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Is the onset of septoria tritici blotch epidemics related to the local pool of ascospores?

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To elucidate the early epidemic stages of septoria tritici blotch, especially the relationship between the onset of epidemics, the local availability of primary inoculum, and the presence of wheat debris, the early disease dynamics and airborne concentration in *Zymoseptoria tritici* ascospores were concomitantly assessed at a small spatiotemporal scale and over two years, using spore traps coupled with a qPCR assay. One plot, with the crop debris left, provided a local source of primary inoculum, while the other plot, without debris, lacked any. According to the assay's limits of detection, daily spore trap samples were classified as not detectable or not quantifiable, detectable, and quantifiable. The proportions of samples assigned to the different classes and numbers of spores in samples classified as quantifiable were significantly different between years, time periods (from November to March) and spore trap location (field with or without debris). The effect of year on the airborne ascospore concentration was high: 22 daily peaks with more than 230 ascospores m^{-3} day^{-1} were identified in the autumn of 2012/13, but none in the autumn of 2011/12. The local presence of wheat debris had no obvious effect on the amount of airborne ascospores or on the earliness of the two epidemics, especially in the year with high inoculum pressure (2012/13). These results suggest that the amount of primary airborne inoculum available in a wheat crop is not a limiting factor for the onset of an epidemic.

Keywords: debris management, *Mycosphaerella graminicola*, primary inoculum, qPCR, spore trap, wheat

Introduction

The ascomycete fungus *Zymoseptoria tritici* (Quaedvlieg *et al.*, 2011), also known as *Septoria tritici* (teleomorph, *Mycosphaerella graminicola*), causes septoria tritici blotch, a worldwide foliar disease of wheat. In the UK, septoria tritici blotch is the most damaging disease of wheat, causing yield losses of 10–40% (Eyal, 1987; HGCA, 2012). Wind-dispersed ascospores resulting from the sexual reproduction of the fungus on infected wheat debris are considered as the main source of primary inoculum (Shaw & Royle, 1989; Bathgate & Loughman, 2001; Suffert *et al.*, 2011). For fungi surviving on infested residues, cropping practices aimed at reducing the amount of primary inoculum will generally reduce the subsequent disease intensity and are often recommended as a method of disease control (Eyal *et al.*, 1987); the quantitative test of this assumption requires accurate quantification of the amount of ascospores acting as primary inoculum. In tan spot disease of wheat, disease severity was positively correlated with the initial amount of ascospores of *Pyrenophora tritici-repentis* released by residue (Adee & Pfender, 1989). A similar relationship was recorded in barley leaf blotch, caused by *Rhynchosporium secalis* (Fountaine *et al.*, 2010), in ascochyta blight of chickpea, caused by *Mycosphaerella rabiei* (Trapero-Casas *et al.*, 1996), in brown spot of pear, caused by *Stemphylium vesicarium* (Rossi *et al.*, 2008), and in South American leaf blight of rubber, caused by *Microcylus ulei* (Guyot *et al.*, 2014); in the latter case, the nature of the ascospore source is, however, still unknown. In septoria tritici blotch, the timing of ascospore release appears to influence, transiently, the earliness of epidemics: disease developed earlier in a wheat field where infected debris from the previous wheat crop had been left than in a neighbouring field from which debris had been removed (Suffert & Sache, 2011); such an effect vanished after winter. The experiment was not fully conclusive because the amount of airborne ascospores was not quantified.

Airborne spores can be routinely captured by high-throughput spore traps (West *et al.*, 2008; Jackson & Bayliss, 2011). Direct counting of spores on trapping slides or tapes under a microscope can be performed only for spores that can be unambiguously assigned to a fungal species. This is not possible when spores lacking specific morphological features, such as ascospores of *Z. tritici*, are caught together with spores of other fungal species similar in shape and size. Methods integrating spore trapping with qPCR assays were developed to quantify DNA extracted from trapped ascospores of the *Leptosphaeria maculans*/*L. biglobosa* complex (Kaczmarek *et al.*, 2009, 2012; Piliponyte-Dzikiene *et al.*, 2014), *Pyrenopeziza brassicae* (Karolewski *et al.*, 2012),

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and *Sclerotinia sclerotiorum* (Rogers *et al.*, 2009; Parker *et al.*, 2014). In these studies, the quantification of airborne inoculum could not be correlated with disease intensity. For *Z. tritici*, qPCR assays were used to monitor fungicide resistance (Fraaije *et al.*, 2005) and the spatiotemporal distribution of inoculum (Duvivier *et al.*, 2013), without specific emphasis on the onset of epidemics.

