European Journal of Plant Pathology (2006) 114: 77–89
DOI 10.1007/s10658-005-2643-4


# Fitness cost associated with loss of the *AvrLm4* avirulence function in *Leptosphaeria maculans* (phoma stem canker of oilseed rape)

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Accepted 1 September 2005

*Key words:* ascospore, avirulence gene, blackleg, *Brassica napus* (canola), near-isogenic isolate, resistance gene

**Abstract**

Near-isogenic isolates of *Leptosphaeria maculans* differing at the AvrLm4 avirulence locus (*AvrLm4* or *avrLm4*) were produced *in vitro*. Methods for inoculation of leaves of oilseed rape with ascospores or conidia were compared. The 'ascospore shower' inoculation was the most efficient method for use when inoculum is limited (e.g. ascospores produced *in vitro*). It was used in controlled environments to compare fitness of *AvrLm4* and *avrLm4* isolates at 5, 10, 15, 20 or 25 °C on leaves of oilseed rape cultivars Eurol and Darmor lacking the resistance gene *Rlm4*, which corresponds to *AvrLm4*. At all temperatures tested, *AvrLm4* ascospores produced more lesions than *avrLm4* ascospores. The diameters of lesions produced by *AvrLm4* ascospores were greater than those of lesions produced by *avrLm4* ascospores. At 15–20 °C, more lesions initiated by *AvrLm4* ascospores produced pycnidia than did lesions initiated by *avrLm4* ascospores. However, there were no differences between *AvrLm4* and *avrLm4* isolates in incubation period (from inoculation to appearance of lesions) or rate of mycelial growth in leaves from lesions towards the stems. In field experiments with winter oilseed rape cultivars lacking *Rlm4*, the frequency of *AvrLm4* isolates increased from 5.7% at the phoma leaf lesion stage (autumn) to 20.5% at the stem canker stage (summer) during 2002/2003 and from 7.9 to 11.5% during 2003/2004 growing seasons. Results of controlled environment and field experiments indicate that *avrLm4* isolates have a fitness cost compared to *AvrLm4* isolates.

*Abbreviations:* AFLP – amplified fragment length polymorphism; BC – back-cross; cv – cultivar; GS – growth stage; NI – near isogenic

## Introduction

*Leptosphaeria maculans* is an important worldwide pathogen of cruciferous crops, especially *Brassica* species, causing phoma stem base canker (blackleg) of oilseed rape in Europe, Canada and Australia (West et al., 2001; Fitt et al., 2006). Use of cultivars (cv) with resistance genes is an efficient and environmentally friendly way to control the disease. However, new major resistance genes often become ineffective after two to three seasons if cultivars carrying them are grown commercially (Li et al., 2003; Rouxel et al., 2003a) or in experimental plots (Brun et al., 2000). There is a need to evaluate *a priori* the durability of new resistance genes to design management strategies that will minimise the risk of rapid resistance breakdown.

One approach to predicting the durability of new resistance genes is through understanding the fitness associated with the corresponding pathogen avirulence genes (Leach et al., 2001). Fitness of an organism is defined as the combined ability for it to survive and reproduce (Crow, 1986; Pringle and Taylor, 2002). Fitness can be measured in absolute or relative terms. For plant pathogens, several traits, such as reproductive rate, infection efficiency or severity of disease caused, have been used to measure fitness (Vera Cruz et al., 2000; Leach et al., 2001; Pringle and Taylor, 2002). Several bacterial avirulence genes possess a function in disease symptom expression (Leach et al., 2001). Therefore, it is suggested that the fitness cost associated with pathogen evolution from avirulence to virulence to overcome host resistance affects the durability of the resistance (Vera Cruz et al., 2000). This has been demonstrated for the *Xanthomonas oryzae*/rice gene-for-gene interaction (Vera Cruz et al., 2000). However, few other experimental data sets are available to test this hypothesis, especially for fungal plant pathogens.

*L. maculans* has a gene-for-gene interaction with *B. napus* (Ansan-Melayah et al., 1998; Balesdent et al., 2001, 2002). Although several major genes conferring resistance to *L. maculans* have been identified (Ansan-Melayah et al., 1998; Delourme et al., 2004, 2006), it has been difficult to investigate *a priori* the durability of these resistance genes through assessing fitness associated with corresponding avirulence genes without having available pairs of *L. maculans* isolates differing only at the corresponding avirulence locus. Since pairs of near isogenic (NI) *L. maculans* isolates differing only at one avirulence locus (Attard et al., 2002) have now been developed, the potential durability of major gene resistance can be examined by comparing the fitness of corresponding avirulent and virulent isolates.

In Europe, including the UK, phoma stem canker epidemics are initiated by ascospores produced on infected debris and there is no evidence that conidia play a role in the development of epidemics (Gladders and Musa, 1980; West et al., 1999; Huang et al., 2005). Effects of major genes for resistance operating during the infection of leaves by germ tubes from these ascospores in autumn can remain for the whole growing season because *L. maculans* is a monocyclic pathogen (Fitt et al., 2006). However, most previous work on the *L. maculans*/*B. napus* interaction has been done with conidial inoculum applied to cotyledons. Only recently have experiments on leaf penetration and infection been done with ascospores (Biddulph et al., 1999; Toscano-Underwood et al., 2001; Huang et al., 2003). To obtain lesions using conidial inoculum, leaves usually have to be wounded before inoculation. Since wounding can induce plant defence responses or allow the pathogen to bypass leaf resistance, this approach is not suitable to compare the fitness of NI *L. maculans* isolates.

Since techniques were published for the *in vitro* production of ascospores of *L. maculans* by crossing the two opposite mating types (Mengistu et al., 1993; Gall et al., 1994), it has become possible to use ascospores from genetically defined isolates as inoculum to study the relative fitness of virulent and avirulent isolates. However, since only small numbers of ascospores are produced *in vitro*, methods for inoculating with the large numbers of ascospores produced on infected debris under natural conditions are not suitable for inoculating with ascospores produced from defined crosses *in vitro*.

