

Effects of *Leptosphaeria maculans* race structure and fitness cost of virulence on management of phoma stem canker in oilseed rape

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Abstract

Fitness of virulent (*avrLm1* and *avrLm4*) or avirulent (*AvrLm1* and *AvrLm4*) isolates of *Leptosphaeria maculans* on *Brassica napus* without the corresponding resistance genes *Rlm1* and *Rlm4* was investigated in controlled environment (CE) and field experiments. Results indicate that there is a measurable fitness cost for virulent isolates compared to avirulent isolates in terms of number of lesions, size of lesions, distance grown through leaf tissue towards the petiole in CE experiments and in terms of systemic growth from leaf lesions to stems in field experiments. There were differences between the *AvrLm1* and *AvrLm4* loci in fitness cost. The optimal temperature for leaf infection was higher for *AvrLm4* isolates than for *AvrLm1* isolates. There was a cultivar effect on fitness cost of virulence at the *AvrLm1* locus but not at the *AvrLm4* locus. Results from field experiments suggest that on the same host without the corresponding *Rlm1* and *Rlm4* genes, *AvrLm4* isolates were more fit than *AvrLm1* isolates in warmer growing seasons. The fitness cost of virulence at the *AvrLm1* locus was generally smaller than that at the *AvrLm4* locus, suggesting that the corresponding resistance gene *Rlm4* is more durable than *Rlm1*. Frequencies of avirulent *AvrLm1* and *AvrLm6* alleles in airborne inoculum for 2006/07, 2007/08 and 2008/09 growing seasons in the UK were investigated using quantitative PCR. There were differences in frequencies of *AvrLm1* and *AvrLm6* alleles in the three seasons. The detection of changes in frequencies of avirulent alleles provides essential information to guide deployment of cultivars with corresponding resistance genes to minimise the risk of breakdown of novel resistance.

Keywords: ascospore, avirulence, blackleg, *Brassica napus* (canola), resistance gene

Introduction

Leptosphaeria maculans is an economically important pathogen of cruciferous crops, especially *Brassica* species, causing phoma stem canker (blackleg) and now responsible for worldwide yield losses worth more than £1000M each growing season (at a price of £300 t⁻¹) (Fitt et al., 2011). Host resistance has been the most economical and effective method for control of the disease (Delourme et al., 2006). Novel sources of resistance to *L. maculans* harbouring race-specific resistance (*R*) genes prove to be very effective when they are first introduced into commercial cultivars. However, *R* gene-mediated resistance is race-specific and often becomes ineffective after two to three growing seasons due to evolution of the pathogen population from avirulence to virulence (Rouxel et al., 2003; Sprague et al., 2006). There is a need to increase the durability of *R* gene resistance and minimise the risk of breakdown. One approach is through understanding the fitness cost associated with pathogen evolution from avirulence to virulence at the corresponding avirulence (*Avr*) loci. Another approach is through investigating seasonal and regional differences in the race structures of *L. maculans* populations to optimise the deployment of cultivars with the corresponding *R* genes and avoid breakdown of novel resistance genes.

Materials and methods

Comparison of fitness of virulent and avirulent isolates during leaf infection in controlled environments

Near isogenic isolates of *L. maculans*, differing in alleles only at the *AvrLm1* (i.e. *AvrLm1*, *avrLm1*) or *AvrLm4* (i.e. *AvrLm4*, *avrLm4*) loci were produced (Huang et al., 2010). Ascospores of virulent (*avrLm1* and *avrLm4*) or avirulent (*AvrLm1* and *AvrLm4*) isolates were produced from *in vitro* crosses. Plants of oilseed rape cultivars Eurol and Darmor (without both *Rlm1* and *Rlm4*, corresponding to *AvrLm1* and *AvrLm4*) were inoculated using the 'ascospore shower' method (Huang et al., 2006). After inoculation, plants were transferred to 5, 10, 15, 20 or 25°C growth cabinets. Incubation period (time from inoculation to the appearance of the first lesion) was recorded. Diameters of 10 lesions were measured 1420 days post inoculation (dpi). The symptomless growth of virulent or avirulent isolates in leaf tissues along the leaf petioles after initial infection was assessed by culture isolation.

Comparison of fitness of virulent and avirulent isolates in natural populations

Three cultivars (Pactol, Darmor and Mohican) without the corresponding resistance genes *Rlm1* and *Rlm4* were used in field experiments in the 2002/03 and 2003/04 growing seasons at Versailles, France. Isolates of *L. maculans* were recovered from individual phoma leaf spot lesions in autumn, from stem cankers before harvest in summer and from ascospores produced on the stem debris in the autumn following the growing seasons (Huang et al., 2006). The isolates were characterised at the *AvrLm1* locus (as *AvrLm1* or *avrLm1*) and *AvrLm4* locus (as *AvrLm4* or *avrLm4*) using cotyledon test on cultivars Columbus (*Rlm1- Rlm3*), Jet Neuf (*Rlm4*) and the susceptible controls Westar (no resistance genes) and 00.22.1.1 (*Rlm3* only). In total, 501 isolates from the 2002/03 growing season and 689 isolates from the 2003/04 growing season were characterised.

