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Research article

Comparing single- vs. mixed-genotype infections of *Mycosphaerella graminicola* on wheat: effects on pathogen virulence and host tolerance

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Abstract. Mixed-genotype infections (infections of a host by more than one pathogen genotype) are common in plant-pathogen systems. However their impact on the course of the infection and especially on pathogen virulence and host response to infection is poorly understood. We investigated the effects of mixed-genotype infections on several parameters: host resistance and tolerance, as well as pathogen aggressiveness and virulence. For these purposes, we inoculated three wheat lines with three *Mycosphaerella graminicola* genotypes, alone or in mixtures, in a greenhouse experiment. For some of the mixtures, disease severity and virulence were lower than expected from infection by the same genotypes alone, suggesting that competition between genotypes was reducing their aggressiveness and virulence. One host line was fully resistant, but there were differences in resistance in the other lines. The two host lines that became infected differed slightly in tolerance, but mixed-genotype infections had no effect on host tolerance.

Key words: co-infection, multiple infection, resistance, *Septoria tritici*, *Triticum aestivum*

Introduction

In plant pathosystems, hosts are often infected by several genotypes of a single pathogen species. With the development of molecular markers, such mixed-genotype infections (also called co-infections) have been demonstrated for several plant pathogenic fungi (e.g. *Mycosphaerella graminicola* on wheat (McDonald and Martinez, 1990a), *Alternaria alternata* on pear (Adachi and Tsuge, 1994), *Sclerotinia sclerotiorum* on canola (Maltby and Mihail, 1997), *Aspergillus flavus* on cotton (Bayman and Cotty, 1991), *Cryphonectria parasitica* on chestnut (Anagnostakis and Kranz, 1987), *Gibberella fujikuroi* on maize

(Kedera *et al.*, 1994) and *Fusarium moniliforme* on asparagus (La Mondia and Elmer, 1989). For animal and human parasites, mixed-infections have also been reported (e.g. hepatitis C virus (Mueller *et al.*, 1993) or malaria infection (Day *et al.*, 1992)).

Even though mixed-infections on plants are frequent in nature, they have been the subject of only a few studies. The primary finding has been that co-infecting strains compete with each other (Zelikovitch and Eyal, 1991; Eyal, 1992; Weeds *et al.*, 2000), in some cases to the point of competitive exclusion (Wille *et al.*, 2002). The presence of multiple pathogen genotypes may also affect the host response to the infection. This response may be stimulated by the presence of multiple genotypes, but it may also be less effective by necessitating more resources (Taylor *et al.*, 1998) and result in increased virulence (defined here as reduction in host fitness).

At another time scale, mixed infections are suspected to have an important effect on the evolution of virulence. At the moment, only theoretical models are available for exploring the impact of mixed-genotype infections on the evolution of virulence. Under mixed-genotype infections, natural selection may favour different host exploitation strategies than under single-genotype infections thereby leading to higher virulence (van Baalen and Sabelis, 1995). However, another model for pathogens with sub-lethal effects suggests that virulence may evolve towards a lower level under mixed-genotype infections than under single-genotype infections (Schjorring and Koella, 2002). Yet another model comes to similar predictions if parasites collaborate or engage in the production of a collective resource, e.g. a cell-wall degrading enzyme in the case of a necrotrophic fungus (Brown *et al.*, 2002). Which of these models best describes reality is not known. In general, a model's significance depends on its assumptions and on how well these fit reality. More knowledge on the interactions among pathogen genotypes and between host and pathogen under mixed-genotype infections will improve the realism of the assumptions.

Mixed-genotype infections are common and may play an important role in evolution of virulence, but more information is needed to better appreciate their impact. We investigated mixed-genotype infections in the *M. graminicola* – wheat pathosystem. Mixed-genotype infections have been reported for this pathogen (McDonald and Martinez, 1990a) and even within the same lesion, different genotypes were present in about one fourth of the lesions assayed (McDonald *et al.*, 1995). This means that *M. graminicola* genotypes can be in direct contact with other genotypes. Earlier studies with a few genotypes of this pathogen have demonstrated a reduction in pycnidial coverage, suggesting that interstrain competition during mixed infections reduces pathogen fitness (Zelikovitch and Eyal, 1991; Eyal, 1992). Here we extend these earlier results by simultaneously comparing mixed and single-genotype infections for pathogen aggressiveness (host tissue colonisation), pathogen virulence, host

resistance (ability to reduce the extent of the infection), and host tolerance (ability to reduce the fitness consequences of infection).

