCO-EMWA	4 (7) [5-EMCO]	5 (8) [7-EMWA]	
EMCO-WACO	5 (8) [5- EMCO]		3 (4) [3-WACO]
EMWA-WACO		5 (10) [10- EMWA]	3 (5) [4- WACO]
3M	4 (9) [8- EMCO]	5 (12) [10- EMWA]	2 (4) [3- WACO]

Some strains were used in different experiments at different times. For example, EMCO and WACO were used twice and EMWA three times. Sporulation success and latent period for EMWA in the different experiments were quite consistent. WACO infected badly when inoculated alone in both experiments, in one not leading to any sporulation. Therefore, WACO infected poorly consistently in the two experiments. However, there was a large difference observed for EMCO, which infected well for one experiment but badly for the other (Table 6.2). This suggests that infection with EMCO was poorly repeatable between the general environmental conditions of the two experiments whereas infection with EMWA was more repeatable. In the following I will consider the results for each experiment separately.

## **1. Infection success, number of sporulating leaves, latent period of infection and rate of increase in sporulating leaves varied among inoculation treatments**

Except for the EMWA-FRI5 combination, for which every inoculated plant sporulated, the different inocula caused different infection success. Infection success of the different individual strains differed in most cases, i.e., except for between EMCO and EMWA in the three-strain mix experiment: EMCO-ORS3 ( $\chi^2 = 48.8$ ,  $df = 1$ ,  $p < 0.0001$ ), EMWA-WACO ( $\chi^2 = 76.1$ ,  $df = 1$ ,  $p < 0.0001$ ), three-strain mix: EMCO - EMWA ( $\chi^2 = 0.15$ ,  $df = 1$ ,  $p = 0.70$ ), EMCO-WACO ( $\chi^2 = 14.6$ ,  $df = 1$ ,  $p = 0.00013$ ), EMWA-

WACO ( $\chi^2 = 12.1$ ,  $df = 1$ ,  $p = 0.00050$ ). This implies that Gb ecotype is differently susceptible to infection of these different strains. Similarly, except for the three-strain combination, the number of sporulating leaves on infected plants varied among treatments, and differed between single inoculations (Table 6.2). Even for the EMWA-FRI5 combination, where infection success did not vary among treatments, the number of sporulating leaves per plant did vary, and vice versa for the three-strain combination, where there was no significant variation in number of sporulating leaves on infected plants despite heterogeneity for infection success.

In the experiments presented here, using a single host genotype, different strains of *H. arabidopsidis* often showed different latent periods when inoculated alone (Table 6.2), suggesting genetic differences for latent period among these strains. However, there was also some heterogeneity observed for the same strains among the different experiments, indicating an effect of general environmental conditions on latent period.

If strains do not interact or interfere with each other in the inoculum, one would expect that the mixed inoculations would infect similarly to the more successful single inoculation in the mix. Similarly, mixed inoculations would sporulate as fast as the more rapid strain in the mix. This is what I find in almost all cases. However, for some combinations, mixed inoculations showed lower infection success and longer latent period than the more successful inoculum. For example, the high dose mixed inoculum ORS3-EMCO infected less well ( $\chi^2 = 22.0$ ,  $df = 1$ ,  $p < 0.0001$ ) and sporulated later than ORS3 alone (Table 6.2). In the three-strain experiment some two-strain mixes had poorer infection success than the individual strains alone. Nevertheless, these results depend on the identities of the strains. The presence of WACO in a two-strain mixture depressed the infection success of EMCO ( $\chi^2 = 7.85$ ,  $df = 1$ ,  $p = 0.0051$ ) but not of EMWA ( $\chi^2 = 0.039$ ,  $df = 1$ ,  $p = 0.84$ ). But the combination of EMCO and EMWA infected far worse than either of those strains alone, compared to EMCO alone ( $\chi^2 = 10.8$ ,  $df = 1$ ,  $p = 0.0010$ ) and EMWA alone ( $\chi^2 = 8.54$ ,  $df = 1$ ,  $p = 0.0034$ ). This rather confusing result suggests that some combinations of strain mixtures interfere with each other and reduce infection success. The three-strain mix, on the other hand, had equivalent infection success to that observed for EMCO and EMWA, the two strains that infected well, alone. Once a plant became successfully infected in this experiment, number of sporulating leaves did not differ among the inoculation treatments (Table 6.2). Every two-strain mix in three-strain combination seemed to show longer latent period, although it was not significantly different from the single inoculations, but not for three-strain mix treatment.

The number of sporulating leaves increased over time for all combinations. Except for three-strain combination cumulative sporulating leaves varied among inoculation treatments (Tables 6.2-6.3; Figures 6.1-6.4). The rate of increase in number of sporulating leaves varies among treatments for the

EMCO-ORS3 and EMWA-WACO combinations (Table 6.3). This suggests genetic difference in sporulation success of the different strains, but I note that there were also differences for the same strains in different experiments. For example, EMCO alone in three-strain combination sporulated better than in EMCO-ORS3 combination (Figures 6.1 and 6.4).

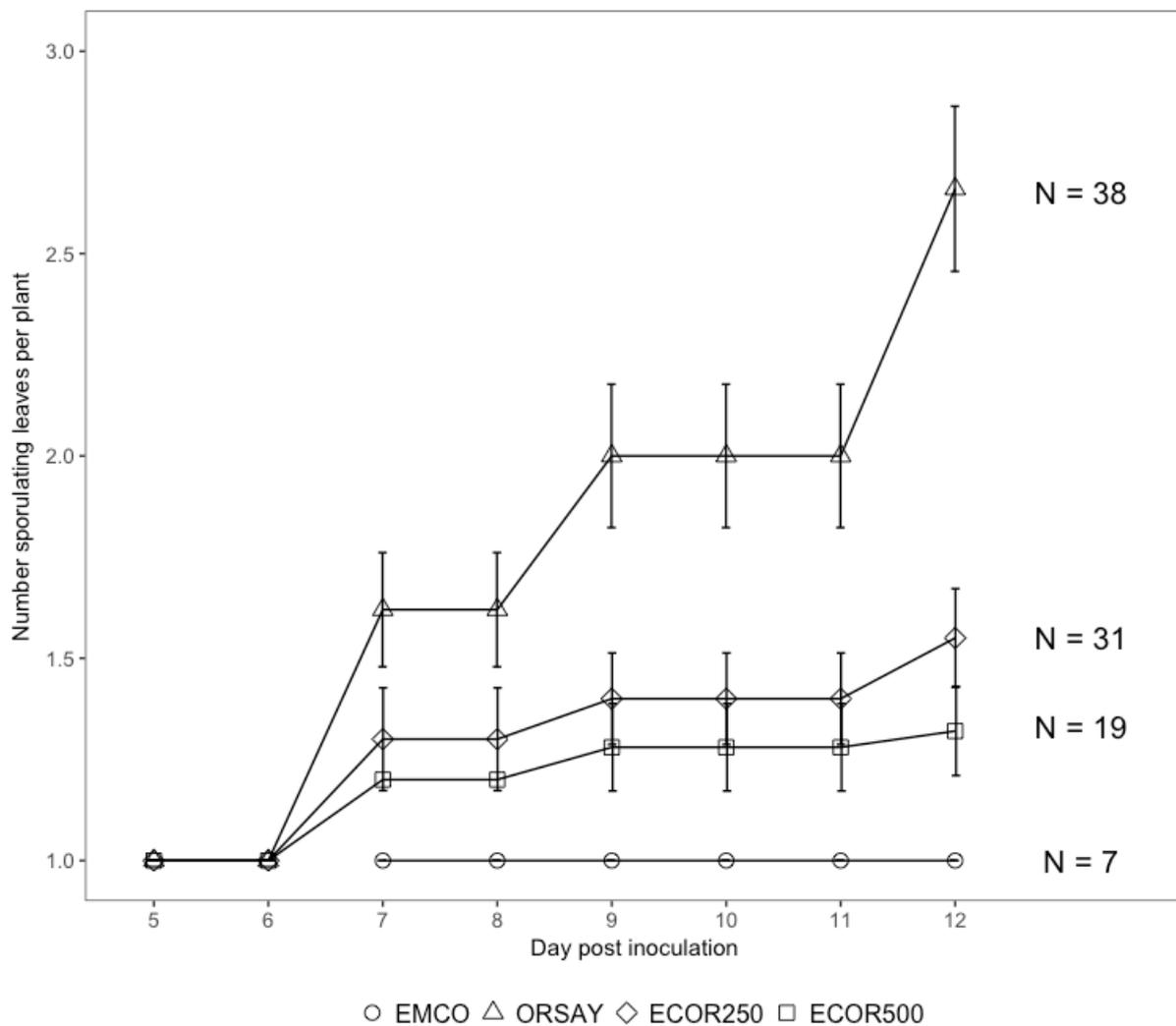
As mentioned above for infection success and latent period, if there is no interaction or interference between inoculated strains within a host after inoculation, mixed inoculations should show similar patterns of sporulation over time as the more infectious single strain in the mixture, as I observed for the EMWA-FRI5 combination (Figure 6.2). On the other hand, there was evidence of interference, with mixed inoculations showing less increase in sporulation over time than the more infectious single strain for the EMCO-ORS3 and EMWA-WACO combinations (Figures 6.1 and 6.3).

**Table 6.2** Summarised infection success i.e. whether an inoculated plant sporulated or not, average number of sporulating leaves on day last observed (day 10 for EMWA-WACO, day 12 for EMCO-ORS3 and EMWA-FRI5, day 14 for three-strain mix combination), and latent period (observed first sporulation), for different strain combinations with their inoculation treatments. Average latency and average number of sporulating leaves of each treatment indicated with different letters shows significant difference in each combination. “†” indicates number of sporulating leaves per pot containing at least one sporulating plant, while average number of sporulating leaves was determined per sporulating plants in the other all experiments.

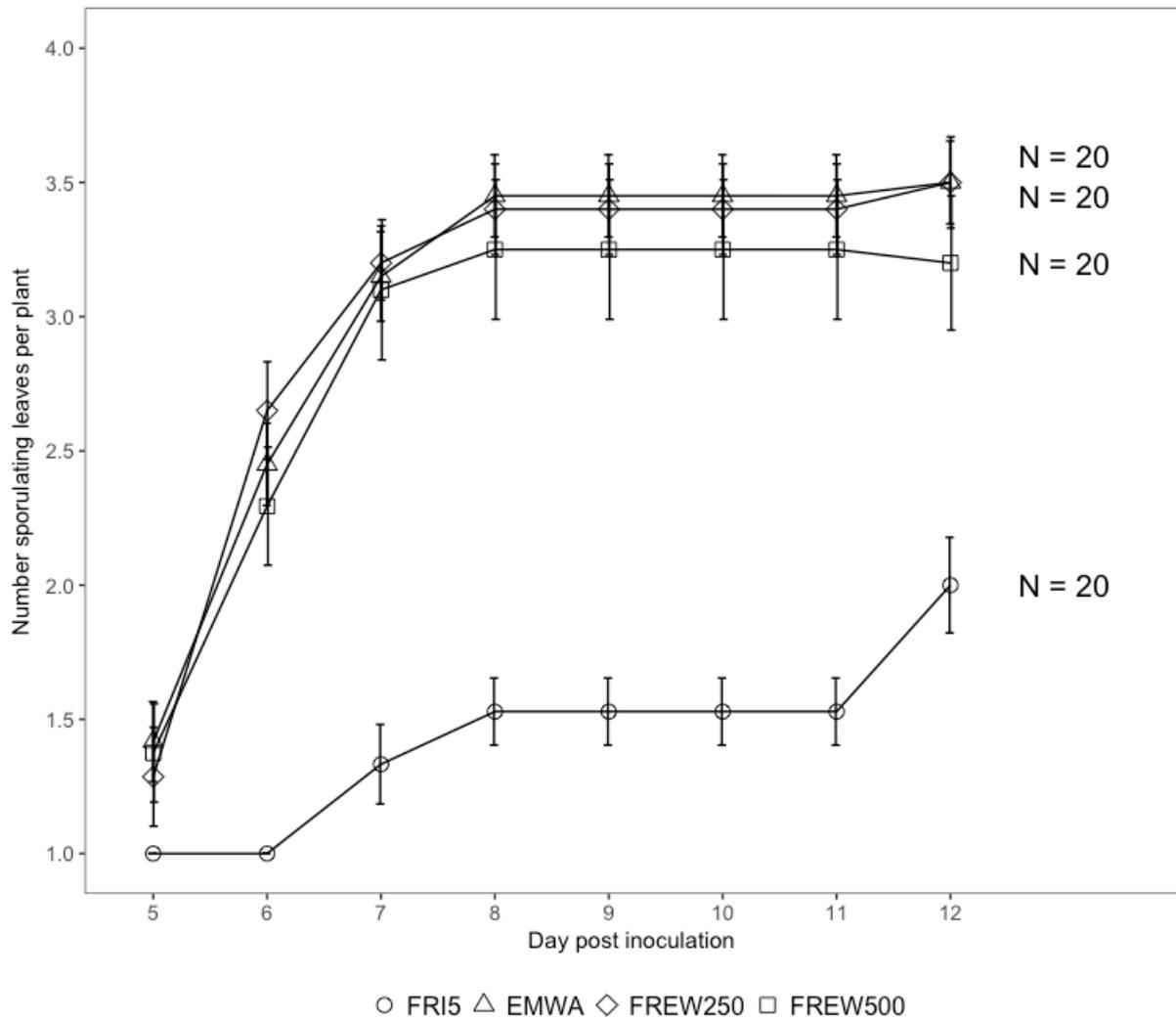
Strain combination	Treatment	Sporulating plants [total plants]	Average cumulative number of sporulating leaves ± SD	Average latency ± SE
EMCO-ORSAY	EMCO	7 [40]	1.00 ± 0.00b	10.33 ± 0.48a
	ORSAY	38 [40]	2.66 ± 1.38a	7.10 ± 0.21c
	ECOR250	31 [40]	1.55 ± 0.63b	7.47 ± 0.23bc
	ECOR 500	19 [40]	1.32 ± 0.52b	9.11 ± 0.29a
		$\chi^2 = 57.9$ , df= 3, $p < 0.0001$	$F_{(3, 91)} = 15.0$ , $p < 0.0001$	$F_{(3, 91)} = 11.9$ , $p < 0.0001$
EMWA-FRI5	FRI5	20 [20]	2.00 ± 0.79a	7.80 ± 0.43a
	EMWA	20 [20]	3.50 ± 0.69b	5.40 ± 0.11b
	FREW250	20 [20]	3.50 ± 0.76b	5.65 ± 0.11b
	FREW 500	20 [20]	3.20 ± 1.11b	5.74 ± 0.16b
		$\chi^2 = 0$ , df= 3, $p = 1$	$F_{(3, 75)} = 14.7$ , $p < 0.0001$	$F_{(3, 75)} = 20.0$ , $p < 0.0001$
EMWA-WACO	EMWA	39 [40]	2.39 ± 1.09a	6.15 ± 0.069a
	WACO	0 [40]	0 b	No sporulation
	EWWC250	33 [40]	1.88 ± 0.89a	6.27 ± 0.12a
	EWWC 500	38 [40]	1.71 ± 0.80a	6.13 ± 0.067a
		$\chi^2 = 119.7$ , df= 3, $p < 0.0001$	$F_{(3, 126)} = 34.3$ , $p < 0.0001$	$F_{(2, 107)} = 0.764$ , $p = 0.468$
Three-strain mix	EMCO (EC)	21 [60]	2.69 ± 2.18†	7.36 ± 0.31a
	EMWA (EW)	19 [60]	3.00 ± 1.89†	7.00 ± 0.23a
	WACO (WC)	4 [60]	1.50 ± 0.58†	9.33 ± 0.33b
	EC-EW	6 [60]	1.40 ± 0.89†	8.25 ± 0.63ab
	EC-WC	6 [60]	2.33 ± 1.15†	8.20 ± 0.37ab
	EW-WC	18 [60]	1.67 ± 0.98†	8.73 ± 0.24ab
	3M	24 [60]	2.46 ± 1.26†	7.61 ± 0.35a
		$\chi^2 = 38.9$ , df= 6, $p < 0.0001$	$(F_{(6, 54)} = 1.33$ , $p = 0.261$	$F_{(6, 58)} = 10.4$ , $p < 0.0001$

**Table 6.3** Analyses of Covariance with repeated measures testing how the number of sporulating leaves per plant for EMCO-ORS3 and EMWA-WACO combination or per pot for EMWA-FRI5 and three-strain mix combination varied among inoculation treatments, over time post-inoculation (in days) and with the interaction from generalised linear mixed-effect model with penalized quaslikelihood method using Poisson error distribution for count data. No sporulation was observed for the WACO treatment of EMWA-WACO combination. No slope estimate is given for the time effect when there was the significant interaction that indicated the slopes differed among the different treatments.

