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Splash dispersal of *Leptosphaeria maculans* pycnidiospores and the spread of blackleg on oilseed rape

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Abstract

The fungus *Leptosphaeria maculans* causes blackleg (phoma stem canker), one of the most serious diseases of oilseed rape. The role of pycnidiospores produced during asexual reproduction is poorly documented and limits the understanding of the pathogen's population dynamics. The objectives of this study were to assess rain-splash dispersal of pycnidiospores of *L. maculans* from phoma leaf spots, and transmission of the disease from oilseed rape stubble carrying pycnidia. The work was conducted in still air with either a drop generator or a rain simulator. The impact of simulated incident drops on phoma leaf spots resulted in the dispersal of *L. maculans* pycnidiospores within splash droplets. Ninety per cent of the spores were collected within 14 cm of the source and a few were regularly observed up to 40 cm. Pycnidiospores produced on oilseed rape stubble and dispersed by simulated rain infected oilseed rape trap plants in a spatial pattern that matched the spatial dispersal of the pycnidiospores. In the field, rain-splash dispersal of pycnidiospores could increase the pathogen population and may enhance sexual reproduction by facilitating the mating of initially spatially separated isolates of opposite mating type.

Keywords: *Brassica napus*, dispersal gradient, *Phoma lingam*, quantitative epidemiology, rain-splash

Introduction

Phoma stem canker (blackleg) caused by the fungus *Leptosphaeria maculans* (anamorph *Phoma lingam*) is a world-wide disease of oilseed rape (*Brassica napus, B. rapa* and *B. juncea*), causing serious losses in Europe, Australia and North America (Fitt *et al*., 2006). The disease is initiated by airborne ascospores, released from pseudothecia on infected oilseed rape stubble, that produce phoma leaf spots in autumn (McGee, 1977; Hall, 1992; West *et al*., 2001; Howlett, 2004). Initial leaf infections are followed by systemic growth of the fungus via the leaf petiole to the stem where stem canker develops, and in severe cases, causes lodging and death of the plant (Hammond & Lewis, 1987; Xi *et al*., 1991; West *et al*., 1999; Evans *et al*., 2004). Pycnidiospores are produced in abundance within phoma leaf spots and are involved in the localized spread of disease (Barbetti, 1976). However, the disease is generally regarded as monocyclic (West *et al*., 2001) and the importance of pycnidiospores has not been quantified.

Pycnidiospores are cylindrical and hyaline and measure $3\text{–}6 \times 1\cdot5\text{–}2\ \mu$m (Smith & Sutton, 1964). They are contained in black, globose and ostiolate pycnidia produced in necrotic lesions (phoma leaf spots) of recently killed tissue; under suitable environmental conditions, pycnidiospores exude through pycnidial ostioles in red or pinkish mucilage (Boerema, 1976; Williams, 1992). Pycnidiospores of *L. maculans* display typical characteristics of splash-borne spores (Fitt *et al*., 1989), and are believed to be exclusively dispersed by rain-splash (Salisbury *et al*., 1995; Howlett *et al*., 2001). This hypothesis is confirmed in the field by the association of peaks of pycnidiospore counts with rain events (Guo & Fernando, 2005). In places with no observed ascospore contamination, pycnidiospore dispersal was shown by the spread of blackleg up to 216 cm from plants grown from infected seeds (Hall *et al*., 1996), and up to 105 cm from inoculated rows (Barbetti, 1976). This passive dispersal during rain events is putatively the only dispersal mechanism for pycnidiospores. This mechanism and the resulting spread of disease have not yet been investigated in detail under controlled conditions. Rain simulators or simulated raindrops are commonly used to study the splashing of fungal spores and have provided techniques for understanding the mechanisms involved (Fitt *et al*., 1989; Madden, 1992; Geagea *et al*., 1999).

The role of secondary infections by pycnidiospores of *L. maculans* could be more important than currently believed. This mechanism could increase the size of the population, and may facilitate the mating of

isolates of opposite mating type which are initially spatially separated. These two processes can be important for pathogen-population dynamics (McDonald & Linde, 2002). For this reason a better understanding of the pycnidiospore splash dispersal mechanism is required. The objectives of this study were: (i) to demonstrate under controlled conditions that rain-splash is an effective dispersal mechanism of pycnidiospores of *L. maculans* from phoma leaf spots and from oilseed rape stubble; (ii) to characterize in still air the dispersal gradient of pycnidiospores from phoma leaf spots following the impact of incident water drops; and (iii) to characterize the disease gradient resulting from a simulated rain event on pycnidiospore-bearing stubble.

Materials and methods
Pycnidiospore dispersal gradients
Pycnidiospore sources
Leaves of oilseed rape (*Brassica napus*, cv. Drakkar) with phoma leaf spots, and oilseed rape stubble of unknown cultivar were sampled in the field. Phoma leaf spots were cut from the leaves and categorized according to the number of pycnidia per leaf spot (0–50, 50–100, 100–150 or 150–200). The spore content of the pycnidia was estimated by vortexing 10 phoma leaf spots from each category in $0·5$ mL water and counting the pycnidiospores with a haematocytometer (Malassez cell) under an optical microscope (Leitz® Dialux 22, magnification ×400).

Drop generator
A water-drop generator consisting of a 20 mL syringe (Terumo®) was filled with lactophenol cotton blue (LCB) solution (15%) and connected to a vertically-oriented hypodermic needle (Terumo®). The stain was required to visualize both the splash droplets collected and the pycnidiospores within these droplets. It was assumed that the addition of LCB did not change the physical properties of the liquid from that of water. The volumes of the drops generated by two needles (Terumo®, NN-1938R and NN-2516R) were measured by collecting and weighing six series of 100 drops. Spherical drops were assumed and the diameters estimated as $2·1$ mm for NN-1938R (small) and $2·8$ mm for NN-2516R (large), which simulated drop sizes of natural rainfall (Ulbrich, 1983). Since kinetic energy depends mainly on drop diameter, variation in the physical properties of the two drop sizes could be tested. In all experiments, drops were released from a vertical height of 1 m to demonstrate the mechanism of splashing, even if drops released from this height do not reach the terminal velocity of natural rain drops (Geagea *et al*., 1999). Liquid flow through the syringe and the number of drops released was controlled manually to produce drops of consistent diameter.

