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

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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^{-1} 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^{-1} and 0.53 mm day^{-} °C^{-1}. In addition, the incidence and severity of phoma stem canker *in planta* over a natural growing cycle at four temperatures (16°C, 20°C, 24°C 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*.

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 non-heritable (environmental and non-additive genetic) variance influencing it. This is simpler for haploid organisms which 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 (additive genetic + non-additive genetic+ environmental and their interactions) 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 (Stonard *et al.*, 2010; West *et al.*, 2001; Fitt *et al.*, 2006). 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 wind-borne ascospores that germinate and then penetrate the leaf through stomata or wounds.

Hyphae grow asymptomatically from these lesions down the leaf petioles into the stem, where cankers form as the crop develops and matures. Further saprophytic colonisation 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 & McDonald (2011) explored relative growth rates of isolates of *Zymoseptoria 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 which 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 focussed 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 (Huang *et al.,* 2007*;* Toscano-Underwood *et al.,* 2003).

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. Evans *et al.* 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. Evans *et al.* 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. Evans *et al*. were careful to restrict predictions to periods when the climate models encompassed conditions which had occurred historically. However, evolutionary change will alter the relationships and cause predictions to fail if the pathogens are able to adapt to unfavourable conditions. Also, projected change in climate suggests increases in maximum temperatures and the frequency of very hot weather much greater than changes in mean temperature, so the exact shape of temperature response curves is important. In particular, the Evans *et al.* model implicitly assumes - because it uses thermal time (i.e. temperature sums) - that the response curves are linear in the temperature ranges relevant to projected future climate.

The linear rates of growth of *Leptosphaeria maculans* and *L. biglobosa* across the leaf and along the petiole to the stem are critical components of fitness for these pathogens. Unless this relocation is completed before the leaf abscises, the fungi are unable to reproduce sexually and contribute to the next generation. Measurement of variation in temperature response of this character is therefore critical to understanding likely evolutionary response to temperature change. However, the character of interest is growth *in planta*, and it is unclear whether this is well correlated, in all life stages, with growth *in vitro*.

If it is assumed that variation in the developmental stages prior to asymptomatic growth along petioles is limited, then single-point measurements for the location of leading hyphae could be used to assess growth rates in biologically realistic conditions. However, this requires large numbers of replicates, both to allow destructive sampling and to overcome the problems of residual variation in host plants, environmental conditions and variation in previous developmental stages. Since *Leptosphaeria* spp. are haploid, genotypic variance is closer to measurement of additive genetic variance than in diploid organisms; dominance (but not epistatic) effects are absent. It is plausible that growth along the petiole would be related to the rate of growth in agar culture. Measurement of linear growth rate on solid growth medium therefore offers a more robust, and more cost-effective, methodology for investigating the temperature response of linear growth in these pathogens. However, it is unknown whether there is a correlation between the two systems for *Leptosphaeria* species, as has been demonstrated for other plant pathogens.

The studies reported here therefore had three aims: (1) to clarify the shape of the temperature response curve for radial growth on agar (2) to measure the genotypic variance (that is, the variance in phenotype due to additive and non-additive differences in genotype between individuals: Falconer & Mackay, 1996) in temperature response parameters for the radial growth on agar in representative samples of *Leptosphaeria maculans* and *L. biglobosa* populations, and (3) to test whether *in vitro* radial growth temperature parameters predict *in planta* growth, represented by canker incidence and severity. In order to allow a more robust dataset with a manageable number of plants, fungal isolates and temperatures, *in planta* growth was represented by canker incidence and severity, the growth measure most strongly correlated with yield loss rather than growth rate in petioles.

Methods

Samples of cankered *Brassica napus* stems were collected from eight sites evenly spread over the oilseed-rape growing area of England in 2011-12 (supplementary information A). Isolates were made from stems of cv. Drakkar, a spring rape cultivar of *Brassica napus* with no recognised quantitative or *R* gene resistance. Pure isolates were obtained from three types of material. If visible on the surface of the stem, pink cirrhi 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 c. 20 °C for 24 h. Small pieces of blackened stem material approximately 5 mm x 2 mm x 2 mm were cut from lesions or cankers, surface sterilised for 2 minutes in sodium hypochlorite solution (1% available chlorine),

rinsed twice in sterile water, blotted dry and placed on PDA agar plates with streptomycin and penicillin (both at 50 mg l^{-1}). After two 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 colonised. Plates were then moved to the laboratory bench so that they received natural daylight at 19 to 23°C.