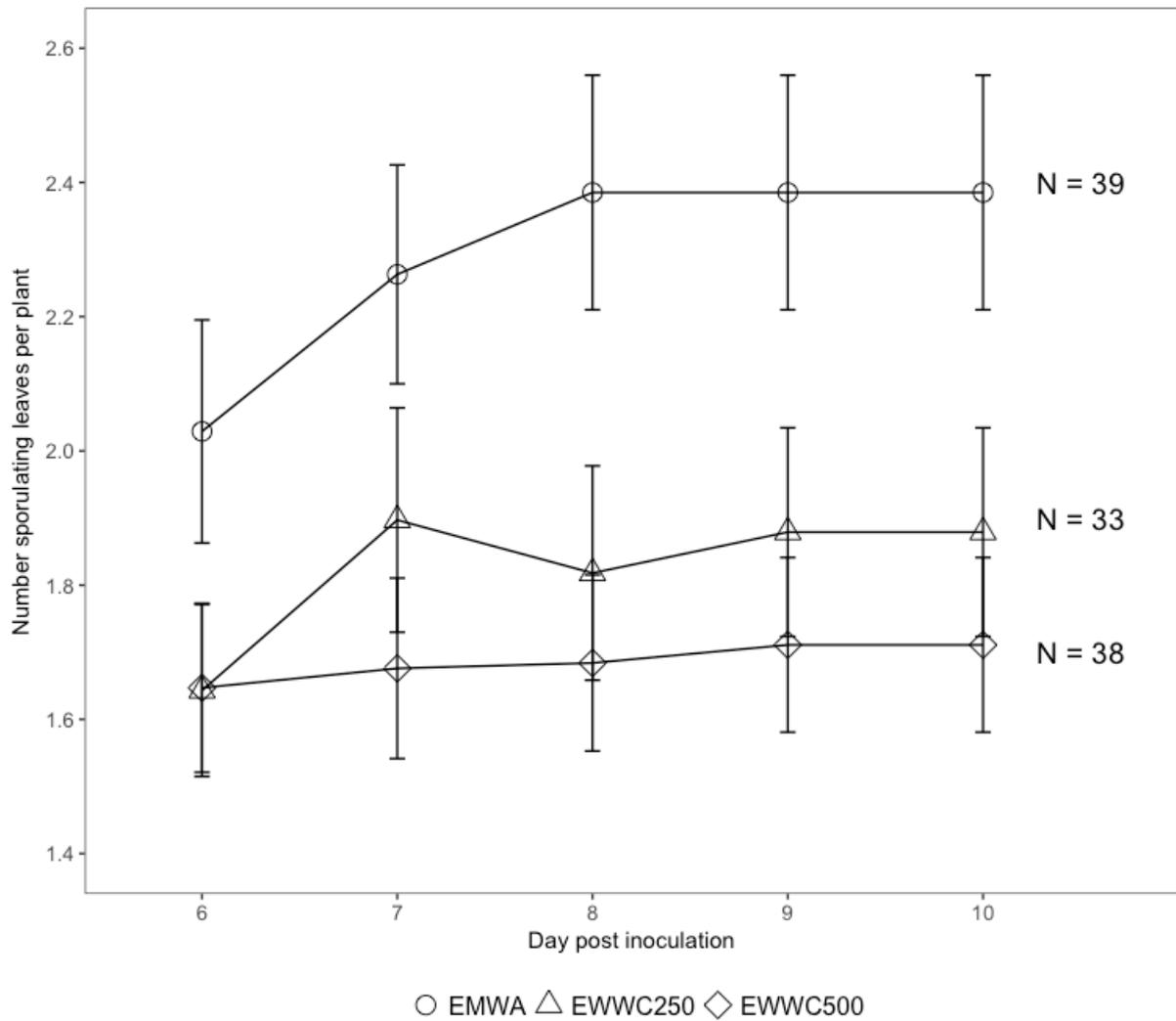
Combination	Effect	Df	Likelihood ratio $\chi^2$	<i>p</i>	Slope $\pm$ SE
EMCO-ORS3	Inoculation treatment	3	23.5	0.000032	
	Day post-inoculation	1	129.1	< 0.0001	
	Inoculation treatment x Day post-inoculation	3	38.5	< 0.0001	
EMWA-FRI5	Inoculation treatment	3	106.2	< 0.0001	
	Day post-inoculation	1	244.2	< 0.0001	0.62 $\pm$ 0.0042
	Inoculation treatment x Day post-inoculation	3	2.010	0.57	
EMCO-WACO	Inoculation treatment	3	7.01	0.030	
	Day post-inoculation	1	79.2	< 0.0001	
	Inoculation treatment x Day post-inoculation	3	19.4	0.000062	
Three-strain mix	Inoculation treatment	6	11.8	0.067	
	Day post-inoculation	1	146.9	< 0.0001	0.025 $\pm$ 0.0034
	Inoculation treatment x Day post-inoculation	6	7.87	0.25	



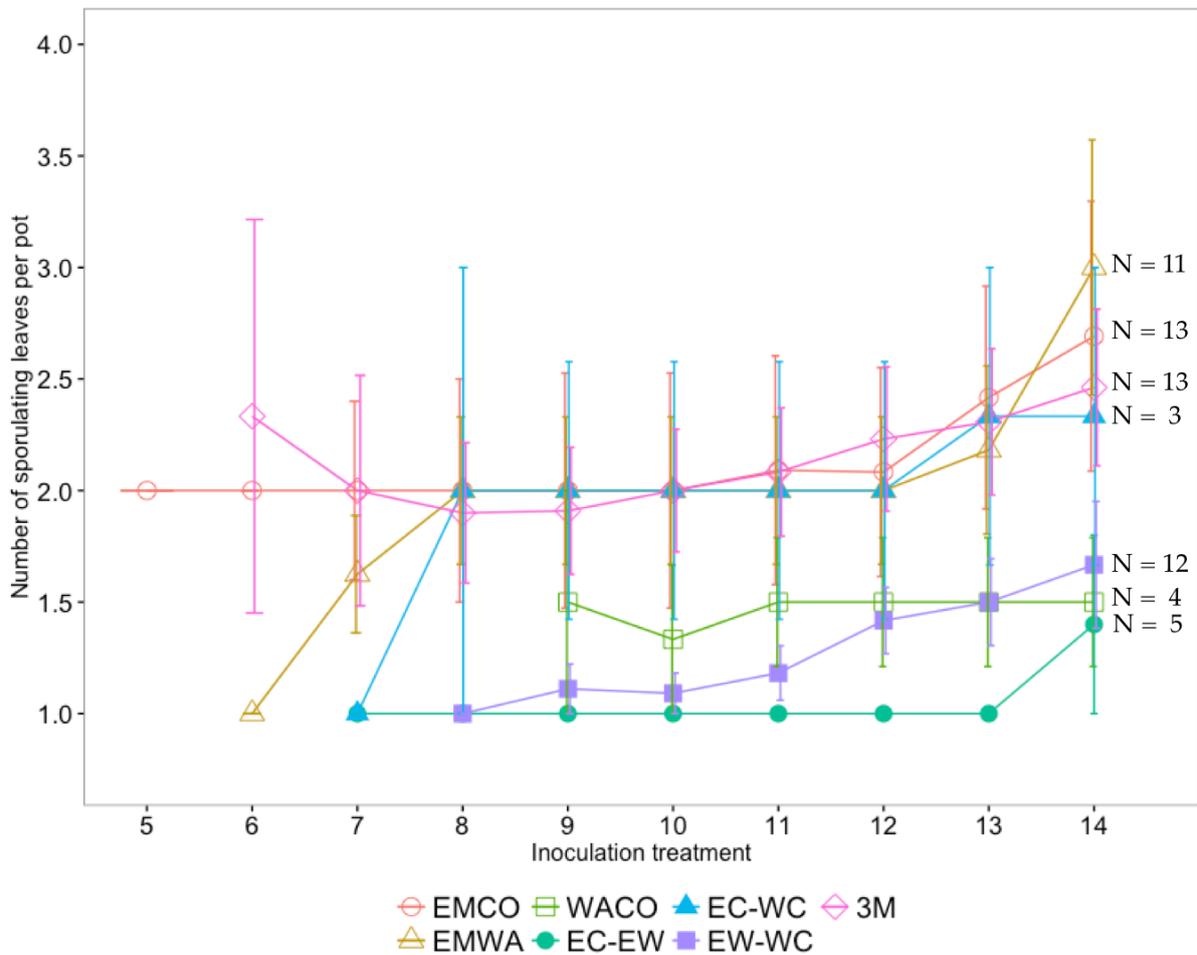
**Figure 6.1** Mean ( $\pm$  S.E.) cumulative number of sporulating leaves per sporulating plant for the different inoculation treatments of EMCO-ORS3 combination. Forty plants per treatment were initially inoculated. No sporulation was observed on control plants. N indicates the number of sporulating plants in each inoculation treatment at day 12 post-inoculation. The number of sporulating leaves increased over time for three treatments and differed in mean among the inoculation treatments, being highest for ORS3 (Table 6.3). Treatments differed in how the number of sporulating leaves increased over time (Table 6.3), with a significant slope ( $\pm$ SE) of ORS3 ( $0.26 \pm 0.012$ ) and ECOR250 ( $0.11 \pm 0.0096$ ) treatment.



**Figure 6.2** Mean ( $\pm$  S.E.) cumulative number of sporulating leaves per sporulating plant for the different inoculation treatments of the EMWA- FRI5 combination. Twenty plants per treatment were initially inoculated and all spore-inoculated plants sporulated. No sporulation was observed on control plants. N indicates the number of sporulating plants in each inoculation treatment at day 12 post-inoculation. The number of sporulating leaves differed among the inoculation treatments but increased over time for all treatments similarly, since there was no significant interaction between treatment and day post-inoculation (Table 6.3).



**Figure 6.3** Mean ( $\pm$  S.E.) cumulative number of sporulating leaves per sporulating plant for the different inoculation treatments of the EMWA-WACO combination, excluding the WACO treatment that did not sporulate at all. Forty plants per treatment were initially inoculated. No sporulation was observed on control plants. N indicates the number of sporulating plant in each inoculation treatment at day 10 post-inoculation. The number of sporulating leaves differed among the inoculation treatments and increased over time differently for the different treatments (Table 6.3), with a significant slope ( $\pm$ SE) for the EMWA ( $0.21 \pm 0.0078$ ) and EWWC500 ( $0.10 \pm 0.011$ ) treatments.



**Figure 6.4** Mean ( $\pm$  S.E.) cumulative number of sporulating leaves per pot containing at least one sporulating plant for the different inoculation treatments of three-strain mixed combination. No sporulation was observed on control plants. Twenty pots each containing three plants were used for each inoculation treatment. N indicates the number of pots containing at least one sporulating plant in each inoculation treatment at day 14 post-inoculation. Sporulation increased over time similarly for all treatments (see Table 6.3).

### *Variation in infection phenotypes among single strain inoculations*

The phenotypes of infection, including infection success, timing of parasite reproduction, or latent period, number of sporulating leaves and transmission success, may depend on the identity of both host and parasite. The expression of these phenotypes includes both the parasite effects and the host response (Shykoff & Kaltz, 1998). For example, host genotype-by-parasite genotype interactions explain variation in infection success in other parasite-host systems (Gray & Gill, 1993; Carius et al., 2001; Lambrechts et al., 2005; Carpenter et al., 2012; Råberg et al., 2014) as well as in the system studied here (Salvaudon et al., 2005, 2007, 2008). This is a general finding whenever multiple genotypes of hosts and/or parasites are used for experimental investigation of the interaction (van Mølken & Stuefer, 2011; Pariaud et al., 2013; Susi et al., 2015a; Susi et al., 2015b). Similarly, latent

period, an important life-history trait and fitness component of parasites that determines age at first reproduction, can vary with parasite and/or host identity in this (Héraudet et al., 2008) and other host-parasite systems (Carlisle et al., 2002; Montarry et al., 2010; Cooke et al., 2012). Furthermore the correlations among these component phenotypes, such as the trade-off between latent period and transmission or spore production (Héraudet et al., 2008; Pariaud et al., 2013) and the relationship between parasite fitness and virulence may also vary (Salvaudon et al., 2005) in a host-parasite genotype-specific manner.

In order to minimise the variation due to specific interactions with different plant genotypes, in all experiments I used a single *A. thaliana* genotype- Gb, which was shown to be susceptible to a large range of genotypes of *H. arabidopsidis*. This genotype was chosen to maximise the possibilities to obtain multiple infections (Salvaudon et al., 2007), to allow us to compare the consequence of infection of different strains alone and in combination. The findings presented here, therefore, are valid for this single host genotype, but details may differ for other hosts. Therefore I will mainly discuss differences due to parasite genotypic variation without considering the effects of host genotypic variation.

In these experiments there was a great deal of variation in the effects both among sets of parasite strains and among experiments. Here (Table 6.2), as has been previously observed (Héraudet et al., 2008, Salvaudon et al., 2005, 2007), different pathogen strains varied in latent period, number of sporulating leaves and disease transmission success, but this was not the case for all experiments. In general the strains with the poorer infection success also had longer latent period, but I found differences in latent period and number of sporulating leaves even for strains that did not differ in overall infection success. This shows that the different phenotypes of the infection, though related, are not completely dependent one on the other.

Some infection phenotypes are sensitive to various aspects of the environment, for example, temperature (see Scharsack et al., 2016 for a review). Indeed, infection success (Leicht & Seppälä, 2014; Coelho & Bezerra, 2006), parasite reproduction (Paull et al., 2015) and latent period (Shaw, 1990; Xu, 1999; Mariette et al., 2016) all can vary with temperature, implying that temperature may influence host response and/or parasite success. Because our different experiments were conducted at different times of the year, such background temperature differences could therefore explain some of the variation among experiments in absolute and relative phenotypes of the different parasite strains.

### ***Variation in infection phenotypes between single and co- inoculation***

In addition to temperature, the presence of another strain of pathogen may also represent an aspect of the environment that could influence infection phenotypes. Here I could compare infection phenotypes resulting after single versus mixed inoculation to address whether the presence of another strain influenced latent period, overall infection success (number of infected plants), overall parasite reproduction (number of sporulating leaves), and rate of increase in number of sporulating leaves.

If parasite strains do not interfere or compete then I would expect that mixed inoculations should have similar infection phenotypes to the more infectious, more virulent or more rapid single strain inoculations. However, different parasite strains may interfere with or facilitate each other. For latent period, if there is no interaction, I should see the first sporulating strains at approximately the same time whether they are inoculated alone or with another strain. This is indeed what I found for most but not all of the mixed inoculations. This was the case for EMWA-FRI5, EMWA-WACO and the three strain mix, where all mixed inoculations showed latent periods consistent with the more or most rapid strain in the inoculum. It was not, however, always the case for EMCO-ORS3, where the higher-dose mixed inoculation showed a latent period similar to that of the later- rather than the earlier-sporulating strain (Table 6.2). For the malaria pathogen *Plasmodium vivax*, incubation times (= latent period) following mixed inoculations were intermediate between those of the faster and the slower strains (Lover & Coker, 2015), implying that the presence of a slower strain interferes with the more rapid one.

