# Temperature and leaf wetness duration affect phenotypic expression of *Rlm6*-mediated resistance to *Leptosphaeria maculans* in *Brassica napus*

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## Summary

• Near-isogenic *Brassica napus* lines carrying/lacking resistance gene *Rlm6* were used to investigate the effects of temperature and leaf wetness duration on phenotypic expression of *Rlm6*-mediated resistance.

• Leaves were inoculated with ascospores or conidia of *Leptosphaeria maculans* carrying the effector gene *AvrLm6*. Incubation period to the onset of lesion development, number of lesions and lesion diameter were assessed. Symptomless growth of *L. maculans* from leaf lesions to stems was investigated using a green fluorescent protein (GFP) expressing isolate carrying *AvrLm6*.

• *L. maculans* produced large grey lesions on Darmor (lacking *Rlm6*) at 5–25 °C and DarmorMX (carrying *Rlm6*) at 25°C, but small dark spots and 'green islands' on DarmorMX at 5–20°C. With increasing temperature/wetness duration, numbers of lesions/spots generally increased. GFP-expressing *L. maculans* grew from leaf lesions down leaf petioles to stems on DarmorMX at 25°C but not at 15°C.

• We conclude that temperature and leaf wetness duration affect the phenotypic expression of *Rlm6*-mediated resistance in leaves and subsequent *L. maculans* spread down petioles to produce stem cankers.

**Key words:** ascospores, *Brassica napus* (oilseed rape), GFP (green fluorescent protein), major resistance gene, phoma stem canker, temperature-sensitivity.

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## Introduction

The effects of temperature on host plant resistance responses have been reported in several pathosystems but there is little information on the effect of leaf wetness. The tobacco *N* gene-mediated resistance to tobacco mosaic virus (TMV) is temperature-sensitive. Below 27°C, the *N* resistance gene mediates a hypersensitive response (HR); above 27°C, there is no HR and the virus is able to infect the plant systemically (Wright *et al.*, 2000). Wheat cultivars with the resistance gene *Sr6* are resistant to the fungal pathogen *Puccinia graminis* f.sp. *tritici* when grown at 18°C, but are susceptible at 27°C (Moerschbacher *et al.*, 1989). Tomato *Cf-4* gene-mediated resistance to *Cladosporium fulvum* is also sensitive to temperature. Transformed tomato seedlings expressing both the *Cf-4* gene and the corresponding effector (formerly termed avirulence) gene *Avr4* died rapidly as a result of systemic HR at 20°C but grew normally at 33°C (de Jong *et al.*, 2002). The HR is commonly associated with a gene-for-gene interaction. *Leptosphaeria maculans*, causing phoma stem canker of oilseed rape, has a gene-for-gene interaction with *Brassica napus* (Ansan-Melayah *et al.*, 1998; Balesdent *et al.*, 2001). Several single locus resistance genes (e.g. *Rlm1*, *Rlm3*, *Rlm7*, *Rlm9*) (Ansan-Melayah *et al.*, 1998; Delourme *et al.*, 2004) and the corresponding effector genes (*AvrLm1*, *AvrLm3*, *AvrLm7*, *AvrLm9*) (Ansan-Melayah *et al.*, 1995; Balesdent *et al.*, 2002) have been identified and *AvrLm1* has recently been cloned (Gout *et al.*, 2006). However, little is known about the

influence of temperature and leaf wetness duration on the interactions between *B. napus* resistance genes and *L. maculans* effector genes.

Phoma stem canker epidemics are initiated by airborne *L. maculans* ascospores (West *et al.*, 1999; Huang *et al.*, 2005), which infect leaf laminas and produce leaf lesions from which the pathogen grows systemically down the petiole into the stem to cause stem canker (Hammond *et al.*, 1985). However, most previous studies on resistance to *L. maculans* operating at the leaf infection stage have been done with *L. maculans* conidia. When cotyledons (cv. Quinta) were inoculated with *L. maculans* conidia (race A2 isolates), the response was resistant at 18°C but susceptible at 27°C (Badawy *et al.*, 1992). The interaction between Quinta and the A2 isolates of *L. maculans* has now been characterised as a specific *Rlm1/AvrLm1* interaction (Ansan-Melayah *et al.*, 1995; Balesdent *et al.*, 2001). In these experiments using a conidial inoculum, the leaves had to be wounded before inoculation. This methodology is not ideal for studying the interactions between a pathogen and its host, since wounding alone can induce plant defence responses. The development of an ascospore inoculation method (i.e. simulating natural infection; Huang *et al.*, 2006) now provides an appropriate method for investigating the effects of temperature and leaf wetness duration on the interaction between *L. maculans* and *B. napus*. Studies on the development of phoma leaf lesions on oilseed rape plants inoculated with *L. maculans* ascospores suggest that temperature and leaf wetness duration both affect the number of leaf lesions and incubation period (time from inoculation to the appearance of lesions) (Toscano-Underwood *et al.*, 2001). Work is now needed to examine the effects of temperature and wetness on the expression of resistance to *L. maculans* in *B. napus* leaves.

As phoma stem canker is monocyclic, and the timing of leaf infection on young plants in autumn affects the severity of phoma stem canker before harvest in the following summer, the systemic growth of *L. maculans* from leaf lesions to stems is a crucial phase in the development of epidemics. However, it has been difficult to study such growth because this phase is symptomless (Hammond *et al.*, 1985). There is now the opportunity to study this symptomless growth using green fluorescent protein (GFP)-expressing isolates of *L. maculans* (Sexton & Howlett, 2001; Eckert *et al.*, 2005). Such GFP-expressing isolates can be used to investigate whether the expression of resistance in *B. napus* leaves can prevent the subsequent symptomless spread of *L. maculans* to stems. This paper describes a series of experiments using near-isogenic *B. napus* lines carrying/lacking a resistance gene *Rlm6*, which was introduced into the *B. napus* genome from *B. juncea* (Chèvre *et al.*, 1997) but has not been used in commercial cultivars, to investigate effects of temperature and leaf wetness on the phenotypic expression of *Rlm6*-mediated resistance to *L. maculans* in *B. napus* leaves and the use of GFP to observe the symptomless growth of *L. maculans* from a leaf lesion down the petiole to the stem.

## Materials and Methods

Seven experiments were carried out in controlled environment cabinets, using *Brassica napus* L. near-isogenic lines Darmor (lacking *Rlm6*) and DarmorMX (carrying *Rlm6*) (Table S1) to avoid the potential effects associated with host genetic background. Ascospores or conidia of *L. maculans* with the corresponding effector gene *AvrLm6* were used as an inoculum for these experiments.

### Production of near isogenic DarmorMX and Darmor

The major specific resistance gene *Rlm6* (previously named *Jlm1*) from *B. juncea* was introduced into *B. napus* (cv. Darmor) by crossing SamouraïMX (Chèvre *et al.*, 1997) with Darmor, followed by five back-crosses (B) to Darmor (Table 1). At each generation, plants with resistance to *L. maculans* were selected by using a cotyledon pathogenicity test and/or molecular markers carried by the introgression from *B. juncea*. These plants were checked for the stability of their meiotic behaviour. The selection of plants homozygous for the resistance gene *Rlm6* was done in successive generations after the $B_3$ generation by using molecular markers for the *B. napus* genomic region replaced by the introgression (Barret *et al.*, 1998). In all cases, no significant differences from the segregation expected for a single dominant major gene were observed; 50% of plants from back-crossing were resistant and 75% of plants from selfing were resistant, and 25% of plants from selfing were homozygous for the introgression (Table 1). According to theoretical values, DarmorMX plants had 96.9% of the Darmor genome at the $B_4$ generation and 98.4% of the Darmor genome at the $B_5$ generation. Therefore, plants of DarmorMX homozygous for *Rlm6* selected from two generations of self-pollination of $B_5$ plants and its near-isogenic line Darmor were used in this work.