Conidia were harvested once pycnidia were abundant by flooding the colony with 5 ml of 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, Nottingham, UK), 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 sterilised 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.

Growth rate *in vitro*.

The temperature gradient plate used was constructed at the University of Reading on a base manufactured by Grant Instruments (Cambridge, UK; www.grantinstruments.com). A thick aluminium plate is 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 by 14 grid of compartments (each 51 mm x 51 mm and 45 mm tall) is 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 Limited, Hadleigh, Suffolk, UK) 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, Thames Ditton, Surrey, UK) 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 h in an insulated box on the laboratory bench (mean temperature of room 20°C to 23°C) before transfer to the temperature gradient plate. This allowed mycelia to re-orientate 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, San Jose, CA 95134, USA) placed on the agar in boxes as for an experimental run. They were set to record temperature every 10 minutes. 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 h then moved to subsequent rows each for 12 h. 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 non-significant variation across 24 hour 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 pre-test 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°C and 35°C; preliminary tests showed that no isolate grew at all above 35°C. Temperature loggers were set to record temperature every 10 minutes 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 x 14 grid.

Boxes were photographed at roughly 24 h intervals for seven 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 to 6) or a Canon EOS 600D camera (runs 7 to 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 minutes. 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 minimise temperature changes in other positions.

Photographs were measured in ImageJ (download from imagej.nih.gov/ij/). Since 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 photos 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 photos 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 + photo.

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 non-linear 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 $\log_e$(rate + 0.5); this equalised inter-replicate variances in growth-rate at different temperatures. The estimated growth rates were finally analysed with a linear mixed model using GenStat v13 (VSNI, Hemel Hempstead UK), treating the fixed model as temperature*isolate and the random model as run/block.

T_{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 $R=bT+s+g(1-\exp(rT^2))$ to the observations at temperatures between 10°C 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°C, 31.8°C, and 34.3°C, since estimates of a single T_{max} from line-fitting to the last 2-4 growth rates, or a single model of the whole data-set, were very unstable and dominated by individual data-points.

Disease development *in planta*

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

Plants and growth conditions

Seeds were pre-germinated on moist filter paper in Petri dishes for 48 h in the dark at 20 °C ± 1 °C prior to sowing in a dampened 1:1 mix of MiracleGro™ (Scotts MiracleGro Co, Marysville, Ohio, USA) and John Innes N° 3 composts. Three seeds were sown in each 3L 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, 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 400W high pressure mercury vapour bulbs to extend the photoperiod to an intended 12 h day (see below). In the second replicate, plants were germinated and initially grown in a controlled environment room (Weiss Gallenkamp, Loughborough, Leicestershire, UK) at a constant temperature of 20 °C ± 1 °C with a 16 h day at 220 µmol m^{-2} sec^{-1} PAR from Phillips TL-D reflex 58W/840 white fluorescent lamps.

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 (1 x 10^7 spores ml^{-1}) was placed over each wound. Control plants were inoculated with sterile distilled water. Pots were inoculated in three replicate batches, a total of nine plants per isolate per temperature treatment. Each batch was immediately enclosed within a polythene covered frame for 72 h. 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 h to encourage successful infection.

In replicate 1, 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 °C ± 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 °C ± 1 °C.

Temperature treatments

To ensure we had consistent initial infection before testing subsequent development, pots were transferred to a series of controlled environment temperature regimes seven days after inoculation. 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_{opt} and

the linear relationship found, at temperatures below T_{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 °C, 20 °C, 24 °C and 28 °C ± 0.2 °C, in separate controlled environment rooms (Weiss Gallenkamp, Loughborough, UK) with 80% ± 1% relative humidity and mean light intensity of 220 µmol m^{-2} sec^{-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 h photoperiod; plants at 20 °C were exposed to a 16 h photoperiod for the first 22 days and a 12 h photoperiod thereafter; plants at 24 °C were exposed to a 12 h photoperiod for the first 22 days and a 16 h photoperiod thereafter; and plants at 28 °C were exposed to a 12 h photoperiod. Water was provided by ensuring that the capillary matting beneath pots was constantly wet. Pots in each controlled environment were randomised to minimise 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 °C, 20 °C, 22 °C, 24 °C and 28 °C ± 0.3 °C, in controlled environments (Large Saxil Growing Cabinets, RK Saxon Ltd, no longer trading) 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 h photoperiod. Relative humidity was adjusted as close as possible to 80% in each controlled environment (85% ± 3, 83% ± 2, 81% ± 2, 75% ± 2, 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 randomised 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 maximise the level of photosynthetically active radiation reaching leaves and stems. Spaces between pots were equal to the diameter of the pots.