For infection success, I found examples of interference but none of facilitation, though for some combinations infection success was similar between the single and the mixed treatments (Table 6.2). Similarly, mixed inoculations of the fungal endophyte *Epichloë bromicola* on the host plant *Bromus erectus* produced less infection than that single inoculations of some focal strains (Wille et al., 2002). This mixed inoculation experiment also documented that infection success of individual strains differed between single and mixed inoculation. Similar results were also found for *Plasmodium vivax* malaria (Lover & Coker, 2015). In contrast, facilitation, with higher infection success following mixed than single inoculations has been found for trematode parasites of snails (Karvonen et al., 2012, Gleichsner et al., 2018). This implies some interaction between co-infecting strains either in mixed inoculum or within a host in mixed infection, and its effect on overall infection success.

Similarly, being with another strain in the same host may influence overall parasite reproduction or individual genotype reproduction compared with when it infects alone. In my experiments, I found examples of interference, with fewer sporulating leaves and slower increase in number of sporulating leaves from mixed than single inoculation treatments, but not for all experiments. In other experimental systems interference has also been observed for parasite reproduction in mixed

experiments (Gower & Webster, 2005; Balmer et al., 2009; Ojosnegros et al., 2010). However sometimes there is no evidence of interference (de Roode et al., 2003; Wargo et al., 2007, Thompson et al., 2017), competitive suppression by more competitive parasites or by more virulent parasites (Taylor et al., 1997; de Roode et al., 2005a; de Roode et al., 2005b; Bell et al., 2006; Ben-Ami et al., 2008; Ben-Ami & Routtu, 2013), or even evidence for facilitation, with higher parasite reproduction from mixed than single inoculation (López-Villavicencio et al., 2010; Buono et al., 2014; Susi et al., 2015a; Susi et al., 2015b; Lindsay et al., 2016; Gleichsner et al., 2018).

## **2. Infection success of individual strains observed on inoculated leaves after mixed inoculations, and multiple infections found in few cases**

Success of the inoculation, presented above, tells whether inoculation led to infection or not. For mixed inoculations I identified the genotype(s) to determine which strain had infected the plant in two different ways. For the three-strain mixed experiment I collected all sporulating leaves from every inoculated plant and genotyped the spores with the discriminating microsatellite marker. Table 6.4 gives the results of which genotypes were detected from which sporulating leaves, and from which sporulating plants for three-strain mix combination. For the three-strain combination I found only nine leaves that contained two *H. arabidopsidis* genotypes at the same time (Table 6.4A). In the three-strain mix treatment, I also found co-infection of two inoculated strains on a single infected plant but from different sporulating leaves (Table 6.4B). This suggests that there might be the interference in mixed inoculum such that one strain suppresses the other at the level of each individual leaf.

## **3. Individual strains varied in infection success among different mixed inoculations when co-inoculated with different partner strains**

That co-inoculation of two strains resulted in reduced overall infection success compared with the more infectious single strain in some cases above suggests that there was interaction or interference between some inoculated strains in the mixed inoculum. Furthermore, this interaction or interference effect depended on the strain identities. Here, I examined whether infection success of individual strains varied among inocula differing in partner strains.

In the three-strain mixed inoculation experiment the infection success of the strains EMCO and EMWA differed depending on which partner strains it was co-inoculated with (EMCO: Wald  $\chi^2 = 13.5$ ,  $df = 2$ ,  $p = 0.0012$ ; EMWA: Wald  $\chi^2 = 10.6$ ,  $df = 2$ ,  $p = 0.0050$ ). This confirms that interference or competition depends on the identity or number of the interacting strains. EMCO co-inoculated with EMWA or with WACO showed lower infection success than when it was in the three-strain mixture inoculum. EMWA co-inoculated with EMCO had lower infection success than when it was mixed with WACO or in the three-strain mixture. However, infection success of WACO, which was overall

very low, did not vary among different mixed inocula (Wald  $\chi^2 = 2.13$ ,  $df = 2$ ,  $p = 0.34$ ; see Table 6.4). This suggests that in the mixed inoculation with more than two-strains some negative interactions can cancel each other out.

**Table 6.4** Summary of strain detection using the Co 2-2 microsatellite marker for all inoculation treatments of the three-strain mixed experiment: A. data are from each individually collected sporulating leaf; B. data from each plant. Some plants showed co-infection within the same leaves, as can be seen from A. Others had two strains but on different leaves, (shown in parentheses). No sporulation was observed on control plants. I performed up to 6 PCRs for each spore sample. I stopped when I obtained two clear consistent results or after 6 unsuccessful PCRs.

<b>A.</b>		Leaves revealing the specific Co 2-2 marker phenotype						
Treatment	Total sporulating leaves	EMCO	EMWA	WACO	EMCO&EMWA	EMCO&WACO	EMWA&WACO	3M
EMCO	34	32						
EMWA	33		27					
WACO	6			2				
EMCO-EMWA	7	2	2		1			
EMCO-WACO	7	4		1		1		
EMWA-WACO	20		13	4			2	
3M	32	14	10	1	2	1	2	0

<b>B.</b>		Plants revealing the specific Co 2-2 marker phenotype						
Treatment	Total Sporulating plants	EMCO	EMWA	WACO	EMCO&EMWA	EMCO&WACO	EMWA&WACO	3M
EMCO	21	21						
EMWA	19		19					
WACO	4			2				
EMCO-EMWA	6	2	2		1			
EMCO-WACO	6	4		1		1		
EMWA-WACO	18		12	4			2	
3M	24	10	7		1 (3)	1 (1)	1	0

### ***Strains interfere with each other in mixed inocula***

Every co-inoculation from the three-strain mix led to infection with both or all three strains but only in few cases did I find two strains on the same plant or in the same leaf. However, the mixed inoculations reduced infection success for all parasite strains, though the strains vary in the strength of their interference. For example, WACO interfered less with EMWA than it did with EMCO and the three-strain mixed inoculation showed less interference. Such reduction in infection success with genotype-specific effects are similar to what was found for *Epichloë bromicola* on its host *Bromus erectus* (Wille et al., 2002). However interference by growth-inhibition of the *Flavobacterium*

*columnare* bacteria increased with increasing number of strains (Kinnula et al., 2017) unlike what I found here.

#### **4. Transmission success of individual strains differs between plants infected by mixed inocula at low versus high dose**

For the other three experiments (EMCO-ORS3, EMWA-FRI5, EMWA-WACO) I used inoculated plants for transmission experiments and collected the sporulating leaves from recipient plants after transmission, for genotyping as above. For these two-strain combinations (Tables 6.5-6.7), though I found plants infected with two strains, I never detected more than one genotype in a single leaf. The poor infection success observed for WACO in single inoculation was reflected by its success in combination. WACO was always in the minority when present in a mix. However, although WACO failed to infect in the two-strain combination of EMWA-WACO, there was successful transmission from both mixed-inoculation treatments (Table 6.7), proving the viability of the spores.

In these experiments I could also compare transmission from plants that had been inoculated with mixed inoculum of high or low dose. If the interaction or interference between inoculated strains occurs when they confront each other, higher spore numbers of both strains will enhance strain-strain contact, and thus the probability and intensity of the interaction. Hence, there would be strong effect of interaction or interference on infection success of individual strains from mixed inoculum with higher spore concentration than the other with lower spore concentration. I tested the infection success of individual strains in every two-strain combination. Because in two of the two-strain combination experiments only one of the strains successfully transmitted to the recipient plants (respectively ORS3 and EMWA), I analysed infection success only for those strains from the EMCO-ORS3 and EMWA-FRI5 experiments (Table 6.5-6.6). Infection success of both individual strains in EMWA-WACO combination was examined between high- and low dose mixed inoculum.

Transmission success of ORS3, EMWA (when inoculated with FRI5) and WACO did not differ between low- and high dose inoculum (ORS3: Wald  $\chi^2 = 1.49$ , df= 1,  $p = 0.22$ ; EMWA: Wald  $\chi^2 = 3.44$ , df= 1,  $p = 0.063$ ; WACO: Wald  $\chi^2 = 0.21$ , df= 1,  $p = 0.65$ ). However, I found significantly more leaves infected with EMWA from the low dose mixed inoculum source plants than from either the high dose mixed inoculum source plants (Wald  $\chi^2 = 10.1$ , df= 1,  $p = 0.0014$ ) or from source plants inoculated with EMWA alone (Wald  $\chi^2 = 7.29$ , df= 1,  $p = 0.0069$ ; see Table 6.7). This shows a dose effect on infection success of an individual strain from mixed inoculum in one case, with low dose mixture stimulating EMWA.

**Table 6.5** Summary of strain detection using the Co 2-2 microsatellite marker for the EMCO-ORS3 experiment, on collected sporulating leaves of recipient plants after transmission. Transmission was from each sporulating source plant to a single recipient plant in an individual pot (4, 18, 14 and 10 sporulating source plants for EMCO, ORS3, ECOR250 and ECOR500 respectively) and from each of 20 controls. Five leaves from control plants, each leaf from a randomly chosen plant from a different pot, were tested by PCR. Two separate PCRs were performed for each sample and at least one revealed a clear reaction for all tested sporulating leaves. In no case did I find co-occurrence of inoculated strains on a single sporulating leaf. The mixed-inoculated source plants only transmitted the ORS3 strain. No PCR product was detected from leaves of control plants.

Treatment	Sporulating recipient plants	Total sporulating leaves on all recipient plants	Leaves revealing the specific Co 2-2 marker phenotype	
			EMCO	ORSAY
EMCO	1	2	2	
ORSAY	12	24		24
ECOR250	9	18	0	18
ECOR500	5	12	0	12

**Table 6.6** Summary of strain detection using the Co 2-2 microsatellite marker for the EMWA-FRI5 experiment, on collected sporulating leaves of recipient plants after transmission. Transmission was from each sporulating source plant to three recipient plants in an individual pot (each of 20 sporulating source plants for each treatment) and from each of 20 controls. Ten leaves from control plants, each leaf from a randomly chosen plant from a different pot, were tested by PCR. Two separate PCRs were performed for each sample and at least one revealed a clear reaction for all tested sporulating leaves. In no case did I find co-occurrence of inoculated strains on a single sporulating leaf. The mixed-inoculated source plants only transmitted the EMWA strain. No PCR product was detected from leaves of control plants.

Treatments	Sporulating recipient plants	Total sporulating leaves on all recipient plants (failed detection)	Leaves revealing the specific Co 2-2 marker phenotype	
			FRI5	EMWA
FRI5	45	80 (12)	68	
EMWA	48	136 (25)		101
FREW250	47	144 (35)	0	109
FREW500	45	106 (17)	0	89

**Table 6.7** Summary of strain detection using the Co 2-2 microsatellite marker for the EMWA-WACO experiment, on collected sporulating leaves of recipient plants after transmission. Transmission was from each sporulating source plant to a single recipient plant in an individual pot (19, 18 and 19 sporulating source plants for EMWA, EWWC250 and EWWC500 respectively) and from each of 20 controls. Five leaves from control plants, each leaf from a randomly chosen plant from a different pot, were tested by PCR. Two separate PCRs were performed for each sample and at least one revealed a clear reaction for all tested sporulating leaves. In no case did I find co-occurrence of inoculated strains on a single sporulating leaf. The mixed-inoculated source plants transmitted both the EMWA and WACO strain separately, found on different sporulating leaves of respective different plants. No PCR product was detected from leaves of control plants. EMWA was transmitted significantly better from source plants inoculated with the low-dose mixed inoculum.

Treatments	Sporulating recipient plants	Total sporulating leaves on all recipient plants	Leaves revealing the specific Co 2-2 marker phenotype	
			EMWA	WACO
EMWA	13	30	30	
WACO	0	-	-	-
EWWC250	15	50	47	3
EWWC500	11	29	27	2

### ***Dose effect on transmission success of individual strain***

In two of the three experiments, only the more infectious of the two strains transmitted successfully from either dose of original inoculum. Though we can not know that the other strain did not infect the plants following the mixed inoculation, this result is strongly suggestive of competitive exclusion or suppression of the less infectious by the more infectious strains in these two cases (Gleichsner et al., 2018). However, in the third case, even the less infectious WACO, which failed to sporulate after single inoculation, transmitted successfully and appeared to facilitate transmission of its partner EMWA at low-dose co-inoculation.

### ***Co-existence from co-inoculations***

I found no effect of dose on the coexistence of two strains. For two strain combinations there was no co-existence, at either initial dose, for the third strain combination there was coexistence at both. Coexistence of co-infecting strains might be influenced by several factors, i.e. competitive exclusion (Nowak & May, 1994; May & Nowak, 1995; Mosquera & Adler, 1998, Wille et al., 2002), relatedness of infecting parasites (López-Villavicencio et al., 2007), and host immune response (Choisy & de Roode, 2010). Often, the more virulent strain excludes the less virulent one (Gleichsner et al., 2018).

## 5. Relationship between transmission and latent period

When testing how the number of sporulating leaves on recipient plants varied with latent period of infection on source plant, I found the number of sporulating leaves on recipient plants increased with increasing latent period of infection only in the EMCO-ORS3 combination (Tables 6.8), suggesting that newly produced spores from later sporulation infected better.

**Table 6.8** Analyses of Covariance with repeated measures testing how the number of sporulating leaves per recipient plant for EMCO-ORS3 and EWWA-WACO combinations or per pot containing at least one sporulating recipient plant for EMWA-FRI5 combination varied among inoculation treatments, as a function of latent period (in days) of the infection of the source plant and with the interaction from generalised linear model using Poisson error distribution for count data. The analysis of EMWA-WACO excluded the WACO treatment that did not sporulate on source plant and transmit at all. Slope estimate is given for the latent period effect when it was significant.

Combination	Effect	Df	Likelihood ratio $\chi^2$	<i>p</i>	Slope $\pm$ SE
EMCO-ORS3	Inoculation treatment	3	5.15	0.16	
	Latent period	1	3.87	0.049	0.21 $\pm$ 0.11
	Inoculation treatment x Latent period	3	1.90	0.59	
EMWA-FRI5	Inoculation treatment	3	9.66	0.022	
	Latent period	1	3.20	0.074	
	Inoculation treatment x Latent period	3	3.92	0.27	
EMCO-WACO	Inoculation treatment	2	8.58	0.014	
	Latent period	1	0.55	0.46	
	Inoculation treatment x Latent period	2	5.91	0.052	

### *The relationship between transmission and latent period from mixed inoculations*

Since transmission is an important component of parasite fitness that might correlate with virulence of infection, many studies have explored how this relationship varies with host and parasite genotype (Alizon & van Baalen, 2005; Bull & Luring, 2014). For this plant-pathogen system, we already know from single strain infection that both host and parasite genotypes influence the relationship between transmission and virulence (Salvaudon et al., 2005, 2007) or transmission and latent period, (Héraudet et al., 2008). Nonetheless, how the relationship between infection phenotypes differs between mixed and single infections is rarely explored.

Here I found that transmission increased with increasing latent period only in the EMCO-ORS3 experiment, but there was no effect of inoculation treatments on this relationship, i.e. there was no

significant interaction (Table 6.8). Furthermore, only the more infectious strain, ORS3, successfully transmitted, even from the mixed inoculation treatments, which may explain the absence of a treatment effect. I have found no studies that test the relationship between transmission and latent period in mixed infections, though there are examples that find no effect of mixed infections on the relationship between transmission and virulence (Doumayrou et al., 2013; Ben-Ami, 2017).

## **Effect of infection and co-infection of *H. arabidopsidis* strains on the fitness and some vegetative phenotypes of *A. thaliana* Gb ecotype**

I estimated several different fitness-related and vegetative phenotypic traits, though not all for all experiments. Overall, infected plants from some treatments of all three experiments differed in some measured phenotypes from the control plants (Table 6.9). Below I also compared the different inoculation treatments by analysing the difference in phenotype between each infected plant and the mean of the controls. For reproductive traits this is an estimate of virulence.