This paper investigates the fitness cost associated with the loss of avirulence function of the *AvrLm4* allele. For this purpose, NI *L. maculans* isolates carrying either the avirulent (*AvrLm4*) or the virulent (*avrLm4*) allele of the avirulence locus AvrLm4 were compared for specific components of fitness (i.e. infection efficiency, latent period, size of the lesions, ability to sporulate on the lesions, systemic growth down the petiole) in controlled environments using a new 'ascospore shower' inoculation method. To support results from controlled environment experiments, changes in frequencies of *AvrLm4* isolates in natural *L. maculans* populations were assessed under field conditions using winter oilseed rape crops lacking the corresponding resistance gene *Rlm4*.

## Materials and methods

### *Production of near-isogenic isolates of* Leptosphaeria maculans

Near-isogenic isolates of *L. maculans*, differing in their alleles at the AvrLm4 locus, were produced by a back-crossing (BC) scheme. Two sister isolates of opposite mating-type, v23.1.2 (*avrLm4, MAT1–1*) and v23.1.3 (*AvrLm4, MAT1–2*), were

crossed *in vitro* and the $F_1$ progeny isolates were characterised at the AvrLm4 locus by inoculation of cotyledons of cv Jet Neuf (carrying *Rlm4*) and cv Westar (susceptible with no resistance genes) (Balesdent et al., 2001; Attard et al., 2002). The mating types of progeny isolates were determined by PCR (Cozijnsen and Howlett, 2003). An *avrLm4*, *MAT1–1* isolate was selected at random from the $F_1$ progeny and back-crossed to v23.1.3 (*AvrLm4*, *MAT1–2*). The $BC_1$ progeny isolates were characterized on cv Jet Neuf and an *avrLm4*, *MAT1–1* isolate was selected and crossed to v23.1.3 for the second back-cross ($BC_2$). This procedure was repeated until the fifth BC generation ($BC_5$), obtained by crossing v23.1.3 and v37.1.4. Ninety-five progeny isolates recovered from the fifth BC were characterised at the AvrLm4 and *MAT* loci.

AFLP markers were used to confirm the increase in isogenicity at each step of the BC scheme. AFLP bands were amplified using 21 primer pairs (Kuhn et al., 2006) with genomic DNA of the $F_1$ parental isolates v23.1.2 and v23.1.3, along with the selected parental isolate for each subsequent round of BC. Bands were separated on CastAway 5.5% acrylamide gels (Stratagene) and visualized by silver nitrate staining (Kuhn et al., 2006). Bands that were polymorphic between parental isolates of each BC generation were recorded.

### In vitro *growth rates of NI* L. maculans *isolates*

The *in vitro* growth rates of the $F_1$ parental isolates and six avirulent (*AvrLm4*) and six virulent (*avrLm4*) NI isolates from $BC_5$ progeny were compared. Plugs (8 mm diameter) were taken from the margin of actively growing colonies of each isolate and placed in the centre of Petri dishes (9 cm diameter) filled with 20 ml of V8 juice agar. Petri dishes were incubated in darkness at 4, 12 or 22 °C, with three or four replicate Petri dishes per isolate. Radial growth was assessed by measuring two perpendicular colony diameters after 6, 11 and 17 days (12 and 22 °C) or 10, 24 and 32 days (4 °C). To estimate the growth rate, a linear regression of colony diameter on incubation time was done for each isolate. Analyses of variance were done to assess differences between *AvrLm4* and *avrLm4* NI isolates in growth rate *in vitro* at different temperatures, using GENSTAT statistical software (Payne et al., 1993).

### In vitro *crosses of NI isolates to produce ascospores*

To produce ascospores of isolates with either *AvrLm4* or *avrLm4* alleles, four *AvrLm4* (two *MAT1–1* and two *MAT1–2*) and four *avrLm4* (two *MAT1–1* and two *MAT1–2*) isolates from the fifth BC progeny were randomly selected for subsequent crossing. Conidial suspensions of the eight isolates were prepared and adjusted to a concentration of $10^6$ spores $ml^{-1}$. Equal volumes of spore suspensions of the four *AvrLm4* isolates were mixed, and 50 $\mu$l of the mixture were spread over 20% V8 juice agar in Petri dishes (5 cm diameter). Petri dishes were incubated for 7 days at 25 °C, under mixed white (500 nm) and near-UV light (360 nm) for 12 h of light per day, then flooded with 5 ml of 1.5% water agar (cooled to 45 °C) and incubated for 5–6 weeks at 12 °C under alternating near-UV light (12 h per day)/darkness. Pseudothecia produced under these conditions contained ascospores of only the *AvrLm4* genotype. Similarly, pseudothecia containing *avrLm4* ascospores were produced by incubating four *avrLm4* isolates together.

### *Development of the 'ascospore shower' method for inoculation with ascospores produced* in vitro

Seeds of cv Lipton were sown in pots (7 cm diameter) containing peat-based compost and a soluble fertiliser (1·5 kg PG mix $m^{-3}$; Petersfield Products, Cosby, Leicester, UK). Plants were grown in a glasshouse and thinned to one plant per pot 10 days after sowing. Pots were then placed in seed trays (37 cm×23 cm) and transferred to a 15 °C controlled-environment cabinet. Plants were inoculated when they reached growth stage (GS) 1,3 (Sylvester-Bradley and Makepeace, 1985) and kept in the growth cabinet throughout the experiment.