### Experiments with ascospore inoculum

*L. maculans* ascospore inoculum was obtained from naturally infected winter oilseed rape stem base debris. Winter oilseed rape (cv. Lipton) stem bases (< 5 cm above ground level, including tap roots) with basal phoma canker were collected after harvest from fields at Rothamsted, UK, in August 2000. It was confirmed that these stem pieces produced only ascospores of *L. maculans* (Huang *et al.*, 2003). Stem pieces containing mature pseudothecia were stored dry at −20°C until required. To determine whether the ascospores produced on this stem base debris were appropriate for studying the phenotypic expression of *Rlm6*-mediated resistance, 25 single ascospore isolations were made from the debris and tested for pathogenicity on DarmorMX and Darmor. All these isolates were avirulent on DarmorMX and virulent on Darmor; thus the ascospores from that debris carried the effector gene *AvrLm6*. A further characterisation of the *L. maculans*

 

**Table 1** Procedure for selection of DarmorMX (carrying resistance gene *Rlm6*[1]) and Darmor (lacking *Rlm6* ) near-isogenic lines of *Brassica napus* (oilseed rape)[2]

| Back-cross generation | | | Selfing generation of the back-crossed plants[4] | | | |
|---|---|---|---|---|---|---|
| Generation | No. plants | % plants resistant[3] | Generation | No. plants | % plants resistant[3] | % plants homozygous for *Rlm6*[5] |
| $B_1$ | 13 | 46.1 | | | | |
| $B_2$ | 7 | 57.1 | | | | |
| $B_3$ | 15 | 46.7 | $B_3F_1$ | 106 | 71.7 | 28.3 |
| $B_4$ | 36 | 44.4 | $B_4F_1$ | 102 | 72.5 | 27.4 |
| $B_5$ | 20 | 50.0 | $B_5F_1$ | 160 | 68.8 | 23.7 |

[1]*Rlm6*, a major gene for resistance to *Leptosphaeria maculans*, was introgressed into *B. napus* from *B. juncea* (Chèvre *et al*., 1997).
[2]The $F_1$ was produced by crossing SamouraïMX (Chèvre *et al*., 1997) with Darmor. The back-crossing generations were produced by back-crossing to Darmor.
[3]At each generation, plants with resistance to *L. maculans* were selected by a cotyledon test and/or molecular markers carried by the introgression, and checked for the stability of their meiotic behaviour.
[4]Some of the resistant back-cross plants were selfed to produce the next generation.
[5]The selection of plants homozygous for *Rlm6* was done on successive generations after the $B_3$ generation by using molecular markers for the *B. napus* genomic region replaced by the introgression (Barret *et al*., 1998).

population indicated that the frequencies of *AvrLm6* and *avrLm9* alleles in the natural population at Rothamsted were both 100% (Stachowiak *et al*., 2006). Thus, unlike *Rlm6* in DarmorMX, the only major resistance gene found in Darmor (*Rlm9*) was not effective against the *L. maculans* population at Rothamsted. The debris was therefore used as a source of ascospore inoculum for these experiments.

Plants of Darmor and DarmorMX were grown in pots (5 cm diameter) containing peat-based compost and a soluble fertiliser. Plants were initially grown in a glasshouse (20–23°C) with one plant per pot and placed in seed trays (37 cm × 23 cm) with 14 plants (seven plants of Darmor and seven plants of DarmorMX) per tray. Three weeks after sowing, the plants were transferred to a 15°C controlled environment cabinet (12 h light: 12 h darkness, light density 210 μe $m^{-2}$ $s^{-1}$) until each plant had three expanded leaves (growth stage (GS) 1,3; Sylvester-Bradley & Makepeace, 1985) and were ready for inoculation.

To inoculate the plants with ascospores, pieces of stem base debris bearing mature pseudothecia were removed from storage at −20°C, cut into smaller pieces (2–3 cm long) and mixed thoroughly. Six stem pieces were chosen at random and evenly attached to the underside of a tray lid (37 cm × 23 cm × 14 cm) with Vaseline (Chesebrough-Pond's Ltd, London). In total, 20 tray lids (five temperatures × four wetness treatments) were prepared. The pieces of stem were sprayed with distilled water until run-off to induce the release of ascospores and the lids with attached pseudothecia were placed over the trays with plants to allow ascospores to be naturally deposited onto the plants at 15°C from 15 cm above the plants. After 2 h, the pieces of debris with pseudothecia were removed from the tray lids. The inoculated plants were sprayed with distilled water using a laboratory sprayer and covered immediately with tray lids for wetness duration treatments. Four trays chosen at random were placed in each growth cabinet (at 5, 10, 15, 20 or 25°C). Trays (wetness treatments) were arranged in a completely randomised design in each growth cabinet (temperature treatment). Plants received wetness periods of 12, 24, 48 or 72 h at each temperature. After wetness periods, the tray lids were removed. The experiment was repeated three times, with growth cabinets allocated at random to each of the three experiments, whenever possible (Table S1). To estimate the number of ascospores deposited per unit leaf area, three glass microscope slides (7.5 cm × 2.5 cm) were placed in between the plants in each tray at approximately the same height as the leaves. The number of ascospores deposited on each slide was counted to estimate number of spores deposited per $cm^2$. The maximum length and width of each leaf on 10 plants of each cultivar were measured just before inoculation to estimate the leaf area per plant, and then the number of ascospores deposited per plant was calculated [total leaf area ($cm^2$) per plant × number of spores per $cm^2$]. The number of lesions resulting from inoculation with 100 ascospores was then estimated [(total number of lesions per plant) ÷ (total number of spores deposited per plant) × 100].

## Experiments with conidial (wild type) inoculum

To investigate whether the inoculation method (i.e. naturally depositing ascospores on leaves without wounding or inoculating with conidial suspension after wounding) affected the phenotypic expression of *Rlm6* mediated resistance, Darmor and DarmorMX were inoculated with conidia using the traditional wound-and-inoculate method (Huang *et al*., 2006). Conidial inoculum was produced from *L. maculans*

isolate ME24, obtained from a stem base canker on an oilseed rape plant sampled in July 2002 from a field experiment in Yorkshire, UK. A pathogenicity test on DarmorMX confirmed that ME24 carries the effector gene *AvrLm6*; thus ME24 was used to investigate the effects of temperature on the phenotypic expression of *Rlm6*-mediated resistance. A conidial suspension of ME24 was prepared from a 12-d-old culture on V8 agar (Balesdent *et al.*, 2001) and the concentration of conidia was adjusted to $10^6$ conidia $ml^{-1}$ using a haemocytometer slide. Plants of Darmor and DarmorMX were grown in pots (7 cm diameter) with one plant per pot in a 15°C controlled environment cabinet until inoculation at GS1,4 (four expanded leaves). To inoculate the plants, the first three leaves were wounded using a sterile pin and a 10 µl droplet of conidial suspension was placed on the wound. Each plant had 20–25 inoculation sites distributed over the three leaves, depending on the size of each leaf. Trays (37 cm × 23 cm) containing three inoculated plants were covered with lids to maintain leaf wetness for 72 h. These plants were at a lower density than those for ascospore inoculation experiments to prevent loss of inoculum drops through touching of leaves from adjacent plants. One tray with Darmor and one with DarmorMX was placed in each of the 15°C or 25°C growth cabinets. To replicate the temperature treatments, the experiment was repeated three times (Table S1).

