Inter-individual genetic variation in the temperature response of Leptosphaeria species pathogenic on oilseed rape

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Inter-individual genetic variation in the temperature response of *Leptosphaeria* species pathogenic on oilseed rape

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Abstract

It is important to understand the likely response of plant pathogens to increased temperatures due to anthropogenic climate change. This includes evolutionary change due to selection on genetically based variation in growth rate with temperature. We attempted to quantify this in two ways. First, radial mycelial growth rates in agar culture were determined for a collection of 44 English isolates of *Leptosphaeria maculans* and 17 isolates of *L. biglobosa*, at 14 temperatures. For *L. maculans* the genotypic variances in four parameters were measured: minimum temperature allowing growth, optimum temperature, growth rate at the optimum temperature, and growth rate at the highest usable temperature, 31.8°C. The standard deviations were 0.068°C, 1.28°C, 0.21 mm/day, and 0.31 mm·day−1·°C−1, respectively. For *L. biglobosa*, these figures were, respectively: immeasurably small, 1.31°C, 0.053 mm/day, and 0.53 mm·day−1·°C−1. In addition, the incidence and severity of phoma stem canker in planta over a natural growing cycle at four temperatures (16, 20, 24, and 28°C) around the average culture optimum were determined. There was no correlation between in vitro and in planta growth, and the decrease in pathogen measures either side of the optimum temperature was much less for in planta growth than for in vitro growth. We conclude that both pathogens have the capacity to evolve to adapt to changes in environmental conditions, but that predictions of the effect of this adaptation, or estimates of heritability in natural conditions, cannot be made from measurements in vitro.

KEYWORDS
adaptation, *Brassica napus*, climate change, heritability, *Leptosphaeria*, temperature

1 | INTRODUCTION

Long-term predictions of plant disease severity, and prevalence, in response to anthropogenic climate change are needed in order to plan appropriate societal and production responses. It is clear that the geographic range of pathogens may be limited by climate, and climatic modelling is extensively used to make predictions about changes in range and severity of pathogens (Sutherst, 2003). However, this approach only makes sense if we assume that there is no evolutionary adjustment of pathogens to changing climate. Such
adjustment can have serious effects on crop production as seen, for example, in the recent expansion of *Puccinia striiformis* on wheat (Milus et al., 2009).

The rate at which selection changes the mean of a phenotypic character is proportional to (a) the strength of net selection on the character, (b) the additive genetic variance influencing it, and (c) inversely proportional to the nonheritable (environmental and non-additive genetic) variance influencing it. This is simpler for haploid organisms that can reproduce clonally, as dominance is less important (absent unless a locus is duplicated) and genotypes can be replicated. If it can be estimated, as in a breeding programme in managed agriculture, the ratio of additive genetic variance to total variance is defined as the heritability of the character. Understanding the environmental variance in a pathogen population growing in a realistic setting is not necessarily easy.

A first step in predicting the extent of adaptation of a pathogen to a changing environment is to understand the genetic architecture of response to environmental change. This has been attempted for temperature adaptation of *Zymoseptoria tritici*, for example, by Zhan and McDonald (2011) and subsequently Lendenmann et al. (2016) using growth rates in vitro at two temperatures, and Boixel et al. (2019) at multiple temperatures.

Phoma stem canker is one of the most important diseases of oilseed rape, causing estimated worldwide losses of €500 million in 2008 (Fitt et al., 2008). It is caused by the ascomycete fungi *Leptosphaeria maculans* and *L. biglobosa*. *L. maculans* is the more aggressive and damaging of the two causal organisms, although both can be associated with severe basal cankers (West et al., 2001; Fitt et al., 2006; Stonard et al., 2010). Symptoms of the disease are first seen a few months after planting as pale brown lesions with multiple black pycnidia on leaves. Infections are initiated by windborne ascospores that germinate and then penetrate the leaf through stomata or wounds. Hyphae grow symptomlessly from these lesions down the leaf petioles into the stem, where cankers form as the crop develops and matures. Further saprophytic colonization occurs in oilseed rape debris left after harvesting. It is followed by the development of pseudothecia from which ascospores are discharged in rainy weather. The ascospores are dispersed by air movement and initiate the leaf lesions, which start the next disease cycle (West et al., 2001). The extent to which two *Leptosphaeria* spp. pathogenic on oilseed rape possess genetic variation in their responses to environmental variables is currently unknown.

An increase in average temperature, or in temperature variability, is likely to alter disease prevalence and severity. On the other hand, a common, sexually reproducing pathogen might possess sufficient heritable variation to adapt to some extent to environmental change. This has been shown in *Phytophthora infestans* in comparisons of latent period, lesion expansion, and sporulation in planta within clones isolated from warmer or cooler regions of Europe (Mariette et al., 2016); rather more complex patterns of adaptation were shown for growth rate on agar of isolates from different regions of China (Yang et al., 2016). A similar pattern was shown in *Cryphonectria parasitica*, again on agar (Robin et al., 2017). Zhan and McDonald (2011) explored relative growth rates of isolates of *Z. tritici* on agar at two temperatures and found evidence of thermal adaptation to warmer and colder environments. Further extensive experimental work on *Z. tritici* has established that variation in in planta fitness traits with temperature is reasonably correlated with in vitro multiplication of cultured spores (Zhan et al., 2016; Boixel et al., 2019). Lendenmann et al. (2016) mapped some quantitative trait loci that affect relative growth on agar at two temperatures. Most of these loci had pleiotropic effects, so that adaptation to temperature is likely to affect other aspects of phenotype, including pathogenicity.