The objective of the present study was to assess the influence of the amount of airborne ascospores of *Z. tritici* on the earliness of septoria tritici blotch. To this end, the incidence and severity of disease were recorded for two consecutive growing seasons, in two neighbouring wheat plots, one with infected debris left to provide a local source of ascospores and the other with no debris. The DNA content of ascospores either caught using a spore trap or produced by infected wheat debris was quantified using the specific qPCR assay proposed by Duvivier *et al.* (2013), taking into account the technical limits of the qPCR assay when very small amounts of spores are to be quantified.

Materials and methods

Experimental field design

A field experiment derived from that described in Suffert & Sache (2011) was carried out during two successive growing seasons (2011/12 and 2012/13, hereafter referred to as the first and second season, respectively) at the Grignon experimental station (France; 48°51′N, 01°58′E; Fig. 1). Two plots were sown on 17 October 2011 and 16 October 2012, respectively, with wheat cv. Soissons, which is moderately susceptible to septoria tritici blotch (rated 5 on a 1–10 scale of decreasing susceptibility, Arvalis Institut du Végétal-CTPS). No fungicide was applied. In the first plot (D+, 20 × 100 m), cropped with wheat since 2007, straw was chopped at harvest (mid-July) and left on the soil surface; debris was chopped and partially buried to a depth of 10 cm 6 weeks later (late September). Debris naturally contaminated with *Z. tritici* was expected to act in autumn as a local source of primary inoculum. The second plot (D_0, 40 × 100 m), located 300 m away from plot D+, did not contain any wheat debris because it had been cropped during the previous season with faba bean.

Air temperature at a height of 2 m above soil level and rainfall were recorded hourly by an automated weather station (Enerco 516i, Cimel Électronique, France) located *c.* 500 m from the plots. The thermal time *t*, expressed in degree-days, was calculated, starting from the sowing date, by summing the daily mean air temperature using 0°C as a base temperature.

Disease assessment

Disease was assessed at 12 sampling dates in the first season and 10 sampling dates in the second one, twice or thrice monthly at the beginning of the epidemics (autumn and winter) and thereafter monthly during the epidemic phase (spring) until May. At each sampling date, five plants were randomly collected in five 1-m^2 quadrats per plot, located 20 m away from each other, and washed to remove soil particles. Disease severity and incidence were calculated on the main stem and the first tiller of each plant sample. The leaf layers were identified according to their order of emergence (L1 is the first leaf, L2 the second, etc.). For a given layer, disease incidence was estimated as the percentage of leaves with visible lesions (Suffert *et al.*, 2013). Severity was estimated as the average percentage of necrotic leaf area covered by pycnidia using a diagrammatic scale (1, 2, 3 and 5%, then 10, 15, 20% until 100%).

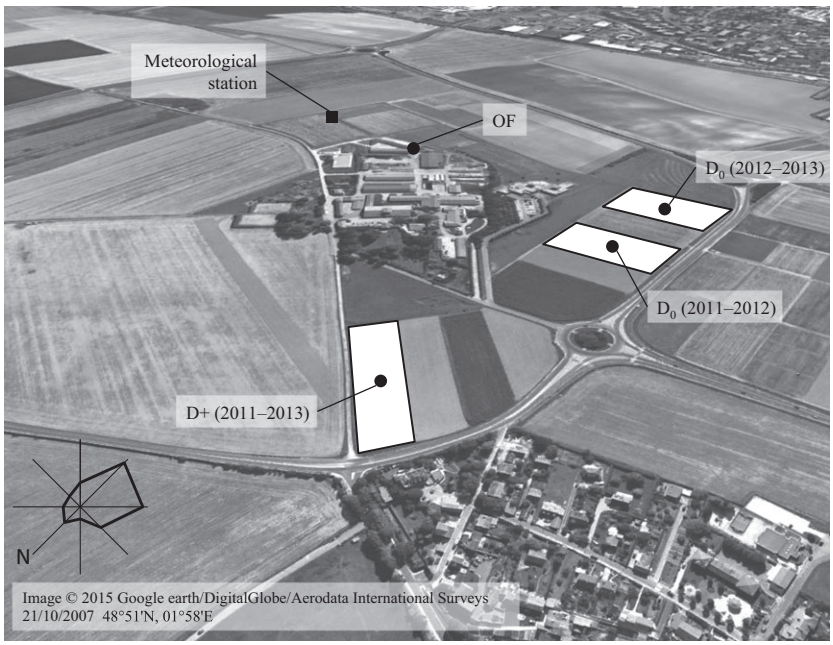

Figure 1 Experimental set-up. Plots (D_0 and D+) and spore traps (black dots, D_0, D+ and OF) used during the two years of the experiment at the Grignon experimental station. D+: plot cropped with wheat since 2007, with straw chopped at harvest and left on the soil surface; D_0: plot without wheat debris; OF: out of field. The dominant wind direction during the two years of experiment is shown by the wind rose (bottom left).