Assessment

Plant growth stage assessments used the Sylvester-Bradley (1985) scale. In replicate 2, all pods on the main stem were collected and seeds were weighed as a yield estimate.

Plants were judged to be ready for canker severity assessment when most of the seeds were black and hard in pods on the middle third of the main inflorescence. In replicate 1, plants were assessed at 98 days post-inoculation. In replicate 2, all plants were assessed at 91 days post-inoculation. Each stem was cut 20cm above soil level and removed from the pot. Roots were washed and side-roots were trimmed from the tap root. Stems were stored for up to four days on trays loosely covered with cling film at 5 °C until assessment.

Cankers were assessed using external symptoms without whittling on the Zhou et al. (1999) scale and on a scale used by Huang et al. (2014). The Zhou scale gives good differentiation for severe cankers while the Huang scale gives more differentiation between 1% and 99% girdling. These two scales together allow easy conversion to the French scale proposed by Aubertot et al. (2004). Since spring oilseed rape does not develop a distinct ring of leaf scars at the crown, stems were cut where external symptoms were most severe near the leaf scar(s) of the inoculated leaf or leaves. Otherwise, they were cut at the first leaf scar. Internal symptoms were used to score the higher ratings on the Aubertot scale as specified by that scale. A photograph of each cross-section was taken and percentage girdling was calculated by measuring the damaged and undamaged portions of the circumference of each cross-section photograph using ImageJ (imagej.nih.gov/ij). Finally, stems and tap roots were cut longitudinally and the distances that tissue damage had progressed upwards and downwards from the horizontal cut were recorded.

Yield

Seeds were not collected in replicate 1. In replicate 2, all pods on the main stem were collected during the final assessment, air dried, and split open. Seeds were counted and weighed to give the total yield.

Data analysis

Incidence data were analysed using generalised linear mixed model regression with a binomial error distribution and a logit link function using the package *glmm* in R v3.5.1 (https://www.R-project.org; supplementary information B). Fixed effects were temperature, replicate and species. Random effects were isolate and isolate-temperature interaction. The difference between species was based on a single *L. biglobosa* isolate, but the contrast associated was far larger than any difference between *L. maculans* isolates.

Severity measures were analysed by REML in Genstat v13 (VSN International, www.vsni.co.uk). Canker severity was analysed, in cankered plants only, using REML. Initial analyses with a linear mixed model including blocks as a random effect failed to converge reliably, but did show that block effects were very small. Analyses without the extra random block effects converged stably. Severity was therefore analysed with no random effects related to blocks.

Results

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 ($P < 0.001$; Fig. 2). Interactions between temperature and species, between isolates within species and between temperature and isolate were all significant ($P < 0.001$; Fig. 3). The difference in growth rate was significantly greater between the two species than between isolates within species at all temperatures ($P < 0.001$). 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 ($P < 0.001$). There was weak evidence ($F_{43,16} = 1.6$; $P = 0.15$) for less environmental stability, that is, greater residual variance, in *L. maculans* than in *L. biglobosa.*

T_{min}. T_{min} differed slightly between *L. biglobosa* (species mean 0.11 °C, s.e.m. 0.03) and *L. maculans* (species mean 0.18 °C, s.e.m. 0.02; $F_{1,68} = 6.1$), $P < 0.016$). Genetic variance for T_{min} was 0.0046 $°C^2$ (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 $°C^2$ for *L. maculans* and 0.0090 $°C^2$ for *L. biglobosa* ($F_{16.\,51} = 1.17$, $P = 0.3$).

R_{max}. Mean R_{max} for *L. maculans (*2.60 mm day^{-1}, s.e. 0.05) was less than for *L. biglobosa* (3.69 mm day^{-1}, s.e. 0.05); (comparing means, $F_{(1,60)} = 273$, $P < 0.001$). Mean genetic variance was more for *L. maculans* (0.043 mm^2 day^{-2}, i.e. standard deviation of 0.21 mm day^{-1} or a coefficient of variation of 8%) than for *L. biglobosa* (0.0028 mm^2 day^{-2}, i.e. standard deviation of 0.053 mm day^{-1} or a coefficient of variation of 1.4%; F-ratio between species = $15.3_{(43,16)}$, $P < 0.001$). 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 mm^2 day^{-2} for *L. maculans* and 0.042 mm^2 day^2 for *L. biglobosa* (F-ratio = $1.71_{(16,51)}$, $P = 0.02$).