In *Leptosphaeria* spp. on oilseed rape, there has been much research on the relation of environmental variables to pseudothecial maturation, canker development, and ascospore release, again focused on the average population response. This is because available fungicides have little effect against *Leptosphaeria* spp. once the fungi have invaded the host stem, and advice on the most effective time to control the disease when leaf symptoms are apparent is therefore important. The favourability of weather variables for pseudothecial maturation has been extensively modelled (Aubertot et al., 2006; Salam et al., 2007). For ascospore maturation, release, and dispersal, key variables are temperature and rainfall (Toscano-Underwood et al., 2003; Huang et al., 2007).

Although the time of initial infection is very useful for disease management, later phases of the life cycle also determine long-term abundance and are strongly affected by temperature, and integrated models have been published (e.g., Lô-Pelzer et al., 2010). The model published by Evans et al. (2008), based on Gladders et al. (2006), predicts phoma stem canker progress through the entire growing season from onset to harvest and is of specific interest for the present study. They used three successive regression models, one for each stage of progress. The first model predicts a date in autumn when incidence of phoma leaf spotting reaches 10% of plants with leaf spots. This is the empirical threshold for fungicide spray application in the UK. They found negative linear relationships between date when 10% of plants have leaf spots and both mean daily maximum temperature and total rainfall during a developmental period from 15 July (representing the approximate date of harvest) to 26 September. The second model predicts the date of onset of stem canker symptoms in spring, relative to the date when 10% of plants have leaf spots. This assumes a linear relationship of pathogen growth with thermal time. The thermal time thresholds for canker appearance differ between cultivars “susceptible” or “resistant” to *L. maculans*. Finally, canker severity at harvest is predicted from the date of onset of stem canker symptoms and a linear regression on the subsequent thermal time until harvest.

Given predictions of future temperature and rainfall, these relationships allow predictions of harvest severity of stem canker under diverse climate change scenarios to be made (Evans et al., 2008). Evans et al. (2008) used this empirical model to predict changes in the effect of the disease on UK oilseed rape crops up to the mid-21st century. They were careful to restrict predictions
samples of cankered *Brassica napus* stems were collected from eight sites evenly spread over the oilseed rape-growing area of England in 2011–2012 (Figure S1, Table S1). Isolates were made from stems of cv. Drakkar, a spring oilseed rape cultivar of *B. napus* with no recognized quantitative or *R* gene resistance. Pure isolates were obtained from three types of material. If visible on the surface of the stem, pink cirri were collected into 50 μl of sterile water using a sterile mounted needle. The suspension was spread on distilled water agar (DWA). Otherwise, stems were washed under running tap water to remove all visible soil and then dried at around 20°C for 24 hr. Small pieces of blackened stem material approximately 5 × 2 × 2 mm were cut from lesions or cankers, surface sterilized for 2 min in sodium hypochlorite solution (1% available chlorine), rinsed twice in sterile water, blotted dry, and placed on potato dextrose agar (PDA) plates with streptomycin and penicillin (both at 50 mg/L). After 2 days the plates were examined using an inverted binocular microscope. Hyphal tips from germinating spores or developing colonies were collected by cutting a small wedge of agar bearing the hyphal tip at the point of the wedge and transferring it to the surface of PDA. Incubation was at 20°C in the dark; one colony was retained from each sample, chosen at random (by labelling and using a random number generator) from those with appropriate morphology. Species identifications were confirmed by PCR using the primer pairs developed by Xue *et al.* (1992).

Once colonies reached approximately 60 mm in diameter, a piece of agar was cut from near the centre of the colony and rubbed over the surface of a fresh plate of V8 agar to encourage conidial production. Plates were incubated in the dark at 20°C until either pre-pycnidial clusters of hyphae were observed or most of the agar surface was colonized. Plates were then moved to the laboratory bench so that they received natural daylight at 19–23°C.

Conidia were harvested once pycnidia were abundant by flooding the colony with 5 ml sterile water and rubbing gently with a sterile bent glass rod. The resulting spore suspension was filtered through two layers of Miracloth (Merck Chemicals Ltd), diluted and used to produce single spore isolates for further use. Spore suspensions prepared as above from single spore isolates were stored at ~20°C in 1 ml aliquots in 1.5 ml Eppendorf tubes for DNA extraction. For long-term storage, they were added to an equal quantity of sterilized 50% glycerol solution and stored at ~80°C in 200 μl aliquots; these were thawed and diluted before use by sequential addition of sterile water.

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2 | MATERIALS AND METHODS

The temperature gradient plate used was constructed at the University of Reading on a base manufactured by Grant Instruments. A thick aluminium plate was cooled along one edge and heated along the opposite edge (Murdoch *et al.*, 1989), giving a closely linear trend perpendicular to these edges. A plastic 14 × 14 grid of compartments (each 51 × 51 mm, and 45 mm tall) was placed on top of the plate, giving 14 distinct temperature zones, each containing 14 compartments. The
grid was covered with a sheet of 50 mm Celotex TB4020 (Celotex Ltd) to reduce the temperature gradient between the lid and base of the plastic boxes. On top of this was a sheet of glass, then black polythene, and a layer of corrugated cardboard, in order to exclude sunlight and so prevent the fungal colonies from producing conidia.