A suspension of *L. maculans* conidia was prepared from a 12-day-old culture of isolate L44, which was obtained from a phoma leaf spot collected during November 2001 from winter oilseed rape cv Apex at Rothamsted. A suspension of *L. maculans* ascospores was prepared from naturally infected oilseed rape stem base debris collected during August 2002 at Rothamsted, using the method described by Huang et al. (2001). Ascospores from defined crosses were obtained by crossing *L. maculans* isolates v23.1.2 and v23.1.3 on V8 juice agar (Balesdent et al., 2001). Pieces of agar

with mature pseudothecia were attached to the under-side of a Petri dish lid, then the lid was placed over the base of the dish, which was then placed at 15 °C for 6–8 h in darkness to allow ascospores to discharge into the bottom of the dish. The dish was viewed under a binocular stereo-microscope (Olympus Optical Co., London) to confirm that ascospores had been discharged. Distilled water was added to suspend the ascospores. The concentration of the conidial suspension was adjusted to $10^6$ conidia $ml^{-1}$ and the concentration of the ascospore suspension was adjusted to $10^3$ ascospores $ml^{-1}$.

For inoculation of leaves with conidia, three methods were used: (1) spray the whole plant with conidial suspension; (2) point inoculation with a 10 $\mu$l drop of conidial suspension without wounding; (3) point inoculation with a 10 $\mu$l drop of conidial suspension on a site that had been wounded using a sterile pin. Three plants were inoculated by each method. For point inoculation, only the first and second leaves of each plant were inoculated; 6–8 wounded sites or 10–12 unwounded sites on each plant were inoculated. For inoculation with ascospores, three inoculation methods were used: (1) spray the whole plant with ascospore suspension; (2) point inoculation with a 10 $\mu$l drop of ascospore suspension on the leaf surface without wounding the leaf; (3) 'rain down' dry ascospores onto the plant. To allow ascospores to 'rain down' onto plants ('ascospore shower'), three small pieces (0.5×2 cm) of oilseed rape debris with mature pseudothecia were attached to the under-side of a tray cover (37×23×14 cm) with Vaseline (Chesebrough-Pond's Ltd, London) and the debris was sprayed with distilled water until run-off. Alternatively, for ascospores produced by *in vitro* crosses, pieces of agar with mature pseudothecia produced *in vitro* were attached to the under-side of a tray cover. For both sources of inoculum, the tray cover was then placed over the tray with plants (Figure 1). Three plants were inoculated by each inoculation method. For point inoculation, 4–6 unwounded sites on each plant were inoculated. With 'ascospore shower' inoculation, the duration of the inoculation period was 2 h for ascospores from natural conditions and 24 h for ascospores from *in vitro* crosses. After inoculation, plants were covered immediately with polyethylene bags sprayed inside with distilled water to maintain leaf wetness for 72 h.

The numbers of new phoma leaf spots on each leaf of the inoculated plants were counted daily until no more new leaf spots appeared. The incubation period (from inoculation to the appearance of first phoma leaf spots) for each treatment was recorded. For point inoculation, the infection efficiency was estimated as the percentage of inoculated sites that produced lesions. To estimate the infection efficiency of conidia or ascospores inoculated by spraying, at the time of inoculation three microscope slides were placed among the plants so that conidia or ascospores used for the experiment were also deposited on the slides. The number of conidia or ascospores deposited per $cm^2$ on each of the slides was counted under a light microscope (Olympus Optical Co., London). The leaf areas of five additional uninoculated plants were measured at the time of inoculation. The mean number of conidia or ascospores deposited per plant was estimated [leaf area ($cm^2$) per plant × number of spores per $cm^2$]. The infection efficiency was then estimated as number of spores required to cause one lesion (i.e. total number of spores deposited per plant divided by total number of lesions which developed per plant).

### *Fitness of virulent (avrLm4) and avirulent (AvrLm4)* L. maculans *isolates during leaf infection in controlled environments*

Plants of oilseed rape cultivars Eurol (lacking *Rlm4*) and Darmor (lacking *Rlm4*) were grown in

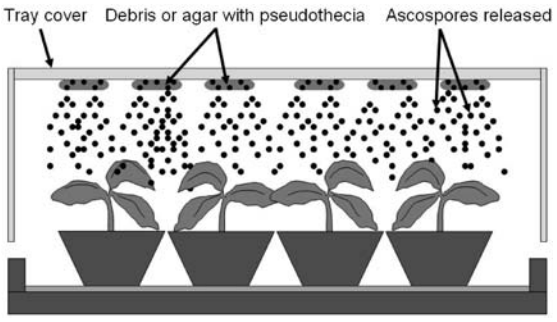


*Figure 1.* 'Ascospore shower' method for inoculation of oilseed rape seedlings with *Leptosphaeria maculans* ascospores produced under natural conditions on oilseed rape debris or *in vitro* by crossing the two opposite mating types on agar medium. Small pieces of oilseed rape debris with mature pseudothecia were attached to the under-side of a tray cover and the debris was sprayed with distilled water until run-off. Alternatively, for defined crosses, pieces of agar with mature pseudothecia produced *in vitro* were attached to the under-side of a tray cover. The tray cover was then placed over the plants to allow dry ascospores to 'rain down' onto the plants.

pots (5 cm diameter) containing peat-based compost and a soluble fertiliser. The pots were put in trays (37×23 cm) in four rows with seven pots in each row. The two cultivars were sown in alternate rows. Plants were grown in a glasshouse, thinned to one plant per pot 10 days after sowing and then transferred to a 15 °C controlled-environment cabinet until they reached GS 1, 3 and were ready for inoculation.

Plants were inoculated using the 'ascospore shower' method with ascospores of either *AvrLm4* or *avrLm4* isolates that had been produced *in vitro*. A total of 10 trays with 28 plants per tray was inoculated. After inoculation, plants were sprayed with distilled water and covered with tray covers. Two trays (one inoculated with ascospores of *AvrLm4* isolates, the other inoculated with ascospores of *avrLm4* isolates) were selected at random and transferred to each of the 5, 10, 15, 20 or 25 °C growth cabinets. The tray covers were kept on for 48 h to maintain leaf wetness. The experiment was repeated three times, with each experiment inoculated with fresh ascospores produced in a new set of crosses. Growth cabinets were allocated at random to each of the three experiments, whenever possible. The temperatures in the growth cabinets were monitored throughout the experiments and varied by ±1 °C.