Quantification of ascospore production from infected wheat debris

Infected wheat debris was collected from plot D+ at five sampling dates (10 October 2012, 12 November 2012, 7 December 2012, 9 January 2013 and 4 March 2013) during the early stages of the epidemics. Debris was cut in 2-cm fragments and kept at 18°C for 24 h. Ten grams of debris were evenly spread on a wet filter in a moist box (Suffert & Sache, 2011) in order to promote the ejection of ascospores from mature pseudothecia. In each box, eight Petri dishes containing potato dextrose agar (PDA) medium (39 g L^{-1}) were placed upside down 1 cm above the debris. The boxes were placed for 6 h at 18°C in the dark; thereafter Petri dishes were closed and incubated in the same conditions. Germination occurred several hours later but the resulting colonies were visually counted 5 days later, assuming that a colony resulted from the germination of a single ascospore (Suffert & Sache, 2011). The potential amount of ascospores produced per m^2 in each field plot was then estimated for each date by dividing the amount of ascospores produced per gram of debris by the density of debris (Table 1).

Airborne ascospore trapping

Three 7-day recording volumetric spore traps (Burkard Manufacturing Co.) were used to catch airborne *Z. tritici* ascospores during the first part of the epidemics (autumn and winter) in the two seasons. The traps were operated in the centre of plot D+, in the centre of plot D_0, and outside the field (OF) 500 m away from D+ and D_0 in a relatively windy location (Fig. 2). The D_0 and OF spore traps were intended to collect mainly ascospores produced from debris of distant wheat crops, while the D+ spore trap was intended to collect ascospores produced either from distant wheat crop debris or from local wheat debris left on the soil of the D+ plot. The orifice of the traps was located 0·7 m above the soil surface, so that only wind-dispersed ascospores were collected, considering that pycnidiospores are not expected to be splash-dispersed up to this height (Shaw, 1987). The spore traps were run continuously at the manufacturer's throughput (10 L min^{-1}/14·3 m^3 day^{-1}) over two periods, from 10 November 2011 to 11 March 2012 and from 9 October 2012 to 3 April 2013. Ascospores were caught on a Melinex impacting tape coated with a thin layer of petroleum jelly. For each 7 day sampling period, the impacting tape was cut into seven 1-day sections (48 × 15 mm) using a Burkard Perspex cutting block. Each section was enclosed in a box in order to prevent cross-contact between tapes. The boxes were kept at −80°C for several months until DNA extraction.

Table 1 Features of the experimental field design

	Debris management[a]	
	D+	D_0
2011/12 (First season)		
Preceding crop	Wheat	Faba bean
Sowing date	17/10/2011	17/10/2011
Seedling emergence date	01/11/2011	01/11/2011
Debris density (g m^{-2}, on 11/01/2012)	238	0
Plant density (plants m^{-2}, on 11/01/2012)	152	154
2012/13 (Second season)		
Preceding crop	Wheat	Faba bean
Sowing date	16/10/2012	15/10/2012
Seedling emergence date	22/10/2012	22/10/2012
Debris density (g m^{-2}, on 11/12/2012)	470	0
Plant density (plants m^{-2}, on 19/03/2013)	114	140

[a]D+ = wheat debris left; D_0 = faba bean debris (traces) and no wheat debris.

Quantification of ascospore DNA

For the sake of comparison between the three trapping locations, the tapes of the three spore traps operated during the same 7-day period were extracted concomitantly. Each 1-day tape section was placed in a Fast-Prep tube (2 mL) containing 80 μL AP1 buffer (DNeasy Plant Mini kit; QIAGEN) and 130 mg glass beads (4·5 mm in diameter; Deutscher). DNA was extracted in sets of 21 tubes. For each set, the positive control consisted of a suspension of pycnidiospores of *Z. tritici* (5000 pycnidiospores/50 μL) in 70% ethanol. All the positive controls came from a single pycnidiospore suspension prepared in January 2012 from infected wheat leaves and aliquoted into tubes that were subsequently kept at −20°C. In order to detect contamination events during DNA extraction, a negative control containing sterile water was also used. The tubes were shaken in a Fast Prep machine (3 × 1 min at 6 m s^{-1}) to disrupt and remove the ascospores from the tape. The DNeasy Plant Mini kit (QIAGEN) was adapted for the material. AP1 buffer (350 μL) and 4 μL RNase A (QIAGEN) were added to each tube, before heating for 5 min at 65°C. Then, 6 μL of proteinase K (20 mg mL^{-1}) was added and tubes were heated again for 10 min at 65°C. After centrifugation for 1 min at 20 000 ***g***, the supernatant was transferred to a new tube. The next steps of the DNA extraction followed QIAGEN standard protocol. Extracted DNA was kept at −20°C.