### **6. Phenotypes of infected plants varied among inoculation treatments**

There was a lot of heterogeneity among the different experiments. Infected plants showed lower phenotypic values for reproductive traits than uninfected ones for two experiments but higher in the third (Table 6.9). Indeed, this pathogen has been observed to induce negative virulence in the past (Salvaudon et al., 2005, 2007). Specific strains did not always lead to similar phenotypes when they were inoculated in different experiments. For example, infection by EMCO, which was used in two different experiments, led to lower total fitness than controls in both experiments (though this was significant in only one). Infection by EMWA, on the other hand, led to a significant decrease in fitness in one experiment and a slight increase in the other. This suggests that environmental conditions, that vary even in the greenhouse, for example with changing time of year, influenced the phenotype of the infection. Overall, however, I found significant effects of infection on plant fitness.

Total fitness, which summarized the number and size of siliques on different branches, varied among experiments, with greater reproduction in the three-strain mix experiment than in the other two experiments. Total fitness varied significantly among inoculation treatments in each experiment as did some vegetative traits in some experiments, e.g. number of branches in EMWA-FRI5 and plant height in three-strain mix experiment, but there was no overall consistent effect either of infection of single versus mixed inoculation (Table 6.9).

**Table 6.9** Average measured phenotypes of infected plants for different strain combinations with their inoculation treatments. Bushiness was determined as number of branches per inflorescence for EMWA-FRI5 combination, and as number of inflorescences per plant for three-strain mix. Average measured phenotypes of each treatment indicated with different letters shows significant difference in each combination.

Strain combination	Treatment	Total fitness $\pm$ SE	Seed weight (g) $\pm$ SE	Total siliques $\pm$ SE	Plant Height (cm) $\pm$ SE	Bushiness $\pm$ SE
EMCO-ORS3 (day inoculation: 24-12-2014)	Control	156.99 $\pm$ 6.90a	148.21 $\pm$ 6.91a	123.65 $\pm$ 5.76a	48.95 $\pm$ 1.03	
	EMCO	148.95 $\pm$ 11.7ab	119.73 $\pm$ 22.9ab	117.33 $\pm$ 6.57ab	52.50 $\pm$ 2.02	
	ORS3	144.99 $\pm$ 7.25ab	151.61 $\pm$ 7.55a	115.58 $\pm$ 4.16ab	48.29 $\pm$ 1.17	Not measured
	ECOR250	120.88 $\pm$ 7.03b	99.90 $\pm$ 9.33b	95.29 $\pm$ 4.43b	45.81 $\pm$ 1.11	
	ECOR500	123.22 $\pm$ 13.9ab	122.90 $\pm$ 14.3ab	93.89 $\pm$ 9.68ab	47.67 $\pm$ 2.67	
			$F_{(4, 63)} = 4.41, p = 0.0033$	$F_{(4, 63)} = 7.17, p < 0.00001$	$F_{(4, 63)} = 4.27, p = 0.0041$	$F_{(4, 63)} = 1.46, p = 0.22$
EMWA-FRI5 (day inoculation: 24-5-2015)	Control	149.21 $\pm$ 10.9 b		124.45 $\pm$ 7.80		12.7 $\pm$ 0.66a
	FRI5	190.64 $\pm$ 10.5 ab		134.85 $\pm$ 3.82		13.2 $\pm$ 0.80a
	EMWA	175.95 $\pm$ 11.9 ab	Not measured	136.15 $\pm$ 7.82	Not measured	10.9 $\pm$ 0.55ab
	FREW250	192.47 $\pm$ 13.4 ab		146.11 $\pm$ 9.64		9.8 $\pm$ 0.43b
	FREW 500	197.50 $\pm$ 8.78 a		146.83 $\pm$ 6.24		12.5 $\pm$ 0.55a
			$F_{(4, 92)} = 3.04, p = 0.021$	$F_{(4, 92)} = 1.58, p = 0.19$		$F_{(4, 92)} = 5.07, p = 0.00097$
Three-strain mix (day inoculation: 25-11-2015)	Control	327.91 $\pm$ 10.9a	120.45 $\pm$ 5.28b	302.68 $\pm$ 14.1b	49.61 $\pm$ 0.74a	5.48 $\pm$ 0.27
	EMCO	288.16 $\pm$ 14.1b	86.83 $\pm$ 7.61c	223.38 $\pm$ 18.2c	47.78 $\pm$ 0.96ab	6.41 $\pm$ 0.33
	EMWA	274.05 $\pm$ 14.8bc	74.94 $\pm$ 8.86c	208.16 $\pm$ 19.2c	44.56 $\pm$ 1.01b	7.00 $\pm$ 0.35
	WACO	355.97 $\pm$ 32.3a	166.63 $\pm$ 15.6a	276.00 $\pm$ 41.7bc	46.80 $\pm$ 2.19ab	6.00 $\pm$ 0.82
	EMCO-EMWA	309.35 $\pm$ 26.3ab	148.62 $\pm$ 12.8a	454.83 $\pm$ 34.1a	46.12 $\pm$ 1.79ab	5.67 $\pm$ 0.56
	EMCO - WACO	237.86 $\pm$ 26.3c	59.48 $\pm$ 12.8cd	177.83 $\pm$ 34.1c	49.45 $\pm$ 1.79ab	6.33 $\pm$ 0.61
	EMWA - WACO	350.38 $\pm$ 15.2a	112.48 $\pm$ 7.36b	261.50 $\pm$ 19.7b	46.77 $\pm$ 1.03ab	5.83 $\pm$ 0.29
	3M	187.52 $\pm$ 13.2d	38.88 $\pm$ 6.38d	273.54 $\pm$ 17.0b	43.78 $\pm$ 0.90b	6.86 $\pm$ 0.59
			$F_{(7, 125)} = 14.0, p < 0.0001$	$F_{(7, 125)} = 22.9, p < 0.0001$	$F_{(7, 125)} = 8.43, p < 0.0001$	$F_{(7, 125)} = 4.85, p < 0.0001$

MANOVAs on combined measured traits revealed variation among inoculation treatments for every experiment (Tables 6.10).

**Table 6.10** Multivariate Analyses of Variance showing that the combined phenotype varied among inoculation treatments for three experiments (combined phenotype for EMCO-ORS3 experiment: total fitness, total seed weight (mg), plant height (cm) and total number of siliques; for EMWA-FRI5 experiment: total fitness, total number of siliques, and bushiness (number of branches per plant); for three-strain mix experiment: total fitness, total seed weight (mg), total number of siliques, plant height (cm), bushiness (number of inflorescences per plant), and rosette diameter (cm)).

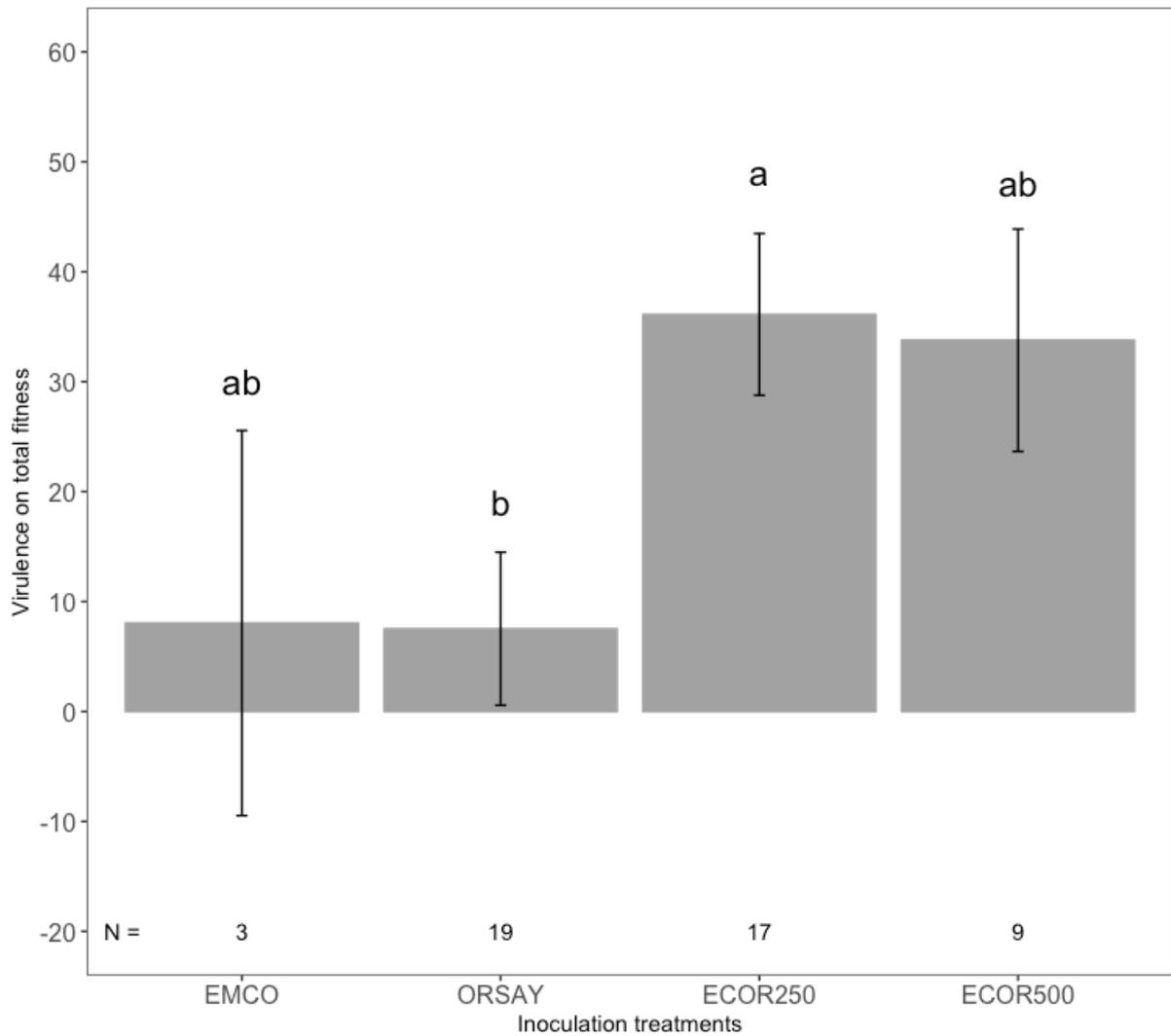
combination	Effect	Df	Wilks'λ	Df (Num, Den)	F	p
EMCO-ORS3	Inoculation treatment	4	0.55	16, 183.9	2.52	0.0016
	Residuals	63				
EMWA-FRI5	Inoculation treatment	4	0.61	12, 238.4	4.11	< 0.001
	Residuals	92				
Three-strain mix	Inoculation treatment	7	0.11	42, 566.3	8.02	< 0.001
	Residuals	125				

### ***Fitness of plants infected with the same individual strains varied among experiments***

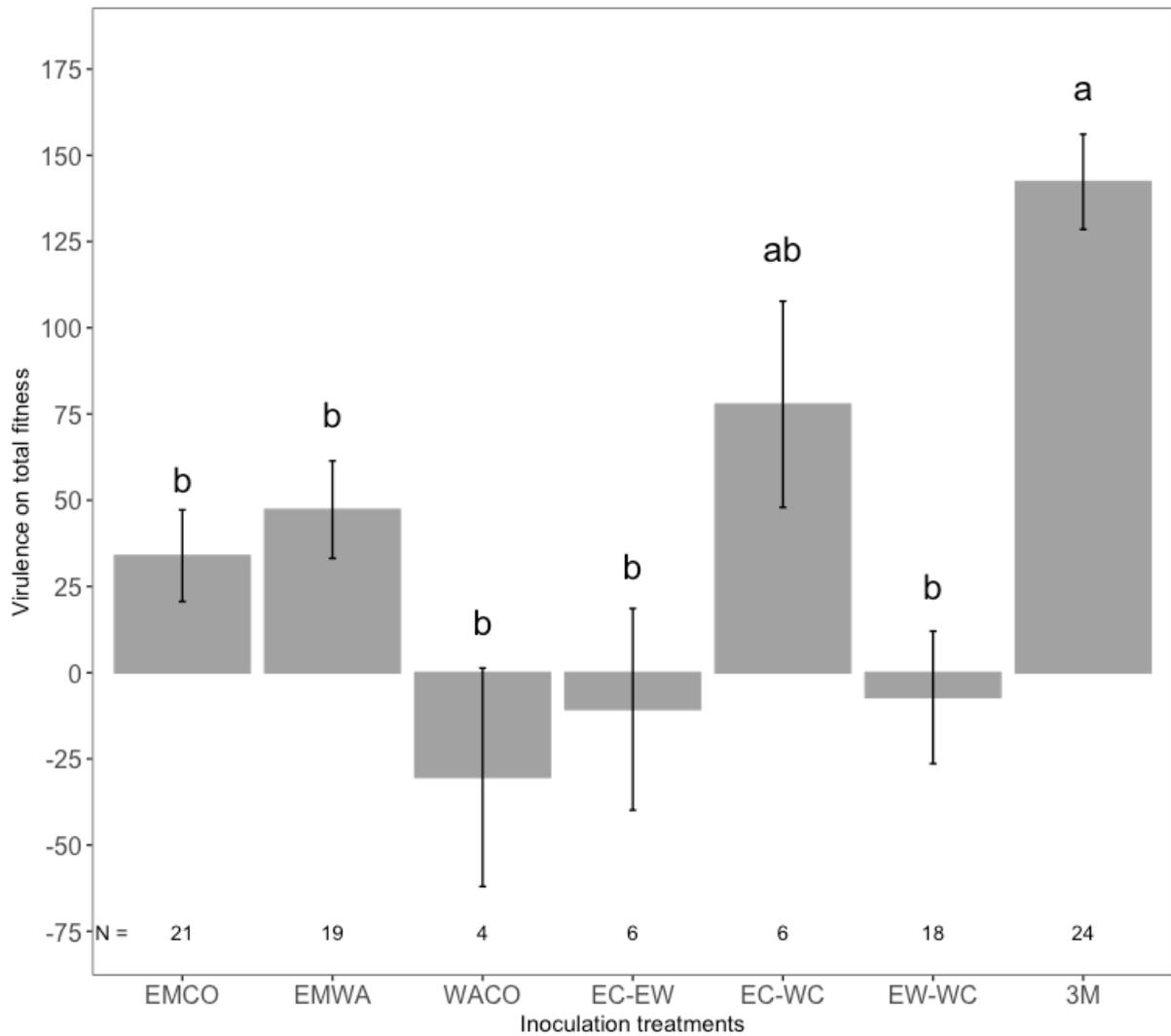
Plant phenotypes and infection phenotypes varied among the different experiments, possibly due to differences in environmental conditions linked to seasonal differences, such as temperature (Guinnee & Moore, 2004; Mitchell et al., 2005; Vojvodic et al., 2011; Paull et al., 2015). In my experiments, even plants infected by the same individual strains showed differences in total plant fitness across different experiments (Table 6.9). For example, plants singly infected with EMCO and EMWA in the two-strain mix experiments had lower total plant fitness than those from three-strain mix experiment (Table 6.9), even though all experiments were conducted in the same growth chamber under the same growth condition. Indeed, each experiment was carried out at different period of the year or in different years. This could generate variation in host fitness infected by the individual strain in different experiments. The effect of infection on plant phenotypes also varied among the measured traits, for example between vegetative and reproductive characters. There was no clear relation between reproductive or fitness traits and vegetative ones although I found differences among the inoculation treatments for some but not all traits. Other studies have found differences between healthy and infected plants for several vegetative (Jerling & Berglund, 1994; Piqueras, 1999; Pan & Clay, 2003; Desprez-Loustau et al., 2014), reproductive (Salvaudon et al., 2005, 2007, 2008; López-Villavicencio et al., 2010; Buono et al., 2014), and both kinds of trait in the same experiments (Pagán et al., 2007; Salvaudon & Shykoff, 2013; Hily et al., 2016) in this and other systems.