Materials and methods

The wheat – M. graminicola pathosystem

We used wheat and *M. graminicola* as a study system, because it offers some major advantages. First, it is a well-known pathosystem. Wheat resistance has been studied extensively, *M. graminicola* is readily cultivable *in vitro*, and genotypes can be characterised with RFLP markers (McDonald and Martinez, 1990a, b). Second, wheat is an inbreeder, which allows one to readily obtain highly homogeneous lines. Third, host fitness is easy to measure, since wheat is an annual species and we do not need to distinguish between male and female fertility because of selfing.

The host

Three spring wheat cultivars, obtained from M. van Ginkel (International Maize and Wheat Improvement Center (CIMMYT), Mexico), were used as hosts: line 1 (TRAP#1/BOW), line 2 (CROC_1/AE.SQUARROSA (205)//BORL95) and line 3 (CATBIRD). The seeds had been treated with fungicides (Carboxin, Captan and Chlorothalonil) to prevent seed borne diseases. Before planting, they were rinsed multiple times with water to remove as much of the fungicide as possible, which could have otherwise influenced the disease response. On March 22, 2000, four seeds of the same cultivar were sown in 11 pots filled with a soil mixture (30% sterilised field soil, 25% bark compost, 20% sand, 15% thin white peat and 10% rice chaff; RICOTER Erdaufbereitung AG) enriched with fertiliser (Osmocote plus 8-9 Mt 16/8/12/1.2; 3 kg/m³). Pots were grouped in plastic trays (45 × 25 cm) and bottom-watered. Within a few days of germination, plants were thinned to one per pot. Plants were grown in a greenhouse under 50 kLux lamps with 15 h day. Temperature was set at 12–15 °C during the night and 16–19 °C during the day, but, because the greenhouse lacks a cooling system, the temperatures were higher on warm days.

The pathogen

Mycosphaerella graminicola (Fückel) J. Schrot. in Cohn infects numerous wild grass species (Eyal, 1999) and causes Septoria leaf blotch of wheat. The disease is named after the anamorph, *Septoria tritici* Roberge in Desmaz. This fungus infects leaf blades and grows exclusively intercellularly (Kema *et al.*, 1996). Necrosis of the leaf tissue results in visible rectangular and brown lesions. On

these, black fructifications (pycnidia) develop in a linear pattern, and about 3 weeks after the beginning of the infection, pycnidiospores are released and dispersed to nearby leaves by raindrops. Asexual as well as sexual reproduction occurs in this pathogen, which overwinters on leaf debris in the field (Eyal *et al.*, 1987). In the present experiment, we used three *M. graminicola* genotypes (1: ST999A9B, 2: ST999H3A, 3: ST999E10C) collected in 1999 in Eschikon (Switzerland) by B. McDonald and characterised as being different genotypes with RFLP's by C. Linde. The experiment was conducted in the greenhouse to avoid contamination by other *M. graminicola* genotypes.

Experimental design

Because the treatment factor (inoculation with one or more pathogen genotypes) had to be applied to bigger experimental units than the host line factor, we used a split-plot design (Fig. 1). Indeed, to avoid cross-contamination between treatments, it was necessary to leave enough space between plants inoculated with different genotypes and therefore, within blocks, we had to spatially group the plants that received the same treatment. The treatment factor had 11 levels and was applied at the plot level. The line factor had three levels and was applied at the subplot level. There were nine plants per subplot, three of each line. Each treatment was present once in each of the seven blocks. In total there were 693 plants (7 blocks $\times$ 11 treatments $\times$ 3 lines $\times$ 3 replicates).

Figure 1. Setup of the experiment in the greenhouse. We used a split-plot design; inoculation treatment was applied at the plot level and wheat line at the subplot level.