Plants were assessed daily until no new leaf spots appeared and the incubation period was recorded. The diameters of 10 lesions on plants grown at 15, 20 and 25 °C were measured 13–14 days after inoculation. To compare the growth of *AvrLm4* and *avrLm4* isolates after initial infection (growth from leaf lesion towards the petiole to reach the stem), affected leaves were detached 18–19 days after inoculation, and *L. maculans* was isolated at 1, 2 and 3 cm from centre of the lesion in the direction of petiole, using the method described by West et al. (2002). To compare the sporulation of *AvrLm4* and *avrLm4* isolates, the number of lesions that produced pycnidia was counted at the end of each experiment (i.e. when no more new lesions appeared).

Analyses of variance using the GENSTAT statistical software compared components of the fitness (e.g. incubation period, number of lesions, size of lesions, growth *in planta* and sporulation ability) of *AvrLm4* and *avrLm4* isolates during infection of leaves of oilseed rape without the resistance gene *Rlm4* (Payne et al., 1993). Linear regressions of incubation period on temperature were calculated separately for the inoculum (*AvrLm4* or *avrLm4*) treatments in each of the three replicate experiments. Analyses of position and parallelism were done to assess whether the data were best fitted by a single line or series of parallel lines or series of non-parallel lines for *AvrLm4* and *avrLm4* isolates.

### *Fitness of virulent (avrLm4) and avirulent (AvrLm4) isolates in natural populations of* L. maculans

Oilseed rape cultivars Darmor, Mohican and Pactol, all lacking resistance gene *Rlm4*, were grown in field experiments during two growing seasons at Versailles, France. During 2002/2003, each of the three cultivars was grown in 6.5×2.3 m plots with four replicates. During 2003/2004, only cv Mohican was grown in 20×3 m plots with 10 replicates. The experimental plots for the two seasons were located in the same large field, but not at the same place in the field. During 2002/2003, 2.87 ha of oilseed rape (cultivars Extra and Pollen, with *Rlm4*) were also grown in this field. Isolates were recovered from individual phoma leaf spot lesions sampled from plots in autumn (5 and 11 November 2002, 24–26 November 2003) or spring (9 April 2004), from stem cankers sampled before harvest during summer (20 June 2003, 8 July 2004) and from ascospores produced on the debris in the following autumn using the method described by West et al. (2002). All isolates were characterised at the AvrLm4 locus using cotyledon inoculation tests on cultivars Jet Neuf (carrying *Rlm4*) and Westar (susceptible control with no resistance genes) (Balesdent et al., 2001).

## Results

### *Production of near-isogenic isolates of* Leptosphaeria maculans

The 21 AFLP primer pairs generated 94 AFLP fragments that were polymorphic between the two parental isolates of the $F_1$ generation. The number of polymorphic markers decreased at each successive BC generation (Table 1), indicating an increase in isogenicity. The level of

polymorphism at each BC generation was greater than expected (Table 1). *AvrLm4* and *avrLm4* isolates from the $BC_5$ generation, which were used to produce near-isogenic ascospores for fitness studies, were identical for more than 90% of the markers tested.

### In vitro *growth rates of NI* L. maculans *isolates*

For the six avirulent (*AvrLm4*) and six virulent (*avrLm4*) NI isolates selected from the $BC_5$ progeny (Table 2), the *in vitro* growth rate of *avrLm4* isolates was greater than that of *AvrLm4* isolates at 4 °C ($P < 0.001$; 78df; SED = 0.01) and 12 °C ($P < 0.01$; 80df; SED = 0.02). However, these differences between *AvrLm4* and *avrLm4* NI isolates were small (0.05 mm per day at 4 °C and 0.09 mm per day at 12 °C), compared to the differences between the two parental isolates of the $F_1$ generation (0.18–0.36 mm per day at 4–22 °C; Table 2). In addition, there was no difference in mean growth rate between *AvrLm4* (3.62 mm per day) and *avrLm4* (3.65 mm per day) isolates at 22 °C ($P = 0.13$; 60df; SED = 0.03). Furthermore, there was no difference in germination patterns between ascospores of *AvrLm4* and *avrLm4* isolates on water agar at 22 °C.

*Table 1*. Estimation of the level of isogenicity of the two parental isolates of each generation of the back-crossing (BC) scheme for producing near isogenic (NI) *AvrLm4* and *avrLm4* isolates of *Leptosphaeria maculans*

| Generation | No. AFLP markers polymorphic between parental isolates[a] | Observed % polymorphic markers[b] | Expected % polymorphic markers |
|---|---|---|---|
| $F_1$ | 94[c] | 100 | 100 |
| $BC_1$ | 50 | 53.2 | 50 |
| $BC_2$ | 30 | 31.9 | 25 |
| $BC_3$ | 15 | 16.0 | 12.5 |
| $BC_4$ | 15 | 16.0 | 6.3 |
| $BC_5$ | 9 | 9.6 | 3.2 |

[a]Parental isolates for the F1 were v23.1.3 (*MAT1–2*) and v23.1.2 (*MAT1–1*); parental isolates for BC5 were v23.1.3 (*MAT1–2*) and v37.1.4 (*MAT1–1*).
[b]Based on the proportion of AFLP markers which were polymorphic between parental isolates.
[c]A total of 94 markers were compared on each pair of parental isolates.