Square polystyrene boxes (internal side length 43 mm, internal depth 15 mm; Allied Plastics) containing an average of 6.7 g agar were used as growth chambers within the grid. Circular blocks of agar 6 mm diameter, taken from the perimeter of actively growing colonies on V8 agar, were placed in the centre of each agar-filled box and stored for either 24 or 48 hr in an insulated box on the laboratory bench (mean temperature of room 20–23°C) before transfer to the temperature gradient plate. This allowed mycelia to reorientate growth and to grow into the fresh agar before transfer to the gradient plate. The boxes in the coolest six positions in the temperature gradient plate were placed lid uppermost and the boxes in the warmest eight positions were inverted, so that slight temperature gradients across the height of the box would in all cases cause any evaporated water to condense back into the agar. The daily opening of boxes for measurement allowed gas exchange with the atmosphere to prevent excessive change in the gaseous environment within the boxes.

The consistency of temperatures perpendicular to the temperature gradient was confirmed using temperature loggers (Thermochron iButton DS1921G; Maxim Integrated) placed on the agar in boxes as for an experimental run. They were set to record temperature every 10 min. The loggers were shown to be consistent with each other in preliminary work. They were placed in the coolest row of the grid for 12 hr then moved to subsequent rows every 12 hr. The mean temperature in each grid position was recorded. Position perpendicular to the temperature gradient showed no detectable trend using linear regression, and temperatures in individual cells showed very small and nonsignificant variation across 24-hr periods or between night and day temperatures.

Following simulation investigation of possible designs, a partially balanced incomplete block experiment was designed with two replicates of 60 isolates within 10 runs of the gradient plate. Each run included 12 isolates, a "standard" isolate, and a set of temperature loggers (Thermochron iButton DS1921G). The standard was a L. maculans isolate (FWD-115-1U) selected for its good linear growth rate over a broad temperature range from a pretest of 13 isolates from several UK locations. Seventeen L. biglobosa isolates and 44 L. maculans isolates (including the standard) were included. The two sides of the plate were set to 0 and 35°C; preliminary tests showed that no isolate grew at all above 35°C. Temperature loggers were set to record temperature every 10 min with an accuracy of 0.5°C. A Latin square design, separately generated and so unique for each run, was used to place the 12 isolates, the standard isolate, and the temperature loggers within the 14 × 14 grid.

Boxes were photographed at roughly 24-hr intervals for 7 days. For photography, the boxes were removed in blocks of one row along the temperature gradient, containing one sample of each isolate. Boxes were opened and photographed daily in a laminar flow cabinet using a Canon EOS 300D camera (runs 1–6) or a Canon EOS 600D camera (runs 7–10), each with a Jessop 58 mm 1A lens at a standard distance between the camera lens and the fungal colonies. On each occasion, boxes were in an environment at room temperature for less than 15 min. Boxes were returned to their positions on the temperature gradient plate in their initial orientation. Any fungal colony that had reached the meniscus at the edge of the agar on which it was growing was removed and replaced with an empty box to minimize temperature changes in other positions.

Photographs were measured in ImageJ (imagej.nih.gov/ij/). Because bright reflections on the surface of the agar were often whiter than thin hyphal layers at the edge of the colony, the software was unable to automatically detect the edge of the fungal colonies. The perimeter of the colony was therefore drawn “by hand” on photographs within the ImageJ software and the enclosed area was recorded in pixels. The number of pixels per mm of colony was calculated from the box width in photographs selected at random from those taken by each camera.

The behaviour of hyphae at the leading edge of colonies differed among species, isolates, and temperatures. The leading hyphae of some colonies formed a distinct sharp edge, while other colonies exhibited more diffuse growth, making interpretation of the colony edge more difficult to define. To standardize measurements, the following precautions were taken: all measurements were made by the same person; all measurements were made on the same computer using the same monitor, mouse, and mouse-mat; and to improve consistency all measuring sessions were initiated by measuring, in random order and orientation, the same 40 photographs, chosen to span the variety of morphology and contrast. Photographs of each colony were measured in times-series order to ensure consistency in the interpretation of colony features; photographs remained labelled with their position in the temperature gradient plate, effectively blinding measurements. All isolates were included in subsequent analyses, regardless of margin behaviour.

The variance in colony area measured that was attributable to operator variation was calculated using a linear mixed model with no fixed factors and a random model of training session + photograph.

### 2.2 Data analysis

Radial growth rates for colonies were well-represented by a straight-line model. For temperatures <18°C or >25°C, the growth rate was calculated omitting the photograph at the time of placement of the colony on the plate to avoid nonlinear growth effects while the agar and colony adjusted to the experimental temperatures. Small negative values for growth rates occurring at very high temperatures were replaced with zero. Variance between replicates was greater at faster growth rates; thus, for further analysis, growth rates were transformed using log(rate + 0.5); this equalized inter-replicate variances in growth rate at different temperatures. The estimated growth rates were finally analysed with a linear mixed model using GenStat v. 13 (VSNI), treating the fixed model as temperature × isolate and the random model as run/block.

$$T_{\text{min}}$$, the minimum temperature at which any growth occurred, was estimated from a fit of a straight-line model to growth at
temperatures <21°C. Fits were good in all cases: mean $R^2$ across all isolates 0.97, SD 0.03. $T_{opt}$, the temperature at which growth was fastest, and $R_{max}$, the radial growth rate at $T_{opt}$, were estimated from a fit of the equation $R = bT + s + g \left[1 - \exp(-T^2)\right]$ to the observations at temperatures between 10 and 33°C, where $R$ is growth rate, $T$ is temperature and $b$, $s$, and $g$ are fitted constants. Mean $R^2$ across all isolates was 0.995, SD 0.005, with no evidence of a difference between the two species ($F[1, 58] = 0.33, p = 0.6$). Growth at the highest temperatures was examined at individual temperatures of 29.4, 31.8, and 34.3°C, because estimates of a single $T_{max}$ from line-fitting to the last two to four growth rates, or a single model of the whole data set, were very unstable and dominated by individual data points.

