Rain-splash is an effective mechanism of dispersal of blackleg *(Leptosphaeria maculans)* pycnidiospores

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Abstract

The fungus *Leptosphaeria maculans* causes blackleg, one of the most serious diseases of oilseed rape. The use of cultivars with major resistance genes is an efficient means of controlling the disease but this strategy fails to remain durably efficient over time. To control more efficiently and durably the disease there is a need to improve our understanding of the adaptation of fungus populations to the host resistance factors. The role of asexual reproduction due to pycnidiospores, which could be involved in this adaptation, is poorly documented. The objectives of this study were (i) to assess rain-splash dispersal of pycnidiospores of *L. maculans* from phoma leaf spots and (ii) to assess rain-splash transmission of the disease from oilseed rape stubble carrying pycnidia. Experimentations were conducted in still air either with a drop generator or a rain simulator. From phoma leaf spots, the impaction of incident drops caused the dispersal of pycnidiospores of *L. maculans* within splash droplets. Ninety per cent of these spores were collected beneath 14 cm from the source and a few of them were regularly found up to 40 cm from the source. The pycnidiospores produced on oilseed rape stubble were dispersed by the simulated rain and were able to infect oilseed rape trap plants. The spatial dispersal of the disease matched the spatial dispersal of the pycnidiospores. For this heterothallic fungus, rain-splash dispersal of isolates of opposite mating types could make it possible to these initially distant individuals to meet on the same plant and so to produce by sexual reproduction ascospores dispersed on long distance by the wind. This mechanism would support the increase in frequency of virulent individuals and would make easier the meeting of sexual partners, a dynamical process partly responsible for major gene resistance overcoming.

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

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 infested oilseed rape stubble that produce phoma leaf spots in autumn (Hall, 1992). 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; West *et al.*, 1999). 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 and the importance of pycnidiospores has not been quantified.

At low pathogen population density, the role of secondary infections by pycnidiospores of *L. maculans* could be more important than hitherto believed because this mechanism could increase the size of the population and facilitate the mating of isolates of opposite mating type initially distant in space. These two dynamic processes have been suggested to increase the adaptation of populations (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 characterize in still air the dispersal gradient of pycnidiospores from phoma leaf spots following the impact of incident water drops, and (ii) to characterize the disease gradient resulting from a simulated rain event on pycnidiospore-bearing stubble.

Materials and methods

Pycnidiospore dispersal gradients

Drop generator

A water drop generator consisting of a 20-mL syringe (Terumo®, Leuven, Belgium) was filled with Lactophenol Cotton Blue (LCB) solution (15%) and connected to a vertically-oriented hypodermic needle (Terumo®). 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). In all experiments, drops were released from a vertical height of 1 m. Water flow through the syringe and the number of drops released were controlled manually to produce drops of consistent diameter.

Pycnidiospore dispersal gradients

Oilseed rape (*Brassica napus*, cv. Drakkar) leaves bearing phoma leaf spots were sampled in the field. Phoma leaf spots were cut from the leaves and categorised according to the number of pycnidia per leaf spot (0-50, 50-100, 100-150, or 150-200). Phoma leaf spots bearing 150-200 pycnidia were exposed either to small or 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 was 32.8 cm from the source. Splash droplets collected on a given slide were counted for pycnidiospores under an optical microscope (Leitz® Dialux 22, magnification × 400). Coordinates 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 stems of oilseed rape in Roux bottles with 100 mL of distilled water were autoclaved twice for 20 min at 120°C, at a 24 h interval. The bottles were autoclaved a third time at 115°C for 20 min 24 h after addition of 50 mL V8-juice medium. A mono-ascospore isolate of *L. maculans* called FCr3 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 (Tec Jet SS 6560, Deltalab, Voreppe, France) with a sweep angle of 180° positioned at a height of 3.8 m. The intensity of the simulated rain was assessed by measuring the volume of water collected in 26 vials placed on the ground 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 was equivalent to a 2 mm rainfall event.

Disease dispersal gradients

Horizontal disease dispersal from infected stubble was assessed by collecting 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. Plants were grown in a glasshouse at 20°C under a 16-h photoperiod and planted into 2×3 parallel rows (six plants 4 cm apart per row) at 5, 10 and 15 cm from the position of the infected stubble during the experiment. Two pieces of infected stubble were placed at the height of the leaves in the centre of the batch and exposed to a simulated 2 mm rain event. After each simulated rain event, the trap plants were dried for 4 h at ambient temperature (18°C). Trap plants were then incubated in a growth chamber at 20°C, in saturated humidity with a 16 h photoperiod. The number of phoma leaf spots was counted on all trap plants for each batch 21 days after the simulated rain event.