Pycnidiospore removal threshold from phoma leaf spots and infected stubble
A phoma leaf spot measuring between $1·5$ and 2 cm in diameter surrounded with 2–3 mm uninfected leaf tissue and bearing 150–200 pycnidia was placed horizontally under the drop generator and submitted to incident drops ($2·8$ mm diameter). The resulting splash droplets were collected on two glass slides ($76 × 26$ mm) laid horizontally $2·5$ cm from the spore source in opposite directions. The slides were changed after each incident drop and checked for splash droplets under an optical microscope (Leitz® Dialux 22, magnification ×400). The pycnidiospore removal threshold, i.e the minimal number of incident drops required to remove and transport pycnidiospores, was achieved as soon as one spore-bearing splash droplet was observed. The experiment was repeated with ten phoma leaf spots and the same protocol was applied to ten infected stubbles measuring about 25 cm in length and 2 cm in diameter.

Pycnidiospore depletion from phoma leaf spots
Phoma leaf spots bearing either 0–50 or 150–200 pycnidia were exposed to large drops as described above. The dispersed spores were collected on two glass slides laid horizontally $2·5$ cm from the spore source in opposite directions. Groups of three drops were collected, released at regular intervals (drops 1–3, 11–13, 21–23, 61–63, 101–103, 141–143). Splash droplets that occurred between these numbered drops were not considered. The numbers of pycnidiospores were counted for the splash droplets. The experiment was repeated with five phoma leaf spots for each of the two leaf spot categories.

Pycnidiospore dispersal gradients

Phoma leaf spots bearing 150–200 pycnidia were exposed to both small and large drops released in sequences of 15 drops. Splash droplets were collected on glass slides laid horizontally in two opposite directions from the spore source, so that the nearest slide was 2·5 cm from the source and the most distant slide 32·8 cm from the source. A regular interval of 2·5 cm was maintained between slides. The extent of the droplet collection area (from 2·5 to 40·6 cm from the source) was determined by preliminary experiments which showed that no splash droplet was collected beyond 40 cm from the source. Glass slides were not placed within 2·5 cm of the source in order to not alter the counting by possible leakage of liquid accumulated on the source near to the glass slide. Because every splash droplet contained pycnidiospores only a subsample of droplets were counted. Each slide was divided longitudinally into three 2·5 cm widths, and a minimum of three droplets were counted per zone, resulting in enumeration of spores in a minimum of nine splash droplets per slide. Splash droplets collected on a given section of the slide were assumed to have travelled the distance from the source to the centre of the section. The number of pycnidiospores in un-enumerated droplets was estimated by multiplying the droplet area by the mean pycnidiospore concentration of the enumerated droplets in the same section of the slide. Cartesian coordinates and the area of every LCB coloured droplet present were determined by analysis of photographs of each slide using image analysis software Assess® (Image Analysis Software for Plant Disease Quantification, APS Press, 2002). The experiment was repeated with five phoma leaf spots for each drop size.

Disease dispersal gradients

Pycnidiospore sources

Eight pieces of mature healthy oilseed rape stem (collected in the field from an unknown cultivar, were autoclaved twice, 24 h apart, for 20 min at 120°C in Roux bottles with 100 mL of distilled water. The bottles were autoclaved a third time at 115°C for 20 min, 24 h after the addition of 50 mL V8-juice medium. A single ascospore isolate of *L. maculans* (FCr3), originally obtained from one oilseed rape stubble collected in 1990 in Saint-Pathus, France was used as the inoculum source. Spherical fungal explants of 5 mm diameter on malt medium were cut from the margin of actively growing colonies, and placed on the oilseed rape stems in the Roux bottles, two explants per stem separated by 10 cm. Optimum pycnidiospore production was obtained by incubating the bottles horizontally for 12 days at 18°C under a 12 h photoperiod 300–400 nm (OSRAM L40 W/75 lamps 40 cm above the culture bottles).

Rain simulator

The Deltalab Microprocessor Controlled Spray System, EID 330, manufactured by Orstom (Asseline & Valentin, 1978), was used to simulate rain events in still air. Rainfall was simulated by a constant speed oscillating nozzle (Deltalab, Tec Jet SS 6560) with a sweep angle of 180° positioned at a height of 3·8 m. Water pressure at the nozzle orifice was 62 kPa. All subsequent measurements were made within the square metre in the centre of the area sprayed, where intensity and drop diameter distribution of the simulated rain were the most homogenous. The intensity of the simulated rain was assessed by measuring the volume of water collected in 26 vials placed at random under the rain simulator for 2 min. With a constant intensity of 40 mm h^{-1}, operation of the rain simulator for 3 min equalled a 2 mm rainfall event. The probability density function (Fig. 1) of drop diameters produced by the nozzle at 62 kPa water pressure was obtained from an optical single-beam disdrometer (CETP). This probability density function was compared to that of natural rainfall by using Ulbrich's characterization of rain:

$$N(D) = N_0 D^\mu \exp(-\Lambda D),$$

((Eqn. 1))

$$\Lambda = \frac{(3\cdot67 + \mu)R^{-\frac{1}{4\cdot67+\mu}}}{10\varepsilon}$$

where , *D* (mm) = drop diameter, *R* (mm h^{-1}) = rain intensity, and N_0, μ and ε are rain characteristics depending on the type of rain (thunderstorm, showers, etc.).

By using a nonlinear equation fitting (Eqn. 1) on the rain simulator drop probability density function, it was found that $\mu = 6\cdot291 \pm 0\cdot655$ and $\varepsilon = 0\cdot119 \pm 0\cdot010$ with $P < 0\cdot001$ (*t*-test). According to Ulbrich (1983),

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such parameters correspond to natural rain showers. Mean drop velocity at ground level was also measured by the disdrometer and was found to be $5 \cdot 02$ m s^{-1} which is slightly higher than the corresponding natural rain ($4 \cdot 38$ m s^{-1} for the same μ and ε).