2.3 Disease development in planta

This experiment was replicated once using the same five fungal isolates. Four isolates of *L. maculans* with different in vitro temperature responses, including the standard isolate used in vitro, and one isolate of *L. biglobosa* were selected (Figure 1). *B. napus* ‘Drakkar’ was used as host because it has no recognized specific resistance against *any L. maculans* or *L. biglobosa* isolate. Flowering in this cultivar is not temperature-sensitive and it does not require a vernalization period to initiate stem extension.

2.4 Plants and growth conditions

Seeds were pregerminated on moist filter paper in Petri dishes for 48 hr in the dark at 20 ± 1°C prior to sowing in a dampened 1:1 mix of MiracleGro (Scotts MiracleGro Co.) and John Innes no. 3 composts. Three seeds were sown in each 3 L pot. The soil surface was covered with a 0.5 cm layer of vermiculite. Spare seedlings, germinated and planted under the same conditions and at the same time, were transplanted to replace plants that failed to emerge.

Pots were placed on capillary matting in trays and watered from beneath. In the first replicate of the experiment, plants were grown in a glasshouse until temperature treatments were applied, at a mean temperature of approximately 20°C, fluctuating approximately 15–25°C. In the first replicate, natural daylight was supplemented with 400 W high-pressure mercury vapour bulbs to extend the photoperiod to an intended 12 hr day (see below). In the second replicate, plants were germinated and initially grown in a controlled environment room (Weiss Gallenkamp) at a constant temperature of 20 ± 1°C with a 16 hr day at 220 μmol m⁻² s⁻¹ PAR from Phillips TL-D reflex 58W/840 white fluorescent lamps.

2.5 Inoculation and incubation

Previous work has shown that stem lesions can be reproducibly established in a reasonably natural way through inoculation of the petiole close to the stem (Sprague et al., 2007; Huang et al., 2014). Similar methodology was therefore adopted for this study. At 21 days old, when plants had one to two true leaves (growth stage 1.2 to 1.3 [Sylvester-Bradley, 1985]) the upper surface of the petiole of each leaf to be inoculated was gently rubbed close to the stem with clean, moist tissue paper to remove wax from the cuticle. Three small wounds were made approximately 5 mm from the stem with a sterile mounted needle. A 10 μl drop of spore suspension (10⁷ spores/ml) was placed over each wound. Control plants were inoculated with sterile distilled water. Three pots were inoculated with each isolate as a batch, a total of nine plants per isolate per temperature treatment. Each batch was immediately enclosed within a polythene-covered frame for 72 hr. Both plants and the inside of the polythene coverings were sprayed daily with distilled water to maintain high humidity. A layer of dark plastic was used to exclude light for the first 24 hr to encourage successful infection.

In Replicate 1 of the experiment, the petioles of the lowest two leaves of the three plants in each pot were all inoculated with the same isolate. Temperature in the glasshouse was high on the day of inoculation, reaching a maximum of 30°C. Mean glasshouse temperature (mean of minimum and maximum temperatures) during the following week was 26.6 ± 2.1°C. In Replicate 2, only the lowest leaf petiole was inoculated to initiate one canker per plant. Individual plants within each pot were inoculated with a different isolate in a partially balanced block design. Pots with inoculated plants were placed in a Latin square design within the plastic coverings. Separate pots containing three control plants, inoculated with sterile water, were incubated beneath a separate plastic covering to prevent contamination from inoculated plants. Plants remained in a controlled environment at 20 ± 1°C.

2.6 Temperature treatments

To ensure we had consistent initial infection before testing subsequent development, pots were transferred to a series of controlled environment temperature regimes 7 days after inoculation (dai). To
make results comparable to the constant temperatures used for in vitro experimentation, constant temperature regimes were used. Temperatures were selected to test the value of $T_{\text{opt}}$ and the linear relationship found, at temperatures below $T_{\text{opt}}$ between radial growth in culture and temperature.

For Replicate 1, one pot per isolate per batch was placed at each of four constant temperatures, 16, 20, 24, and $28 \pm 0.2^\circ\text{C}$, in separate controlled environment rooms (Weiss Gallenkamp) with 80% ± 1 relative humidity and mean light intensity of 220 μmol-m⁻²-s⁻¹ PAR 3 cm above the soil surface, from Phillips TL-D reflex 58W/840 white fluorescent lamps. Due to problems with cabinet control software, plants at 16°C were exposed to a 16 hr photoperiod; plants at 20°C were exposed to a 16 hr photoperiod for the first 22 days and a 12 hr photoperiod thereafter; plants at 24°C were exposed to a 12 hr photoperiod for the first 22 days and a 16 hr photoperiod thereafter; and plants at 28°C were exposed to a 12 hr photoperiod. Water was provided by ensuring that the capillary matting beneath pots was constantly wet. Pots in each controlled environment were randomized to minimize position effects and placed adjacent to each other in a single block.