Fungal DNA was quantified using the quantitative PCR assay developed for *Z. tritici* (Duvivier *et al.*, 2013). The specific set of primers included a forward primer (5′-ATTGGCGAGAG GGATGAAGG-3′), a reverse primer (5′-TTCGTGTCCCAGTG CGTGTA-3′), both giving an amplification product of 101 bp, and a TaqMan fluorogenic probe (5′-ACGACTCGCGGCTTTC ACCCAACG-3′). The probe was labelled with a FAM fluorescent reporter dye and a BHQ-1 quencher. The quantification reaction was performed with the CFX96 Real-time System C1000 thermal cycler (Bio-Rad), using hard shell PCR 96-well WHT/CLR plates. The reaction mixture was composed of reverse and forward primers at 500 nM per reaction, the probe at 500 nM per reaction in a final volume of 25 μL, which contained 5 μL of DNA sample per well. All samples (standard DNA, ascospore DNA from tapes, positive and negative controls) were analysed with three replicates. The amplification programme was 95°C for 10 min and (95°C for 15 s, 60°C for 20 s, 72°C for 40 s) repeated for 40 cycles.

The concentration of DNA in the unknown samples was calculated by comparing cycle threshold (C_t) values of the samples with known standard quantities of *Z. tritici* genomic DNA, using a tenfold serial dilution from 0·5 ng to 5×10^{-5} ng per well. C_t values of the dilution samples were plotted against the log of the initial concentration of *Z. tritici* genomic DNA to produce a standard curve. The validity of the standard curve of the quantification method was tested using a linear regression model coupled with an ANOVA analysis and a Fisher test.

The specificity of the quantification method (Duvivier *et al.*, 2013) was confirmed by including DNA extracts from 11 *Z. tritici* isolates collected in the D+ plot (one of which was used as the qPCR standard) and eight *Septoria passerinii* isolates.