Figure 1 *Drop probability density function of the rain simulator (bars), measured by an optical disdrometer. The solid line corresponds to the fit of the experimental data upon Ulbrich's (1983) diameter distribution of natural rain.*
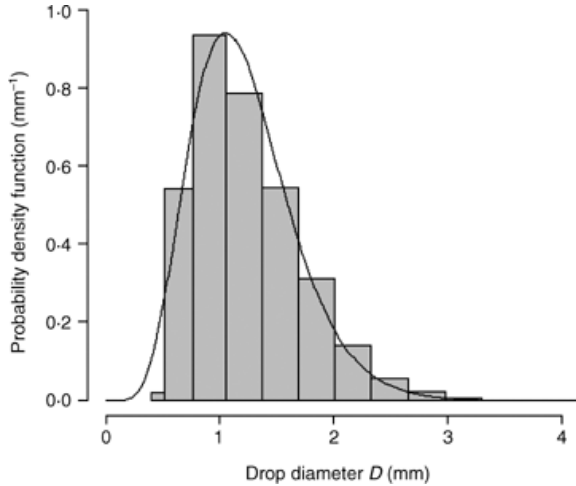
Disease dispersal gradients
Horizontal disease dispersal from infected stubble was assessed by collecting the spore-bearing splash droplets on trap plants placed at various distances from the source. The trap plants were susceptible oilseed rape plants (cv. Westar) at the two-leaf stage, grown in a batch ($30 \times 44 \times 10$ cm) filled with a mix of 40% blond peat, 20% black peat, 20% sand and 20% topsoil (silt and clay) (Terreaux Armoricains). Plants were grown in a glasshouse at 20°C under a 16 h photoperiod and planted into 2×3 parallel rows (six plants per row, 4 cm apart) at 5, 10 and 15 cm from the position of the infected stubble. Two pieces of infected stubble were placed at the height of the leaves in the centre of the batch (Fig. 2) and exposed to a simulated 2 mm rain event. After each simulated rain event, the trap plants were allowed to dry for 4 h at ambient temperature (18°C). Trap plants were then incubated in a growth chamber at 20°C, with a 16 h photoperiod and saturated humidity. The number of phoma leaf spots was counted on all trap plants for each batch, 21 days after the simulated rain event.

Figure 2 *Schematic layout of infection source (shaded rolls: diseased oilseed rape stems) and trap plants (boxes) in the experiment with the rain simulator in still air.*

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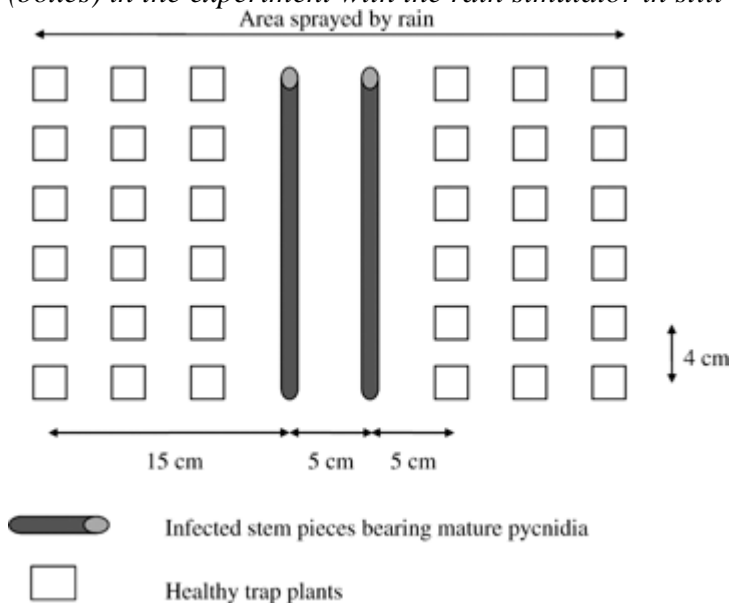
Statistical analysis of the experimental spore and disease gradients

An exponential equation was fitted for spatial experimental gradients (Kiyosawa, 1972; Fitt *et al.*, 1987):

$$y = a \cdot \exp(-b \cdot d) \tag{Eqn. 2}$$

where y is either the proportion of spores (spore dispersal gradient) or the number of phoma leaf spots counted on trap plants (disease dispersal gradient) recorded at distance d (cm) from the source.

For spore dispersal gradients, parameters a (intercept) and b (slope) were estimated by regression after linearization of equation (Eqn. 2):

$$\ln(y) = \ln(a) - b \cdot d \tag{Eqn. 3}$$

The distance from the source at which the proportion of counted pycnidiospores decreased by 50% and 90% was calculated as $\alpha = -\ln(0{\cdot}5) / b$ and $\alpha' = -\ln(0{\cdot}1) / b$, respectively.

For disease dispersal gradients, the parameters a and b were estimated by nonlinear regression between the number of phoma leaf spots and the distance from the source (SAS Institute Inc.), assuming that the source of pycnidiospores was at the centre of the two stubble pieces. This nonlinear regression method was used because of the great number of trap plants without symptoms (i.e. a null y).

The confidence interval (IC) of the slope parameter (b) was calculated as:

$$IC = b \pm se \; t \; (P/2; \, n - 2) \tag{Eqn. 4}$$

In order to test equalities for slope values between experimental gradients, confidence intervals for slope differences between gradients were calculated as:

$$IC = (b1 - b2) \pm se \; t \; (P/2; \, n1 + n2 - 4) \tag{Eqn. 5}$$

Results

Numbers of pycnidiospores borne by phoma leaf spots

The mean number of pycnidiospores for each class of pycnidia-bearing leaf spots (0–50; 50–100; 100–150; 150–200 pycnidia) increased with the number of pycnidia per leaf spot (Fig. 3). The number of spores per pycnidium was relatively constant and was not related to the number of pycnidia per leaf spot. From an analysis of variance (ANOVA), the spore content was not significantly different (F-test, $P < 0{\cdot}05$) between the two lowest classes of leaf spots. Significant differences were found between the 100–150 and the 150–200 pycnidia classes, as well as between the 50–100 and the 100–150 pycnidia classes. Leaf spots bearing 100–150 pycnidia showed a good compromise between the quantity and the variability of their spore load, and were therefore used as the spore source to characterize pycnidiospore-horizontal dispersal gradients.