For Replicate 2, one replicate set of inoculated pots was placed at each of five temperatures, 16, 20, 22, 24, and $28 \pm 0.3^\circ\text{C}$, in controlled environments (Large Saxil Growing Cabinets) with mean light intensity of 700 μmol-m⁻²-s⁻¹ 3 cm above the soil surface from 58 W cool white fluorescent tubes (OSRAM L58W/840) with a 16 hr photoperiod. Relative humidity was adjusted as close as possible to 80% in each controlled environment (85% ± 3, 83% ± 2, 81% ± 2, 75% ± 2, and 75% ± 2, respectively). Water was provided by timed irrigation on the soil surface, with the frequency and duration of watering periods adjusted to ensure that the surface of the compost was continually damp in each chamber. Pot position was randomized within each chamber and positions were classified to allow for position effects to be excluded during analysis. Pots were spaced out across the available space to maximize the level of photosynthetically active radiation reaching leaves and stems. Spaces between pots were equal to the diameter of the pots.

### 3.1 | Growth rate in vitro

The effects of run (each use of the temperature gradient plate) and block (each row of boxes parallel to the temperature gradient that were removed together for photography) were extremely small. Run accounted for 1.6% of the variation in the data set. The estimated variance component for block was negative and block was not used in further analyses.

The main effects of temperature and isolate on growth rate were substantial ($p < .001$; Figure 2). Interactions between temperature and species, between isolates within species, and between temperature and isolate were all significant ($p < .001$; Figure 3). The difference in growth rate was significantly greater between the two species than
between isolates within species at all temperatures (p < .001). Separate analyses for each species showed that the effects of temperature, isolate, and the interaction between them were significantly greater than residual variation within each species (p < .001). There was weak evidence (F[43, 16] = 1.6, p = .15) for less environmental stability, that is, greater residual variance, in L. maculans than in L. biglobosa.

### 3.1.1| $T_{\text{min}}$

$T_{\text{min}}$ differed slightly between L. biglobosa (species mean 0.11°C, SEM = 0.03) and L. maculans (species mean 0.18°C, SEM = 0.02, F[1, 68] = 6.1, p < .016). Genetic variance for $T_{\text{min}}$ was 0.0046°C² (i.e., genetic SD = 0.068°C and coefficient of variation 38%) in L. maculans but immeasurably small for L. biglobosa. The environmental (residual and run) variance was larger than the genetic variance and similar in the two species: 0.0076°C² for L. maculans and 0.0090°C² for L. biglobosa (F[16, 51] = 1.17, p = .3).

### 3.1.2| $R_{\text{max}}$

Mean $R_{\text{max}}$ for L. maculans (2.60 mm/day, SE = 0.05) was less than for L. biglobosa (3.69 mm/day, SE = 0.05); (comparing means, F[1, 60] = 273, p < .001). Mean genetic variance was more for L. maculans (0.043 mm²/day², i.e., SD of 0.21 mm/day or a coefficient of variation of 8%) than for L. biglobosa (0.0028 mm²/day², i.e., SD of 0.053 mm/day or a coefficient of variation of 1.4%; F[43, 16] ratio between species = 15.3, p < .001). Environmental variance was comparable to genetic variance for L. maculans but much larger for L. biglobosa.

There was weak evidence for a difference in environmental variance between species: 0.025 mm²/day² for L. maculans and 0.042 mm²/day² for L. biglobosa (F[16, 51] ratio = 1.71, p = .02).

### 3.1.3| Sensitivity of growth rate to temperature

At temperatures below 21°C there was an approximately linear and positive temperature response of growth rate to temperature (Figure 2). There was a substantial difference in the size of this effect between the two species: L. maculans, 0.119 mm-day⁻¹°C⁻¹, SE = 0.002°C; L. biglobosa, 0.157 mm-day⁻¹°C⁻¹, SE = 0.003°C (F[1, 62] = 159, p < .001). The two species differed in the genetic variance for this parameter: L. maculans 6.6 × 10⁻⁵ mm²·day⁻²°C⁻² (i.e., genetic standard deviation 0.008 mm·day⁻¹°C⁻¹, 6.7% of the mean); L. biglobosa 3.0 × 10⁻⁵ mm²·day⁻²°C⁻² (v.r. F[95, 33] = 2.2, p = .006). The environmental (run plus residual) variance was not significantly different between species, at approximately 9.0 × 10⁻⁵ mm²·day⁻²°C⁻² (F[95, 33] = 1.1, p = .4).

### 3.1.4| $T_{\text{opt}}$

There was no significant difference in $T_{\text{opt}}$ between the two species (F[1, 60] ratio = 3.28, p = .075). Mean $T_{\text{opt}}$ for L. maculans was 26.25°C (SEM = 0.25) and for L. biglobosa was 25.52°C (SEM = 0.36). Genetic variance for $T_{\text{opt}}$ was similar for both species (L. maculans 1.65°C², i.e., genetic standard deviation 1.22°C; L. biglobosa 1.72°C²) but the environmental variance was significantly different between species: 1.81°C² for L. maculans and 0.36°C² for L. biglobosa (F[95, 33] ratio = 7.2, p < .001).

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**FIGURE 2** Ranges of radial growth rate (as mm per day) for all (a) *Leptosphaeria maculans* and (b) *L. biglobosa* cultures tested on V8 agar, at each nominal temperature used.
between species (was slight evidence for a difference in environmental variance between species, in the opposite direction to that systematically bias in estimates of \(T_{opt}\) and \(R_{max}\). Dashed line shows temperature below which growth rate responds approximately linearly to temperature.