### Experiments with conidial (GFP-expressing) inoculum

To investigate whether the effects of temperature on phenotypic expression of *Rlm6*-mediated resistance on leaves are reflected in the effects on subsequent symptomless growth of *L. maculans* from leaf to stem, the isolate ME24 (carrying *AvrLm6*) was transformed using plasmid pCAMBgfp (Sesma & Osbourn, 2004) and *Agrobacterium tumefaciens*-mediated transformation (Eckert *et al.*, 2005). GFP-expressing transformants were tested for the strength of their fluorescent protein expression and avirulence on leaves of DarmorMX and virulence on leaves of Darmor. Based on these tests, the transformant ME24/3.13 was selected. Since a Defra licence (i.e. a licence from the UK Department for Environment, Food and Rural Affairs) for producing ascospores of GFP-expressing *L. maculans* has not yet been obtained by Rothamsted Research, the plants were only inoculated with GFP-expressing conidia. The conidial suspension of ME24/3.13 was prepared from a 12-d-old culture on V8 agar and the concentration of conidia was adjusted to $10^6$ conidia $ml^{-1}$ using a haemocytometer slide. Each oilseed rape plant (Darmor, DarmorMX) was grown in a pair of Magenta vessels (77 mm × 77 mm × 97 mm) connected with a Magenta coupler in a 15°C controlled environment cabinet until inoculation at GS1,4. For inoculation, three leaves of each plant were wounded using a sterile pin and a 10 µL droplet of conidial suspension was placed on the wound. Each leaf had three inoculation sites. After inoculation, the plants were kept in the Magenta vessels until the end of the experiment.

### Disease assessment and microscopy

In experiments with ascospore inoculum, on each plant the numbers of new large (> 2 mm diameter) grey phoma leaf lesions on Darmor at 5–25°C and DarmorMX at 25°C, or small (< 2 mm diameter) dark spots on DarmorMX at 5–20°C, were counted daily until no new lesions (or dark spots) appeared, or the leaf died. The time from inoculation until the first lesion/spot appeared (the incubation period) was recorded at each temperature. At 15, 20 and 25°C, the diameters of 10 lesions/spots (the largest lesion on each of 10 randomly selected plants) were measured 14 d after inoculation. The number of lesions per ascospore was estimated as the number lesions/spots resulting from inoculation with 100 ascospores.

In experiments with conidial inoculum (*L. maculans* isolate ME24), the numbers of inoculated sites which produced grey lesions or dark spots on each plant were counted daily until no new lesions/spots appeared or the leaf died. The time from inoculation until the first lesion/spot appeared was recorded at each temperature. The diameters of 10 lesions/spots were measured 17 d after inoculation at each temperature. The percentage of inoculated sites which produced phoma leaf lesions/spots was calculated.

To test whether the small dark spots or green islands on DarmorMX (inoculated with ascospores) were caused by *L. maculans*, isolations were done from these small dark spots or green islands using the method described by West *et al.* (2002). Similarly, to test whether inoculation with conidia after wounding of DarmorMX had been successful when no symptoms developed, isolations were done from these symptomless wounded inoculation sites.

For plants inoculated with GFP-expressing *L. maculans* ME24/3.13, leaves were sampled 10 and 16 d after inoculation, and stems were sampled 31 and 36 d after inoculation when the inoculated leaves had dropped off. Leaf or stem samples were viewed directly using a Leica MZ FLIII stereomicroscope equipped with a mercury lamp. For the detection of GFP fluorescence, a GFP2 filter from Leica Microsystems (Milton Keynes, UK) was used. To view the symptomless growth of the pathogen, the petioles were embedded in 6% agar, and then cut into 0.15 mm thick sections using a vibrating blade microtome (Leica VT 1000s). Slides with petiole cross-sections were viewed under a Zeiss Axiophot fluorescence microscope equipped with a mercury lamp. For the detection of GFP fluorescence, a GFP1 filter from Carl Zeiss Ltd (Welwyn Garden City, UK) was used. Photomicrographs were taken with a digital camera (Leica DC 300FX) operated with IM50 software (Leica DC Twain, ver. 4.1.5.0).

 

### Statistical analysis

In experiments with ascospore inoculum, analyses of variance were done to assess the effects of temperature and leaf wetness duration on incubation period, number of lesions, number of lesions per ascospore and lesion diameter on leaves of DarmorMX (carrying *Rlm6*) and Darmor (lacking *Rlm6*). The times to the appearance of 50% of the maximum number of lesions or dark spots for each treatment were estimated by linear interpolation between points just below and just above 50%, to estimate the 50% value. Linear regressions of incubation period (time from inoculation to the appearance of first lesions or 50% of lesions) on temperature were calculated separately for Darmor and DarmorMX in the three replicate experiments. Analyses of position and parallelism were done to assess whether the data were fitted best by single straight lines, pairs of parallel lines or pairs of nonparallel lines for Darmor and DarmorMX. To assess the relationships between the number of lesions or the number of lesions per 100 spores and temperature or wetness, linear regressions (with a quadratic term if it significantly improved the relationship) were done separately for Darmor and DarmorMX in the three replicate experiments. In experiments with the conidial inoculum (wild type ME24), analyses of variance were done to assess the effects of temperature on the number of lesions, per cent inoculated sites which produced lesions, and lesion diameter on leaves of Darmor and DarmorMX. All the analysis was done using GENSTAT statistical software (Payne *et al.*, 1993).

## Results

### Phoma leaf spot phenotype

In experiments with the ascospore inoculum, disease symptoms differed between DarmorMX (carrying *Rlm6*) and Darmor (lacking *Rlm6*) at temperatures of 5–20°C. On Darmor for all temperature and wetness duration treatments, large grey lesions (> 2 mm in diameter; Fig. 1a; typical grey phoma leaf lesions) had developed by 5 (25°C) to 18 d (5°C) after inoculation. On DarmorMX, the symptoms differed between temperatures. At 5–10°C, no visible symptoms were observed up to 18 d after inoculation, when the leaves were still green (as in Fig. 1b). However, a few small dark spots (< 2 mm in diameter; as in Fig. 1d, typical small dark spots) or 'green islands' (i.e. leaf areas around sites of successful penetration with delayed senescence in comparison with the rest of the leaf tissue; as in Fig. 1c) were observed on some leaves 21–30 d after inoculation, when the leaves had turned yellow and senesced. At 15°C, by 11 d after inoculation no visible symptoms had developed on DarmorMX (Fig. 1b), whereas large grey lesions had developed on Darmor (Fig. 1a). Small dark necrotic spots were not observed on DarmorMX at 15°C until 14 d after inoculation. At 20°C, small dark necrotic spots (Fig. 1d) were observed on DarmorMX 8 d after inoculation, when the leaves were green. At both 15 and 20°C, by 18–23 d after inoculation when the leaves had senesced, the small dark spots remained small (< 2 mm in diameter) and 'green islands' were also observed (Fig. 1c). At 25°C, large grey lesions developed on DarmorMX (Fig. 1e) by 6 d after inoculation. *L. maculans* isolates were obtained from both small dark spots and 'green islands' which developed on DarmorMX at 5–20°C (Table S2).