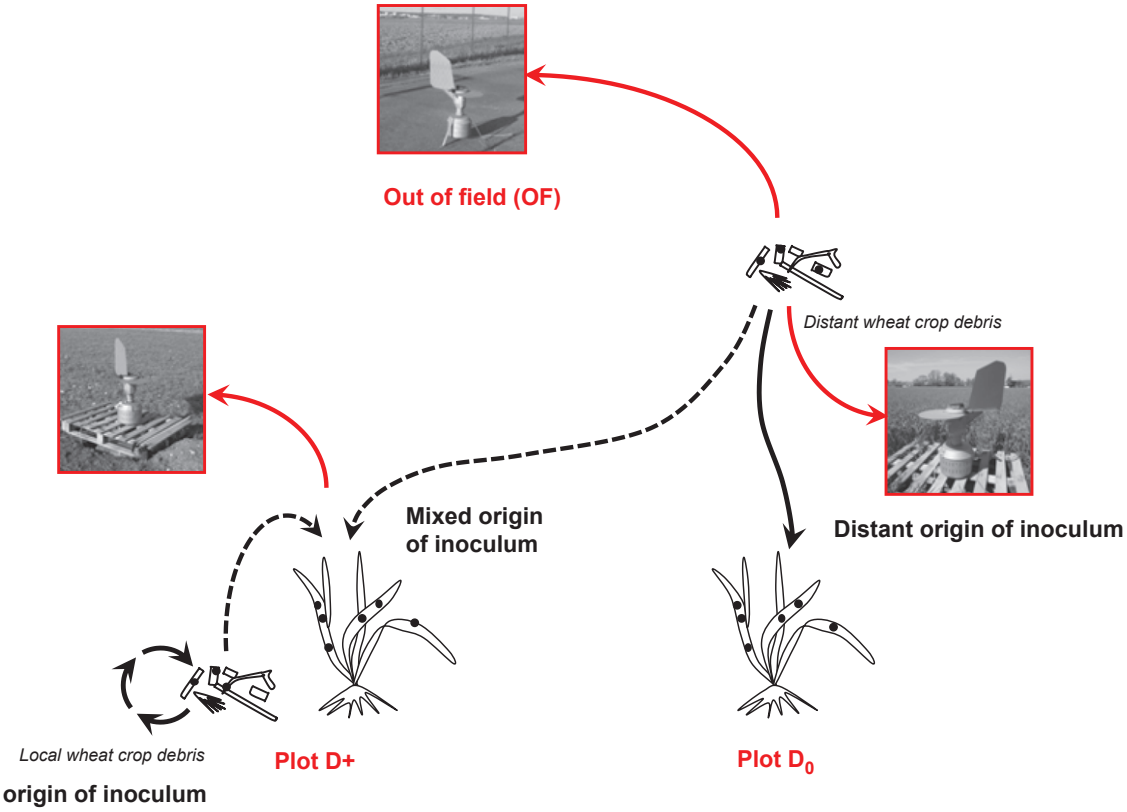

Figure 2 Schematic representation of the origin of the *Zymoseptoria tritici* ascospores collected by the three spore traps according to their location. The spore traps from the plot without debris (D_0) and out of field (OF) were expected to collect mainly distant primary inoculum (ascospores produced on distant wheat crop debris). The spore trap from the plot with wheat debris (D+) was expected to collect both distant and local primary inoculum (ascospores produced on local wheat debris left on the soil of the D+ plot), in an unknown proportion.

The limit of detection (LOD) of the qPCR assay was determined using a DNA standard curve consisting of a tenfold serial dilution from 0·5 to 5×10^{-5} ng of DNA. Dilutions 1, 2 and 3 (0·5, 0·05 and 0·005 ng of DNA) were performed in triplicate whereas dilutions 4 and 5 (5×10^{-4} and 5×10^{-5} ng of DNA) were repeated 30 times. The LOD of a qPCR assay is usually considered to be reached when 90% of the replicates (here, 27/30) are detected (AFNOR, 2006). Here 100% of the replicates were detected for a given dilution D and less than 90% were detected for the dilution D/10. Accordingly, the theoretical LOD of the qPCR assay was between those two C_t values but could not be determined precisely. Consequently, two LODs were defined and used; the upper limit of detection (LOD_{up}) corresponded to the mean C_t value for dilution D and the lower limit of detection (LOD_{low}) corresponded to the mean C_t value for dilution D/10.

The limit of quantification (LOQ) of the qPCR assay was calculated using standard curves of all the plates considered as usable (see Results section). To this end, a Student's *t*-test was used (AFNOR, 2006).

Each sample (1-day tape section) was assigned to a class according to the position of the three C_t values of the sample compared to the limits LOQ, LOD_{up} and LOD_{low}. Thus, samples with three replicated C_t values under LOQ were assigned to class I; ascospores collected in these samples were considered as 'truly quantifiable'. Samples with three replicated C_t values ranging from LOQ to LOD_{up} were assigned to class II; ascospores collected in these samples were considered as 'quantifiable'. Samples with three replicated C_t values ranging from LOD_{up} to LOD_{low} were assigned to class III; ascospores collected in these samples were considered as 'detectable but not quantifiable'. Samples with three replicated C_t values higher than LOD_{low} or with one or two undetermined C_t values were assigned to class IV; ascospores collected in these samples were considered as 'not quantifiable or detectable'. Samples with three undetermined C_t values were assigned to class V; these samples were considered as 'ascospore-free'. Samples were then assigned to one of three time periods (P1, P2, P3), specific for each year, according to the collection date. The limit between P1 and P2 in the first season and the limit between P2 and P3 in the second season were defined by the peak in the quantity of *Z. tritici* DNA. The limit was established by fitting a quadratic equation to the C_t values of all samples ('quantifiable', 'detectable', and 'not detectable') collected during the year. The limit between P2 and P3 for the first season, and between P1 and P2 for the second season, was fixed so that the same number of samples was assigned to each period. For the first season, P1 lasted from 10 November 2011 to 2 January 2012, P2 lasted from 3 January 2012 to 16 February 2012, and P3 lasted from 17 February 2012 to 11 March 2012. For the second season, P1 lasted from 9 October 2012 to 15 December 2012, P2 lasted from 16 December 2012 to 1 February 2013, and P3 lasted from 2 February 2013 to 3 April 2013.

Statistical analysis

The number of samples assigned to each class (I–V) and the quantity of DNA obtained by qPCR for a given sample were hereafter considered as explained variables. The time period (P1, P2, P3) and the spore trap location (D+, D_0, OF) were considered as explanatory variables. First, the effect of spore trap location on the number of samples assigned to each class was investigated for the two seasons. A nonparametric Kruskal–Wallis test was used to determine if there were significant differences in the number of samples assigned to each class between all spore traps or between two spore traps. Secondly, the effects of spore trap location and of the time period on the quantity of DNA were investigated for samples assigned to classes I, II (samples in which *Z. tritici* DNA was considered as 'quantifiable') and V (samples quantified as ascospore-free) in a factorial analysis of variance for the two seasons. The variance was partitioned into sources attributable to period (P), and spore trap location (T) according to the model:

$$Y_{ipt} = M + P + T + P \times T + \epsilon_{ipt}$$

where Y_{ipt} is the quantity of DNA of sample i, collected in period p, in spore trap t. M indicates the overall mean and ε_{ipt} the variance between replicates.