### 3.1.5 Growth at high temperature

At the 12th temperature position (29.4°C, SD = 0.5°C) genetic variance for \(L.\, maculans\) was 0.154 mm² day\(^{-2}\)°C\(^{-2}\) and for \(L.\, biglobosa\) was 0.366 mm² day\(^{-2}\)°C\(^{-2}\) (F[33, 95] ratio = 2.37, \(p < .001\)). There was slight evidence for a difference in environmental variance between species (\(L.\, maculans\): 0.084 mm² day\(^{-2}\)°C\(^{-2}\); \(L.\, biglobosa\): 0.144 mm² day\(^{-2}\)°C\(^{-2}\), F[33, 95] ratio = 1.71, \(p = .02\)). At the 13th temperature position (31.8°C ± 0.5), genetic variance was greater in \(L.\, biglobosa\) (0.28 mm² day\(^{-2}\)°C\(^{-2}\)) than in \(L.\, maculans\) (0.096 mm² day\(^{-2}\)°C\(^{-2}\), F[33, 95] ratio = 2.9, \(p < .001\)). There was slight evidence for a difference in environmental variance between species, in the opposite direction to that of the 12th temperature position (\(L.\, maculans\): 0.102 mm² day\(^{-2}\)°C\(^{-2}\); \(L.\, biglobosa\): 0.060 mm² day\(^{-2}\)°C\(^{-2}\); \(v_r = 1.7[93.333], p = .04\)). At the 14th temperature position (34.3°C ± 0.5), \(L.\, maculans\) was unable to grow. The genetic and environmental variances for \(L.\, biglobosa\) were 0.0382 mm² day\(^{-2}\)°C\(^{-2}\) and 0.0034 mm² day\(^{-2}\)°C\(^{-2}\), respectively.

### 3.2 Disease development in planta

#### 3.2.1 Host growth

In Replicate 1, effects on growth stage due to temperature could not be separated from effects due to differences in photoperiod in each regime.

In Replicate 2, growth stage reached varied according to temperature treatment at both 49 and 91 dai. At 49 dai, the proportion of plants with fully developed buds (GS 3.9) and at early flowering (GS 4.1 onwards) declined with increasing temperature (\(p < .001\)). However, among plants that had begun to flower, the proportion of plants in later flowering stages increased with increasing temperature (\(p < .001\)). At 91 dai, no seed had set at 28°C and development into flowering was partially inhibited at 24°C (Figure 4). However, in plants that flowered, progress in all three stages—completion of flowering, seed development, and pod senescence—increased with temperature (\(p \leq .001\) in each case: generalized linear model with binomial error).

#### 3.2.2 Disease

In both replicates, inoculation of the petiole produced visible necrotic lesions that progressed in both directions along the length of the petiole (Figure 5). Necrosis occurred on all petioles where stems were subsequently found to be infected with \(L.\, maculans\) spp. The extent of necrosis was very variable, both in the amount of tissue damage through a cross-section of the petiole and also in the distance grown along the petiole. There were no observable differences in symptoms between plants inoculated with \(L.\, maculans\) or \(L.\, biglobosa\). The most frequently encountered symptom following stem infection resulting from petiole inoculation was stem lesions beneath the leaf scar at which the fungus entered the stem. These lesions frequently extended above the leaf scar but the majority of surface damage was always below the leaf scar. Similarly, in most cases, at all temperatures tested, the internal symptoms extended much further into the hypocotyl and root than up the stem. There was an association between the proportion of stems that had developed cankers at final assessment and the proportion of inoculated leaves that had abscised at 35 dai in Replicate 1 (\(\chi^2 = 22.8, p < .001\)) or 28 dai in Replicate 2 (\(\chi^2 = 23.0, p < .001\)). More cankers developed in plants where leaves remained attached. The association was independent of temperature.

Incidence of disease was greater after inoculation with all \(L.\, maculans\) isolates than after inoculation with the selected \(L.\, biglobosa\) isolate (Figure 6) in both replicates. Incidence of disease was much greater in Replicate 1 than in Replicate 2. The variance component...
representing variation between isolates of *L. maculans* was not significant (Table 1, Figure 6). There were no significant average effects of temperature (*p* > 0.2 for all contrasts). The variance component representing isolate interaction with temperature was significant, with a standard deviation difference in incidence due to the more aggressive of two random isolates at different temperatures representing a 34% increase (Table 1).

There was no apparent trend with temperature in canker severity in infected stems for any measure. No isolate differences were detected (Figure 7, Table 2).

Yields were measured in Replicate 2 but not in Replicate 1. Because of the host developmental differences, yield per plant depended very strongly on temperature and was close to zero at 28°C (Figure 8). There were no significant differences in yield between healthy plants and plants with phoma stem canker symptoms at any temperature. There was no observable average effect of canker severity on yield; however, the sensitivity of the experiment was small because of inevitable plant to plant variation. Based on the observed plant to plant variation, differences in yield of approximately 20% between infected and uninfected plants would have been detectable.

**4 | DISCUSSION**

The in vitro experiment on radial growth rate on agar was precisely controlled and several sources of bias or scatter were carefully eliminated. Nonetheless, the "environmental" variance for measured temperature response parameters was often larger than the variance attributable to genetic differences between isolates. There were some species differences between both genetic and environmental variances for some of the temperature response parameters studied. This suggests that the two *Leptosphaeria* species causing phoma stem canker on oilseed rape may have differing evolutionary potentials for temperature adaptation.