Figure 3 *Number of pycnidiospores of Leptosphaeria maculans borne by phoma leaf spots according to the number of pycnidia per symptom. Means and standard errors of ten repetitions are presented. Means followed by the same letter are not significantly different according to the F-test (P < 0·05).*
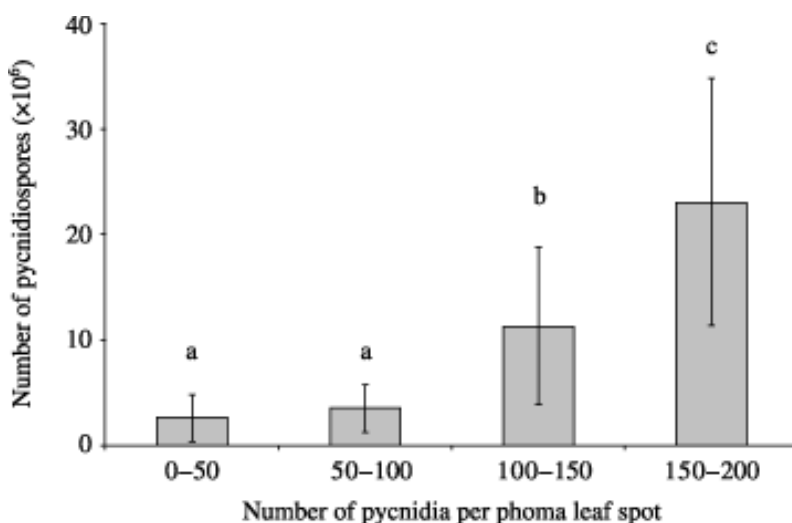
Pycnidiospore removal threshold
Pycnidiospores were removed and transported in splash droplets with the first drop from pycnidia borne either by phoma leaf spots or by oilseed rape stubble. Ten repeated experiments were conducted using large drops for both spore sources namely phoma leaf spots and stubble.

Pycnidiospore depletion from phoma leaf spots
Splash droplets containing removed pycnidiospores were collected after the impact of 143 large drops onto phoma leaf spots bearing either 0–50 or 150–200 pycnidia (Fig. 4). Most pycnidiospores were removed after the first 63 drops for both categories of leaf spots. Splash droplets resulting from the impact of the 1st–3rd, 11th–13th, or 21st–23rd drops contained pycnidiospores, whereas the first splash droplets collected that did not contain pycnidiospores were observed after the impact of the 61st–63rd drop for both categories of leaf spots. Therefore, a series of 15 drops were released to characterize pycnidiospore horizontal dispersal gradients.

Figure 4 *Effect of increasing number of large (2·8 mm) incident drops released from a height of 1 m on* Leptosphaeria maculans *pycnidiospore depletion from two groups of phoma leaf spots (0–50 pycnidia and 150–200 pycnidia.). Collecting slides were in place during each sequence of three drops. Means and standard errors of five repetitions are presented. Bars represent the mean total number of pycnidiospores counted in all the splash-droplets deposited on the two glass slides after each sequence of three incident drops.*
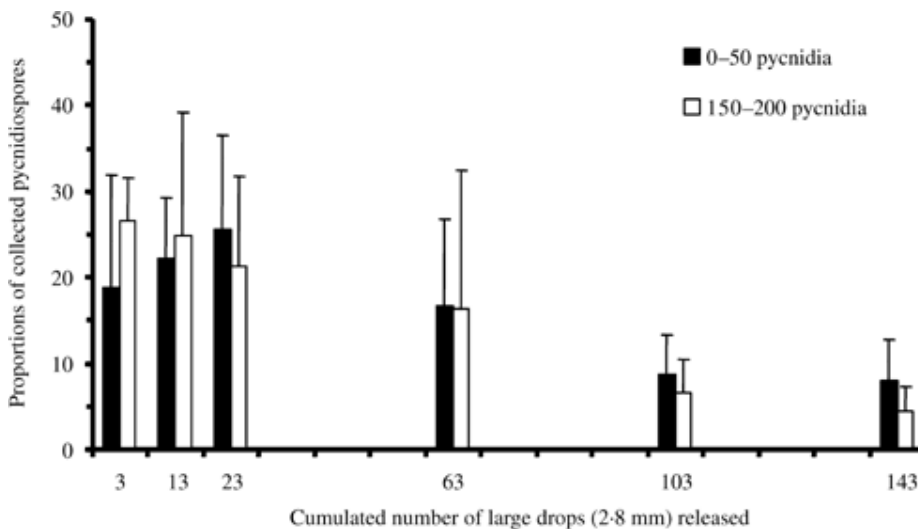
*Pycnidiospore horizontal dispersal gradients*
The number of pycnidiospores in the rain-splash droplets was estimated after the impact of the first 15 drops for both drop sizes on five phoma leaf spots (called *Pls1* to *Pls5*). Surprisingly the total number of pycnidiospores collected was greater after the impact of the small (23 928) rather than the large (19 020) drops (Table 1). This result can be explained by the great number of pycnidiospores removed from a single leaf spot (*Pls4*) tested with the small drops.

Table 1. *Numbers (n) and proportions (%) of pycnidiospores from* Leptosphaeria maculans *removed and collected from each phoma leaf spot (Pls1 to 5) on oilseed rape, after the release of 15 incident water drops of diameter D from a height of 1 m*

D (mm)		*Pls1*	*Pls2*	*Pls3*	*Pls4*	*Pls5*	Total
2·8	*n*	866	908	2119	8047	7080	19 020
	%	4·6	4·8	11·1	42·3	37·2	100
2·1	*n*	570	172	2225	17 095	3234	23 298
	%	2·4	0·7	9·6	73·4	13·9	100