For each of the two seasons, the means of quantity of DNA of each sample belonging to classes I, II and V were then compared between periods and between spore traps with a Student–Newman–Keuls test. Statistical analyses were performed with the S-PLUS v. 6.3 software (Lucent Technologies, Inc.).

Estimation of the airborne ascospore concentration

For the sake of comparison with the literature, airborne ascospore concentrations were derived from the quantity of DNA measured by the qPCR assay. A standard curve using *Z. tritici* pycnidiospore suspensions at different concentrations was established. From a unique pycnidiospore suspension, three batches of dilutions were prepared separately. Seven dilutions were performed for each batch: 5000, 1250, 500, 250, 125, 50 and 25 spores in 50 μL of 70% ethanol. Pycnidiospores were then disrupted and DNA extracted and quantified as described above. Considering that pycnidiospores are made of four to eight cells (Shaner, 2010), it was assumed they are made of six cells on average (Duvivier *et al.*, 2013), whereas ascospores are bicellular. The relationship between the approximate amount of ascospores (y) and the total quantity of DNA (x) was established by using a linear equation. The airborne ascospore concentration (m^{-3} day^{-1}) was then obtained by dividing the estimated amount of ascospores by the volume of air sucked in by the spore trap (14·3 m^3 day^{-1}).

Results

Disease assessment

No difference in plant development was observed between D+ and D_0 plots. At each sampling date, there was no difference in the biomass of plants sampled in the two plots (data not shown). Accordingly, differences in epidemic development between the two plots could not be due to differences in the development of the plant stand.

The earliness of the septoria tritici blotch epidemic was quite similar for the two seasons in plot D_0 (Table 2). The first lesions were detected on 1 December (430°C-days post-sowing) and 29 November (390°C-days post-sowing) during the first and second season, respectively. Six weeks later (on 9 January 2011 at 706°C-days post-sowing and on 7 January 2012 at 625°C-days post-sowing, respectively), the uppermost diseased leaf layers were leaf L5 of the main stem and leaf L1 of the first tiller in the first season, and leaf L3 of the main stem in the second season. This small difference in disease development was due to lower temperatures in December 2011 than in December 2012. Similarly, differences in disease development were low between the two seasons in plot D+ (data not shown).

In the first season, the epidemic developed earlier in plot D+ than D_0. Until mid-December (500°C-days post-sowing), there was no difference between the two plots in disease severity (Fig. 3a) or incidence (Fig. 3b), as assessed on leaf L1 and L2 of the main stem. Later on, disease severity and incidence were consistently higher in D+ than in D_0. The difference in disease severity peaked (at 20%) on 9 January 2011 (706°C-days post-sowing),

Table 2 Wheat leaf layers infected by *Zymoseptoria tritici*, but still partially green[a], on main stem (MS) and first tiller (T1) in plot D_0, at each sampling date during seasons 2011/12 (d1–d12) and 2012/13 (d1–d10)

	Date	Dd[b]	MS	T1
First season (2011/12)				
Sowing date	15/10/2011	0		
d1	16/11/2011	311		
d2	22/11/2011	358		
d3	01/12/2011	430	0–1	
d4	08/12/2011	488	1–2	
d5	20/12/2011	555	1–3	
d6	09/01/2012	706	1–5	1
d7	31/01/2012	815	1–6	1–3
d8	23/02/2012	865	2–6	1–3
d9	28/03/2012	1174	3–7	2–5
d10	24/04/2012	1390	5–8	4–7
d11	30/05/2012	1891	8–11	6–10
d12	21/06/2012	2224	10–11	9–10
Second season (2012/13)				
Sowing date	15/10/2012	0		
d1	20/11/2012	320		
d2	29/11/2012	390	0–1	
d3	11/12/2012	420	1–2	
d4	17/12/2012	453	1–3	
d5	07/01/2013	625	1–3	
d6	06/02/2013	727	2–5	1–2
d7	20/03/2013	861	4–6	1–4
d8	25/04/2013	1147	4–7	2–4
d9	22/05/2013	1446	5–8	2–5
d10	01/07/2013	2022	7–11	4–8

[a]For example, '2–6' means that layer 1 is completely senescent and that layers 2–6 have at least one infected leaf (neither completely senescent nor completely healthy) among the 25 plants sampled (five plants randomly collected in five 1 m^2 quadrats).
[b]Degree days post-sowing, base 0°C.

both on leaves L1 and L2. The difference in disease incidence peaked (at more than 30%) on 8 December 2011 (488°C-days post-sowing) on leaf L1 and on 20 December 2011 (555°C-days post-sowing) on leaf L2.