### *Development of the 'ascospore shower' method for inoculation with ascospores produced* in vitro

All the plants inoculated with ascospores that had been produced on oilseed rape stem base debris under natural conditions developed leaf lesions, regardless of the inoculation method (Table 3). However, no lesions developed on unwounded plants inoculated with droplets of conidial suspensions, and very few lesions developed on plants sprayed with conidia. Plants inoculated with conidia on wounded sites developed lesions. All plants inoculated with ascospores produced *in vitro* developed lesions. The incubation period (from inoculation to appearance of the first lesion) was longer when conidia were inoculum (15–18 days) than when ascospores were inoculum (5–9 days). For ascospores, the incubation period was longer for the 'ascospore shower' method (9 days) than for droplet inoculation (5–7 days). For conidia, the incubation period was longer for spray inoculation (18 days) than for droplet inoculation (15 days).

The infection efficiency of ascospores was greater than that of conidia (Table 3). For droplet inoculation, only 56% of sites inoculated with conidia developed lesions, while 100% of sites inoculated with ascospores, either produced under natural conditions or from crosses *in vitro*, developed lesions. In treatments where leaves were sprayed with spore suspensions, about nine ascospores were required to produce one lesion, while about $2.4 \times 10^6$ conidia were necessary to produce one lesion. The 'ascospore shower' inoculation method was more efficient than the ascospore suspension method. To inoculate three plants with ascospores from *in vitro* crosses between v23.1.2 and v23.1.3, three pseudothecia were used to produce the ascospore shower and each plant developed an average of 3.5 lesions (Table 3). By contrast, ten pseudothecia were needed to make only 200 $\mu$l of ascospore suspension at the concentration required ($10^3$ ascospores $ml^{-1}$). This volume was not sufficient for spray inoculation, and could be used only for point inoculation. To inoculate three plants with ascospores produced under natural conditions, three small pieces of debris were used to produce the ascospore shower. By contrast, 10 pieces of debris were required to make an ascospore suspension for spray inoculation.

*Table 2*. *In vitro* growth rate of the *Leptosphaeria maculans* parental isolates of the $F_1$ and $BC_5$ generations and of near-isogenic isolates (*AvrLm4* and *avrLm4*) from the $BC_5$ progeny

| Isolate[a] | AvrLm4 allele[b] | *MAT* allele[c] | Growth rate (mm day$^{-1}$)[d] | | |
|---|---|---|---|---|---|
| | | | 4 °C | 12 °C | 22 °C |
| Parents | | | | | |
| v23.1.3 | *AvrLm4* | *MAT1–2* | 0.98 | 2.00 | 3.51 |
| v23.1.2 | *avrLm4* | *MAT1–1* | 1.16 | 2.36 | 3.80 |
| v37.1.4 | *avrLm4* | *MAT1–1* | 1.00 | 1.88 | 3.44 |
| Progeny | | | | | |
| v41.1.2 | *AvrLm4* | *MAT1–1* | 1.01 | 2.09 | 3.81 |
| v41.2.1 | *AvrLm4* | *MAT1–1* | 0.99 | 1.89 | 3.75 |
| v41.5.3 | *AvrLm4* | *MAT1–1* | 0.99 | 1.78 | 3.59 |
| v41.2.4 | *AvrLm4* | *MAT1–2* | 0.94 | 1.88 | 3.51 |
| v41.2.5 | *AvrLm4* | *MAT1–2* | 1.00 | 1.87 | 3.71 |
| v41.3.9 | *AvrLm4* | *MAT1–2* | 0.96 | 2.14 | 3.64 |
| v41.1.1 | *avrLm4* | *MAT1–1* | 1.00 | 2.11 | 3.68 |
| v41.2.8 | *avrLm4* | *MAT1–1* | 0.97 | 1.84 | 3.62 |
| v41.3.3 | *avrLm4* | *MAT1–1* | 0.98 | 2.18 | 3.57 |
| v41.3.1 | *avrLm4* | *MAT1–2* | 0.98 | 1.87 | 3.64 |
| v41.3.10 | *avrLm4* | *MAT1–2* | 1.10 | 1.83 | 3.58 |
| v41.5.6 | *avrLm4* | *MAT1–2* | 1.03 | 2.22 | 3.63 |
| SED | | | 0.006 | 0.021 | 0.027 |
| (df) | | | (99) | (95) | (75) |

[a]The parental isolates were v23.1.3 and v23.1.2 for $F_1$, v23.1.3 and v37.1.4 for $BC_5$.
[b]Isolates were characterised at the AvrLm4 locus by inoculation onto cotyledons of oilseed rape seedlings of cv Jet Neuf (with resistance gene *Rlm4*) and Westar (susceptible control with no resistance genes).
[c]The mating type (*MAT1–1* or *MAT1–2*) of isolates was determined by PCR.
[d]Radial growth of isolates grown on V8-juice agar in darkness was measured for two perpendicular diameters (mm) per plate 6, 11 and 17 days (12 and 22 °C) or 10, 24 and 32 days (4 °C) after inoculation. The growth rate was the slope of the regression line calculated from data for four times (0, 6, 11 and 17 days for 12 and 22 °C; 0, 10, 24 and 32 days for 4 °C). Data are means from three or four replicate plates per isolate.

*Table 3*. Effects of inoculum type (ascospores or conidia) and inoculation method on incubation period, number of lesions and infection efficiency of *Leptosphaeria maculans* (phoma leaf spot) on leaves of oilseed rape cv. Lipton

| Inoculum | Inoculation method | Incubation period (days)[a] | No. lesions per plant | Infection efficiency |
|---|---|---|---|---|
| Conidia[b] | Point droplet | –[c] | 0 | 0 |
| | Wounding/point | 15 | 4.1 | 56.1%[d] |
| | Spray droplets | 18 | 0.6 | 1/($2.4\times10^6$)[e] |
| Ascospores (produced naturally)[f] | Point droplet | 5 | 5.4 | 100%[d] |
| | Spray droplets | 7 | 45.5 | 1/(8.7)[e] |
| | Ascospore shower | 9 | 6.5 | –[c] |
| Ascospores (produced *in vitro*)[g] | Point droplet | 7 | 4 | 100%[d] |
| | Ascospore shower | 9 | 3.5 | –[c] |

[a]Time (days) from inoculation to appearance of the first lesion.
[b]The conidial suspension was made from a *L. maculans* isolate derived from a phoma leaf spot sampled during November 2001 from an oilseed rape (cv Apex) field experiment at Rothamsted.
[c]Not available or not tested.
[d]For point inoculation, infection efficiency is defined as the percentage of inoculated sites that developed lesions.
[e]For spray inoculation with spore suspension (conidia or ascospores), infection efficiency is defined in terms of the number of spores required to cause one lesion (e.g., 9 ascospores required to cause one lesion for spray ascospores droplets inoculation).
[f]Ascospores were obtained from naturally infested winter oilseed rape stem base debris (cv Apex) collected during August 2002 at Rothamsted.
[g]Ascospores were obtained from *in vitro* crosses between isolates v23.1.2 and v23.1.3.