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For each drop size, the proportion of pycnidiospores collected after the impact of 15 drops decreased with increasing distance from the source (Fig. 5). The proportion of pycnidiospores collected peaked between 2·5 and 5 cm from the source both for the large and small drops. Less than 250 pycnidiospores were recorded beyond 10 cm of the source for either drop size, although they were still observed at 30–40 cm, the greatest distance interval measured. Beyond 10 cm from the source, pycnidiospores were not found at all distance intervals. In order to fit the linearized exponential model to the observed spore dispersal gradient, it is assumed that these intervals had received 0·001% of the total number of pycnidiospores collected (Campbell & Madden, 1990). The number of pycnidiospores collected at the 5–10 cm distance interval was much higher for the small drops than the large. However, phoma leaf spots bearing unexpectedly high numbers of pycnidiospores (*Pls4*) strongly biased the average after the impact of the small drops. Conversely, certain distance classes exhibited small peaks in spore counts, which corresponded to splash droplets of large diameter (approximately 1 mm). Moreover, the impact of the small drops on *Pls4* leaf spot resulted in large splash droplets of up to 21 mm^2 in area at 7·5–10 cm. The small peaks (low spore counts) observed 7·5–10 cm can be explained by the integration of the area of the drops and the method of enumeration. When spore counts of *Pls4* were excluded from the analysis, the proportion of pycnidiospores recorded decreased sharply for distances greater than 10 cm from the source and the proportion of pycnidiospores recorded within 15 cm of the source was lower for the small drops than the large (Fig. 5).

Figure 5 *Proportion of splash droplets and pycnidiospores of Leptosphaeria maculans recorded and distance travelled from the source. Both were collected under the action of 15 drops of both diameters (2·8 mm and 2·1 mm) from a height of 1 m.*
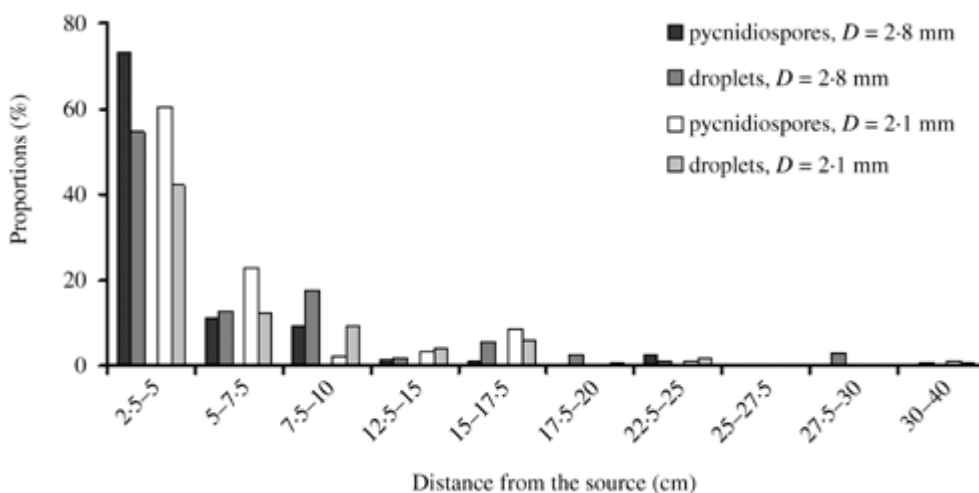
Most of the pycnidiospores that were impacted by the simulated rain drops were transported only at short distances; i.e. 94% of the pycnidiospores were recorded within 10 cm of the source for the large drops and 86% for the small (Fig. 5). At the 2·5–5 cm distance interval, 73% of the pycnidiospores were collected for the large drops and 60% for the small. The proportion of splash droplets collected at each distance interval followed a similar distribution to that of the proportion of removed pycnidiospores (Fig. 5). Differences observed between the distributions reflect variation in the area of the splash droplets.

The exponential model explains 63% ($R^2 = 0·63$, $P < 0·05$) of the variation observed for the spore dispersal gradient of the large drops and 57% ($R^2 = 0·57$, $P < 0·05$) for the small, excluding the *Pls4* leaf spots (Table 2). The high proportion of pycnidiospore-carrying droplets collected between 2·5 and 5 cm from the source (Fig. 5) revealed a very rapid reduction in the number of pycnidiospores recorded within 5 cm of the source and explained the major portion of the variance unaccounted for by the model. Model parameters and the derived 50% (half) and 90%-distances were estimated (Table 2). The slope of the gradient (*b*) did not significantly differ between the drop sizes (confidence interval for the difference between slopes: IC = [−14·66; 13·63], $t = 2·120$, $P = 0·05$, 16 df). Estimation of the travel distances confirmed that most of

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the pycnidiospores were collected very close to the source: the 50% (half-distance) and 90%-dispersal distances did not exceed 4 cm and 14 cm, respectively.

Table 2. *Parameters obtained by fitting Eqn. (2) to the relationships between proportions of pycnidiospores of* Leptosphaeria maculans *or numbers of phoma leaf spots (y) collected or trapped under the action of incident drops of diameter D or simulated rain of 2 mm, and distance (x) (x in first column, two intervals used for comparison).* α *is the 'half-distance' where y decreases by half,* α' *is the distance where y decreases by 90%, and the standard error (se) followed by confidence intervals for values of slopes b.* R^2 *is the coefficient of determination of the exponential model*

x (cm)	D (mm)	b (se) +/− (m-1)	α (cm)	α' (cm)	R^2
0 to 40	2·8	−16·65 (4·5) + /− 8·37	4	14	0·63
	2·1	−16·14 (4·9) + /− 9·17	4	14	0·57
0 to 17·5	2·8	−32·69 (6·8) + /− 10·6	2	7	0·94
	2·1	−16·46 (14·52) + /− 27·5	4	13	0·4
	Rain simulator	−21·89 (5·5) + /− 10·8	3	11	0·96

Disease dispersal gradients
A simulated 2 mm rain shower was able to remove pycnidiospores from infected oilseed rape stubble and transport them to trap plants up to 17·5 cm from the source. Twenty-one days after infection, the trap plants exhibited a total of 45 phoma leaf spots. An exponential function was fitted to the experimental disease dispersal gradients (Table 2). For comparison of spore and disease dispersal on the same scale, the pycnidiospore dispersal gradients were re-estimated (Table 2) using only the spore counts up to 17·5 cm from the source (maximum distance between stubble and trap plants for disease dispersal gradients). The slopes estimated for the two drop sizes did not differ significantly (confidence interval for the difference between slopes: IC = [−38·35; 8·28], $t = 2 \cdot 776$, $P = 0 \cdot 05$, 4 df). The slopes of the disease and spore dispersal gradients evaluated up to 17·5 cm from the source did not differ significantly for either drop size (confidence intervals for the difference between slopes: ($t = 3 \cdot 182$; $P = 0 \cdot 05$; 3 df), both for the large drops: IC = [−42·12; 13·25] or for the small: IC = [−57·64; 41·06]) Fig. 6).