In experiments with conidial inoculum, at 15°C large grey lesions developed on Darmor 14 d after inoculation, but very few visible symptoms developed on DarmorMX, even when

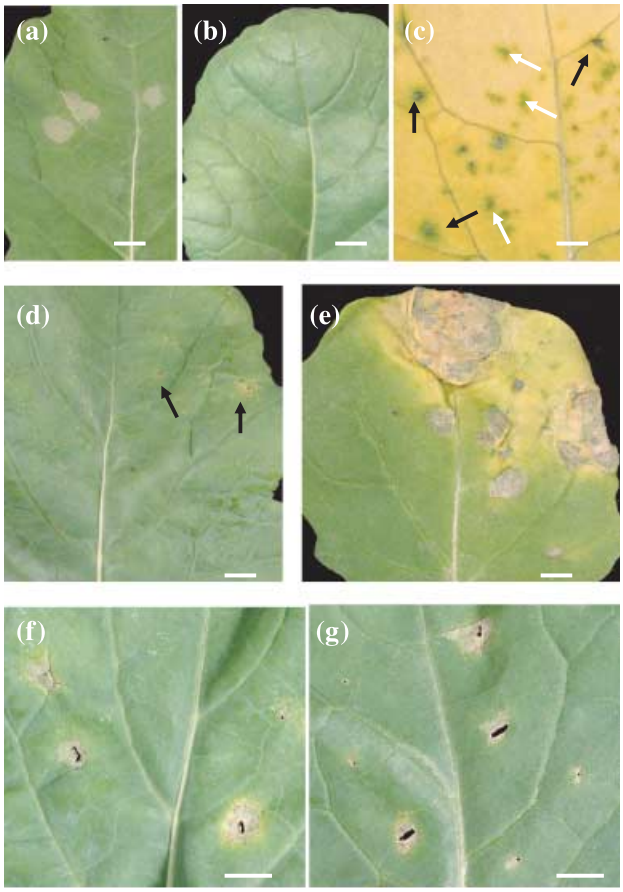


**Fig. 1** Symptoms on the leaves of *Brassica napus* Darmor (lacking *Rlm6*) and DarmorMX (carrying *Rlm6*) inoculated with ascospores (without wounding, a,b,c,d,e; with 48 h wetness) or conidia (after wounding, f,g; with 72 h wetness) of *Leptosphaeria maculans* carrying the effector gene *AvrLm6*. (a) Large grey lesions on Darmor 11 d after inoculation at 15°C; (b) no visible symptoms on DarmorMX 11 d after inoculation at 15°C; (c) small dark spots (black arrows) and green islands (white arrows) on DarmorMX 18 d after inoculation at 15°C; (d) small dark spots (arrows) on DarmorMX 11 d after inoculation at 20°C; (e) large grey lesions on DarmorMX 16 d after inoculation at 25°C; (f) large grey lesions on Darmor 16 d after inoculation at 25°C; (g) large grey lesions on DarmorMX 16 d after inoculation at 25°C. Bar, 5 mm.

the leaves senesced; however, *L. maculans* was re-isolated from the inoculated sites on symptomless leaves (Table S2). At 25°C, large grey lesions developed both on Darmor (Fig. 1f) and DarmorMX (Fig. 1g) by 10 d after inoculation.

## Incubation period

When the leaves were inoculated with ascospores, temperature ($P < 0.001$; SED = 0.25; 8 d.f.) affected the incubation period of *L. maculans*, estimated as the time from inoculation to the appearance of the first typical grey lesions on Darmor or small dark spots (5–20°C)/grey lesions (25°C) on DarmorMX. However, there was no difference in the time to the appearance of the first grey lesions on Darmor or dark spots/grey lesions on DarmorMX between wetness durations ($P = 0.19$; SED = 0.4; 29 d.f.). The time to the appearance of the first lesions/spots decreased with increasing temperature from 5 to 25°C for both Darmor and DarmorMX. The incubation period decreased from 18 d at 5°C to 6 d at 25°C for Darmor, and from 27 d at 5°C to 7 d at 25°C for DarmorMX (Table 2). The incubation period was longer for DarmorMX than Darmor ($P < 0.001$; SED = 0.6; 29 d.f.). For example, at 15°C, 11 d after inoculation, large grey lesions developed on Darmor, while no visible symptoms developed on DarmorMX. Similarly, temperature ($P < 0.001$; SED = 0.3; 8 d.f.) affected the time from inoculation to the appearance of 50% of the lesions/spots. As the temperature increased, the time to the appearance of 50% of lesions decreased from 21 d at 5°C to 7 d at 25°C for Darmor and from 26 d at 5°C to 9 d at 25°C for DarmorMX (Table 2). Over this temperature range the relationships between incubation period ($f$) and temperature ($T$) were fitted best by a pair of nonparallel lines for Darmor or DarmorMX (Fig. 2). The relationships between $1/f$ and temperature ($T$) were fitted best by a pair of parallel lines for the time to the first lesion/spot and by a pair of nonparallel lines for the time to 50% of lesion/spots for Darmor and DarmorMX.

When leaves were inoculated with conidia, the effects of temperature on the incubation period were similar to those with ascospore inoculum. As temperature increased, the time from inoculation to the appearance of the first lesions on Darmor decreased from 15 d at 15°C to 9 d at 25°C. However, the incubation period of *L. maculans* was longer with conidial inoculum than that with ascospore inoculum.

**Table 2** Effects of temperature and leaf wetness duration on the incubation period on leaves of *Brassica napus* DarmorMX (carrying *Rlm6*) or Darmor (lacking *Rlm6*) inoculated with ascospores of *Leptosphaeria maculans* with the effector gene *AvrLm6*

| Temperature (°C) | Wetness duration (h) | Time to first lesion/spot[1] (d) | | Time to 50% lesions/spots[2] (d) | |
|---|---|---|---|---|---|
| | | Darmor | DarmorMX | Darmor | DarmorMX |
| 5 | 12 | 18.0 | 26.3 | 20.0 | 25.5 |
| | 24 | 18.3 | 27.3 | 21.1 | 26.1 |
| | 48 | 18.3 | 26.3 | 19.9 | 25.5 |
| | 72 | 18.3 | 23.8 | 21.2 | 24.3 |
| 10 | 12 | 12.3 | 21.4 | 15.3 | 33.9 |
| | 24 | 13.3 | 20.3 | 14.7 | 21.7 |
| | 48 | 12.0 | 21.0 | 13.9 | 21.9 |
| | 72 | 12.0 | 20.7 | 14.1 | 22.6 |
| 15 | 12 | 7.7 | 13.7 | 10.2 | 18.2 |
| | 24 | 7.7 | 14.3 | 8.7 | 16.0 |
| | 48 | 7.3 | 13.7 | 9.2 | 16.3 |
| | 72 | 7.3 | 10.7 | 9.4 | 15.7 |
| 20 | 12 | 7.0 | 11.0 | 8.0 | 13.1 |
| | 24 | 6.7 | 9.3 | 7.4 | 13.2 |
| | 48 | 6.3 | 8.7 | 7.5 | 12.0 |
| | 72 | 7.0 | 8.3 | 7.8 | 12.4 |
| 25 | 12 | 5.7 | 6.7 | 6.3 | 9.6 |
| | 24 | 5.0 | 6.3 | 6.3 | 8.9 |
| | 48 | 6.0 | 6.7 | 7.2 | 9.6 |
| | 72 | 6.0 | 6.3 | 7.6 | 9.0 |
| SED[3] | | 1.4 | | 1.3 | |
| (d.f.) | | (58) | | (43) | |

[1]The incubation period was estimated as the time from inoculation to appearance of the first large grey lesion on Darmor or small dark spot (5–20°C)/grey lesion (25°C) on DarmorMX. Data are means from three experiments.
[2]The incubation period was estimated as the time from inoculation to appearance of 50% of large grey lesions on Darmor or small dark spots (5–20°C)/grey lesions (25°C) on DarmorMX. This was done by linear interpolation between the two dates on which the percentage of the total number of lesions/spots was just less than or just greater than 50%.
[3]Approximate maximum SED.