The treatments

We inoculated plants with two different spore concentrations to obtain a wider range of disease severity, which improves the ability to measure tolerance. Percentage of the leaf area covered by lesions with fruiting bodies has been shown to increase linearly with $\log_{10}$ of the inoculum concentration (Shearer, 1978).

Around the time when the flag leaf was visible, the host plants were subjected to 11 different treatments, which were different combinations of the three *M. graminicola* genotypes: 1: control, inoculation with water; 2–7: inoculation with each pathogen genotype alone in high (10^6 spores/ml) or low (10^5 spores/ml) inoculum concentration; 8–11: inoculation with mixtures of 2 (1 + 2, 1 + 3, 2 + 3) or 3 pathogen genotypes (1 + 2 + 3) at an inoculum concentration of 10^6 spores/ml.

Genotypes culture and inoculation procedure

Single-spore colonies grown on YMA plates were transferred to 250 ml flasks filled with 80 ml liquid medium (9 g yeast extract, 9 g glucose in 1000 ml ddH₂O). One hundred μ l 25 mg/ml kanamycin was added to each flask. Cultures were grown in the dark at 15 °C on a shaker for 9 days. The milky cultures were centrifuged and the spores diluted in 20 ml water. The spore concentration was measured with a Thoma–Zeiss hemacytometer and adjusted to the required level (10^5 spores/ml for the low inoculum concentration treatments and 10^6 spores/ml for all other treatments). On average, 12.5 ml of inoculum was prepared per plant. A few Tween 20 drops were added to the inoculum that was sprayed onto the plants with a compressed air atomiser. Blocks 1, 3, 5 and 6 were inoculated on May 11, 2000. Blocks 2, 4 and 7 two days later. Plants were moved to a greenhouse where the temperature was between 16 and 20 °C and put for 3 days under plastic tents where humidifiers maintained high humidity. Plants stayed 3 days in these moist chambers. Between treatments, hands, atomiser and other instruments were cleaned thoroughly with ethanol and rinsed with water.

Measurements

On June 6–9, 2000, disease severity was measured as the percentage of the leaf area covered by *M. graminicola* lesions on the top three leaves of each plant. Seeds were harvested at maturity, and total seed weight and seed number recorded as measures of fitness. In August 2000, the above ground plant material was harvested and biomass determined.

Statistical analysis

ANOVAs were used to test whether infection treatment and host line had an effect on disease severity and host fitness. Block and wheat lines were random

effects, whereas treatment was a fixed effect. Disease severity was arcsine square root transformed to improve normality assumptions.

ANCOVAs on host fitness with disease severity as a covariate were run separately for each host line to test for differences in tolerance under single- vs. mixed-genotypes infections. Only plants inoculated with high inoculum concentrations were included in this analysis (7 treatments) since inoculation with genotype mixtures were done only at high concentrations. Additional ANCOVAs on host fitness with disease severity as a covariate were used to test for genetic variation for tolerance and virulence. A significant disease severity-by-line interaction indicates whether host lines differ in tolerance (Simms and Triplett, 1994). The analyses were performed on the plants inoculated with single pathogen genotypes only in low and high inoculum concentrations (6 treatments). Seed weight and seed number were highly correlated ($r_{XY} = 0.915$) and, because analyses on these two response variables gave similar results, only p -values for seed weight (=yield) are mentioned in the results section. Block 1 was removed from the analyses on fitness, because it was located next to the outer wall of the greenhouse and the plants in this block clearly experienced very different environmental conditions than the ones in other blocks (e.g. higher temperatures). Analyses were performed with JMP 4.0.3 (1989–2000 SAS Institute Inc.) and SPLUS 2000 Professional Release 2 (1988–1999 MathSoft, Inc.).

Results

Disease severity

Host line 2 was fully resistant to infection by *M. graminicola* and was therefore excluded from further analyses. The two other host lines differed in susceptibility (Table 1 and Fig. 2). On host line 1, up to one third of the leaf area was covered by lesions, whereas, on the more susceptible host line 3, disease severity ranged from 0 to 70%. Under the control treatment, few plants (8 out of 63) were infected and the lesions that formed were very small (less than 5% of the leaf area). This indicates that cross-contamination between treatments was negligible.