Figure 6 *Exponential dispersal models applied to observed proportions of Leptosphaeria maculans pycnidiospores (y) collected at distances (x) up to 17·5 cm from the source under the action of 15 large (2·8 mm) incident drops (▲ for proportions and — for the exponential model) or small (2·1 mm) incident drops (□ for proportions and - - - for the exponential model). These models were compared with the exponential dispersal model applied on observed mean numbers of symptoms exhibited by trap plants (y) developed at distances (x) up to 17·5 cm from the source of spores (◊ for mean numbers of symptoms and - — - — for the exponential model).*
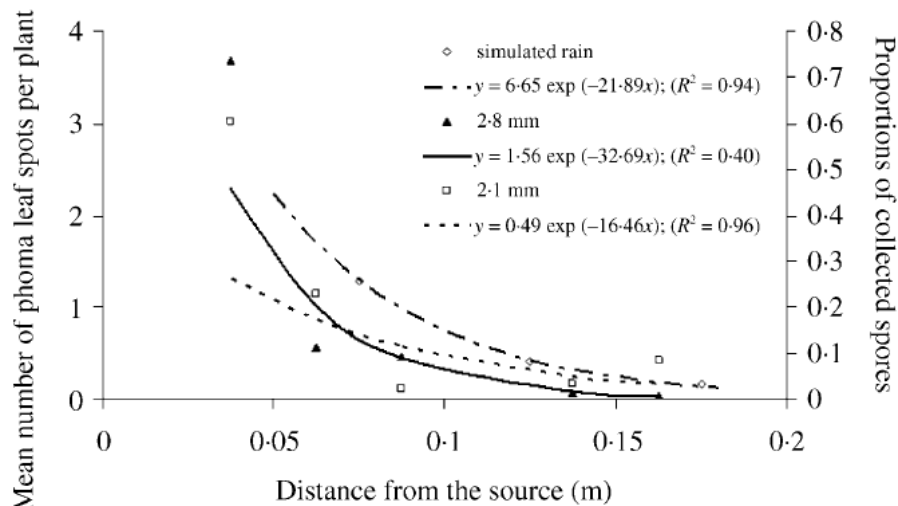
Discussion

This study clearly describes the rain-splash mechanism for *L. maculans* pycnidiospore dispersal but further work is needed to assess this dispersal mechanism and its role in the spread of blackleg disease in field conditions. Ascospore infection is considered to be of major importance in initiating disease epidemics, because ascospores and pycnidiospores have different infection requirements. Ascospore germination, penetration and development of symptoms on cotyledons can occur up to two days earlier than for pycnidiospores (Li *et al*., 2004). Furthermore, fewer ascospores than pycnidiospores are required to produce infection (Wood & Barbetti, 1977). Yet it has been shown that under controlled conditions, co-inoculation with ascospores increased the ability of pycnidiospores to cause disease compared with pycnidiospores alone (Li *et al*., 2006). Pycnidiospore infections could also have a major role during the growing season when there are only few ascospores available.

During the process of splash-dispersal of pycnidiospores, the immediate release of pycnidiospores from pycnidia is partly explained by the morphology of the fructifications of *L. maculans*. Pycnidiospores are released through an ostiole when relative humidity is close to saturation. The closer to the surface of the host tissue the pycnidia is situated, the less energy is required to extract the spores (Rapilly, 1991). Pycnidiospores are exuded from pycnidia in a mucilage (Williams, 1992). Retention of pycnidiospores in the mucilage may explain the incomplete spore depletion from pycnidia as was shown for *Phaeosphaeria* (anamorph *Stagonospora*) *nodorum,* which possesses similar fructifications (Rapilly, 1991). Under field conditions during rain events, pycnidiospore dispersal is expected to occur rapidly. However, during epidemics, exhaustion of pycnidiospores from pycnidia within phoma leaf spots should not be a limiting factor of secondary disease cycles.

The number of pycnidiospores per leaf spot varied between $2·6 \times 10^6$ and $23·0 \times 10^6$ among the four categories of leaf spots assessed (Fig. 3). In this study, the strength of the spore source was standardized by using phoma leaf spots bearing 100–150 pycnidia. Within this category, differences observed in the spore loads of the leaf spots may indicate differences in pycnidia maturation since the source of spores used to characterize pycnidiospore dispersal gradients were heterogeneous. Although the strength of a source affects the amount of disease and therefore alters the constant *a* in the regression equation (Gregory, 1968), it was decided to exclude *Pls4* tested with the small drop diameter from the analysis because the number of pycnidiospores recorded from this spot was on average ten times greater than those observed from the four other spots tested with the same drop diameter.

The slopes of the experimental dispersal gradients obtained from the two drop sizes did not differ significantly. These results are in agreement with those of other studies which have reported that the slope of the spore splash dispersal gradient did not significantly differ with drop characteristics such as diameter and velocity (Geagea *et al*., 1999; S. Saint-Jean, personal communication).

Pycnidiospores were observed to be dispersed by rain-splash over short distances from the source (e.g. a half-distance of 4 cm) and these distances were similar to the travel distances reported for other splashborne fungi. For other conidial spores, half-distance of dispersal obtained in still air is 5–9·8 cm for *Pseudocercosporella capsellae* (Fitt *et al*., 1992); 7 cm for *Oculimacula* (anamorph *Pseudocercosporella*) *herpotrichoides*, 10 cm for *Pyrenopeziza brassicae* and 11 cm for *L. nodorum* (Fitt *et al*., 1987); 15 cm for *Asochyta fabae* f. sp. *lentis* (Pedersen *et al*., 1994); 10·5–13·6 cm for *Fusarium culmorum* and 9·2–16·5 cm for *F. avenaceum* (Jenkinson & Parry, 1994); and 7·7–14·7 cm for *F. poae* (Horberg, 2002). As demonstrated in previous studies (Fatemi & Fitt, 1983; Horberg, 2002), it is assumed that the distance does not depend on spore morphology. Comparing this half-distance with the similar half-distance of *P. capsellae* spores splash-dispersed from white leaf spots on oilseed rape leaves (5–9·8 cm) (Fitt *et al*., 1992), it is concluded that the splash-dispersal process is mainly influenced by the host support characteristics, e.g. leaf surface roughness, age and waxiness. However, the distances reported in this study under controlled conditions for *L. maculans* were much shorter than those reported in field studies. Under field conditions distances of 105 cm (Barbetti, 1976) and 216 cm (Hall *et al*., 1996) have been reported, underlining the importance of splash-dispersal of pycnidiospore from plants grown from infected seeds in the latter case. These greater distances are possibly due to wind which enhances dispersal distances of spore-carrying droplets (Fitt *et al*., 1989) to the succession of generations and to differences in raindrop sizes which may range from 0·5 to 5 mm (Ulbrich, 1983).