 

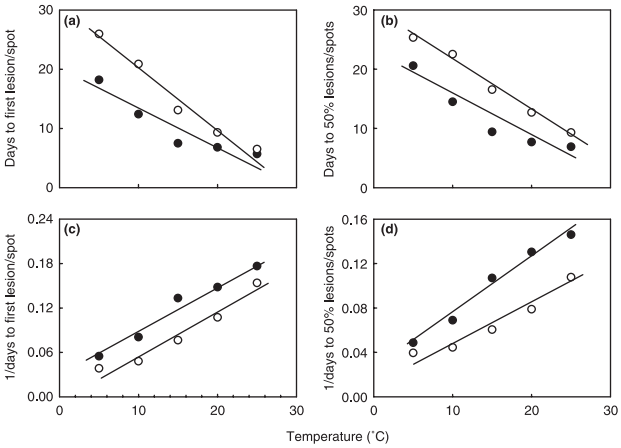


**Fig. 2** Effects of temperature on the incubation period (a,b) and on the inverse of the incubation period (c,d) of *Leptosphaeria maculans*. The incubation period was estimated as time from inoculation with *L. maculans* ascospores to the appearance of the first lesions/spots (a), or to 50% lesions/spots (b), on leaves of Darmor (closed symbols) or DarmorMX (open symbols). Over this temperature range, the relationships between incubation period ($f$) and temperature ($T$) were fitted best by pairs of nonparallel lines for time to first lesions/spots ($f = 19.4 - 0.62T$ for Darmor and $f = 30.3 - 1.01T$ for DarmorMX) and time to 50% lesions/spots ($f = 22.1 - 0.68T$ for Darmor and $f = 29.9 - 0.84T$ for DarmorMX). The relationships between $1/f$ and $T$ were best fitted by a pair of parallel lines for first lesions/spots ($1/f = 0.286 + 0.006T$ for Darmor and $1/f = 0.005 + 0.006T$ for DarmorMX) and by a pair of nonparallel lines for 50% lesions/spots ($1/f = 0.023 + 0.005T$ for Darmor and $1/f = 0.015 + 0.003T$ for DarmorMX). The data points are means of four wetness duration treatments in three replicate experiments but the regression lines were calculated from data for individual experiments.

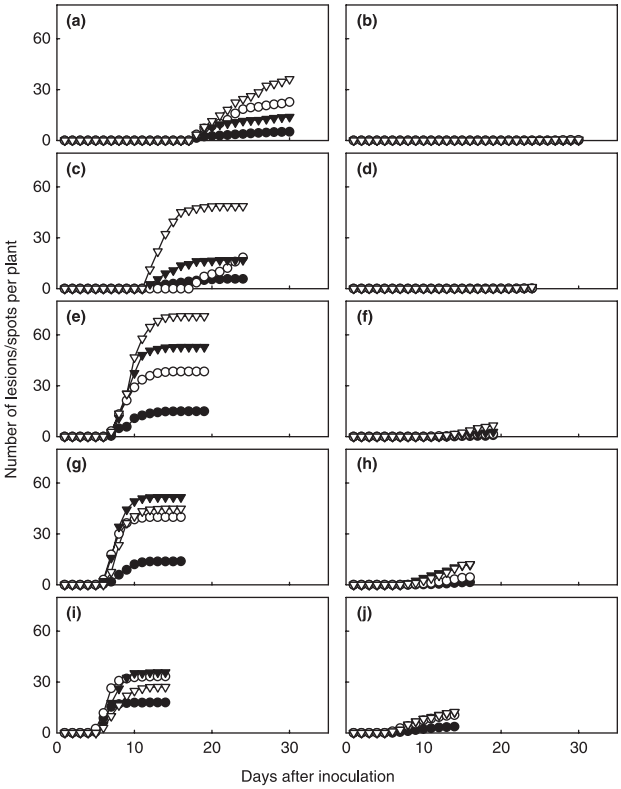


**Fig. 3** Changes with time in number of large grey lesions on leaves of *Brassica napus* Darmor (lacking *Rlm6* ) (a,c,e,g,i) or small dark spots (5–20°C)/grey lesions (25°C) on DarmorMX (carrying *Rlm6* ) (b,d,f,h,j) inoculated with ascospores of *Leptosphaeria maculans* carrying the effector gene *AvrLm6* and with wetness durations of 12 h (closed circles), 24 h (open circles), 48 h (closed triangles) or 72 h (open triangles) at temperatures of 5°C (a,b), 10°C (c,d),15°C (e,f), 20°C (g,h) or 25°C (i,j). Lesions were assessed on seven plants per treatment. The data illustrated are means of three experiments.

For example, at 15°C the time from inoculation to the appearance of the first lesions on Darmor was 15 d with conidial inoculum, in contrast to the 7 d with ascospore inoculum.

## Number of lesions

Temperature ($P < 0.01$; SED = 2.8; 8 d.f.) and wetness duration ($P < 0.001$; SED = 3.4; 30 d.f.) both affected the maximum number of grey phoma lesions that developed on leaves of Darmor (5–25°C) or small dark necrotic spots that developed on DarmorMX (5–20°C) inoculated with ascospores, but the effects of temperature and wetness duration on the pattern of lesion development with time differed between Darmor and DarmorMX (Fig. 3). For all the temperature and wetness treatments, more lesions developed on Darmor than did spots/lesions on DarmorMX ($P < 0.001$; SED = 2.2; 40 d.f.). On Darmor, more lesions developed at 15 and 20°C than at 5, 10 and 25°C with wetness durations of 48 and 72 h (Table 3). The number of lesions decreased as leaf wetness duration decreased from 48 to 12 h; very few lesions developed with a wetness duration of 12 h at 5 or 10°C and more developed at 15, 10 and 25°C (Table 3). On DarmorMX, very few symptoms developed at 5 and 10°C; the number of small dark spots (at 15 and 20°C) or large grey lesions (at 25°C) increased as the temperature increased from 15 to 25°C and wetness duration increased from 12 to 72 h (Fig. 2; Table 3). Temperature ($P < 0.05$; SED = 2.8; 8 d.f.) and wetness duration ($P < 0.05$; SED = 2.5; 30 d.f.) both affected the estimated number of lesions/spots produced by inoculation with 100 ascospores on leaves of Darmor and DarmorMX (Table 3). The number of lesions/spots per 100 ascospores was greater on Darmor than on DarmorMX ($P < 0.001$; SED = 1.4; 40 d.f.). The number of lesions per 100 ascospores on Darmor was generally greater at 15°C and 20°C than at 5, 10 or 25°C. The number of lesions per 100 ascospores on Darmor generally increased as wetness duration increased from 12 to 48 h and was greatest at 15°C with 48 h wetness duration. The number of lesions/spots per 100 ascospores on DarmorMX

**Table 3** Effects of temperature and leaf wetness duration on number of grey lesions/dark spots (maximum number or number per spore) on leaves of *Brassica napus* DarmorMX (carrying *Rlm6*) or Darmor (lacking *Rlm6*) inoculated with ascospores of *Leptosphaeria maculans* carrying effector gene *AvrLm6*

| Temperature (°C) | Wetness (h) | Maximum no. lesions/spots per plant[1] | | No. spores per plant[1,2] | Lesions/spots per ascospore[1,3] | |
|---|---|---|---|---|---|---|
| | | D | DM | | D | DM |
| 5 | 12 | 5.1 | 0 | 336 | 1.4 | 0 |
| | 24 | 22.6 | 0.3 | 951 | 5.2 | 0.1 |
| | 48 | 13.9 | 0.1 | 370 | 4.4 | 0.1 |
| | 72 | 37.1 | 0.6 | 327 | 11.5 | 0.2 |
| 10 | 12 | 5.7 | 0 | 751 | 2.5 | 0 |
| | 24 | 16.9 | 0.1 | 304 | 7.9 | 0.1 |
| | 48 | 48.7 | 0.4 | 666 | 7.4 | 0.1 |
| | 72 | 28.1 | 0.9 | 751 | 7.2 | 0.2 |
| 15 | 12 | 15.0 | 1.0 | 94 | 16.1 | 0.8 |
| | 24 | 38.4 | 1.6 | 619 | 17.3 | 1.1 |
| | 48 | 52.9 | 3.1 | 190 | 40.2 | 2.3 |
| | 72 | 75.8 | 6.9 | 709 | 22.1 | 2.1 |
| 20 | 12 | 13.9 | 1.6 | 734 | 4.6 | 0.4 |
| | 24 | 39.9 | 4.5 | 836 | 8.7 | 0.8 |
| | 48 | 51.7 | 12.1 | 342 | 15.2 | 3.5 |
| | 72 | 44.9 | 12.3 | 778 | 25.9 | 6.6 |
| 25 | 12 | 17.9 | 3.9 | 411 | 3.9 | 1.0 |
| | 24 | 33.2 | 10.8 | 407 | 16.9 | 5.5 |
| | 48 | 35.7 | 12.4 | 309 | 12.2 | 4.4 |
| | 72 | 23.7 | 13.7 | 188 | 13.3 | 8.1 |
| SED[4] | | 8.6 | | | 7.2 | |
| (d.f) | | (137) | | | (62) | |