## **7. Virulence on total fitness differs between single infection and infection from mixed inoculation**

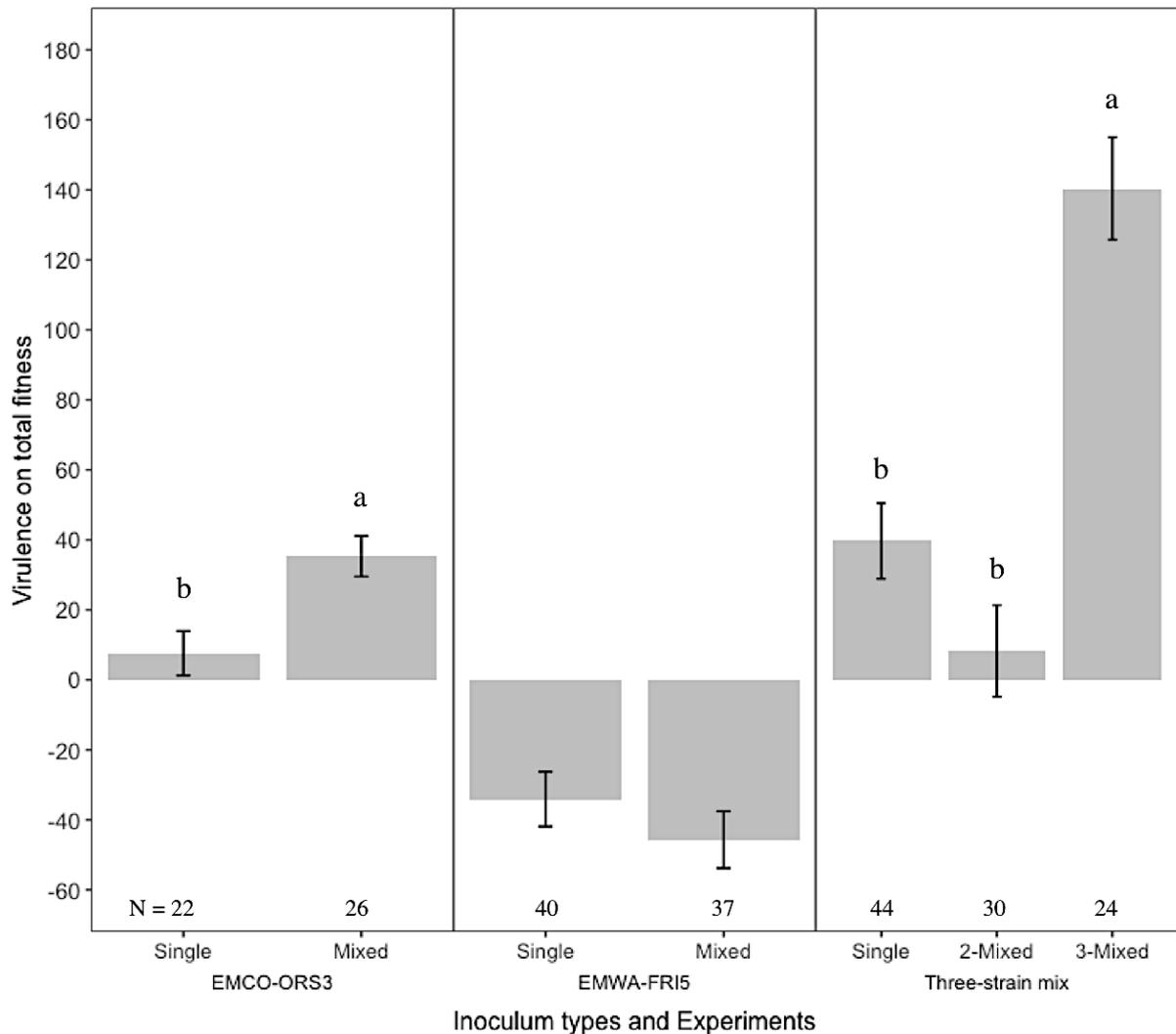
The results presented above can be converted to measures of phenotypic differences between control and infected plants, which is a measure of virulence when it concerns fitness trait, discussed here for total plant fitness. I found that virulence for total fitness showed a lot of heterogeneity across the three experiments varying in magnitude and sign, varying among the inoculation treatments for the two experiments that showed positive virulence (Figures 6.5-6.7). In the EMCO-ORS3 experiment, virulence did not differ between the two different single infections or between the two mixed inoculation treatments at different doses. Nonetheless there was a significant difference in the (low) virulence of infection with the single strain, ORS3, and the (higher) virulence of the infection from the low dose mixed inoculum (Figure 6.5). In the EMWA-FRI experiment virulence was globally negative and did not vary among treatments (data not shown). In the three-strain mix experiment there was no variation in virulence among infected plants from the different single inoculations but infected plants from the three-strain inoculation mixture suffered significantly higher virulence than those infected with any single strain. However this was not the case for two-strain inoculation combinations (Figure 6.6). In summary, mixed inoculation treatments sometimes but not always showed higher virulence than single inoculations (Figure 6.7).



**Figure 6.5** Mean ( $\pm$  SE) for virulence of parasite infection as expressed for total fitness on day 60 post-inoculation among inoculation treatments of EMCO-ORS3 strain experiment. The virulence of infection for each plant was calculated as the mean total fitness over all control plants minus its total fitness. An ANOVA revealed the significant difference of virulence of infection on total fitness among inoculation treatments ( $F_{(3,44)} = 3.32, p = 0.028$ ). Treatments marked with different letter(s) above the bars differed significantly tested by the Tukey multiple comparisons method. N indicates the number of measured plants for each inoculation treatment.



**Figure 6.6** Mean ( $\pm$  SE) for virulence of parasite infection as expressed for total fitness on day 60 post-inoculation among inoculation treatments of three-strain mix experiment. The virulence of infection for each plant was calculated as the mean total fitness over all control plants minus its total fitness. An ANOVA revealed the significant difference of virulence of infection on total fitness among inoculation treatments ( $F_{(6,91)} = 12.3, p < 0.0001$ ). Treatments marked with different letter (s) above the bars differed significantly tested by the Tukey multiple comparisons method. N indicates the number of measured plants for each inoculation treatment.



**Figure 6.7** Mean ( $\pm$  SE) for virulence of parasite infection as expressed for total fitness on day 60 post-inoculation between/among inoculum types of three experiments. The virulence of infection for each plant was calculated as the mean total fitness over all control plants minus its total fitness. An ANOVA revealed the significant difference of virulence of infection on total fitness between inoculum types for EMCO-ORS3 ( $F_{(1, 46)} = 10.4, p = 0.0023$ ) and among inoculum types for three-strain mix experiment ( $F_{(2, 95)} = 24.4, p < 0.0001$ ), but not for EMWA-FRI5 experiment ( $F_{(1, 75)} = 1.06, p = 0.31$ ). Inoculum types marked with different letter(s) above the bars differed significantly tested by the Tukey multiple comparisons method. N indicates the number of measured infected plants for each inoculum type.

### ***Virulence on total fitness differs between single infection and infection from mixed inoculation***

If different parasite strains interact within-host if they co-occur after co-inoculation, overall virulence of infection from mixed inoculation may differ from that from single inoculations (Alizon et al., 2013). Many experiments, from various host-pathogen systems, have shown that virulence from mixed infections differs from that of single infections (Table 1B). For a few examples, multiple infections

may lead to higher virulence than single infections (Taylor et al., 1998; Davies et al., 2002; López-Villavicencio et al., 2010; Susi et al., 2015a; Susi et al., 2015b; Lindsay et al., 2016; Kinnula et al., 2017), intermediate level between two infecting genotypes (Harrison et al., 2006; Ben-Ami et al., 2008; Balmer et al., 2009; Pollitt et al., 2014; Thompson et al., 2017), or lower than both single infections (Schurch & Roy, 2004; Gleichsner et al., 2018), depending on what kind of interaction within-host occurs (Buckling & Brockhurst, 2008), as mentioned in the introduction. Here I found that mixed inoculations from the EMCO-ORS3 experiment caused higher virulence than the single inoculations (Figure 6.7). Furthermore, infected plants of three-strain mixture in three-strain mix experiment suffered higher virulence than all single- and most two-strain mixed inoculations (Figures 6.6 and 6.7). Although I did not find this increase in virulence for infections from mixed inoculations in all experiments, this provides suggestive evidence that the interaction between different parasite strains either in the inoculum or in the plant leads to higher virulence. In latter case, increased number of genotypes in the inoculum contributed higher virulence. Unfortunately I do not know how many strains really infected these plants. However the significant effect of inoculation treatment on virulence suggests that there may indeed have been co-infection and that genetic diversity of conspecific parasites affected virulence (Ben-Ami & Routtu, 2013; Kinnula et al., 2017; Gleichsner et al., 2018). In other words, increasing the number of diversity of infecting genotypes modifies competitive interaction within-host, generating higher virulence.

### ***Virulence of infection from mixed inoculation somehow reflects interaction between strains***

As discussed by Alizon et al. (2013), the overall virulence resulting from multiple infection does not predict the trajectory of virulence evolution, e.g. selection within-host. However, some studies (i.e., Balmer et al., 2009; Inglis et al., 2009; Ojosnegros et al., 2010; Bashey, et al., 2012; Gil-Salas et al., 2012; Lass et al., 2013; Salvaudon et al., 2013; Breyta et al., 2016; Tollenaere et al., 2017) distinguished reproductive performance of each infecting genotype in mixed infection, to explore selection within a host. Indeed, mixed infection may cause higher virulence even though the individual parasite strain with higher virulence may not have the highest within-host fitness (Kinnula et al., 2017). In my three-strain experiment, I genotyped each individual strain after mixed inoculation on the same plants used to estimate virulence. Furthermore, I found no variation in virulence among the three single strain infections. The parasite genotype EMCO was predominant in infections following mixed inoculations, so increase in virulence cannot be associated with an increase in the frequency of a highly virulent strain, but rather in a plastic response in the mixture itself. Indeed, in absence of significant variation in virulence of the individual strains, one cannot predict an evolutionary trajectory for virulence.

## 8. Correlation between virulence and parasite reproduction (infection intensity) and latent period

Within the experiments we saw variation in infection phenotypes such as date of first sporulation (latent period) and infection intensity (number of sporulating leaves). I tested for co-variation between virulence and these phenotypes of infected plants. In the two-strain experiments, I found no significant covariation between infection phenotypes and virulence. In the three-strain mix experiment, virulence increased with increasing number of sporulating leaves and decreased with increasing latent period (Table 6.11). More intense infections that sporulated earlier depressed plant fitness more.

### *Virulence co-varies with parasite reproduction (infection intensity) and latent period*

Many studies have found a relationship between virulence and other infection phenotypes that may reveal trade-offs expressed by different parasite genotypes (see Table 1 in Laine & Barrès, 2013). For mixed infections, however, no relationship between virulence and transmission (Doumayrou et al., 2013; Ben-Ami, 2017) has been observed. However, mixed inoculation can lead to variation in infection phenotypes, in my case, sometimes decreasing infection intensity and extending latent period. Therefore it may cause changes in the relationship between virulence and these infection phenotypes. However I found no evidence for this. In my three-strain mix experiment, virulence increased with infection intensity while decreasing with latent period, with no interactions with treatments (Table 6.11).

**Table 6.11** Analyses of Covariance testing how virulence observed on day 60 post-inoculation varied among inoculation treatments, with cumulative number of sporulating leaves on day last observed (day 14), over latent period (in days) and with the interactions for the three-strain mix experiment. Virulence is measured as the difference in total fitness for each infected plant minus the mean of all control plants.

Effect	df	F	p	Slope ± SE
Inoculation treatment	6	16.4	< 0.0001	
Cumulative sporulating leaves	1	15.7	0.00017	27.4 ± 10.3
Latent period	1	6.08	0.016	-6.07 ± 2.60
Inoculation treatment x Cumulative sporulating leaves	6	1.97	0.080	
Inoculation treatment x Latent period	6	1.79	0.11	
Residuals	77			

## VII. SEQUENTIAL INOCULATION EXPERIMENT

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### Parasite strains

In this experiment I used three laboratory strains, EMCO, EMWA and WACO, described above, which are distinguishable using the Co 2-2 molecular marker. Inoculum of each strain was prepared as described for the co-inoculation experiment and adjusted their concentrations to  $5.0 \times 10^4$  spores per ml.

### Plant material

I used thirty-five pots of Gb, each containing three plants, per treatment. There were an additional ten pots of Gb plants for testing the infection ability of each inoculum, five pots at each inoculation time. Five control pots were inoculated with water at both inoculation times. I also grew five pots of the specific ecotypes, each containing three plants, Cvi for EMWA, Ei-2 for EMCO, and Col-0 for WACO, to additionally test that all inocula were viable and could infect their specific hosts at each inoculation time. All plants were at the four-leaf stage at inoculation their first.

### Sequential inoculation procedure

In this experiment, I carried out sequential inoculations to test whether an infected plant could be infected by an additional strain and to see whether a three-day time lag in inoculation led to more multiple infections than coinoculation. I carried out all factorial combinations of two strains in both orders, giving six inoculation treatments plus a control with sterile tap water. Each inoculum was tested for infection ability as mentioned above on Gb and on its specific plant ecotype (five pot per strain) at both times of inoculations (Table 7.1). I sprayed the spore suspensions by using a glass atomiser fitted with an electric pump onto all leaves of each *A. thaliana* plant in each pot for approximately 2 second per pot to standardize number of spores delivered. At the first inoculation, five control plants were initially sprayed with sterile tap water. Then, I sprayed each strain on i) thirty-five pots of Gb per treatment, ii) five pots of Gb for testing infection ability of each inoculum (on Gb), iii) five pots of its habitual ecotype for testing infection ability of each inoculum (on its own ecotype). In addition, the five pots of Gb that would be used three days later to test the infection ability of the spore suspensions for the second inoculation were sprayed with sterile tap water. All inoculated and control plants were placed in growth chamber at 10:14 light-dark photoperiod,  $18^\circ\text{C} \pm 3^\circ\text{C}$  average temperature and 98-100% hygrometry. Each pot was isolated in a transparent plastic column to prevent spore dispersal and contamination. Plants were randomly rotated in the growth

chamber and sprayed with water twice a day above the column to maintain high humidity. Three days after the first inoculation, I prepared inocula as described above, removed the plastic columns and inoculated plants with the appropriate second strains, different from that used for first inoculation. I sprayed the spore suspensions of the second strain on each of the thirty-five pots of Gb that had been inoculated with a first strain as well as the five plants of Gb that had been inoculated with sterile water three days before and five new pots of each strain's specific ecotype to test the viability and infection ability of the inocula used for the second inoculation. I sprayed using the same procedure as for the first inoculation. Then I replaced the individual transparent plastic column for each plant and cultivated plants, spraying them with water to maintain humidity once a day. I observed all the plants every day starting after the second inoculation treatment, recorded first day when sporulation was seen as an estimate of latent period. I counted the number of sporulating leaves every day until 10 days after the second inoculation, and two additional time points, i.e. on day 17 and 24 after second inoculation. On day 10, 17 and 24 after second inoculation I collected each sporulating leaf from every plant in each pot into an individual 2.0 ml tube.

**Table 7.1** The experimental design for sequential-inoculation of all strain combinations. Each pot contained three plants that were inoculated with the first strain, then, three days later, the second strain. The viability and infection ability of each inoculum was tested on both days of inoculation by inoculating it alone onto five pots of Gb and five pots of the specific ecotype on which it had been previously multiplied. Control inoculations were carried out with sterile tap water.