The different treatments had a significant effect on disease severity. The lower inoculum concentrations resulted in less severe infections (contrast: $t = 5.21, p = 0.0001$). On average, a 10-fold increase in inoculum concentration (10^5 vs. 10^6 spores/ml) resulted in a 5-fold increase in disease severity. The pathogen genotypes may have differed in aggressiveness: genotype 3 tended to be more aggressive than genotype 1 ($t = 1.79, p = 0.096$). Though all treatments led to lower disease severity on line 1 than on line 3, this difference was

Table 1. Analysis of variance of disease severity (arcsine square root transformed) on host lines 1 and 3 subjected to 10 inoculation treatments (control treatment excluded)

Source of variation	df	MS	F value	$p > F$
Treatment	9	0.544	4.16	0.0075
Block	6	0.058		
Error 1	54	0.048		
Line	1	2.510	150.30	<0.0001
Line * Treatment	9	0.099	5.96	<0.0001
Error 2	340	0.017		

Line and block are random effects and treatment is a fixed effect. $n = 420$, $R^2 = 0.66$.

Figure 2. Disease severity (percentage of leaf area covered by lesions) $\pm$ SE on host lines 1 (light grey) and 3 (dark grey) inoculated with each of three *Mycosphaerella graminicola* genotypes, alone (in high and low inoculum concentrations) or in mixtures.

particularly great for inoculation by pathogen genotype 2, hence the significant line-by-treatment interaction. Disease severity was influenced by the host genotype and by the interaction between host and pathogen genotypes, and maybe by the pathogen genotype.

Disease severity caused by each genotype mixture was compared to the mean disease severity caused by the same genotypes inoculated alone using planned contrasts. The mixtures 1 + 2 ($t = 0.61$, NS) and 1 + 3 ($t = 0.94$, NS) caused about the same amount of damage as the same genotypes alone. On the other hand, the mixture 2 + 3 ($t = 2.37$, $p = 0.033$) and the three-genotype mixture 1 + 2 + 3 ($t = 2.34$, $p = 0.035$) caused less disease than expected from the results of the inoculation with the corresponding genotypes alone.

Host fitness

Even though disease severity was different on the two susceptible host lines, ANOVAs on seed weight and seed number (Table 2) did not detect a difference in fitness between the two lines. However, treatment had a significant effect on fitness (Fig. 3). A contrast showed that the control plants had a significantly higher fitness than the plants inoculated with single genotypes ($t = 2.53$, $p = 0.014$; for seed weight). On average, infection reduced fitness by around 15%. Plants infected by pathogen genotype 3 had a lower fitness than plants infected by genotypes 1 or 2 ($t = 2.15$, $p = 0.036$). There was no difference in fitness between the plants inoculated with low spore concentration and the ones inoculated with high spore concentration ($t = 0.85$, NS). These were therefore pooled to test for differences between mixed- and single-genotype infections.

Table 2. Analysis of variance of seed number and seed weight

Source of variation	df	Seed number			Seed weight		
		MS	F value	$p > F$	MS	F value	$p > F$
Treatment	10	9571	2.49	0.0163	7.2	2.19	0.0338
Block	5	18,303			31.9		
Error 1	50	3839			3.3		
Line	1	1483	0.68	0.4117	1.8	0.80	0.3706
Line * Treatment	10	1618	0.74	0.6895	1.8	0.78	0.6513
Error 2	317	2195			2.3		

Block 1 excluded; $n = 394$; $R^2 = 0.36$ for seed number and 0.37 for seed weight.

Figure 3. Effect of the infection on fitness. Seed number $\pm$ SE on host line 1 (light grey) and 3 (dark grey) inoculated with each of three *Mycosphaerella graminicola* genotypes, alone or in mixtures.

Infection by pathogen genotypes 1 + 2 reduced fitness to the same extent that infection by the same genotypes alone did ($t = 0.64$, NS). On the other hand, the fitness of plants infected by the other mixtures (1 + 3, 2 + 3 and 1 + 2 + 3) was, or tended to be, higher than the average fitness of plants infected by the same genotypes alone ($t = 2.02$, $p = 0.048$; $t = 1.70$, $p = 0.095$; $t = 2.61$, $p = 0.012$ respectively).