*Fitness of virulent (avrLm4) and avirulent (AvrLm4)* L. maculans *isolates during leaf infection in controlled environments*

Oilseed rape plants inoculated with ascospores of *AvrLm4* isolates developed more lesions than plants inoculated with ascospores of *avrLm4* isolates ($P < 0.001$; 30df; SED = 0.72) (Figure 2). There was an interaction between temperature and type of inoculum ($P < 0.05$; 15df; SED = 2.2). Plants inoculated with ascospores of *AvrLm4* isolates developed the most lesions at 20 °C (10 lesions per plant) and the fewest lesions at 5 °C (only 1 lesion per plant). However, there was no significant difference in the number of lesions between temperatures on plants inoculated with ascospores of *avrLm4* isolates. There was no significant difference in the number of lesions between cultivars Darmor and Eurol; therefore only mean values are presented.

There was no difference in incubation period (from inoculation to the appearance of first lesion) between *AvrLm4* and *avrLm4* isolates at 5–25 °C ($P = 0.8$; 30df; SED = 0.2). However, there were differences between temperatures for both *AvrLm4* and *avrLm4* isolates. The relationship between incubation period ($f$) and temperature ($T$) was fitted best by a single line for *AvrLm4* and a*vrLm4* isolates: $f = 24.3–0.7\ T$ ($R^2 = 0.87$). As temperature increased, the incubation period of both *AvrLm4* and *avrLm4* isolates decreased, from 21 days at 5 °C to 8 days at 25 °C (Figure 3).

At 13–14 days after inoculation, the diameters of lesions on plants inoculated with ascospores of *AvrLm4* isolates were greater than those on plants inoculated with ascospores of *avrLm4* isolates ($P < 0.001$; 18df; SED = 0.04) (Table 4). For both *AvrLm4* and *avrLm4* isolates, the diameters of lesions increased with increased temperature ($P < 0.05$; 4df; SED = 0.1). The mean diameter of *AvrLm4* lesions increased from 3.7 mm at 15 °C to 9.8 mm at 25 °C, and that of *avrLm4* lesions increased from 3.3 mm at 15 °C to 7.1 mm at 25 °C. More lesions on plants inoculated with ascospores of *AvrLm4* isolates produced pycnidia than did lesions on plants inoculated with ascospores of *avrLm4* isolates ($P < 0.05$; 24df; SED = 1.5). On plants inoculated with ascospores of *AvrLm4* isolates, 18% of lesions produced pycnidia at 15 °C, and 11, 10 and 9% of lesions produced pycnidia at 10, 20 and 25 °C, respectively (Table 4). On plants inoculated with ascospores of *avrLm4* isolates, only 14% of lesions produced pycnidia at 15 °C, and 6, 5 and 7% of lesions produced pycnidia at 10, 20 and 25 °C, respectively. No pycnidia were observed on lesions at 5 °C. There was no difference between *AvrLm4* and *avrLm4* isolates in the percentage of lesions, that produced isolates at 1–3 cm away from lesions towards the petiole at 15–25°C (Table 4).

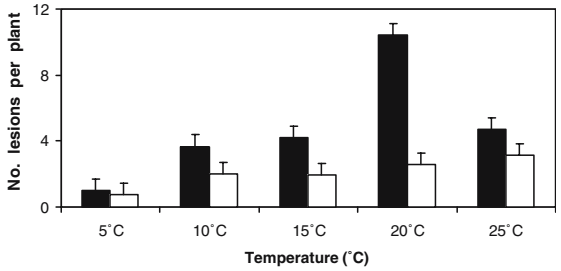


*Figure 2*. Numbers of phoma leaf spot lesions which developed at different temperatures on oilseed rape plants of cultivars Darmor and Eurol that lack resistance gene *Rlm4*, inoculated with ascospores of *AvrLm4* (■) or *avrLm4* (□) isolates of *Leptosphaeria maculans* produced *in vitro*. Data are combined means for cultivars from three replicate experiments ($P < 0.001$; SED = 0.72; 30df).

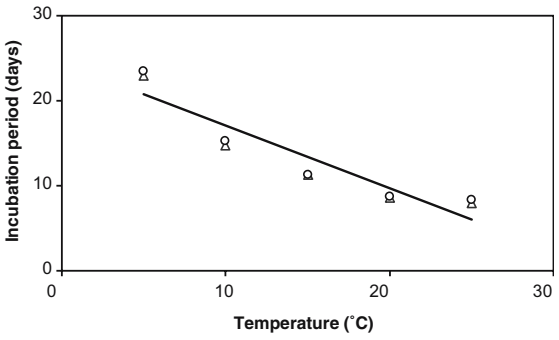


*Figure 3*. Effects of temperature on the incubation period (from inoculation with ascospores to the appearance of the first lesion) of *AvrLm4* (Δ) and *avrLm4* (○) isolates of *Leptosphaeria maculans* on oilseed rape plants (cultivars Darmor and Eurol) lacking the resistance gene *Rlm4*. The relationship between incubation period ($f$) and temperature ($T$) for *AvrLm4* and a*vrLm4* was fitted best by a single line $f = 24.3–0.73\ T$ ($R^2 = 0.87$). Data points are means of three replicate experiments but the regression line was calculated from data for individual experiments.