Statistical analysis of the experimental spore and disease gradients

An exponential equation was fitted for spatial experimental gradients,

$$\text{(Fitt } et\ al.\text{, 1987)}: y = a.\exp(-b.d) \quad (1)$$

where y is either the proportion (number of pycnidiospores recorded at distance d of the total estimated number of pycnidiospores) 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.

Parameters a (intercept) and b (slope) were estimated by regression after linearization of equation (1):

$$\ln(y) = \ln(a) - b.d \quad (2)$$

The distance from the source at which the proportion of counted pycnidiospores decreased by 50% and 90% was calculated as $\alpha = -\ln(0.5) / b$ and $\alpha' = -\ln(0.1) / b$, respectively.

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

$$IC = b \pm se \quad t(P/2;\ n\text{-}2) \quad (3)$$

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

$$IC = (b1 - b2) \pm se \quad t(P/2;\ n_1 + n_2 - 4) \quad (4)$$

Results

Pycnidiospore horizontal dispersal gradients

For each drop size, the proportion of pycnidiospores collected after the impaction of 15 drops decreased with increasing distance from the source (Fig. 1). The proportion of pycnidiospores collected peaked between 2.5 and 5 cm from the source both for the large and small drops. The proportion of splash droplets collected at each distance interval followed a similar distribution to the proportion of removed pycnidiospores (Fig. 1).

The high proportion of pycnidiospores-carrying droplets collected between 2.5 and 5 cm from the source (Fig 1) 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 by model. Model parameters and the derived 50% (half) and 90%-distances were estimated (Table

1). 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 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.

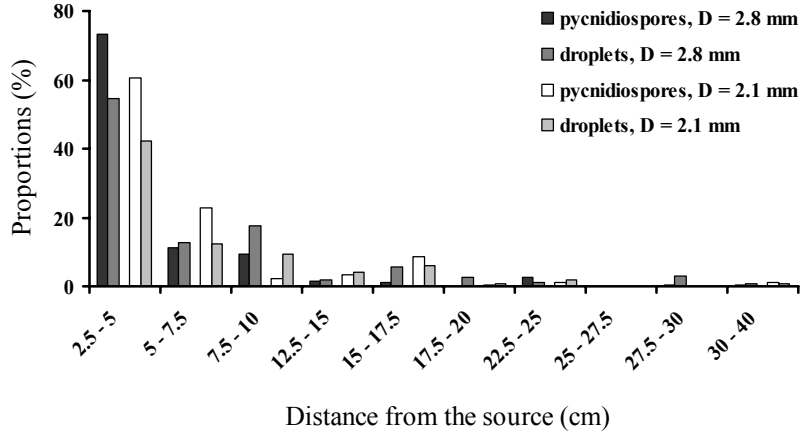

Figure 1 Proportion of splash droplets and pycnidiospores recorded and their travel distance from the source. Both were collected after the impaction of 15 incident drops for both diameters (2.8 mm and 2.1 mm). Height of fall was 1 m.

Table 1 Parameters obtained by fitting Eq. (2) to the relationships between proportions of spores or numbers of phoma leaf spots (*y*) collected or trapped after the impaction of incident drops of diameter *D* or simulated rain of 2 mm and distance (*x*) (*x* in first column, two intervals used for comparisons). *α* is the "half-distance" where y decreases by half, *α'* is the distance where *y* decreases by 90%, and se the standard error 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 event was able to remove pycnidiospores from infected oilseed rape stubble and transport them to trap plants up to 17.5 cm from the source. An exponential function was fitted to the experimental disease dispersal gradients (Table 1). For comparison of spore and disease dispersal on the same scale, the pycnidiospore dispersal gradients were re-estimated (Table 1) 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 of slopes: IC = [- 38.35; 8.28], t = 2.776, *P* = 0.05, 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 = 3.182; *P* = 0.05; 3 df), both for the large drops: IC = [-42.12; 13.25] or for the small: IC = [-57.64; 41.06]).

Discussion

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 splash-borne fungi (Fitt *et al.*, 1992; Horberg, 2002). However, 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 pycnidiospores splash-dispersal from infected seeds in the latter case. These greater distances are possibly due to wind which increase dispersal distances of spore-carrying droplets (Fitt *et al.*, 1989), to the succession of secondary cycles during the growing season or to differences in actual raindrop sizes, which may range from 0.5 to 5 mm (Ulbrich, 1983).

Our 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 (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 if recommended crop rotations are not respected.

Our results support the hypothesis that pycnidiospores may be of more significance that usually believed for the biology of *L. maculans* and for the epidemiology of the blackleg disease in the case of small size pathogen 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 on long distances. Alternately, for isolates missing a partner for sexual reproduction, pycnidiospore dispersal from stubble can be a way to contribute to the next generation. Thus pycnidiospores could have a major role at low population density. The role of pycnidiospores should be considered with respect to their contribution to the adaptation of *L. maculans* populations to selection pressure, e.g. as a consequence of the introduction of major resistance genes in oilseed rape cultivars.

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