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 (Fig 2). There was a substantial difference in the size of this effect between the two species: *L. maculans,* 0.119 mm day^{-1} $°C^{-1}$, s.e. 0.002; *L. biglobosa,* 0.157 mm day^{-1} $°C^{-1}$, s.e. 0.003 °C ($F_{1,62} = 159$, $P < 0.001$). The two species differed in the genetic variance for this parameter: *L. maculans* 6.6×10^{-5} mm^2 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} mm^2 day^{-2} $°C^{-2}$ (v.r. $F_{95,33} = 2.2$, $P = 0.006$). The environmental (run plus residual) variance was not significantly different between species, at approximately 9.0×10^{-5} mm^2 day^{-2} $°C^{-2}$ ($F_{95,33} = 1.1$, $P = 0.4$).

T_{opt}. There was no significant difference in T_{opt} between the two species (F-ratio = $3.28_{(1,60)}$, P = 0.075). Mean T_{opt} for *L. maculans* was 26.25 °C (s.e.m. 0.25) and for *L. biglobosa* was 25.52 °C (s.e.m. 0.36. Genetic variance for T_{opt} was similar for both species (*L. maculans* 1.65 °C^2, i.e. genetic standard deviation 1.22 °C , *L. biglobosa* 1.72 °C^2) but the environmental variance was significantly different between species: 1.81 °C^2 for *L. maculans* and 0.36 °C^2 for *L. biglobosa* (F-ratio = $7.2_{(95,33)}$, $P < 0.001$).

Growth at high temperature. At the 12th temperature position (29.4 °C, sd 0.5 °C) genetic variance for *L. maculans* was 0.154 mm^2 day^{-2} °C^{-2} and for *L. biglobosa* was 0.366 mm^2 day^{-2} °C^{-2} (F-ratio = $2.37_{(33,95)}$, $P < 0.001$). There was slight evidence for a difference in environmental variance between species (*L. maculans:* 0.084 mm^2 day^{-2} °C^{-2}; *L. biglobosa* 0.144 mm^2 day^{-2} °C^{-2}, F-ratio = $1.71_{(33,95)}$, P = 0.02). At the 13th temperature position (31.8 °C ± 0.5), genetic variance was greater in *L. biglobosa* (0.28 mm^2 day^{-2} °C^{-2}) than in *L. maculans* (0.096 mm^2 day^{-2} °C^{-2} (F-ratio = $2.9_{(33,95)}$, $P < 0.001$). 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 mm^2 day^{-2} °C^{-2}; *L. biglobosa* 0.060 mm^2 day^{-2} °C^{-2}; v.r = $1.7_{(95,33)}$, P = 0.04). At the 14th temperature position (mean temperature: 34.3 °C ± 0.5) *L. maculans* was unable to grow. The genetic and environmental variances for *L. biglobosa* were 0.0382 mm^2 day^{-2} °C^{-2} and 0.0034 mm^2 day^{-2} °C^{-2} respectively.

Disease development *in planta*

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 days after inoculation. At 49 days after inoculation, the proportion of plants with fully developed buds (GS 3,9) and at early flowering (GS 4,1 onwards) declined with increasing temperature ($P <$ 0.001). However, among plants which had begun to flower, the proportion of plants in later flowering stages increased with increasing temperature ($P < 0.001$). At 91 days after inoculation, no seed had set at 28 °C and development into flowering was partially inhibited at 24°C (Fig. 4). However, in plants that flowered, progress in all three stages - completion of flowering, seed development and pod senescence - increased with temperature ($P \leq 0.001$ in each case: generalised linear model with binomial error).

Disease. In both replicates, inoculation of the petiole produced visible necrotic lesions which progressed in both directions along the length of the petiole (Fig. 5). Necrosis occurred on all petioles where stems were subsequently found to be infected with *Leptosphaeria* 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 symptoms following stem infection resulting from petiole inoculation were 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 which had developed cankers at final assessment and the proportion of inoculated leaves which had abscised at 35 d post-inoculation in replicate 1 ($\chi^2_1 = 22.8$, $P < 0.001$) or 28 d post-inoculation in replicate 2 ($\chi^2_1 = 23.0$, $P < 0.001$). 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 (Fig. 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; Fig 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 (Fig. 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 (Fig. 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-plant variation. Based on the observed plant to plant variation, differences in yield of *c.* 20% between infected and uninfected plants would have been detectable.