In the second season, no clear trend in the earliness of the epidemic could be observed. There was no difference between plots D+ and D_0 in disease severity (Fig. 3c) on leaf L1 and L2, except on 7 January 2013 (625°C-days post-sowing), when severity on L2 was 20% lower in D+ than D_0. There was no difference, either, in disease incidence (Fig. 3d) on leaves L1 and L2, except on 11 December (420°C-days post-sowing; Fig. 3d), when severity on L1 was 20% lower in D+ than D_0 while severity on L2 was 20% higher in D+ than D_0.

Quantification of ascospores on wheat debris

In the first season, no ascospores were collected on Petri dishes from the wheat debris in plot D+. This does not necessarily mean that ascospore production from debris was null; rather, spore production was very low and fell below the limit of detection of the collection method. In the second season, the production of ascospores during the early epidemic stage in plot D+ (Fig. 4) was estimated to be highest (15 000 ascospores m^{-2}) in October 2012, taking into account the mean debris surface density in the plot (470 g m^{-2}), then decreased, and eventually stopped in March 2013.

Validation of the qPCR analysis

The published performance of the qPCR assay (Duvivier *et al.*, 2013) was confirmed in the experimental conditions of the present study. The genomic DNA (gDNA) standard curve, established using the 29 plates (see below) considered as valid, had the following regression equation: $C_t = -3{\cdot}374 \ \log(x) + 24{\cdot}766$ ($R^2 = 0{\cdot}992$), where x is the quantity of DNA to be determined. The efficiency of the quantification method was estimated at 97·9%. As expected, no amplification was obtained using gDNA of eight different isolates of the barley pathogen *S. passerinii* (data not shown), whereas the gDNA of the 11 *Z. tritici* isolates was amplified.

For the fourth dilution (5×10^{-4} ng DNA) of the DNA standard curve used to establish the limits of detection of the qPCR assay, 100% positive signals were obtained, with a mean C_t value of 36·41, while only 67% positive signals were obtained for the fifth dilution (5×10^{-5} ng DNA), with a mean C_t value of 37·84. Consequently, the C_t values 36·41 (about 19 ± 6 ascospores m^{-3} day^{-1}) and 37·84 (about 13 ± 4 ascospores m^{-3} day^{-1}) were considered as the upper limit of detection (LOD_{up}) and the lower limit of detection (LOD_{low}), respectively. The limit of quantification (LOQ) was established at the C_t value 32·94 (about 121 ± 40 ascospores m^{-3} day^{-1}), although the theoretical LOQ ranged between a C_t of 36·41 (fourth dilution, Student's