To test for differences in tolerance under single- and mixed-genotype infections, ANCOVAs on fitness measures were run separately for host lines 1 and 3. In none of the analyses was the disease severity-by-treatment interaction term significant. This means that the slopes of the regressions of fitness on disease severity were not significantly different from each other and therefore also not between single- and mixed-genotype infections.

To test whether the two susceptible host lines differed in tolerance, ANCOVA's on fitness measures were run on plants inoculated with single genotypes. The level of disease severity did not influence fitness significantly (Table 3). Whether the lines differed in tolerance was measured by the line-by-disease severity interaction. This term was significant for seed number but not for seed weight. On line 1 there was a tendency for a negative relationship between disease severity and seed number and on line 3 for a positive relationship. The line term indicates whether the host lines differed in their general vigour and it was significant. Thus, the general vigour of line 1 was significantly but only slightly (around 3%) higher than that of line 3. Because the difference was weak, it was not detected in the previous analysis on fitness.

Just as including disease severity as a covariate in the analysis controls for differences in resistance among host genotypes, it also controls for differences in aggressiveness among pathogen genotypes. The pathogen genotype and the

Table 3. Analysis of covariance of seed number and seed weight on the plants inoculated with single pathogen genotypes

Source of variation	df	Seed number			Seed weight		
		MS	F value	$p > F$	MS	F value	$p > F$
Path. genotype	2	2116	1.18	0.3467	1.91	0.74	0.5015
Block	5	1857				5.68	
Error 1	10	1789				2.57	
Line	1	24,511	97.80	<0.0001	26.13	34.56	0.0083
Line * PG	2	130	0.14	0.8659	0.71	0.70	0.4964
Disease severity	1	405	0.45	0.5040	0.74	0.73	0.3928
Line * D. severity	1	3611	4.00	0.0470	1.31	1.30	0.2563
PG * D. severity	2	649	0.72	0.4891	0.07	0.07	0.9323
Line * PG * D. sev.	2	1437	1.59	0.2064	0.57	0.56	0.5719
Biomass	1	243,679	269.76	<0.0001	232.93	230.86	<0.0001
Error 2	186	903				1.01	

Block 1 excluded; $n = 214$; $R^2 = 0.67$ for seed number and 0.66 for seed weight.

pathogen genotype-by-disease severity terms indicate then whether genotypes differ in other traits than aggressiveness that may affect host fitness (e.g. toxin production, diversion of host resources). Neither of these two terms was significant.

Discussion

The main purpose of the present study was to investigate the effects of mixed-genotype infections on pathogen aggressiveness and virulence, and host resistance and tolerance. Under single-genotypes infections, the host lines differed in resistance and slightly in tolerance. Wheat line 3 was more susceptible and maybe more tolerant, but it nevertheless had a slightly lower fitness under infection. Whereas there is a consensus about the definition and measurement of resistance to plant pathogens, tolerance is less understood. Ecologists have focused their attention much more on tolerance to herbivory (reviewed in: Agrawal *et al.*, 1999; Strauss and Agrawal, 1999; Stowe *et al.*, 2000), than on tolerance to disease (Simms and Triplett, 1994; Roy *et al.*, 2000; Kover and Schaal, 2002). Although there is a long history of interest in tolerance to disease by plant pathologists, they have, unfortunately, usually confounded it with resistance by defining it as the ability to minimise fitness loss under infection (e.g. Brönnimann, 1974). The problem is that resistance, by limiting the extent of the infection, also minimises fitness loss. Although he has not been widely cited by plant pathologists, Clarke (1986) did separate resistance from tolerance when he defined tolerance to disease as the ability of a plant to endure a certain level of parasitic infection, which, if it occurred in other plants of the same or similar species, would cause greater impairment of yield. That is, whereas resistance prevents infection, or stops or slows down its development, tolerance reduces the fitness consequences of this infection. To be even more accurate, tolerance to disease should be regarded, as it has been for tolerance to herbivory, as a reaction norm across a gradient of disease severity (Mauricio *et al.*, 1997; Simms, 2000) and measured, for each host genotype, as the slope of a regression of fitness on disease severity. Our study after those of Kramer *et al.* (1980) and Simms and Triplett (1994), is one of the first attempts to measure disease tolerance in this way. We found weak evidence for a difference in tolerance between the two lines for one of the fitness measures, seed number. This result corroborates other evidence that crop cultivars can differ in tolerance. Some wheat lines were found to be more tolerant than others to *M. graminicola* infection (Ziv *et al.*, 1981; Zuckerman *et al.*, 1997) when comparing yield loss under similar levels of disease severity. Kramer *et al.* (1980) also found differences in tolerance to leaf rust among barley cultivars with a regression approach.