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 *Leptosphaeria maculans* and *L. biglobosa* has focussed on population averages, and much pre-dates 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.*, 2006, 2003; Naseri *et al.*, 2008). The temperature at which growth rate is maximal (T_{opt}) 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_{min}, T_{opt} and T_{max} for *L. maculans* s.l. as 0-3°C, 26-27°C, 32-35°C, respectively (Ndimande, 1976) and as <10°C, 25°C, 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 mm day^{-1} at 20°C and 2.7 mm day^{-1} at 25°C for isolates growing on V8 agar in the dark. Working with *L. maculans sensu stricto*, Huang *et al*. (2010) reported mean radial growth rates in the dark at 22 °C of 2.9 ± 0.2 mm day^{-1}. (However, Huang *et al.* (2006) quotes 1.8 ± 0.1mm day^{-1} 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 & Fitt, 1999). McGee and Petrie (1978) reported mean colony radii of 3.5 ± 0.3 mm (i.e. *c.* 0.5 mm day^{-1}) for "virulent" isolates (i.e. *L. maculans*) and 5.8 ± 0.1 mm (i.e. *c.* 0.8 mm day^{-1}) 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 asymptomatic 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^{-1} at 3 °C and 5.0 mm day^{-1} at 18/12 °C. The faster rate is roughly double the agar R_{max} measured here. A similar study by Sosnowski (2002, pp. 77–78) using isolation from 2 mm sequential slices of petiole found great variability between replicates, but a maximum growth rate around 3-3.5 mm day^{-1} including lag, at an unspecified temperature favourable for growth of oilseed rape; this is reasonably consistent with our R_{max} on agar. Taken together, it is plausible that radial growth of agar colonies, measured here, is representative of growth along a *B. napus* petiole, and therefore that variation in colony growth is informative about that variation in that life-stage in crop conditions. Incorporating infection processes, the distance

grown across the leaf lamina and through the petiole at two temperatures has been measured directly using a green florescent protein (GFP) expressing isolate of *L. maculans* (Huang *et al.*, 2009, 2014). Huang *et al.* (2009) found that the distance grown along leaf petioles towards the stems was greater in plants growing at a constant 25 °C than at a constant 15 °C for one *L. maculans* isolate growing in two winter oilseed rape cultivars. However, whether this was due to differences detected in virulence and leaf lesion development rates or to different hyphal growth rates along the leaf petioles is unclear.

The consequences that our measurements have for predicting future levels of disease in an altered climate are indeterminate, since we cannot put bounds on heritability in a realistic agricultural setting, and we have limited data that takes host growth into account. It is reasonable to argue that growth on agar is likely to be correlated to some extent with fitness in a natural setting because of the need for rapid asymptomatic growth along the petiole of the infected leaf. However, overall generational fitness in *Leptosphaeria* spp. depends on host responses, canker growth, and spore release. These are not easy to experiment on, and the problem applies to other well-studied species. *Zymoseptoria 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 sporulation in a single generation of *in planta* growth (Pijls *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 which 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 since 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 ^{o}C than at 20 ^{o}C or 24 ^{o}C, the isolates selected for testing *in planta* had equal or greater incidence and severity at 28 ^{o}C in live hosts. Normal host development was damaged at this temperature, as shown by failure to flower and reduction in yield. We hypothesise that the increased canker incidence and severity reflects the interaction of the pathogen with host physiology rather than intrinsic pathogen temperature dependence. This emphasises the point 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 counter-example, 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 *Brassica 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

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

FIGURE LEGENDS

Figure 1. Fitted temperature response curves for growth *in vitro* (as mm day^{-1}) of the isolates used for *in planta* studies. Red: *Leptosphaeria maculans* (fastest to slowest isolate codes inset); Blue *L. biglobosa* (isolate code inset).

Figure 2. Ranges of radial growth rate (as mm day^{-1}) for all a) *Leptosphaeria maculans* and b) *L. biglobosa* cultures tested on V8 agar, at each nominal temperature used.