Published work on temperature adaptation in *L. maculans* and *L. biglobosa* has focused on population averages, and much predates the taxonomic distinction between the species. In agar culture at temperature ranges below 25°C, hyphal growth rate increases with increased temperature in both species (Huang *et al.*, 2003, 2006; Naseri *et al.*, 2008). The temperature at which growth rate is maximal (*T*<sub>opt</sub>) was therefore usually above 25°C. Earlier studies of radial growth rate in culture, done before the taxonomic separation of *L. maculans* and *L. biglobosa*, recorded *T*<sub>min</sub>, *T*<sub>opt</sub>, and *T*<sub>max</sub> for *L. maculans s. l.* as 0–3°C, 26–27°C, and 32–35°C, respectively (Ndimande, 1976) and as <10°C, 25°C, and 35°C (Maguire *et al.*, 1978). Ndimande made no record of number or characteristics of isolates used, while Maguire recorded growth rates of *L. maculans s. l.* from infected seed and was therefore testing a natural population of isolates. Rates calculated from Maguire *et al.* (1978) are 2.5 ± 0.2 mm/day at 20°C and 2.7 mm/day at 25°C for isolates growing on V8 agar in the dark. Working with *L. maculans s. s.*, Huang *et al.* (2010) reported mean radial growth rates in the dark at 22°C of 2.9 ± 0.2 mm/day. However, Huang *et al.* (2006) quotes 1.8 ± 0.1 mm/day for the same isolates; the reason for the discrepancy is unclear. *L. biglobosa* has been widely reported to grow more rapidly than *L. maculans* on all media types (Williams and Fitt, 1999). McGee and Petrie (1978) reported mean colony radii of 3.5 ± 0.3 mm (i.e., c. 0.5 mm/day) for "virulent" isolates (i.e., *L. maculans*) and 5.8 ± 0.1 mm (i.e., c. 0.8 mm/
day) for "avirulent" isolates (i.e., L. biglobosa) after 7 days' growth on V8 agar at 20°C, which suggests rapid decline in growth rate away from the optimum temperature—as seen in our experiments and in many other organisms—and possibly substantial dependence on medium or experimental protocol.

Hammond et al. (1985) published symptomless growth rates for L. maculans in petioles at two temperatures using single-point assessments. Although their methodology is somewhat unclear, they reported hyphal growth rates of 1.4 mm/day at 3°C and 5.0 mm/day at 18/12°C. The faster rate is roughly double the agar $R_{\text{max}}$

Note: Parameter estimates are on the logit scale.

*a*Logit scale.

*b*Square root of variance component.

*c*Exp(SD), factor by which the ratio of cankered to healthy stems is typically multiplied in comparing two isolates or isolate × temperature combinations.

*d*Not relevant to fixed effect contrast.

**FIGURE 7** Leptosphaeria stem canker severity on the Aubertot scale, in plants with symptoms only, at host maturity, in relation to temperature. Blue: Leptosphaeria biglobosa; red: L. maculans. SED applies to comparisons between isolates within a temperature

**TABLE 1** Variances in incidence of phoma stem canker following inoculation with Leptosphaeria isolates in Replicate 1 and Replicate 2, analysed using generalized linear mixed model regression with a binomial error distribution and a logit link function

| Effect | Parameter or contrast$^a$ | SE of parameter estimate | SD$^b$ | Odds-ratio multiplier$^c$ | $p$ |
|--------|--------------------------|--------------------------|--------|---------------------------|-----|
| Replicate 2 versus Replicate 1 | -1.68 | 0.288 | - | - | <.001 |
| L. biglobosa versus L. maculans | -2.43 | 0.313 | - | - | <.001 |
| Variance between isolates | 0.015 | 0.012 | 0.12 | ×1.13 | .13 |
| Isolate × temperature variance | 0.087 | 0.032 | 0.29 | ×1.34 | .003 |

Note: Parameter estimates are on the logit scale.

*a*Logit scale.

*b*Square root of variance component.

*c*Exp(SD), factor by which the ratio of cankered to healthy stems is typically multiplied in comparing two isolates or isolate × temperature combinations.

*d*Not relevant to fixed effect contrast.

**TABLE 2** Effect of isolate on measures of canker severity in successfully inoculated plants grown at one of four temperatures

| Canker assessment method$^a$ | Replicate 1 | Replicate 2 | | | |
|-------------------------------|-------------|-------------|-------------------|-------------------|-------------------|
|                               | Including L. biglobosa | Excluding L. biglobosa | Including L. biglobosa | Excluding L. biglobosa |
| $F$ ratio (4, 12)$^b$ | $F$ ratio (3, 9)$^c$ | $p$ | $F$ ratio (4, 12) | $F$ ratio (3, 9) | $p$ |
| Huang et al. (2014) | 5.1 | <.001 | 5.2 | .002 | 2.2 | .08 | .67 | .60 |
| Zhou et al. (1999) | 4.8 | .001 | 5.7 | .001 | 1.5 | .20 | 1.2 | .30 |
| Aubertot et al. (2004) | 5.1 | <.001 | 5.1 | .002 | 2.2 | .08 | 0.67 | .60 |
| Total length$^d$ | 3.8 | .006 | 1.3 | .200 | 1.4 | .20 | 0.64 | .60 |
| Percentage girdling$^e$ | 5.0 | <.001 | 5.9 | <.001 | 1.7 | .10 | 0.42 | .70 |

Note: Replicate 1 (n = 150, 4 temperatures, 5 isolates) and Replicate 2 (n = 118). Data from 22°C treatment in Replicate 2 excluded. Analysed using REML without blocking, because of variable numbers of isolates due to failed inoculations.