		Second inoculation (3 days later)				
		Inocula	EMCO	EMWA	WACO	water
First inoculation	EMCO		35 Gb	35 Gb	5 Gb	5 Ei-2
	EMWA	35 Gb		35 Gb	5 Gb	5 Cvi
	WACO	35 Gb	35 Gb		5 Gb	5 Col-0
	water	5 Gb	5 Gb	5 Gb	5 Gb	
		5 Ei-2	5 Cvi	5 Col-0		

### **Collection of sporulating leaves for strain identification and leaf samples preparation**

Each sporulating leaf from every plant in each pot of all inoculation treatments was collected into an individual 2.0 ml tube at 10, 17 and 24 days after the second inoculation. Five leaves of Gb, each from five different pots of the control pots inoculated with sterile water at both first and second inoculation were also collected. The sample preparation and strain identification followed the same method as used in co-inoculation experiments above.

### **Statistical analyses**

Infection success and infection phenotypes, i.e., latent period, cumulative number of sporulating leaves and increase in number of sporulating leaves, were analysed as described above for co-inoculation experiment with the three-strain combination. In this experiment, I also compared infection success and infection phenotypes between two inoculation treatments from the same strain combination that differing in the orders of inoculation.

### **Sequential inoculation leads to multiple infections in some strain combinations**

The co-inoculation experiments, with less infection success of individual strains after mixed inoculation than single inoculation, led us to infer interference between strains in mixed inoculum. Here we tested whether a time lag in the inoculation influenced coinfection success, by using sequential inoculation with all strains used for the three-strain mix experiment. This allowed me to test effect of order of inoculated strains on overall infection phenotypes, infection success of individual strains and on co-existence frequency.

All test inocula were viable and able to infect, as shown by successful sporulation on each habitual plant ecotype used for strain multiplication and on some Gb plants inoculated with each single strain, both at the first and at the second time of inoculation. Furthermore, I was able to detect the specific Co 2-2 marker phenotype of each individual strain from some sporulating sequentially inoculated plants (Tables 7.2). No sporulation was observed on control plants.

**Table 7.2** Test of inoculum viability and ability to infect. Here I show the number of pots containing at least one sporulating plant, in parentheses; (the number of sporulating plants), and in square brackets; [the number of infected plants for which the infecting strain was successfully genotyped at the Co 2-2 microsatellite marker] on day 10 post-inoculation. A: infection success of each inoculum on the habitual ecotype used for strain multiplication (EMWA on Cvi, EMCO on Ei-2, WACO on Col-0). B: Control inoculations followed by (first inoculation) or preceded by (second inoculation) a sterile water inoculation on Gb. On day 10 post-inoculation, each whole sporulating plant from all treatments were collected for strain detection, using the Co 2-2 microsatellite marker. Five pots, each containing three plants, were used for every inoculum. No sporulation was observed on controls.

Treatment	Number of pots containing at least one sporulating plant (sporulating plants) [plants revealing the specific Co 2-2 marker phenotype]							
	A.						B.	
	First inoculation			Second inoculation			First inoculation	Second inoculation
	Col-0	Cvi	Ei-2	Col-0	Cvi	Ei-2	Gb	Gb
EMCO→EMWA			5 (14) [10]		5 (15) [14]		5 (11) [10]	5 (11) [9]
EMWA→EMCO		5 (15) [14]				4 (7) [5]	5 (12) [10]	5 (8) [6]
EMCO→WACO			5 (9) [7]	2 (4) [4]			5 (10) [9]	2 (2) [2]
WACO→EMCO	3 (6) [5]					4 (9) [8]	1 (2) [2]	2 (4) [4]
EMWA→WACO		5 (15) [15]		2 (3) [3]			5 (13) [10]	5 (7) [6]
WACO→EMWA	3 (6) [5]				5 (12) [12]		2 (3) [3]	2 (6) [5]

### 1. Order of inoculating individual strain matters to variation in infection success, number of sporulating leaves, latent period of infection and rate of increase in sporulating leaves among inoculation treatments

When strains invade a host sequentially, rather than being coinoculated, the order of the infections can influence the infection phenotypes. For example, in *Pasteuria ramosa* infecting *Daphnia magna*, when a more virulent strain was inoculated first, hosts suffered similar fitness loss as for single infection with the more virulent strain. In contrast, first inoculation with the less virulent strain attenuated the effects of the second, more virulent, infection as well as reducing the reproductive success of the more virulent strain, implying scramble competition (Ben-Ami et al., 2008). However,

sequential inoculation of *Microbotryum violaceum* strains on *Silene latifolia* demonstrated no effect of order of inoculation on the symptoms, but predomination of the first strain, implying the competitive exclusion by first infecting strains (Hood, 2003). Similarly, non-significant order effect was found on transmission of sequentially acquired viral strains by green peach aphids to potato seedlings (Mondal & Gray, 2017).

Here in my sequential experiment, infection phenotypes varied significantly among treatments. Infection success and intensity (Table 7.3), the rate of increase in number of sporulating leaves (Figure 7.1, Table 7.4), and latent period of infection (Figure 7.2) differed between inoculations with the same strain combinations but in different order, usually being similar between treatments with the same first inoculated strain (Table 7.5). I noted that initial inoculation with WACO significantly reduced infection success and intensity of the subsequently inoculated strain (Table 7.3), suggesting that at least this strain impeded a secondary infection. Furthermore, there was some evidence for interference by the strain EMWA on infection intensity of EMCO but not in the opposite direction.

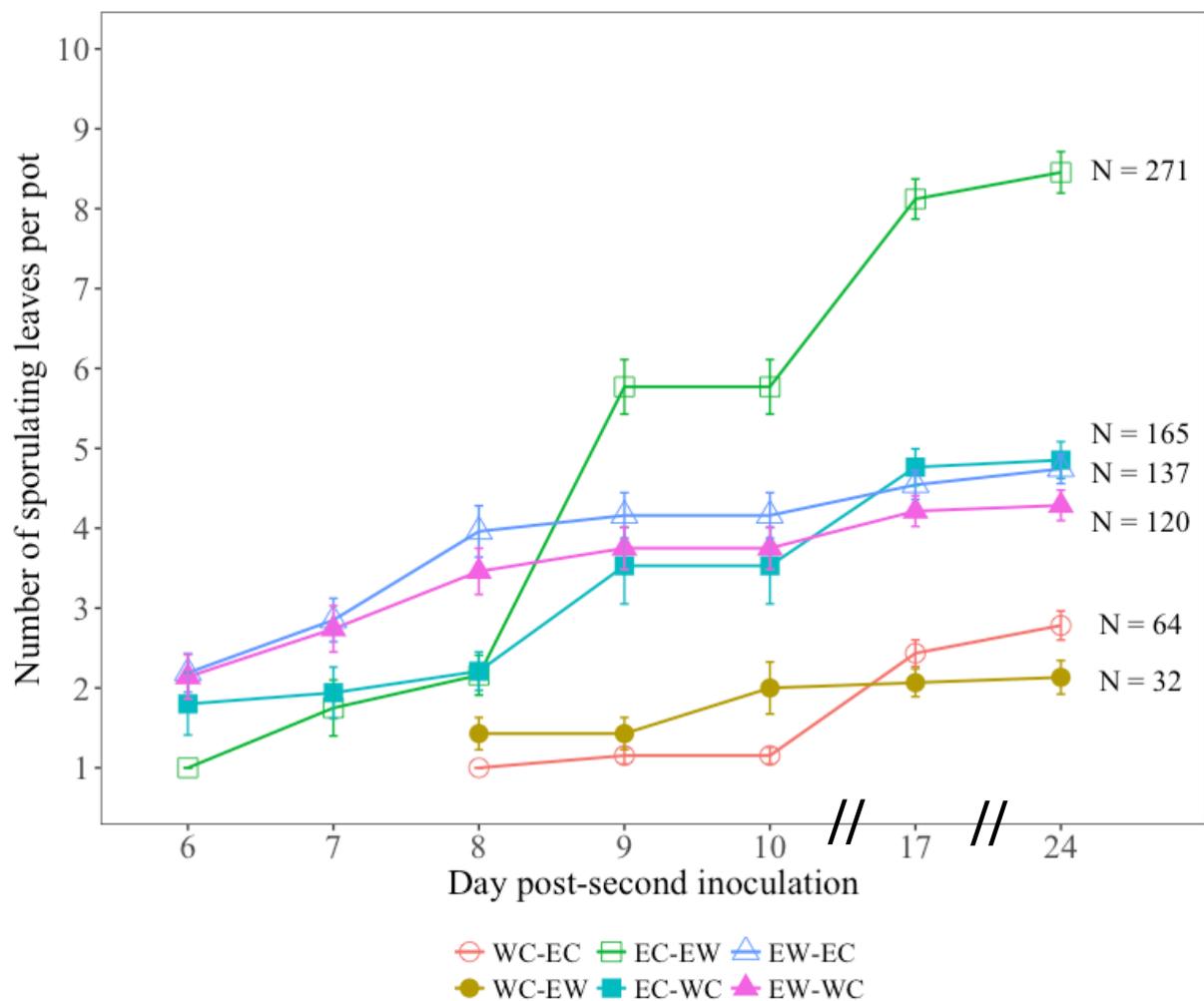
My results from this particular host-pathogen system suggest that sequential inoculation can generate variation in infection phenotypes among inoculation treatments. For example, unsurprisingly, the first inoculated strains determined latent period of the infection. In treatments involving the strain WACO, the order of the inoculation mattered. Infection success was lower overall when WACO was inoculated first than, for the same combination of strains, when WACO was inoculated second (Table 7.3). This result is somewhat similar to what I observed in the coinoculation, where the presence of WACO in the inoculum reduced the success of EMCO, but not of EMWA (Table 7.7). For the coinoculation experiments I inferred direct interactions between the inoculating strains. Here I may also propose a priming effect, by which the first strain activates the host defence system (see Råberg et al., 2006; Conrath et al., 2009; Pastor et al., 2013; Milutinović et al., 2016).

**Table 7.3** Summarised infection success i.e. whether an inoculated plant sporulated or not, and average number of sporulating leaves per pot containing at least one sporulating plant on day 24 after second inoculation. Thirty-five pots each containing three plants were used for every treatment.

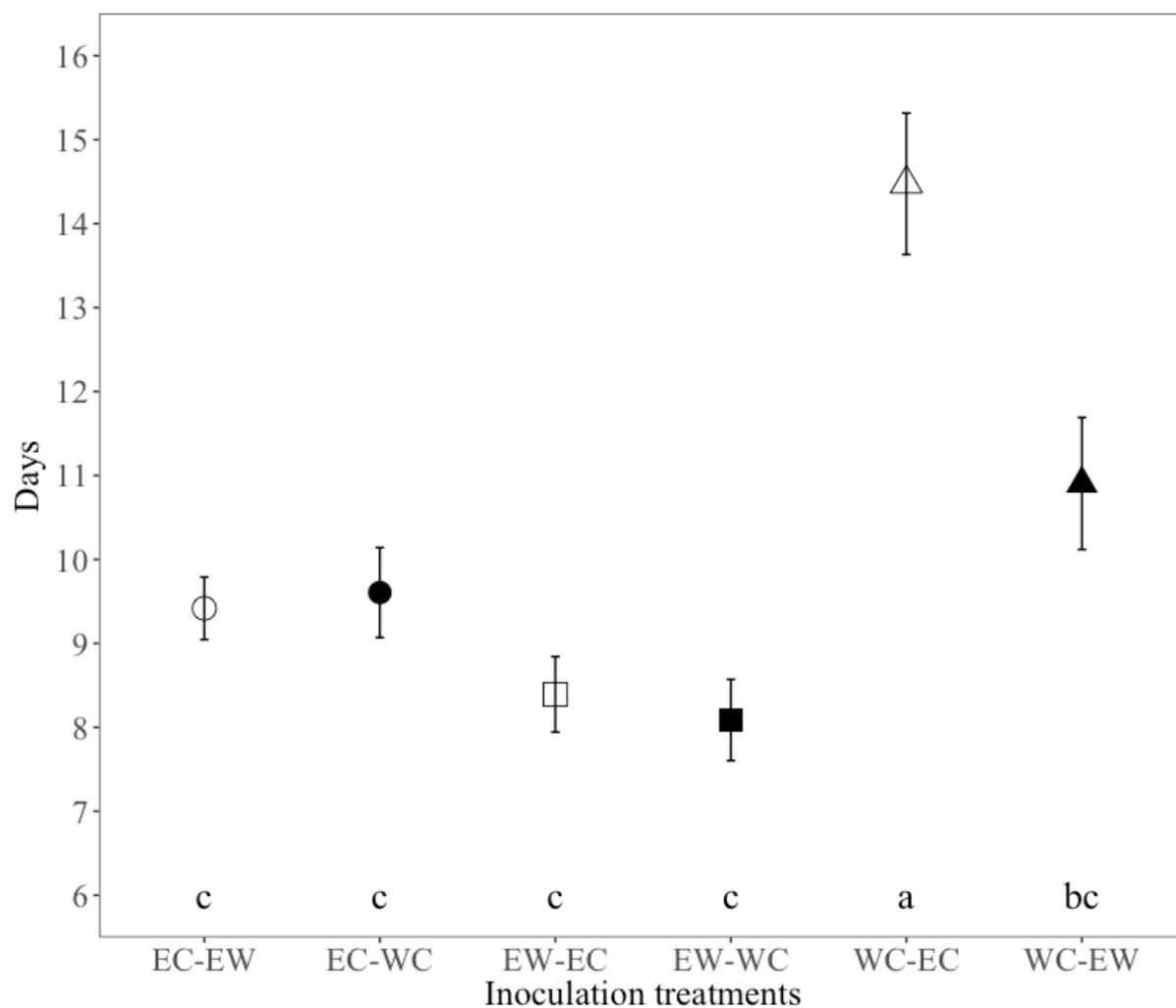
Treatment	Sporulating plants [total plants]	Average cumulative number of sporulating leaves per pot $\pm$ SE
EMCO $\rightarrow$ EMWA	84 [105]	8.21 $\pm$ 0.39 a
EMWA $\rightarrow$ EMCO	78 [105]	4.74 $\pm$ 0.37 b
	$\chi^2 = 0.97$ , df = 1, $p = 0.32$	
EMCO $\rightarrow$ WACO	63 [105]	4.85 $\pm$ 0.38 b
WACO $\rightarrow$ EMCO	32 [105]	2.78 $\pm$ 0.46 c
	$\chi^2 = 18.5$ , df = 1, $p < 0.0001$	
EMWA $\rightarrow$ WACO	61 [105]	4.28 $\pm$ 0.42 b
WACO $\rightarrow$ EMWA	21 [105]	2.13 $\pm$ 0.57 c
	$\chi^2 = 32.0$ , df = 1, $p < 0.0001$	
Among all treatments	$\chi^2 = 120$ , df = 5, $p < 0.0001$	$F_{(5, 162)} = 25.2$ , $p < 0.0001$

**Table 7.4** Analyses of Covariance with repeated measures testing how the number of sporulating leaves per pot varied among inoculation treatments, over time post-inoculation (in days) and with the interaction for the sequential inoculation experiment from generalised linear mixed-effect model with penalized quasilielihood method using Poisson error distribution for count data. Treatments differed in how cumulative number of sporulating leaves per pot increased over time post- second inoculation was shown in Figure 7.1, with significant slope estimate.

Effect	df	Likelihood ratio	$\chi^2$	p-value
Inoculation treatment	5	91.0		< 0.0001
Day post-inoculation	1	410.2		< 0.0001
Inoculation treatment x Day post-inoculation	5	30.6		< 0.0001



**Figure 7.1** Mean ( $\pm$  S.E.) cumulative number of sporulating leaves from all sporulating plants in each pot for the sequential strain combinations, observed on days 6, 7, 8, 9, 10, 17 and 24 after second inoculation. N indicates the total number of sporulating leaves in each inoculation treatment counted on day 24 after the second inoculation. The generalised linear mixed-effect model with penalized quaslikelihood method using Poisson error distribution revealed that treatments differed in how the number of sporulating leaves per pot increased over time post-inoculation (Table 7.4). Significant positive slopes ( $\pm$  SE) were found for the following treatments: EC-EW ( $0.30 \pm 0.0039$ ) and EC-WC ( $0.17 \pm 0.0055$ ).