Pycnidiospores dispersed by large raindrops travel greater distances than those dispersed by the small raindrops (Huber *et al*., 1996; Geagea *et al*., 2000). Therefore, a proportion of splash droplets produced by the impact of large natural raindrops may travel further than the distances observed here.

This study showed that oilseed rape stubble bearing pycnidia constitutes a potential source of inoculum. Since isolates of *L. maculans* are able to survive asexually in the field (Alabouvette & Brunin, 1970; Petrie, 1995; Baird *et al*., 1999), pycnidia-bearing stubble is likely to contribute to the transmission of asexual inoculum (pycnidiospores) between growing seasons. This could happen when oilseed rape is sown in a field where infected stubble from the previous season has remained at the soil surface, and where recommended crop rotations are not respected. The infected stubble could also be a source of inoculum for oilseed rape sown in an adjacent field. Nevertheless, field experimentation is required to quantify its role in pluriannual epidemics; for example, exposing an oilseed rape crop to pycnidia-bearing stubble in a site with no ascospore infection.

The slopes of the disease dispersal and pycnidiospore dispersal gradients under experimental conditions did not significantly differ. Therefore, spatial disease dispersal matches spatial pycnidiospore dispersal. A splash droplet could be regarded as the smallest infectious element required for the reproduction of the disease, that is the infectious unit of dissemination (UD) (Rapilly, 1977, 1979). Hammond & Lewis (1987) showed that the infection efficiency of an 8 μL drop containing four pycnidiospores varied between 5 and 10% according to the pathogen isolates and the cultivars used, but varied between 10 and 60% when the drop contained 40 pycnidiospores. These concentrations are similar or even lower than those observed in the splash droplets, whose estimated volume was mainly lower than 8 μL and which most of the time contained more than four pycnidiospores. Blackleg spread in the field is expected to be very effective since spore carrying droplets result in dissemination of inoculum. This dissemination probably permits the appearance of clusters of secondary phoma leaf spots around an old lesion on the same leaf, or direct stem infection by pycnidiospores. In addition to splash-dispersal, pycnidiospores embedded in water films might be spread by leaf to leaf or leaf to stem contact.

The results reported in this paper support the hypothesis that pycnidiospores may be more significant than usually believed for the biology of *L. maculans* and the epidemiology of the blackleg disease in small populations. Pycnidiospores can potentially increase the size of the fungal population through secondary spread; this may increase the probability of mating between opposite mating types on the same plant, which would result in sexual reproduction and production of pseudothecia containing ascospores that are wind-dispersed over long distances. Alternatively, in the absence of sexual reproduction, pycnidiospore dispersal from stubble provides an alternative source of inoculum. Thus pycnidiospores could have a major role where population densities are low, but field studies are required to quantify the relevance of pycnidiospores in different cropping systems.

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References

Alabouvette C, Brunin B, 1970. Recherches sur la maladie du colza due à*Leptosphaeria maculans* (Desm.) Ces. et de Not. *Annales de Phytopathologie* 2, 463–75.
     Google ScholarServices SFX pour l'INRA
Asseline J, Valentin C, 1978. Construction et mise au point d'un infiltromètre à aspersion. *Cahiers ORSTOM* XV, 321–49.
Baird RE, Phillips DV, Mullinix BG, Alt PJ, 1999. Relative longevity of *Leptosphaeria maculans* and associated mycobiota on canola debris. *Phytoprotection* 80, 1–11.

Barbetti MJ, 1976. The role of pycniospores of *Leptosphaeria maculans* in the spread of blackleg disease in rape. *Australian Journal of Experimental Agriculture and Animal Husbandry* 16, 911–4.

Boerema GH, 1976. The *Phoma* species studied in culture by Dr R. W. Dennis. *Transactions of the British Mycological Society* 67, 289–319.

Campbell CL, Madden LV, 1990. Introduction to Plant Disease Epidemiology. New York, USA: John Wiley & Sons.

Evans N, Baierl A, Steed JS, Huang YJ, Fitt BDL, 2004. Spatial aspects of *Leptosphaeria maculans* (phoma stem canker) epidemiology. *IOBC Bulletin* 27, 139–44.

Fatemi F, Fitt BDL, 1983. Dispersal of *Pseudocercosporella herpotrichoides* and *Pyrenopeziza brassicae* spores in splash droplets. *Plant Pathology* 32, 401–4.

Fitt BDL, Gregory PH, Todd AD, McCartney HA, MacDonald OC, 1987. Spore dispersal and plant disease gradients; a comparison between two empirical models. *Journal of Phytopathology* 118, 227–42.

Fitt BDL, McCartney HA, Walklate PJ, 1989. The role of rain in dispersal of pathogen inoculum. *Annual Review of Phytopathology* 27, 241–70.

Fitt BDL, Inman AJ, Lacey ME, McCartney HA, 1992. Splash dispersal of spores of *Pseudocercosporella capsellae* (white leaf spot) from oilseed rape leaves of different inclination, flexibility and age. *Journal of Plant Diseases and Protection* 99, 234–44.

Fitt BDL, Brun H, Barbetti MJ, Rimmer SR, 2006. World-wide importance of phoma stem canker (*Leptosphaeria maculans* and *L. biglobosa*) on oilseed rape (*Brassica napus*). *European Journal of Plant Pathology* 114, 3–15.