Surprisingly, and despite a reduction in fitness of 15% between infected and uninfected plants, we could not detect a clear negative relationship among the inoculated plants between disease severity and fitness, as if the degree of infection had no effect on fitness, but only whether or not they were infected. The absence in our study of a negative relationship between disease severity and fitness is unusual. For example, King *et al.* (1983) describes equations relating yield to disease severity under infection by *M. graminicola* and *Leptosphaeria nodorum* (the lesions caused by these two fungi cannot be distinguished in the field) and consistently finds a negative relationship between these two measures. In the present experiment, plants experienced low levels of disease severity and it is possible that tolerance may be non-linear, and that it may be more likely to operate at low levels of disease severity.

On the pathogen side, we found genetic variation for virulence and aggressiveness among the different genotypes. Genotype 3 tended to be the most aggressive one, and it reduced host fitness the most. However, when controlling for differences in aggressiveness in ANCOVA on host fitness, the pathogen genotypes did not differ from each other. This means that the higher virulence of genotype 3 can be attributed to its higher aggressiveness and not to a higher toxin production or greater diversion of host resources, for example.

Under mixed-genotype infections, the severity disease of caused by two of the three two-genotype mixtures was not different from the average level of disease caused by each genotype alone. On the other hand, co-infection by genotypes 2 + 3 and also by the three-genotype mixture, 1 + 2 + 3, resulted in lower disease severity, especially on host line 3. The observed lower disease severity could either be due to increased host resistance or to competition among pathogen genotypes. We see no convincing reason why resistance should be higher to the mixtures 2 + 3 and 1 + 2 + 3 and not also to other mixtures including pathogen genotypes 2 or 3. Since lower disease severity was observed only for mixtures including both of these pathogen genotypes, they must have interacted, most probably competed with each other, and this competition inhibited their aggressiveness. It is interesting to note that competition among pathogen genotypes did not occur in every mixture, but depended on which genotypes were present.

Mixed-genotype infections had a similar effect on fitness as on disease severity. Mixture 1 + 2, which caused the same disease severity as the average of the same genotypes alone, also reduced fitness to the same extent as the same genotypes alone. Mixtures 2 + 3 and 1 + 2 + 3, whose aggressiveness was reduced by competition, were less virulent than infections by the same genotypes alone. Whereas for mixture 1 + 3, disease severity was not lower than under single-genotype infection, but virulence under mixed-genotype infection was lower than the mean virulence of the same genotypes alone.

The results of our study are consistent with the two experiments conducted previously with *M. graminicola* on wheat (Zelikovitch and Eyal, 1991; Eyal, 1992) which found a reduction in disease severity in mixtures compared to the mean of the same genotypes alone. Additionally, our results show that this reduction in disease severity depends on the pathogen genotype combination and is accompanied by a reduction in virulence. The results for *M. graminicola* agree with those for other plant pathogens (*Sclerotinia sclerotiorum* on canola (Maltby and Mihail, 1997), *Leptosphaeria maculans* on oilseed rape (Mahuku *et al.*, 1996) and *Botrytis cinerea* on French bean (Weeds *et al.*, 2000)) which found a similar or decreased disease severity in the mixed-genotype infections compared to the single-genotype infections. However, the situation seems to be quite different for animal parasites. An experiment with the rodent malaria *Plasmodium chabaudi* tested whether parasites can alter their host exploitation strategy in mixed-genotype infections and found increased virulence in mixed-genotype infections compared to single-genotype infections (Taylor *et al.*, 1998). Another animal study with schistosome infected snails also found increased virulence in mixed-genotype infections (Davies *et al.*, 2002). These results indicate that when modelling the evolution of virulence under mixed-genotype infections, plant pathogens and animal parasites may have to be considered separately.

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