**Figure 7.2** Mean day of first sporulation (latent period  $\pm$  S.E.) of different inoculation treatments for the sequential strain combinations, presented as “first strain–second strain”, from the experimental design presented in Table 7.1. The abbreviated name of each strain was EMCO (EC), EMWA (EW) and WACO (WC). Latent period represents the day when the first sporulating leaf or leaves were observed on each plant that showed infection. An ANOVA revealed significant differences in latency among the six inoculation treatments,  $F_{(5, 374)} = 17.0$ ,  $p < 0.0001$ ). Treatments marked with a different lowercase letter above the axis labels differed significantly by Tukey multiple comparisons. When analysing latent period as a function of order of inoculated strains, latent period differed significantly across the different first- and second- inoculated strains (Table 7.5).

**Table 7.5** Analyses of Variance testing effect of the identity of first versus second inoculated strains on latent period for the sequential inoculation experiment.

Effect	df	<i>F</i>	<i>p</i>
First inoculated strain	2	36.0	< 0.00001
Second inoculated strain	2	3.73	0.025
Residuals	377		

## **2. Order of inoculation matters to infection success of some individual strains from sequential inoculation**

Genotyping the infections following sequential inoculation revealed that both first and second inoculated strains successfully infected at least one plant (Table 7.6). Infection success detected by PCR varied across the 4 inoculation combinations from 20 – 140 leaves for EMWA ( $\chi^2 = 6.87$ ,  $df = 3$ ,  $p = 0.076$ ), from 12 – 92 leaves for EMCO ( $\chi^2 = 155.8$ ,  $df = 3$ ,  $p < 0.001$ ) and from 1 – 39 leaves for WACO ( $\chi^2 = 48.5$ ,  $df = 3$ ,  $p < 0.001$ , see Table 7.6). EMCO had higher infection success when it was the first strain inoculated ( $\chi^2 = 46.1$ ,  $df = 1$ ,  $p < 0.001$ ), (see de Roode *et al.* (2005); Ben-Ami *et al.* (2008)). WACO, though showing overall poor infection success, had higher infection success when it was the second strain inoculated, suggesting facilitation ( $\chi^2 = 3.65$ ,  $df = 1$ ,  $p = 0.0056$ ; see Table 7.6), as observed by Jäger and Schjørring (2006). This facilitation could result from suppression of host defence by the first arrival strain, and might increase co-infection (Maizels & McSorley, 2016).

## **3. Number of co-existence of two infecting strains from sequential inoculation varied with order of inoculated strain and strain identity**

Sequential inoculation by two strains generated more multiple infections than did co-inoculation, overall, though this varied greatly with the identity of the first inoculated strain, with 93% of the co-infections being concentrated in two inoculation treatments with the same first strain. Co-existence of two infecting strains was more likely when EMCO was the first inoculated strain (Table 7.6). This suggests that different parasite strains interact negatively in the inocula, impeding each other's success, but less when there was a three-day time lag between inocula with the two different strains. Here I cannot test for an effect of genetic relatedness on this interference. However, in another host-parasite system, closely related strains were more able to infect previously infected plants (Koskella *et al.*, 2006).

I found co-infection as a consequence of sequential inoculation but do not know if it would be maintained throughout the host life. One genotype may outcompete the other, leading to competitive exclusion as previously shown with experimental studies on *Microbotryum violaceum* strains sequentially inoculated on *Silene latifolia* (Hood, 2003). Indeed, superinfection, with more virulent strains replacing less virulent ones can also occur (Nowak & May, 1994; Mosquera & Adler, 1998). This indicates that competitive interaction between different genotypes can influence the outcome of sequential infections.

**Table 7.6** Summary of strain detection using the Co 2-2 microsatellite marker for sequential inoculation experiment on collected sporulating leaves of every sporulating plants. Ten leaves from control plants, two leaves from randomly chosen two plants from five pots, were tested by PCR. I performed up to 4 PCRs for each spore sample. I stopped when I obtained two clear consistent results or after 4 unsuccessful PCRs. No PCR product was detected from leaves of control plants. PCR detection failed for some leaves of each treatment.

Treatment	Total sporulating leaves	Leaves revealing the specific Co 2-2 marker phenotype		
		First strain	Second strain	Co-infection
EMCO → EMWA	172	16	95	45
EMWA → EMCO	109	87	9	6
EMCO → WACO	102	54	1	38
WACO → EMCO	16	2	12	0
EMWA → WACO	96	81	1	0
WACO → EMWA	23	1	20	0

## VIII. CONCLUSION

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### CONCLUSION

This thesis aimed at exploring the phenotypic effects of co-infections compared with single genotype infections on hosts. However, co-inoculation did not always lead to co-infection. In fact, co-infections were very rare. Therefore I explored the factors that influenced variation in the success of coinfection, as well as exploring the phenotypes variation of infections resulting from different inoculation treatments.

Mixing inocula influenced overall infection success compared with the infection success of individual strains in some cases, with some strains interfering with the infection success of others. Furthermore, infections resulting from mixed inocula had phenotypes altered in unexpected ways. Though there was no general effect of multiple infection or multiple inoculation on virulence, higher virulence was observed for the infections following the three-strain mixed inoculum. Parasite identity also influenced the reactions of different parasite strains to each other. Sequential inoculation led to more coexistence of strains within hosts than did co-inoculation.

Phenotypic differences under different environmental conditions, for example in the presence of other pathogen strains or for other environmental variables, may represent adaptive plasticity, with the expression of appropriate phenotypes under different environmental conditions. Though all plasticity may not be adaptive, parasites can evolve adaptive plastic responses to the presence of a competing parasite strain (Leggett et al., 2013). Therefore, it is not impossible that the responses to mixed inoculations that I observe are adaptive, having evolved under conditions where multiple infections are common and *H. arabidopsidis* is likely to encounter a competitor within the same host plant.

## **PROBLEMS AND PERSPECTIVES**

First, I would optimise the condition of sequential inoculation, i.e. vary time lag of second inoculation or use other strains to observe co-infections, to gain higher co-infections. Second, it would be important to quantify the fitness of individual strains within-host to determine exact value of fitness cost in multiple infections. I tried quantifying spore production via quantitative PCR, however, molecular markers did not distinguish properly in this method for mixed infections. To solve this problem, comparative genomics of different strains should be assessed to see some polymorphisms among genomes that might provide useful molecular markers. This information will also contribute genetic relatedness between co-inoculated strains to examine kin relationship in mixed infection context of non-lethal biotrophic pathogen on plants. Third, interaction between co-infecting genotypes within the same host would be explored by using transgenic genotypes with different reporter fluorescent dyes. This would illustrate how two different genotypes interact each other within a host and provide information about growth or reproduction of individual genotype in mixed infection compared with when it alone.

# SUMMARY OF THESIS IN FRENCH

(via google translate)

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## Introduction

Les parasites sont des organismes vivant temporairement ou continuellement avec des partenaires interspécifiques (in / on), appelés hôtes, à partir desquels ils obtiennent des ressources pour leur croissance et leur reproduction. Les dommages causés à l'hôte, y compris ceux qui affectent négativement sa morphologie, sa physiologie ou sa reproduction, dus à l'exploitation parasitaire, sont des caractéristiques communes de cette interaction interspécifique extrême appelée parasitisme (voir Araújo et al., 2003).

Par définition, les parasites ont un impact négatif sur la santé de l'hôte et l'effet négatif des parasites sur la croissance, la survie ou la reproduction de l'hôte s'appelle la virulence. Les parasites causent la morbidité et la mortalité à leur hôte en retirant les ressources de l'hôte des fonctions hôtes nécessaires à la production de propagules parasitaires. Les parasites qui ont des taux plus élevés d'exploitation des hôtes devraient avoir une meilleure condition physique, car ils prennent plus de ressources de leurs hôtes et peuvent donc produire plus de progéniture. Dans le même temps, toutes choses étant égales par ailleurs, un parasite capable d'exploiter son hôte plus longtemps devrait également avoir une meilleure condition physique. Cependant, ces deux effets ne sont pas indépendants, car l'exploitation de l'hôte est susceptible de modifier la longévité, les parasites absorbant davantage de ressources endommageant davantage leurs hôtes et les tuant plus rapidement. Par conséquent, la maximisation de la capacité parasitaire représente un compromis entre la maximisation du taux de réplication et la maximisation de la longévité, du moins dans le cas de parasites qui tuent leurs hôtes en détournant d'importantes ressources de l'hôte de la maintenance.

Le compromis entre le taux d'exploitation de l'hôte (prenant beaucoup de ressources hôtes et leur transformation en propagules parasitaires) et le taux de mortalité de l'hôte, qui limite le temps d'exploitation de son hôte, génère une stratégie optimale de virulence intermédiaire, sera sélectionné par sélection naturelle. Les parasites peuvent ne pas être seuls chez les hôtes et la présence d'autres parasites coexistants dans le même hôte peut modifier la virulence optimale de manière importante (Bremermann et Pickering, 1983; Herre, 1993; Knolle, 1989; Mosquera et Adler, 1998; Alizon, 2008). Ebert et Bull, 2008).

La co-infection peut entraîner une coexistence à long terme par plus d'une souche d'agents pathogènes co-infectants ou une surinfection n'ayant qu'une coexistence transitoire parce qu'une souche,

généralement supposée être la plus virulente, déplace l'autre, moins virulente, rapidement (Nowak et May, 1994; May et Nowak, 1995; Mosquera et Adler, 1998) et les hôtes infectés par des souches plus virulentes ne peuvent pas être infectés par un génotype moins virulent (Bremermann & Thieme, 1989). La présence d'autres souches parasitaires peut modifier à la fois le niveau de virulence exprimé par l'infection, c'est-à-dire la virulence globale de l'infection mixte, qui peut différer de la virulence d'une infection avec une seule des souches parasitaires (Alizon et al., 2013) et l'environnement de sélection pour la stratégie optimale de virulence de toutes les souches parasitaires infectantes ou présentes (Ewald, 1983; van Baalen & Sabelis, 1995). Il est important de noter ici qu'une infection mixte peut avoir un effet différent sur l'hôte, exprimé dans la virulence globale de l'infection, par rapport à une infection par un seul génotype. Indépendamment de l'effet sur l'hôte, dans une infection mixte, la présence de plus d'un génotype de parasite peut altérer les bénéfices physiques de l'infection pour chaque génotype présent. Les infections mixtes peuvent entraîner des bénéfices plus faibles, similaires ou peut-être même supérieurs pour une souche de parasite particulière par rapport au fait qu'elle soit le seul génotype présent dans une seule infection. Par conséquent, nous devons tenir compte des effets des infections mixtes sur l'hôte et les parasites.

Lorsque deux souches différentes en virulence co-infectent un seul hôte en même temps, si elles maintiennent la même stratégie d'exploitation en compétition, il est évident que la plus virulente produira plus de propagules que la moins virulente dans l'infection mixte, car la densité combinée des parasites provoquera la mort de l'hôte lorsque le parasite le plus virulent aura produit relativement plus de propagules que le moins virulent. De même, en cas de surinfection, la souche plus virulente éliminera la souche moins virulente lorsque cette dernière souche se sera peu ou pas reproduite. Ainsi, l'infection multiple entraînera une virulence plus élevée parce que la sélection agira contre les souches les plus prudentes avec les stratégies de virulence inférieure, même si cela conduit à une production globale de propagules plus faible (Frank, 1992; Nowak et May, 1994; van Baalen et Sabelis, 1995; May et Nowak, 1995; Mosquera et Adler, 1998; Nowak et Sigmund, 2002).

Les cadres théoriques des effets des infections multiples et de la concurrence intra-hôte ont été décrits ci-dessus. Bien que ce cadre soit assez clair, il existe encore peu d'études expérimentales sur les résultats évolutifs d'infections multiples sur l'évolution de la virulence (Zhan et McDonald, 2013). Ces auteurs discutent des avantages des systèmes microbiens et en particulier des parasites végétaux pour mener des expériences d'évolution afin de tester les prédictions de ces théories. En effet, une gamme d'outils sophistiqués est disponible pour quantifier le nombre d'agents pathogènes et distinguer les souches, ce qui permet d'étudier et de quantifier la concurrence entre différentes souches d'agents pathogènes (voir Zhan et McDonald, 2013). Cependant, il existe des obstacles techniques à l'utilisation d'approches expérimentales d'évolution pour étudier l'évolution de la virulence en cas d'infections multiples, la plus importante étant la difficulté de s'assurer que de multiples souches interagissent

réellement à long terme chez des hôtes individuels. Comme nous le verrons plus loin, l'inoculation avec un mélange de souches, ou une inoculation séquentielle avec différentes souches, ne garantit pas la coexistence de souches multiples au sein des hôtes (voir également Wille et al., 2002).

Le phénotype des infections multiples versus simples par les virus (Ojosnegros et al., 2010; Gil-Salas et al., 2012; Salvaudon et al., 2013; Breyta et al., 2016; Tollenaere et al., 2017), bactéries (Inglis et al., 2009; Bashey et al., 2012; Lass et al., 2013; Pollitt et al., 2014; Stacy et al., 2016), protistes (Balmer et al., 2009; Duncan et al., 2015) et les champignons (López-Villavicencio et al., 2011; Buono et al., 2014; Susi et al., 2015a; Susi et al., 2015b) ont été explorés. Certaines de ces études (par exemple, Balmer et al., 2009; Inglis et al., 2009; Ojosnegros et al., 2010; Bashey et al., 2012; Gil-Salas et al., 2012; Lass et al., 2013; Salvaudon et al., 2013; Breyta et al., 2016; Tollenaere et al., 2017) quantifient les différents génotypes chez des hôtes multi-infectés. Cela fournit des informations sur les effets compétitifs et la sélection au sein de l'hôte, en d'autres termes, quelle stratégie ou quel génotype a le plus de succès, ce qui prédit la sélection de la virulence au moins à court terme.

## **Objectifs de la these**

Dans cette thèse, j'examine les résultats des inoculations, que ce soit avec une ou plusieurs souches d'un agent pathogène naturel, *Hyaloperonospora arabidopsidis*, chez *Arabidopsis thaliana*. Je compare plusieurs aspects des phénotypes résultant des inoculations simples versus multi-souches en termes de:

1. succès de l'infection (que chaque plante inoculée soit infectée ou non)
2. phénotypes d'infection (période de latence, intensité de sporulation, succès de la transmission et virulence sur les plantes sporulantes)
3. Succès de l'infection de souches individuelles

En outre, j'étudie la cooccurrence des souches inoculées résultant à la fois de la co-inoculation et de l'inoculation séquentielle, en utilisant les mêmes combinaisons de souches, afin de tester l'effet du retard sur le succès de la co-infection, la variation des phénotypes d'infection et l'infection succès de souches individuelles.

## **I. Expériences de co-inoculation**

### ***Variation des phénotypes d'infection entre une seule et la co-inoculation***

La présence d'une autre souche d'agent pathogène peut également représenter un aspect de l'environnement susceptible d'influencer les phénotypes d'infection. Ici, j'ai pu comparer les phénotypes d'infection résultant d'une inoculation simple ou mixte pour déterminer si la présence d'une autre souche influençait la période de latence, le succès global de l'infection (nombre de plantes infectées), la reproduction parasitaire globale (nombre de feuilles sporulées) de feuilles sporulantes.

Différentes souches parasitaires peuvent interférer ou se faciliter mutuellement. Pour la période de latence, s'il n'y a pas d'interaction, je devrais voir les premières souches sporulantes à peu près au même moment, qu'elles soient inoculées seules ou avec une autre souche. C'est en effet ce que j'ai trouvé pour la plupart des inoculations mixtes, mais pas toutes. Cela a été le cas pour EMWA-FRI5, EMWA-WACO et le mélange à trois souches, où toutes les inoculations mixtes ont montré des périodes de latence compatibles avec la souche la plus ou la plus rapide de l'inoculum. Ce n'était cependant pas toujours le cas pour EMCO-ORS3, où l'inoculation mixte à dose plus élevée présentait une période de latence similaire à celle de la souche sporulante plus tardive plutôt que plus précoce. Pour le succès de l'infection, j'ai trouvé des exemples d'interférence, mais aucune de facilitation, bien que pour certaines combinaisons, le succès de l'infection était similaire entre les traitements simples et les traitements mixtes. De même, être avec une autre souche chez le même hôte peut influencer la reproduction globale du parasite ou la reproduction du génotype individuel par rapport à la seule infection. Dans mes expériences, j'ai trouvé des exemples d'interférence, avec moins de feuilles sporulantes et une augmentation plus lente du nombre de feuilles sporulées provenant de traitements mixtes que d'une seule inoculation, mais pas pour toutes les expériences.