*Table 4*. Fitness of *Leptosphaeria maculans* near-isogenic isolates *AvrLm4* (avirulent) or *avrLm4* (virulent) in terms of lesion diameter, % lesions producing pycnidia or growth from leaf spot lesions towards the petiole on leaves of *Brassica napus* cultivars Darmor and Eurol without the corresponding resistance gene *Rlm4*[a]

| Temperature (°C) | AvrLm4 allele | Lesion diameter (mm)[b] | % lesions producing pycnidia[c] | % lesions producing hyphae at 1 – 3 cm away from the lesion towards the petiole[d] | | |
|---|---|---|---|---|---|---|
| | | | | 1 cm | 2 cm | 3 cm |
| 5 | *AvrLm4* | –[e] | 0 | – | – | – |
| | *avrLm4* | – | 0 | – | – | – |
| 10 | *AvrLm4* | – | 10.5 | – | – | – |
| | *avrLm4* | – | 6.0 | – | – | – |
| 15 | *AvrLm4* | 3.7 | 17.5 | 78.3 | 61.3 | 58.0 |
| | *avrLm4* | 3.3 | 14.0 | 75.0 | 66.0 | 70.6 |
| 20 | *AvrLm4* | 8.8 | 9.5 | 83.3 | 66.2 | 60.1 |
| | *avrLm4* | 6.0 | 4.7 | 75.7 | 39.2 | 46.6 |
| 25 | *AvrLm4* | 9.8 | 9.4 | 64.7 | 53.9 | 39.6 |
| | *avrLm4* | 7.1 | 6.8 | 41.2 | 23.8 | 22.4 |
| SED | | 0.04 | 1.56 | 15.38 | 16.61 | 16.60 |
| (df) | | (18) | (24) | (10) | (10) | (14) |

[a]Data are the mean values for cultivars Darmor and Eurol from three replicate experiments. In each experiment, plants were inoculated with fresh ascospores of *AvrLm4* or *avrLm4* isolates produced *in vitro* on a new set of crosses.
[b]Lesion diameters were measured 13–14 days after inoculation.
[c]Number of lesions which produced pycnidia was counted at the end of each experiment.
[d]Presence of *L. maculans* hyphae shown by isolations, made on affected leaves 18–19 days after inoculation.
[e]Not measured, since lesions were too small to measure at 10 °C and no lesions had developed at 5 °C 13–14 days after inoculation.

### *Fitness of virulent (avrLm4) and avirulent (AvrLm4) isolates in natural populations of* L. maculans

During the 2002/2003 growing season, 284 isolates were recovered from leaf lesions in autumn, 49 isolates were recovered from stem cankers in summer and 171 isolates were recovered from ascospores produced on stem debris collected from the field at Versailles. These 504 isolates were classified as *AvrLm4* or *avrLm4*. During the growing season, the frequency of *AvrLm4* isolates increased from 5.7% at the phoma leaf spot stage in autumn to 20.4% at the stem canker stage before harvest and 15.2% on debris after harvest (Figure 4). A similar trend was observed during 2003/2004, when 280 isolates (139 from leaf lesions in autumn and 141 from leaf lesions in spring) were recovered from leaf lesions, 166 from stem cankers and 244 from ascospores produced on stem debris. These 690 isolates were classified as *AvrLm4* or *avrLm4*. The frequency of *AvrLm4* isolates increased from 7.9% at the phoma leaf spot stage (in autumn/spring) to 11.5% at the stem canker stage before harvest and 12.3% on debris after harvest.

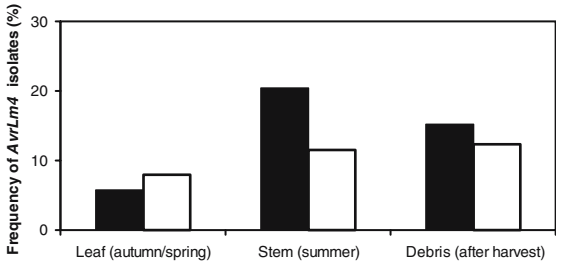


*Figure 4*. Frequency (%) of the avirulent allele (*AvrLm4*) in natural populations of *Leptosphaeria maculans* sampled from oilseed rape cultivars lacking the resistance gene *Rlm4* during 2002/2003 (■) and 2003/2004 (□) field experiments at Versailles, France from phoma leaf spots [sampled during autumn (2002/2003) or autumn and spring (2003/2004)], stem cankers (summer) and stem debris (after harvest).

## Discussion

Results of controlled-environment experiments, indicating that *AvrLm4* and *avrLm4 L. maculans* isolates differ for some, but not all, components of

phoma leaf spot development, suggest that there is a fitness cost of virulence. Firstly, plants inoculated with ascospores of *AvrLm4* isolates produced more lesions than those inoculated with ascospores of *avrLm4* isolates at all five temperatures tested, suggesting that infectivity of *AvrLm4* ascospores is greater than that of *avrLm4* ascospores. Secondly, there was no difference between *AvrLm4* and *avrLm4* isolates in the incubation period from inoculation to lesion development. Similarly, there was no difference between *AvrLm4* and *avrLm4* isolates in growth rate within the leaf within 3 cm from the infection site at 15–25 °C, as for growth on V8-agar medium. This suggests that fitness differences between avirulent and virulent isolates in field conditions may not be related to the speed of colonization of the leaf tissues. Thirdly, at all temperatures tested, *AvrLm4* ascospores produced larger leaf spot lesions than *avrLm4* ascospores and more lesions initiated by *AvrLm4* ascospores produced pycnidia than did lesions initiated by *avrLm4* ascospores. The larger leaf spot lesions may reflect a more extensive colonization of the plant tissue at the infection site by avirulent isolates, or a greater ability of these isolates to induce plant cell death and necrosis. These larger lesions may allow such isolates to benefit from a larger source of nutrients for subsequent systemic colonization of the petiole and the stem and/or sporulation.