Figure 3. Best fit individual temperature response curves of all *Leptosphaeria maculans* (red) and *L. biglobosa* (blue) cultures on V8 agar, using the equation $R = s + bT + g\left(1 - e^{rT^2}\right)$ where R is the radial growth rate (mm day^{-1}), T is the temperature throughout growth (°C) and b, s, and g are parameters fitted individually to each curve. For fitting, T was restricted to the range 10°C to 33 °C to avoid systematic bias in estimates of T_{opt} and R_{max}. Dashed line shows temperature below which growth rate responds approximately linearly to temperature

Figure 4. Cumulative proportion of oilseed rape cv. Drakkar plants which had flowered and set seed at 16h daylength, in relation to temperature and expression of symptoms of *Leptosphaeria* stem canker, after (a) 49 d and (b) 91 d for *in planta* experiment 2. Green: proportion of non-symptomatic plants at or beyond GS 5 (population16 °C; gray: proportion of cankered plants at or beyond GS 5. (Population size of non-symptomatic plants 31, 24, 35 ,33, 28 and of cankered plants 23, 29 ,19, 19, 26 at 16 °C, 20 °C, 22 °C, 24 °C, 28 °C).

Figure 5. Symptoms in oilseed rape cv. Drakkar inoculated with *Leptosphaeria maculans* on the petiole (a) 21d after inoculation, isolate FWD-11S-1U growing at 16 °C; pycnidia (arrowed) are visible on petiole and stem (b) external appearance of stem canker 98 d after inoculation; isolate FDD-11S-6U, growing at 16 °C. All scale bars: 10 mm.

Figure 6. Incidence (as the proportion of affected plants with *Leptosphaeria* stem canker symptoms at maturity) on inoculated oilseed rape cv. Drakkar plants, in relation to temperature, for *in planta* replicate 2. Blue: *Leptosphaeria biglobosa*; red: *L. maculans*. Isolate codes inset.

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

Figure 8. Yield of seed from plants of oilseed rape cv. Drakkar with (black) and without (green) symptomatic infection by *Leptosphaeria* isolates for experiment 2, in relation to plant growth

temperature. White lines represent median values, dashed lines indicate range (with circles indicating data-points more than 2 SD above the mean), boxes extend from lower to upper quartile. Red bars represent mean values from separate ANOVA analyses of healthy and diseased plants. Healthy plants (sample sizes denoted n_h), s.e.d. = 0.21 and d.f. = 112. Diseased plants (sample sizes denoted n_p), s.e.d. = 0.24 and d.f. = 95. At 16 °C $n_h = 31$, $n_p = 23$, at 20 °C $n_h = 23$, $n_p = 30$, at 24 °C $n_h = 34$, $n_p = 20$, at 28 °C $n_h = 28$, $n_p = 26$.

TABLES

Table 1. Variances in incidence of phoma stem canker following inoculation with Leptosphaeria isolates in experiment 1 and experiment 2. Analysed using generalised linear mixed model regression with a binomial error distribution and a logit link function; parameter estimates are on the logit scale.

Effect	Parameter or contrast[a]	SE of parameter estimate	SD[b]	Odds-ratio multiplier[c]	P
Experiment 2 vs. experiment 1	-1.68	0.288	-[d]	-	<0.001
L. biglobosa vs. *L. maculans*	-2.43	0.313	-	-	<0.001
Variance between isolates	0.015	0.012	0.12	×1.13	0.13
Isolate × temperature variance	0.087	0.032	0.29	×1.34	0.003

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

Canker assessment method[a]	Experiment 1				Experiment 2			
	Including *L biglobosa*		Excluding *L biglobosa*		Including *L biglobosa*		Excluding *L biglobosa*	
	F-ratio (4, 12)[b]	*P*	F-ratio (3,9)[c]	P	F-ratio (4,12)	P	F-ratio (3,9)	P
Huang (2014)	5.1	*<0.001*	5.2	*0.002*	2.2	*0.08*	0.67	*0.6*
Zhou(1999)	4.8	*0.001*	5.7	*0.001*	1.5	*0.2*	1.2	*0.3*
Aubertot (2004)	5.1	*<0.001*	5.1	*0.002*	2.2	*0.08*	0.67	*0.6*
Total length[d]	3.8	*0.006*	1.3	*0.2*	1.4	*0.2*	0.64	*0.6*
Percentage girdling[e]	5.0	*<0.001*	5.9	*<0.001*	1.7	*0.1*	0.42	*0.7*

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

[b] 5 isolates (4 *L. maculans*, 1 *L. biglobosa*), so 4 df between isolates, 12 df for variation between replicates of an isolate

[c] 4 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