[1]Data presented are means of three experiments. D: Darmor; DM: DarmorMX. The maximum number of lesions was assessed 14–31 d after inoculation, depending on temperature, when no more new lesions appeared and before the inoculated leaf dropped off.
[2]Glass slides were placed in between the plants at the same height as the leaves in each experiment; the numbers of spores deposited on slides were counted to estimate number of spores per $cm^2$ and the number of spores deposited per plant was calculated [total leaf area ($cm^2$) per plant × number of spores per $cm^2$].
[3]Lesions/spots per ascospore was estimated as the number of lesions/spots produced by inoculation with 100 spores [(no. lesions) ÷ (no. ascospores) × 100] for each experiment.
[4]Approximate maximum SED.

generally increased with increasing temperature and wetness duration and was greatest at 25°C with 72 h wetness duration.

Over this temperature range, the relationships between the number of lesions/spots ($y$) or number of lesions/spots per 100 ascospores ($s$) and temperature ($T$) were fitted best by quadratic curves for Darmor ($y = -7.0 + 5.62\,T - 0.168\,T^2$; $s = -10.2 + 3.23\,T - 0.095\,T^2$), and were fitted best by straight lines for DarmorMX ($y = -3.8 + 0.54\,T$; $s = -1.7 + 0.24\,T$) (Fig. 4). The relationships between the number of lesions/spots ($y$) or the number of lesions/spots per 100 ascospores ($s$) and wetness ($W$) were fitted best by a quadratic curve and a straight line for Darmor ($y = -5.1 + 1.69\,W - 0.015\,W^2$; $s = 5.8 + 0.17\,W$) and were fitted best by straight lines for DarmorMX ($y = 0.8 + 0.09\,W$; $s = 0.1 + 0.05\,W$).

Temperature affected the number of lesions ($P < 0.001$; SED = 3.8; 4 d.f.) and percentage of inoculated sites which developed lesions ($P < 0.001$; SED = 4.4; 4 d.f.) on Darmor and DarmorMX inoculated with conidia (Table 4). On Darmor, more lesions developed at 25°C than at 15°C and the percentage of sites which developed lesions was greater at 25°C (73%) than at 15°C (56%). At 25°C, although symptoms on DarmorMX (Fig. 1g) were similar to those on Darmor (Fig. 1f), the percentage of sites which developed lesions on DarmorMX was less than on Darmor ($P < 0.05$; SED = 4.4; 4 d.f.).

## Lesion diameter

Temperature ($P < 0.001$; SED = 0.3; 4 d.f.) affected lesion diameter on leaves of Darmor and DarmorMX inoculated with ascospores at 15–25°C, but the effects of wetness duration on lesion diameter were not significant ($P = 0.1$;

 

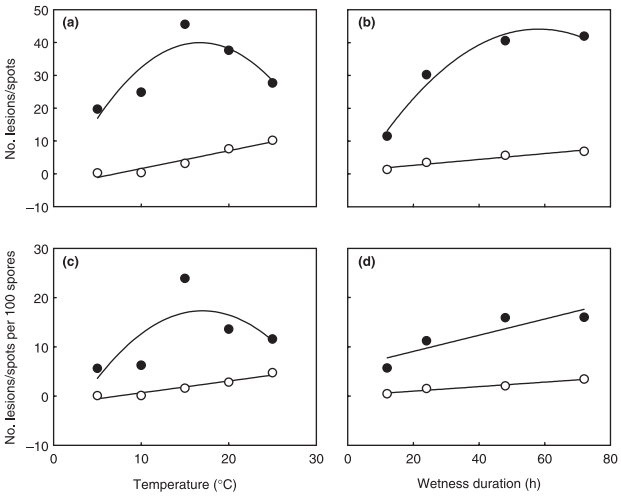


**Fig. 4** Effects of temperature (a,c) and leaf wetness duration (b,d) on number of lesions/spots (a,b) and number of lesions/spots resulting from inoculation with 100 ascospores (c,d) on leaves of *Brassica napus* Darmor (lacking *Rlm6*; closed circles) or DarmorMX (carrying *Rlm6*; open circles) inoculated with ascospores of *Leptosphaeria maculans* carrying the effector gene *AvrLm6*. Over this temperature range, the relationships between number of lesions/spots ($y$) or number of lesions/spots per 100 ascospores ($s$) and temperature ($T$) were fitted best by quadratic curves for Darmor ($y = -7.0 + 5.62T - 0.168T^2$; $s = -10.2 + 3.23T - 0.095T^2$), and were fitted best by straight lines for DarmorMX ($y = -3.8 + 0.54T$; $s = -1.7 + 0.24T$). The relationships between number of lesions/spots ($y$) or number of lesions/spots per 100 ascospores ($s$) and wetness ($W$) were fitted best by a quadratic curve and a straight line for Darmor ($y = -5.1 + 1.69W - 0.015W^2$; $s = 5.8 + 0.17W$) and were fitted best by straight lines for DarmorMX ($y = 0.8 + 0.09W$; $s = 0.1 + 0.05W$).

SED = 0.1; 18 d.f.) (Table 5). On Darmor, 14 d after inoculation, lesion diameter was smaller at 15°C than at 20°C or 25°C, but there was no difference in lesion diameter between 20°C and 25°C. On DarmorMX, 14 d after inoculation, the diameter of the lesions at 25°C was greater than the diameter of spots at 15°C or 20°C. Lesion/spot diameters on DarmorMX (1.0–1.5 mm at 15–20°C, 2.7–3.5 mm at 25°C) were much smaller than lesion diameters on Darmor (4.1–8.6 mm). At the same temperature, increasing wetness duration did not increase lesion/spot diameter on either Darmor or DarmorMX. At 25°C, 17 d after inoculation with conidia, lesion diameters on DarmorMX were similar to those on Darmor.