Results from the field experiments during two seasons support the conclusions from controlled-environment experiments that there is a fitness cost of virulence. In both growing seasons, there was an increase in the frequency of *AvrLm4* isolates between the autumn (leaf spot stage) and summer (stem base canker and post-harvest stages). These data indicate that *AvrLm4* isolates may be fitter than *avrLm4* isolates in systemic growth from leaf lesions to stems under natural conditions. Growth from leaf lesions to stems is a crucial stage in the life cycle of *L. maculans* (Fitt et al., 2006). Experiments under both controlled and field conditions therefore suggest that the *avrLm4* allele may have a fitness cost associated with loss of function of the avirulence gene. Such a fitness cost of the *avrLm4* allele might explain why the cv. Jet Neuf, with the resistance gene *Rlm4*, has been successfully used as a source of resistance for 10–15 years. This also might explain why *AvrLm4* isolates are still present in field populations in France (Balesdent et al., 2006) and other parts of Europe (Stachowiak et al., 2006), although *Rlm4* has been used widely in past cultivars and is still present in more than 25% of current commercial French cultivars (Rouxel et al., 2003b; MH Balesdent, INRA and X Pinochet, CETIOM, France, unpublished data).

A pathogen "cost of virulence" parameter is often a component in models of factors affecting durability of host resistance (Pietravalle et al., 2006). This parameter is based on the hypothesis that loss of pathogen functions linked with the loss of avirulence will decrease the competitiveness of virulent isolates as compared to avirulent isolates. However, this hypothesis had not been demonstrated experimentally for a fungal plant pathogen, although one bacterium–plant interaction has been analysed (Vera-Cruz et al., 2000). Our paper therefore outlines the first experimental evidence that loss of one avirulence function in a fungal pathogen can alter its ability to produce leaf symptoms and sporulate on lesions, as compared to an avirulent isolate. Loss of the *AvrLm4* function also may affect the ability of the virulent isolates to compete with avirulent isolates during growth down the petiole to reach the stem base and to colonize the basal plant tissues in natural field conditions. This work, using both the novel ascospore shower inoculation method and NI isolates, will now be expanded to two other avirulence genes, *AvrLm1* and *AvrLm6*, to evaluate if this is a feature specific to *AvrLm4* or a more general feature of avirulence genes in *L. maculans*.

The development of near-isogenic (NI) isolates of *L. maculans* suggests that the back-crossing (BC) scheme is an efficient procedure for increasing isogenicity between *AvrLm4* and *avrLm4* isolates, since less than 10% of the AFLP markers that were polymorphic between parental isolates at the beginning of the BC scheme were still polymorphic in the fifth BC generation. In theory, the frequency of polymorphism should be halved at each BC generation, but the observed decrease in polymorphism at each generation was less than expected. This result may be linked to the need to maintain polymorphism at another genomic region (the *MAT* locus), independent of *AvrLm4*, to allow subsequent fertile crosses. The increase in isogenicity of *AvrLm4* and *avrLm4* isolates with BC also was shown by the changes in *in vitro* growth rate of the isolates. There were larger

differences in *in vitro* growth rate between the parental isolates (15%) of the $F_1$ generation at 4 and 12 °C than between parental isolates (4%) of the fifth BC generation. These results not only illustrate the efficiency of the inbreeding process, but also indicate that these NI isolates are appropriate for fitness studies, because quantitative differences between avirulent and virulent isolates during host infection and colonization will not be a simple consequence of major differences in general growth of the isolates, but will show differences in the pathogenic fitness of the isolates. Furthermore, the crossing of NI isolates *in vitro* offers a method to produce ascospores of NI *L. maculans* for use as inoculum to compare virulent and avirulent isolates for their ability to infect and colonize plants by natural means.

As an alternative to the production of NI isolates by classical back-crossing, genetically modified isolates resulting from the transformation of a virulent isolate by the cloned avirulent allele can be used. However, it is then necessary to first clone the avirulent allele, which is often difficult, as for *AvrLm1* in *L. maculans* (Attard et al., 2002; L. Gout, INRA, France, unpubl.) and other fungi (Farman and Leong, 1998). In addition, the random integration of the cloned fragment may produce a deleterious effect on the isolate, as random integration of alien DNA is also a method for identifying mutants with decreased pathogenicity, for pathogens such as *L. maculans* (Meyer et al., 2004). Therefore, such an approach necessitates analyzing a number of transformed isolates to obtain useful results.

The 'ascospore shower' method is an efficient procedure for inoculation with *L. maculans* ascospores when inoculum is limited; for instance, ascospores produced from *in vitro* crosses. Three times as many pseudothecia were required to inoculate the same number of plants with ascospore suspension as were required with the 'ascospore shower' method. Furthermore, when using the 'ascospore shower' method, it was not necessary to wound leaves, whereas inoculation with conidial suspensions without wounding the leaves resulted in few lesions. As stem canker epidemics are initiated by air-borne ascospores, this method more accurately simulates natural conditions. Thus, it is suitable for investigating the first stages of interactions between *Brassica* species and *L. maculans*. This new 'ascospore shower' method can be combined with NI *L. maculans* isolates to investigate the fitness of other pairs of virulent and avirulent isolates, and thus the potential durability of novel resistance genes (Brun et al., 2001; Leach et al., 2001; Rouxel et al., 2003a; Sprague et al., 2006).

## Acknowledgements

We thank the European Union (SECURE, QLRT−2001–01813), the UK Department for Environment, Food and Rural Affairs (OREGIN, AR0703), the Biotechnology and Biological Sciences Research Council, the Chadacre Agricultural Trust and the Royal Society for supporting this work. We thank A. D. Todd for statistical analyses of the data.

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