*a*The three assessment methods differ slightly in how scores based on percentage girdling and cross-sectional area of the canker are derived, but are closely related.

*b*Five isolates (four L. maculans, one L. biglobosa), so 4 df between isolates, 12 df for variation between replicates of an isolate.

*c*Four isolates of L. maculans, so 3 df between isolates, 9 df for variation between replicates of an isolate.

*d*Canker length measured from lowest visible to highest visible point after sectioning longitudinally.

*e*At the level of the first leaf scar.
These are not easy to experiment on, and the problem applies to other well-studied species. *Z. tritici* is the best-studied example of variation in temperature responses. This has been shown to have reasonable correlation between growth in liquid culture and some within-season fitness characters (Boixel et al., 2019). Passing to wider aspects of phenotype in this fungus, triazole fungicide insensitivity in liquid culture was correlated, though imperfectly, to sporeulation in a single generation of in planta growth (Pijs et al., 1994). However, even in *Z. tritici* there have been no reports of the relationship between individual variation in over-seasoning characters and temperature.

The rate of evolutionary adaptation to an altered temperature depends on how large the additive variance is in relevant phenotypic traits, relative to the variance caused by the differences between the environments experienced by each individual. The additive genetic variance will be less than the variance due to all genotypic differences that we measured in the work reported here, because epistatic effects will be only partly inherited following sex, which breaks up associations between loci. In a natural setting, the rate of evolution of temperature response depends on the environmental variance in growth rate, which determines heritability. This is presumably affected by host cultivar, position of the initial infection on the leaf, and numerous factors such as fertility, rainfall, age of leaf at infection, insect damage, and the extent of host defence triggering. Even if we further assume the genotypic variance between isolates was mostly due to additive effects, with negligible epistatic effects, the heritability is likely to be small and, therefore, the likely change between years in the population mean of *T* _opt_, *R* _max_, *T* _min_ or in ability to grow at high temperatures is small.

In planta, there was significant, if small, variation between temperature responses of isolates of *L. maculans* (Table 1). However, this was not simple variation in *T* _opt_ and the outcome of evolution—to the extent that the variation is due to additive genetic variance—would depend on the fine detail of how temperatures varied during the growing season.

In the UK, damage caused by *Leptosphaeria* species in the stem in the latter part of the growing cycle is usually the only pathogen effect that contributes to yield loss. Concentrated ascospore showers that occur during seedling emergence have been documented to result in complete crop loss due to severe cotyledon infections, but this is uncommon, especially because compensatory growth in oilseed rape is strong (Leach et al., 1999). Incidence and severity of final stem canker growth necessarily depends on interactions between host and pathogen physiological processes. In the present study, while all isolates grew more slowly on agar at 28°C than at 20 or 24°C, the isolates selected for testing in planta had equal or greater incidence and severity at 28°C than at 20 or 24°C, the isolates selected for testing in planta had equal or greater incidence and severity at 28°C than at 20 or 24°C. The rate of evolutionary adaptation to an altered temperature depends on how large the additive variance is in relevant phenotypic traits, relative to the variance caused by the differences between the environments experienced by each individual. The additive genetic variance will be less than the variance due to all genotypic differences that we measured in the work reported here, because epistatic effects will be only partly inherited following sex, which breaks up associations between loci. In a natural setting, the rate of evolution of temperature response depends on the environmental variance in growth rate, which determines heritability. This is presumably affected by host cultivar, position of the initial infection on the leaf, and numerous factors such as fertility, rainfall, age of leaf at infection, insect damage, and the extent of host defence triggering. Even if we further assume the genotypic variance between isolates was mostly due to additive effects, with negligible epistatic effects, the heritability is likely to be small and, therefore, the likely change between years in the population mean of *T* _opt_, *R* _max_, *T* _min_ or in ability to grow at high temperatures is small.

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that growth in culture is not necessarily related to growth in host in a straightforward way.

Once stem infection is achieved, the severity of internal damage that occurs will also depend on host resistance and environmental factors that cause variation in canker growth and damage caused. Deductions about the variance in disease severity between isolates based on the variance in colony growth on agar depend on the two phenotypic characteristics being correlated. As mentioned, this has been shown (for a limited part of the life cycle) for Z. tritici on wheat. However, as a counterexample, measurements of the growth of the chytridiomycete Batrachochytrium dendrobatidis in culture were uncorrelated with growth on the host (frogs; Raffel et al., 2013). Our experiments demonstrate that in at least one case the temperature response curves of disease severity and radial growth of pathogens on agar are substantially different and one cannot be used to predict the other. While these results are for Leptosphaeria species in B. napus, there is no reason to suppose they are exceptional. In general, it should not be assumed that temperature response curves for growth on agar and for disease severity or life-cycle stages in planta are similar.

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DATA AVAILABILITY STATEMENT
The data that support the findings of this study are available from the corresponding author upon reasonable request.

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**SUPPORTING INFORMATION**

Additional supporting information may be found online in the Supporting Information section.

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