## Systemic growth from leaf lesions towards stems

On leaves of DarmorMX inoculated with the conidia of GFP-expressing *L. maculans* ME24/3.13 at 15°C, localised cell necrosis around the inoculation site was observed. Growth of *L. maculans* was confined within these small necrotic areas (Fig. 5a). Necrosis was not observed on DarmorMX at 25°C (Fig. 5d) or on Darmor at either 15°C or 25°C (Fig. 5b). Whereas on DarmorMX small dark spots with no pycnidia were produced at 15°C (Fig. 5a), large lesions with pycnidia developed on DarmorMX at 25°C (Fig. 5d). On Darmor at both 15°C and 25°C, large lesions with pycnidia were produced (Fig. 5b). On Darmor

**Table 4** Effects of temperature on maximum number of phoma leaf lesions, per cent inoculated sites which developed lesions and lesion diameter on leaves of *Brassica napus* DarmorMX (carrying *Rlm6*) or Darmor (lacking *Rlm6*) inoculated with conidia of *Leptosphaeria maculans* (isolate ME24) carrying the effector gene *AvrLm6*

| Parameter | Cultivar | Temperature 15°C | Temperature 25°C | SED[4] (4 d.f.) |
|---|---|---|---|---|
| Max no. of lesions[1] | Darmor | 15.4 | 17.9 | 3.8 |
| | DarmorMX | 0 | 14.2 | |
| % sites with lesions[2] | Darmor | 55.8 | 73.3 | 4.4 |
| | DarmorMX | 0 | 63.4 | |
| Lesion diameter[3] (cm) | Darmor | 0.6 | 0.6 | 0.12 |
| | DarmorMX | 0 | 0.4 | |

[1]After inoculation with conidia, grey lesions developed on Darmor at 15 and 25°C and on DarmorMX at 25°C, but very few symptoms developed on DarmorMX at 15°C before the inoculated leaves dropped off. Maximum number of lesions was assessed 18–22 d after inoculation, when no more new lesions appeared and before the inoculated leaf dropped off.
[2]Percentage of inoculated sites which developed grey lesions.
[3]Diameters of lesions were measured 17 d after inoculation in each experiment.
[4]Approximate maximum SED.

**Table 5** Effects of temperature and leaf wetness duration on diameter of grey lesions on *Brassica napus* Darmor (carrying *Rlm6*) or dark spots (5–20°C)/grey lesions(25°C) on DarmorMX (lacking *Rlm6*) inoculated with ascospores of *Leptosphaeria maculans* carrying effector gene *AvrLm6*

| Temperature (°C) | Wetness duration (h) | Lesion/spot diameter[1] (mm) Darmor | Lesion/spot diameter[1] (mm) DarmorMX |
|---|---|---|---|
| 15 | 12 | 4.1 | 1.1 |
| | 24 | 4.7 | 1.1 |
| | 48 | 5.1 | 1.0 |
| | 72 | 4.8 | 1.3 |
| 20 | 12 | 8.1 | 1.1 |
| | 24 | 8.1 | 1.5 |
| | 48 | 8.0 | 1.4 |
| | 72 | 8.1 | 1.4 |
| 25 | 12 | 8.2 | 2.7 |
| | 24 | 7.7 | 3.5 |
| | 48 | 8.6 | 3.4 |
| | 72 | 8.2 | 3.1 |
| SED[2] (62 d.f.) | | 0.6 | |

[1]Mean of data from three experiments, assessed 14 d after inoculation; lesions were too small to measure at 10°C and no lesions had developed at 5°C 14 d after inoculation.
[2]Approximate maximum SED.

at 15°C and 25°C and on DarmorMX at 25°C, growth of GFP-expressing hyphae down the leaf petiole towards the stem was observed (Fig. 5c). GFP-expressing hyphae growing down the petiole towards the stem were mainly travelling through xylem vessels or between cells of the xylem parenchyma and cortex (Fig. 5c). At 15°C, 36 d after inoculation, after the inoculated leaves had dropped off, GFP-expressing hyphae were observed on leaf scars of Darmor (Fig. 5f) but not on DarmorMX (Fig. 5h). However, at 25°C, 31 d after inoculation, after the inoculated leaves dropped off, GFP-expressing hyphae were observed on leaf scars of both Darmor (Fig. 5j) and DarmorMX (Fig. 5l).

## Discussion

In this paper, for the first time we have reported that both temperature and leaf wetness duration affect the phenotypic expression of *Rlm6*-mediated resistance to *L. maculans* in *B. napus* (DarmorMX, carrying *Rlm6*), whereas on susceptible Darmor (lacking *Rlm6*) no differences in the type of symptom between temperature and wetness duration treatments were observed. The differences in symptom development on DarmorMX between temperatures and leaf wetness durations suggest that these environmental factors may affect interactions between *L. maculans* effector genes and *B. napus* resistance genes (Fig. 6). The development of large grey lesions on DarmorMX at 25°C but not at 5–20°C suggests that the *Rlm6*-mediated resistance to *L. maculans* is sensitive to temperature. Whereas there was a resistant phenotype (small dark necrotic spots or 'green islands') on DarmorMX at 5–20°C, there was a susceptible phenotype (grey lesions) on DarmorMX at 25°C. These phenotypes suggest that *L. maculans* ascospores depositing on the leaf surfaces of DarmorMX can germinate, and hyphae from ascospores can penetrate the leaf stomata, but the pathogen fails to colonise the surrounding tissue at 5–20°C whereas it can successfully colonise the tissue at 25°C (Fig. 6). Although temperature-sensitive resistance has been reported in several pathosystems (Moerschbacher *et al.*, 1989; Wright *et al.*, 2000; de Jong *et al.*, 2002), there has been little information on the effects of leaf wetness. The observation that, as leaf wetness duration increased more small necrotic spots (5–20°C) or more large grey lesions (25°C) developed on DarmorMX, suggests that leaf wetness duration may also affect the interaction between *L. maculans* and *B. napus* after penetration during the early stage of leaf colonisation. 'Green islands' are commonly associated with compatible interactions between biotrophic pathogens and their hosts (Panstruga, 2003; Hückelhoven *et al.*, 2003). Thus the observation of 'green islands' associated with an incompatible interaction between the hemibiotrophic pathogen *L. maculans* and its *B. napus* host requires further investigation.

The differences between treatments in the numbers of large grey lesions (Darmor, 5–25°C; DarmorMX, 25°C) or small dark spots (DarmorMX, 5–20°C) suggest that both