Determining frequencies of avirulent alleles in airborne inoculum

Airborne ascospores of *L. maculans* were collected during the 2006/07, 2007/08 and 2008/09 growing seasons at Rothamsted in the UK using a Burkard 7-day volumetric spore sampler, which was surrounded radially by oilseed rape stems affected by phoma stem canker from previous growing season as described by Huang et al. (2005). Airborne inoculum was collected each day between the end of August and the following March each season. Weekly strips of Melinex tape were divided into daily pieces (14 x 48 mm) (Kaczmarek et al., 2009). These were then cut longitudinally, with one piece used for extraction of DNA whilst the other was mounted on a microscope slide for counting ascospores. To estimate frequencies of avirulent alleles, DNA was extracted from spores collected on four selected days within the period of maximum ascospore release each season and the frequencies of avirulent alleles of *AvrLm1* and *AvrLm6* were determined using quantitative PCR (Van de Wouw et al., 2010).

Results

Fitness of virulent/avirulent isolates during leaf infection in controlled environments

For colonisation of leaves, there were differences between the pairs of isolates *AvrLm1/avrLm1* and *AvrLm4/avrLm4* in the optimal temperature for leaf infection. The number of lesions that developed was maximum at 15°C for *AvrLm1* isolates but maximum at 20°C for *AvrLm4* isolates (Table 1). Furthermore, there was a cultivar effect on *AvrLm1/avrLm1* isolates but not on *AvrLm4/avrLm4* isolates; more *AvrLm1* or *avrLm1* lesions developed on Darmor than on Eurol but there was no significant difference in number of *AvrLm4* or *avrLm4* lesions between Darmor and Eurol. There was no difference between *AvrLm1/avrLm1* and *AvrLm4/avrLm4* isolates in the length of the incubation period. For systemic growth in leaf tissue towards the petiole after initial infection, the largest difference between *AvrLm1* and *avrLm1* isolates was at 15°C, while the largest difference between *AvrLm4* and *avrLm4* isolates was at 25°C.

Table 1 Comparative fitness of *L. maculans* near-isogenic isolates (*AvrLm1/avrLm1*, *AvrLm4/avrLm4*) in development of number of phoma leaf spot lesions on leaves of oilseed rape cultivars Darmor and Eurol without the corresponding resistance genes *Rlm1* and *Rlm4*

Temperature (°C)		<i>AvrLm1</i>	<i>avrLm1</i>	<i>AvrLm4</i>	<i>avrLm4</i>
5	Darmor	1.2	1.4	0.9	1.0
	Eurol	1.6	0.9	1.1	0.5
10	Darmor	3.5	1.9	3.3	2.0
	Eurol	2.2	1.4	4.0	2.0
15	Darmor	6.7	3.6	5.6	2.6
	Eurol	3.1	2.4	2.8	1.3
20	Darmor	4.1	2.2	10.9	2.1
	Eurol	4.7	1.3	9.9	3.0
25	Darmor	5.1	2.1	4.0	1.6
	Eurol	3.0	2.1	5.4	4.7

Comparison of fitness of virulent and avirulent isolates in natural population

In 2002/03, the frequency of the *AvrLm1* allele in the *L. maculans* population did not change greatly (Huang et al., 2010). By contrast, the frequency of the *AvrLm4* allele increased from 6% at the phoma leaf spot stage in autumn to 20% at the stem canker stage before harvest and 15% on stem debris after harvest (Huang et al., 2006). On average, over the two growing seasons, there was no difference

between the *AvrLm1* (7.1%) and *AvrLm4* (6.8%) loci in the frequency of avirulent alleles at the leaf lesion stage (initial leaf infection). However, at the stem canker stage (growth from leaf lesion to stem) and on debris after harvest (sexual spore reproduction) the frequencies of the *AvrLm4* allele (16% and 13.8%, respectively) were greater than those of the *AvrLm1* allele (9.1% and 8.4%, respectively).

Determining frequencies of avirulent alleles in airborne inoculum

Airborne inoculum samples from four selected days were used to determine the average frequency of avirulent *AvrLm1* or *AvrLm6* alleles in 2006/07, 2007/08 and 2008/09. Although the specific days changed each year, the samples used were all collected between mid-October and mid-December each season, with a minimum of 1000 ascospores collected on each of the four days. Within the three seasons, the frequency of *AvrLm6* was greater than that of *AvrLm1*. The frequency of *AvrLm1* remained consistently small, between 9 and 16%, over all years. Conversely, the frequency of *AvrLm6* fluctuated from 66% in 2006/07 to 35% in 2007/08 and then 49% in 2008/09.

Discussion

Results of controlled environment and field experiments suggest that there is a fitness cost of virulence at the *AvrLm1* and *AvrLm4* loci in *L. maculans* but that there are differences in terms of fitness cost of virulence between the *AvrLm1* and *AvrLm4* loci. There was a cultivar effect on fitness of *AvrLm1* isolates but not on fitness of *AvrLm4* isolates for the two cultivars tested. There are differences between the *AvrLm1* and *AvrLm4* loci in effects of temperature on fitness cost. The maximum number of leaf lesion produced at 20°C for *AvrLm4* isolates and 15°C for *AvrLm1* isolates suggests that current global warming may benefit *AvrLm4* isolates. Greater fitness cost of virulence at the *AvrLm4* locus than at the *AvrLm1* locus suggests that the corresponding resistance gene *Rlm4* may be more durable than *Rlm1* when it is deployed in commercial crops. Results of airborne inoculum analysis suggest that molecular techniques can be used to monitor the changes in frequency of specific alleles in pathogen populations. Following the sequencing of the *L. maculans* genome (Rouxel et al., 2011), more molecular markers to distinguish avirulent and virulent alleles will be developed and qPCR assays of airborne inoculum will play a critical role in disease management strategies. Furthermore, the qPCR assays can be used to assess the potential risk of severe epidemics for crop cultivars with corresponding *R* genes.

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