Geagea L, Huber L, Sache I, 1999. Dry-dispersal and rain-splash of brown (*Puccinia recondita* f. sp. *tritici*) and yellow (*P. striiformis*) rust spores from infected wheat leaves exposed to simulated raindrops. *Plant Pathology* 48, 472–82.

Geagea L, Huber L, Sache I, Flura D, McCartney HA, Fitt BDL, 2000. Influence of simulated rain on dispersal of rust spores from infected wheat seedlings. *Agricultural and Forest Meteorology* 101, 53–66.

Gregory PH, 1968. Interpreting plant disease dispersal gradients. *Annual Review of Phytopathology* 6, 189–212.

Guo XW, Fernando WGD, 2005. Seasonal and diurnal patterns of spore dispersal by *Leptosphaeria maculans* from canola stubble in relation to environmental conditions. *Plant Disease* 89, 97–104.

Hall R, 1992. Epidemiology of blackleg of oilseed rape. *Canadian Journal of Plant Pathology* 14, 46–55.

Hall R, Chigogora JL, Phillips LG, 1996. Role of seedborne inoculum of *Leptosphaeria maculans* in development of blackleg on oilseed rape. *Canadian Journal of Plant Pathology* 18, 35–42.

Hammond KE, Lewis BG, 1987. The establishment of systemic infection in leaves of oilseed rape by *Leptosphaeria maculans*. *Plant Pathology* 36, 135–47.

Horberg HM, 2002. Patterns of splash dispersed conidia of *Fusarium poae* and *Fusarium culmorum*. *European Journal of Plant Pathology* 108, 73–80.

Howlett BJ, 2004. Current knowledge of the interaction between *Brassica napus and Leptosphaeria maculans*. *Canadian Journal of Plant Pathology* 26, 245–52.

Howlett BJ, Idnurum A, Pedras MSC, 2001. *Leptosphaeria maculans*, the causal agent of blackleg disease of Brassicas. *Fungal Genetics and Biology* 33, 1–14.

Huber L, Fitt BDL, McCartney HA, 1996. The incorporation of pathogen spores into rain-splash droplets: a modelling approach. *Plant Pathology* 45, 506–17.

Jenkinson P, Parry DW, 1994. Splash dispersal of conidia of *Fusarium culmorum* and *Fusarium avenaceum*. *Mycological Research* 98, 506–10.

Kiyosawa S, 1972. Mathematical studies on the curve of disease increase – a technique for forecasting epidemic development. *Annals of the Phytopathological Society of Japan* 38, 30–40.

Li H, Tapper N, Dean N, Barbetti MJ, Sivasithamparam K, 2006. Enhanced pathogenicity of *Leptosphaeria maculans* pycnidiospores from paired co-inoculation of *Brassica napus* cotyledons with ascospores. *Annals of Botany* 97, 1151–6.

Li H, Sivasithamparam K, Barbetti MJ, Kuo J, 2004. Germination and invasion by ascospores and pycnidiospores of *Leptosphaeria maculans* on spring-type *Brassica napus* canola varieties with varying susceptibility to blackleg. *Journal of General Plant Pathology* 70, 261–9.

Madden LV, 1992. Rainfall and the dispersal of fungal spores. *Advances in Plant Pathology* 8, 39–79.

McDonald BA, Linde C, 2002. Pathogen population genetics, evolutionary potential, and durable resistance. *Annual Review of Phytopathology* 40, 349–79.

McGee DC, 1977. Black leg (*Leptosphaeria maculans* (Desm.) Ces. et de Not.) of rapeseed in Victoria: sources of infection and relationships between inoculum, environmental factors and disease severity. *Australian Journal of Agricultural Research* 28, 53–62.

Pedersen EA, Morrall RAA, McCartney HA, Fitt BDL, 1994. Dispersal of conidia of *Ascochyta fabae* f. sp. *lentis* from infected lentil plants by simulated wind and rain. *Plant Pathology* 43, 50–5.

Petrie GA, 1995. Long-term survival and sporulation of *Leptosphaeria maculans* (blackleg) on naturally-infected rapeseed/canola stubble in Saskatchewan. *Canadian Plant Disease Survey* 75, 23–34.

Rapilly F, 1977. Réflexions sur les notions de propagule et d'unité de dissémination en épidémiologie végétale: cas des champignons parasites des organes aériens des plantes. *Annales de Phytopathologie* 9, 161–76.

Rapilly F, 1979. Yellow rust epidemiology. *Annual Review of Phytopathology* 17, 59–73.

Rapilly F, 1991. L'Épidémiologie en Pathologie Végétale, Mycoses Aériennes. Paris, France: INRA.

Salisbury PA, Ballinger DJ, Wratten N, Plummer KM, Howlett BJ, 1995. Blackleg disease on oilseed Brassica in Australia: a review. *Australian Journal of Experimental Agriculture* 35, 665–72.

Smith HC, Sutton BC, 1964. *Leptosphaeria maculans* the ascogenous state of *Phoma lingam. Transactions of the British Mycological Society* 47, 159–65.

Ulbrich CW, 1983. Natural variations in the analytical form of the raindrop size distribution. *Journal of Climate Applied Meteorology* 22, 1764–75.

West JS, Biddulph JE, Fitt BDL, Gladders P, 1999. Epidemiology of *Leptosphaeria maculans* in relation to forecasting stem canker severity on winter oilseed rape in the UK. *Annals of Applied Biology* 135, 535–46.

West JS, Kharbanda PD, Barbetti MJ, Fitt BDL, 2001. Epidemiology and management of *Leptosphaeria maculans* (phoma stem canker) on oilseed rape in Australia, Canada and Europe. *Plant Pathology* 50, 10–27.

Williams PH, 1992. Biology of *Leptosphaeria maculans. Canadian Journal of Plant Pathology* 14, 30–5.

Wood PMR, Barbetti MJ, 1977. A study on the inoculation of rape seedlings with ascospores and pycnidiopsores of the blackleg disease causal agent *Leptosphaeria maculans. Journal of the Australian Institute of Agricultural Science* 4, 79–80.

Xi K, Morrall RAA, Gugel RK, Verma PR, 1991. Latent infection in relation to the epidemiology of blackleg of spring rapeseed. *Canadian Journal of Plant Pathology* 13, 321–31.