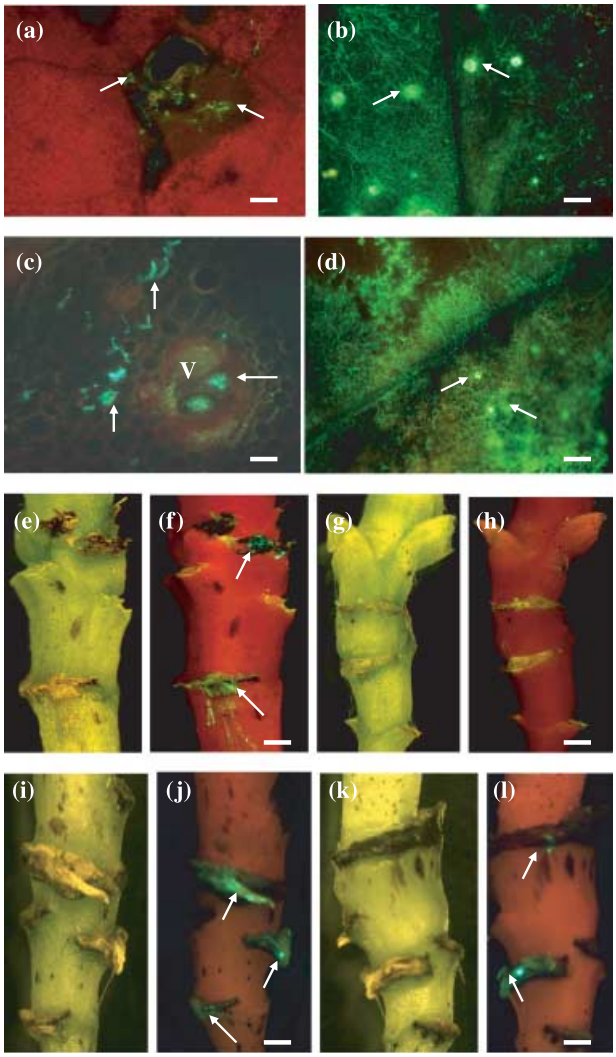


**Fig. 5** Development of *Leptosphaeria maculans* in leaves and stems of *Brassica napus* DarmorMX (carrying *Rlm6*) or Darmor (lacking *Rlm6*), demonstrated by the inoculation of leaves with conidia of *L. maculans* isolate (carrying *AvrLm6*) ME24/3.13 expressing green fluorescent protein (GFP) at 15°C or 25°C, viewed under a fluorescence (a–d,f,h,j,l) or white light (e,g,i,k) microscope. (a) necrotic response on leaves of DarmorMX at 15°C, GFP *L. maculans* (arrows) associated with dead plant cells (brown) but not healthy plant cells (red auto-fluorescence); (b) hyphae and pycnidia (arrows) of GFP *L. maculans* within a leaf lesion on Darmor at 15°C; (c) leaf petiole cross-section of Darmor, showing colonisation of vascular bundle (V) and surrounding tissue by GFP *L. maculans* (arrows) at 25°C; (d) hyphae and pycnidia (arrows) of GFP *L. maculans* within a leaf lesion on DarmorMX at 25°C. *L. maculans* had spread down petioles to reach stems of Darmor (e,f) but not DarmorMX (g,h) at leaf scars (arrows) by 36 d after inoculation at 15°C; *L. maculans* had spread down petioles to reach stems of both Darmor (i,j) and DarmorMX (k,l) at leaf scars (arrows) by 31 d after inoculation at 25°C. Bar, 200 μm (a,b,d), 100 μm (c) or 1 mm (e–l).

 

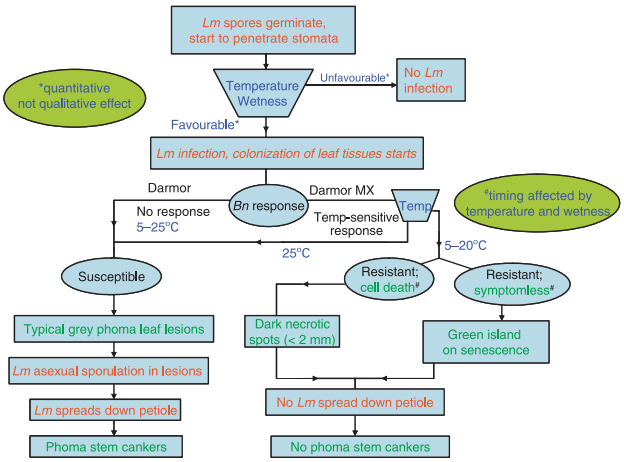


**Fig. 6** Effects of temperature and leaf wetness duration on *Leptosphaeria maculans* (*Lm*) ascospore germination and colonisation of *Brassica napus* (*Bn*) leaf lamina (to produce phoma leaf spots), leaf petiole and stem (to produce stem cankers) tissues in near-isogenic lines Darmor (lacking *Rlm6*) and DarmorMX (carrying *Rlm6*).

temperature and leaf wetness affect the probability that an ascospore deposited on a leaf surface will produce a disease symptom. To colonise the host tissue and produce lesions, ascospores of *L. maculans* need first to germinate and hyphae/germ tubes from ascospores need to penetrate through stomata. Evidence that more typical lesions were produced on Darmor at 15°C with 48–72 h wetness duration than with other temperature/wetness treatments suggests that the probability that ascospores landing on Darmor leaf surfaces can germinate, penetrate and successfully colonise the leaf tissue is greatest at 15°C with 48–72 h wetness duration (Fig. 4). This is consistent with previous results, suggesting that the optimum temperature/wetness duration for *L. maculans* ascospore germination, penetration and the formation of lesions is near 20°C with a 48 h wetness duration (Toscano-Underwood *et al.*, 2001; Huang *et al.*, 2003). However, the relationships between temperature/wetness and number of spots/lesions on DarmorMX, with a linear increase in the number of spots/lesions on DarmorMX with increasing temperature or increasing wetness duration, were different from those on Darmor (Fig. 4). Furthermore, at all temperature and wetness duration treatments, the number of necrotic spots (15–20°C) or grey lesions (25°C) on DarmorMX was smaller than the number of lesions on Darmor. It is not clear whether these differences were the result of an effect of *Rlm6* on *L. maculans* ascospore germination, penetration or colonisation. Li *et al.* (2004) reported that there were no differences between resistant and susceptible cultivars in *L. maculans* ascospore attachment, germination and penetration, but there were differences between them in colonisation after penetration. This suggests that the total number of small dark necrotic spots plus 'green islands' on DarmorMX might have been similar to the total number of large grey lesions on Darmor. This hypothesis can be tested in future work by counting the numbers of both small dark necrotic spots (on green leaves) and 'green islands' (after senescence) on DarmorMX.

By using a GFP-expressing isolate, for the first time we provided direct evidence that resistance mediated by *Rlm6*, expressed at the phoma leaf spot stage, can prevent the subsequent spread of *L. maculans* to stems where it may cause damaging stem base cankers. On DarmorMX, the necrotic reaction observed on leaves at temperatures below 20°C, which confined the growth of *L. maculans* to the area around the infection site, prevented subsequent growth of the pathogen to reach the stem (Fig. 5). The necrotic reaction on DarmorMX at temperatures below 20°C, characterised by cell death, was consistent with previous observations on *B. napus–B. juncea* recombinant lines (Roussel *et al.*, 1999). On Darmor (lacking *Rlm6*), the growth of *L. maculans* down the petioles along the xylem vessels and through the intercellular spaces confirmed previous observations using scanning electron microscopy (Hammond *et al.*, 1985). The use of GFP-expressing isolates provided a convenient method for observing the symptomless growth of *L. maculans* from leaf lesions to stems and showed that *L. maculans* hyphae were present in symptomless leaf scars on stems. The method also showed that *L. maculans* reached the stems 5 d earlier at 25°C than at 15°C, suggesting that early leaf infection may produce severe stem cankers by harvest; since in early autumn, temperatures are higher and plants are smaller, the times required for *L. maculans* to reach the stems are shorter than in late autumn/winter. This confirms the results of UK winter oilseed rape field experiments, in which severe stem canker epidemics before harvest were associated with the early development of phoma leaf spot in autumn (Zhou *et al.*, 1999; Sun *et al.*, 2001).

The influence of temperature on the effectiveness of *Rlm6* against *L. maculans* may help to explain why phoma stem canker is most severe on oilseed rape in Australia, where temperatures during the growing season are higher than in Europe (minimum/maximum mean daily temperature during oilseed rape growing seasons are 6/30°C in Western Australia and 1/21°C in western Europe, respectively) (West *et al.*, 2001). The results of these controlled environment experiments indicate that *Rlm6*-mediated resistance to *L. maculans* is not effective at 25°C; similarly *Rlm1*-mediated resistance to *L. maculans* is not effective at 27°C (Badawy *et al.*, 1992). If oilseed rape cultivars with such temperature-sensitive resistance genes are deployed in areas where the temperatures are lower, such as in the UK, there will be less risk of severe stem canker epidemics than in areas where the temperatures are greater, such as in Australia. Furthermore, this work suggests that, should there be a rapid climate change with increasing temperature in Europe (Thuiller *et al.*, 2005), there is the potential for more severe phoma stem canker

epidemics. Therefore, there is a need to investigate the mechanisms by which temperature affects the interactions between *L. maculans* effector genes and *B. napus* resistance genes.

## Acknowledgements

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## Supplementary material

The following supplementary material is available for this article online.

**Table S1** Type of *Leptosphaeria maculans* inoculum, *Brassica napus* near-isogenic lines (NIL), temperatures and controlled environment cabinets used in each of seven experiments to examine effects of temperature/leaf wetness duration on the phenotype of the Rlm6-mediated resistance to *L. maculans* in leaves of *B. napus* (oilseed rape).

**Table S2** Re-isolation of *Leptosphaeria maculans* from leaves of *Brassica napus* DarmorMX (carrying resistance gene Rlm6) inoculated with ascospores or conidia of *L. maculans* carrying the corresponding effector gene AvrLm6

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