### ***Les souches interfèrent les unes avec les autres dans les inocula mixtes***

Chaque co-inoculation du mélange à trois souches a entraîné une infection par les deux ou les trois souches, mais dans quelques cas seulement, j'ai trouvé deux souches sur la même plante ou dans la même feuille. Cependant, les inoculations mixtes ont réduit le succès de l'infection pour toutes les souches parasitaires, bien que la force de leurs interférences varie. Par exemple, WACO a interféré moins avec EMWA qu'avec EMCO et l'inoculation mixte à trois souches a montré moins d'interférence.

## ***La virulence sur la condition physique totale diffère entre une infection unique et une infection par inoculation mixte***

Ici, j'ai trouvé que les inoculations mixtes provenant de l'expérience EMCO-ORS3 provoquaient une virulence plus élevée que les inoculations uniques. En outre, les plantes infectées du mélange à trois souches dans une expérience de mélange à trois souches ont présenté une virulence plus élevée que toutes les inoculations mixtes à une et à deux souches. Bien que je n'ai pas trouvé cette augmentation de la virulence pour les infections dues à des inoculations mixtes dans toutes les expériences, cela fournit des preuves suggestives que l'interaction entre différentes souches de parasites soit dans l'inoculum soit dans la plante conduit à une plus grande virulence. Dans le dernier cas, un nombre accru de géotypes dans l'inoculum a contribué à une plus grande virulence. Malheureusement, je ne sais pas combien de souches ont réellement infecté ces plantes. Cependant, l'effet significatif du traitement d'inoculation sur la virulence suggère qu'il pourrait effectivement y avoir co-infection et que la diversité génétique des parasites conspécifiques affecte la virulence (Ben-Ami et Routtu, 2013; Kinnula et al., 2017; Gleichsner et al., 2018). En d'autres termes, l'augmentation du nombre de diversité des géotypes infectants modifie l'interaction compétitive au sein de l'hôte, générant une plus grande virulence. Dans mon expérience sur trois souches, j'ai génotypé chaque souche individuelle après inoculation mixte sur les mêmes plantes utilisées pour estimer la virulence. De plus, je n'ai trouvé aucune variation de la virulence parmi les trois infections à souche unique. Le géotype du parasite EMCO était prédominant dans les infections consécutives à des inoculations mixtes. Une augmentation de la virulence ne peut donc pas être associée à une augmentation de la fréquence d'une souche hautement virulente, mais plutôt à une réponse plastique dans le mélange lui-même. En effet, en l'absence de variation significative de la virulence des souches individuelles, on ne peut prédire une trajectoire d'évolution de la virulence.

## **II. Inoculation séquentielle**

### ***L'ordre d'inoculation de la souche individuelle entraîne une variation des phénotypes d'infection***

Ici, dans mon expérience séquentielle, les phénotypes d'infection variaient significativement entre les traitements. Le succès et l'intensité de l'infection, le taux d'augmentation du nombre de feuilles sporulées et la période d'infection latente différaient entre les inoculations avec les mêmes combinaisons de souches mais dans un ordre différent, généralement similaire entre les traitements avec la même première souche inoculée. J'ai noté que l'inoculation initiale de WACO réduisait de manière significative le succès de l'infection et l'intensité de la souche inoculée par la suite, suggérant

qu'au moins cette souche empêchait une infection secondaire. De plus, il y avait des preuves d'interférence par la souche EMWA sur l'intensité de l'infection de l'EMCO mais pas dans la direction opposée.

Mes résultats sur ce système hôte-pathogène particulier suggèrent que l'inoculation séquentielle peut générer une variation des phénotypes d'infection parmi les traitements d'inoculation. Par exemple, sans surprise, les premières souches inoculées ont déterminé la période de latence de l'infection. Dans les traitements impliquant la souche WACO, l'ordre d'inoculation importait. Le succès de l'infection était globalement inférieur lorsque WACO était inoculé en premier lieu, pour la même combinaison de souches, lorsque WACO était inoculé en second lieu. Ce résultat est quelque peu similaire à ce que j'ai observé dans la coinoculation, où la présence de WACO dans l'inoculum réduit le succès de l'EMCO, mais pas celle de l'EMWA. Pour les expériences de coinoculation, j'ai inféré des interactions directes entre les souches d'inoculation. Ici, je peux également proposer un effet d'amorçage, par lequel la première souche active le système de défense de l'hôte (voir Råberg et al., 2006; Conrath et al., 2009; Pastor et al., 2013; Milutinović et al., 2016).

### ***L'ordre d'inoculation a une incidence sur le succès de l'infection par séquençage de certaines souches individuelles***

Le génotypage des infections à la suite d'une inoculation séquentielle a révélé que les premières et deuxièmes souches inoculées infectaient avec succès au moins une plante. Le succès de l'infection détecté par PCR variait entre les 4 combinaisons d'inoculation de 20 à 140 feuilles pour EMWA ( $\chi^2 = 6.87$ ,  $df = 3$ ,  $p = 0.076$ ), de 12 à 92 feuilles pour EMCO ( $\chi^2 = 155.8$ ,  $df = 3$ ,  $p < 0.001$ ) et de 1 à 39 feuilles pour WACO ( $\chi^2 = 48.5$ ,  $df = 3$ ,  $p < 0.001$ ). L'EMCO a eu un plus grand succès d'infection lorsqu'il s'agissait de la première souche inoculée ( $\chi^2 = 46.1$ ,  $df = 1$ ,  $p < 0.001$ ) (voir de Roode et al. (2005); Ben-Ami et al. (2008)). WACO, bien que montrant un succès globalement médiocre de l'infection, avait un succès d'infection plus élevé lorsqu'il s'agissait de la deuxième souche inoculée, ce qui suggère une facilitation ( $\chi^2 = 3.65$ ,  $df = 1$ ,  $p = 0.0056$ ), comme l'ont observé Jäger et Schjørring (2006). Cette facilitation pourrait résulter de la suppression de la défense de l'hôte par la première souche d'arrivée et pourrait augmenter la co-infection (Maizels & McSorley, 2016).

### ***Nombre de coexistence de deux souches infectieuses provenant d'une inoculation séquentielle varié avec l'ordre de la souche inoculée et l'identité de la souche***

L'inoculation séquentielle par deux souches a généré plus d'infections multiples que la co-inoculation, bien que cela varie beaucoup avec l'identité de la première souche inoculée, 93% des co-infections

étant concentrées dans deux traitements d'inoculation avec la même première souche. La coexistence de deux souches infectieuses était plus probable lorsque l'EMCO était la première souche inoculée. Cela suggère que différentes souches de parasites interagissent négativement dans les inoculums, entravant le succès des uns et des autres, mais moins quand il y a un décalage de trois jours entre les inoculums et les deux souches différentes. Ici, je ne peux pas tester un effet de parenté génétique sur cette interférence. Cependant, dans un autre système hôte-parasite, des souches étroitement apparentées étaient plus susceptibles d'infecter des plantes précédemment infectées (Koskella et al., 2006).

J'ai trouvé la co-infection à la suite d'une inoculation séquentielle mais je ne sais pas si elle serait maintenue tout au long de la vie de l'hôte. Un génotype peut surpasser l'autre, conduisant à une exclusion compétitive, comme cela a été montré précédemment avec des études expérimentales sur des souches de *Microbotryum violaceum* inoculées séquentiellement sur *Silene latifolia* (Hood, 2003). En effet, une surinfection, avec des souches plus virulentes remplaçant des souches moins virulentes, peut également se produire (Nowak et May, 1994; Mosquera et Adler, 1998). Cela indique que l'interaction compétitive entre différents génotypes peut influencer l'issue d'infections séquentielles.

## **Conclusion**

Cette thèse visait à explorer les effets phénotypiques des co-infections par rapport aux infections à un seul génotype chez les hôtes. Cependant, la co-inoculation n'a pas toujours conduit à une co-infection. En fait, les co-infections étaient très rares. Par conséquent, j'ai exploré les facteurs qui ont influencé la variation dans le succès de la co-infection, ainsi que l'exploration de la variation des phénotypes des infections résultant de différents traitements d'inoculation.

Le mélange des inocula a influencé le succès global de l'infection par rapport au succès de l'infection de certaines souches dans certains cas, certaines souches interférant avec le succès de l'infection chez d'autres. De plus, les phénotypes provoqués par des infections causées par des inoculums mixtes ont été modifiés de manière inattendue. Bien qu'il n'y ait eu aucun effet général d'infection multiple ou d'inoculation multiple sur la virulence, une virulence plus élevée a été observée pour les infections après l'inoculum mixte à trois souches. L'identité parasitaire a également influencé les réactions des différentes souches parasitaires les unes aux autres. L'inoculation séquentielle a conduit à une plus grande coexistence des souches au sein de l'hôte que la co-inoculation.

Les différences phénotypiques dans différentes conditions environnementales, par exemple en présence d'autres souches pathogènes ou pour d'autres variables environnementales, peuvent

représenter une plasticité adaptative, avec l'expression de phénotypes appropriés dans différentes conditions environnementales. Bien que toute plasticité puisse ne pas être adaptative, les parasites peuvent développer des réponses plastiques adaptatives à la présence d'une souche parasite concurrente (Leggett et al., 2013). Par conséquent, il n'est pas impossible que les réponses aux inoculations mixtes que j'observe soient adaptatives, ayant évolué dans des conditions où de multiples infections sont courantes et que *H. arabidopsidis* est susceptible de rencontrer un concurrent dans la même plante hôte.

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**Title :** Virulence and Multiple infections of *Hyaloperonospora arabidopsidis* (Gäum.) Göker, Riethm., Voglmayr, Weiss & Oberw. on *Arabidopsis thaliana* (L.) Heyhn.

**Keywords :** co-inoculation, evolution, interference, overall virulence, sequential inoculation

**Abstract :** Multiple infections are common in nature, and are considered very important in the evolution of parasite life-history traits. Theoretically, multiple infections should lead to evolution of higher levels of virulence both as an adaptive and as a plastic strategy. In this thesis I use *Hyaloperonospora arabidopsidis*, a natural parasite of *Arabidopsis thaliana*, which has proven a useful tool for unlocking some evolutionary ecology questions, to investigate: i) multiple infections following co-inoculation and sequential inoculation, ii) number of infected plants, infection success and transmission success of individual strain (genotyping via PCR), and infection phenotypes including virulence between after single- and mixed inoculation, iii) effect of time lag of inoculation and order of inoculated strain on infection phenotypes and individual strain infection success. Here I found that sequential inoculation contributed higher frequency of co-infection than co-inoculation of the same strain combinations. Mixed inoculum of some strain combinations led to modification of overall infection phenotypes, often with poorer infection success of individual strains compared with that of the more infectious strains. This result implies interference between strains in mixed inoculum. Overall virulence of infection after mixed inoculation was not always higher than that of single strain infection. Furthermore the single strains used in these experiments did not always differ from each other in virulence.

The one test of a three-strain mixture of genotypes caused higher overall virulence than the three respective single strain infections. Higher overall virulence in this case might be caused by plasticity of inoculated parasite strains response to the presence of other strains in mixed inoculum or an effect of multiple strains suppressing the host defence system. When strains were inoculated sequentially instead of together, infection success of individual strains differed between different orders of inoculation, which could be due to indirect effects via the host defence system. In summary, sequential inoculation seemed to reduce interference between parasite strains, with effect of time lag and order of inoculated strain on infection success of individual strains. Interference in mixed inoculum can generate different infection success and infection phenotypes from the respective single inoculations. I found one clear case of higher overall virulence in infections caused by mixed inoculations. Thus higher overall virulence can occur despite our not finding higher performance of more virulent genotypes from infections following mixed inoculations. Thus these findings do not predict the evolution of higher virulence among these strain combinations tested. However, plasticity of phenotypes of inoculated strains in mixed inoculum did generate higher overall virulence of infection. These findings can help to understand how the parasite genotypes respond to in mixed infections.

**Titre :** Virulence et infections multiples de *Hyaloperonospora arabidopsidis* (Gäum.) Göker, Riethm., Voglmayr, Weiss & Oberw. chez *Arabidopsis thaliana* (L.) Heyhn.

**Mots clés :** co-inoculation, évolution, inoculation séquentielle, interférence, virulence globale

**Résumé :** Les infections multiples sont courantes dans la nature et sont considérées comme très importantes dans l'évolution des caractéristiques biologiques des parasites. Théoriquement, les infections multiples devraient entraîner une évolution de la virulence à la fois comme stratégie adaptative et comme stratégie plastique. Dans cette thèse, j'utilise *Hyaloperonospora arabidopsidis*, un parasite naturel d'*Arabidopsis thaliana*, qui s'est avéré un outil utile pour découvrir certaines questions d'écologie évolutive, pour étudier: i) les infections multiples consécutives à la co-inoculation et à l'inoculation séquentielle; succès de l'infection et succès de transmission de la souche individuelle (génotypage par PCR) et phénotypes d'infection, y compris virulence entre inoculation unique et mixte, iii) effet du délai d'inoculation et d'ordre de la souche inoculée sur les phénotypes d'infection et le succès de l'infection individuelle. Ici, j'ai trouvé que l'inoculation séquentielle contribuait à une fréquence plus élevée de co-infection que la co-inoculation des mêmes combinaisons de souches. L'inoculation mixte de certaines combinaisons de souches a entraîné une modification des phénotypes d'infection, souvent avec un succès d'infection plus faible chez certaines souches que chez les souches plus infectieuses. Ce résultat implique une interférence entre les souches dans l'inoculum mixte. La virulence globale de l'infection après l'inoculation mixte n'était pas toujours supérieure à celle de l'infection à souche simple. De plus, les souches uniques utilisées dans ces expériences ne différaient pas toujours les unes des autres en termes de virulence. Le seul test d'un mélange de génotypes à trois souches a provoqué une virulence globale - plus élevée que les trois infections à souche unique respectives. Une plus grande virulence globale dans ce cas pourrait être due à la plasticité des souches parasitaires inoculées, à la réponse à la présence d'autres souches dans l'inoculum mixte ou à l'effet de multiples souches supprimant le système de défense de l'hôte. Lorsque les souches ont été inoculées de manière séquentielle et non ensemble, le succès de l'infection de souches individuelles différait entre les différents ordres d'inoculation, ce qui pourrait être dû à des effets indirects via le système de défense de l'hôte. En résumé, l'inoculation séquentielle a semblé réduire l'interférence entre les souches parasitaires, avec un effet de décalage temporel et d'ordre de la souche inoculée sur le succès de l'infection de souches individuelles. Une interférence dans un inoculum mixte peut générer différents succès d'infection et phénotypes d'infection à partir des inoculations individuelles respectives. J'ai trouvé un cas évident de virulence globale plus élevée dans les infections causées par des inoculations mixtes. Par conséquent, une virulence globale plus élevée peut se produire malgré le fait que nous ne trouvions pas de meilleures performances de génotypes plus virulents à la suite d'infections à la suite d'inoculations mixtes. Ainsi, ces résultats ne permettent pas de prédire l'évolution de la virulence supérieure parmi ces combinaisons de souches testées. Cependant, la plasticité des phénotypes des souches inoculées dans l'inoculum mixte a généré une virulence globale de l'infection plus élevée. Ces résultats peuvent aider à comprendre comment les génotypes de parasites répondent aux infections mixtes.