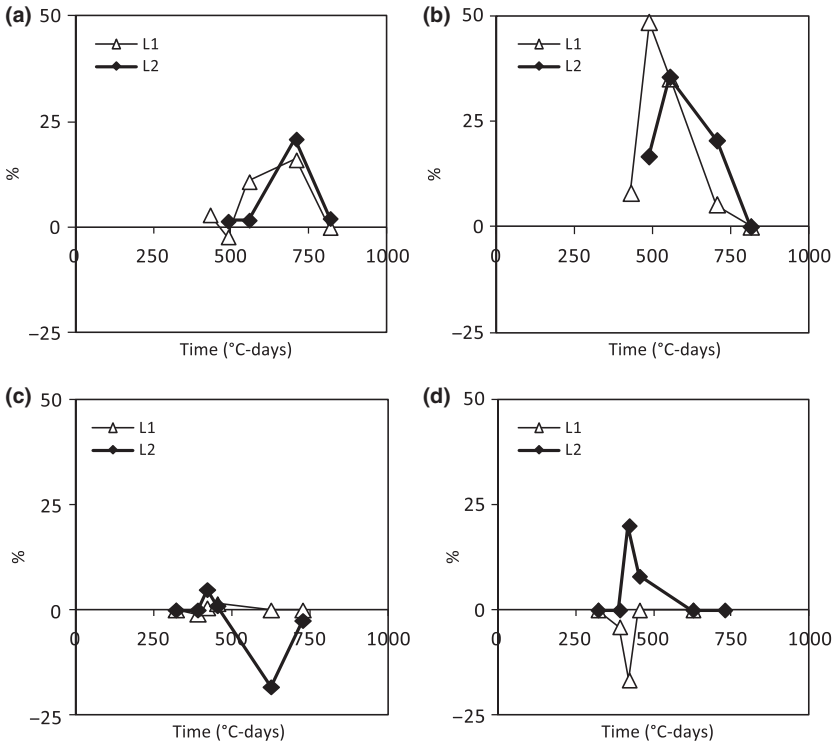

Figure 3 Difference (%) in septoria tritici blotch severity (a, c) and incidence (b, d) between D+ and D_0 plots for the first (2011/12; a, b) and second (2012/13; c, d) seasons, where D+ is a plot with wheat debris and D_0 is a plot without wheat debris. Disease severity and incidence were assessed on the first (△) and second leaf (♦) of the main tiller. Time, expressed in degree-days (°C-days), was calculated starting from the sowing date.

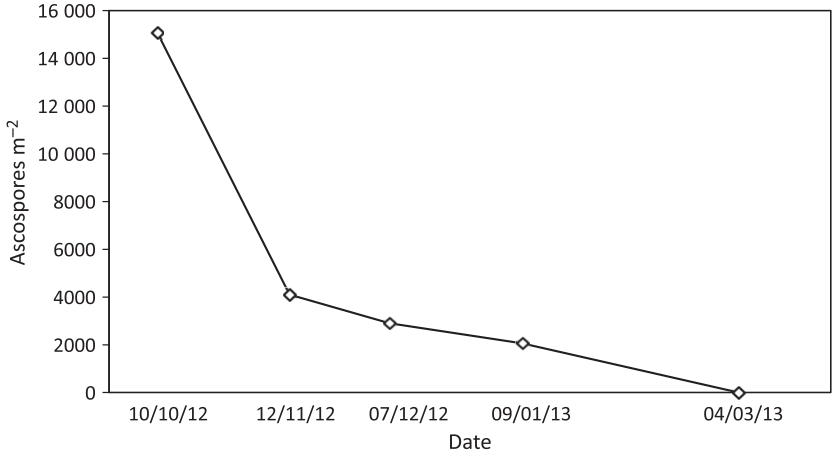

Figure 4 Production of *Zymoseptoria tritici* ascospores on wheat debris collected in the plot with wheat debris (D+) during the early stages of the epidemic (season 2012/13). The potential amount of ascospores was estimated by counting *Z. tritici* colonies resulting from ascospore ejection onto Petri dishes (potato dextrose agar). Mean debris density in the plot was 470 g m^{-2}.

$t = 1{\cdot}012$) and a C_t of 32·94 (third dilution, Student's $t = 0{\cdot}369$)).

The qPCR plates were validated against both positive and negative controls. When the mean C_t values of the three replicates of the positive control ranged between 29 and 31 (absence of false negative), the plate was declared valid. The other plates were not used. When the C_t value of at least one of the three replicates of the negative control was less than 38 (false positive), the plate was declared contaminated and non-valid, and so excluded from the analysis. The application of these two criteria to the 40 plates run during the whole experiment left 29 plates considered as valid and were subsequently used for the quantitative analysis. The remaining 11 plates (seven for the first season and four for the second season) were excluded.

The relationship between the amount of ascospores (y) and the quantity of DNA (x) was described by the linear equation $y = 7438{\cdot}6x + 41{\cdot}2$ (Table 3). This equation gives an estimate of an airborne ascospore concentration (in ascospores m^{-3} day^{-1}) in relation to a quantity of DNA (ng).

Spatiotemporal variations in the amount of ascospore DNA

The qPCR assay was used to analyse 160 samples (1-day tape sections) in the first season and 427 samples in the second season (Fig. 5). In the first season, 15·7% of the samples were considered as quantified (classes I and II), only two samples (1·3%) being assigned to class I (Table 4). Slightly more than half of the samples were considered as neither quantifiable nor detectable (class IV). Only one sample (0·6%) was ascospore-free. In the second season, 67·7% of the samples were considered as quantified, 53 of them (12·4%) being assigned to class I.

Table 3 Quantification of *Zymoseptoria tritici* pycnidiospore DNA by qPCR assay

Amount of deposited pycnidiospores (y)	Mean C_t value	Standard deviation	DNA quantity (x) (ng)	Quantified amount of pycnidiospores[a]	Estimated amount of ascospores[b]	Airborne concentration (ascospores m^{-3} day^{-1})[c]	Sample status
5000	29·93	0·08	0·669	5022	15066	1054	Class I
1250	31·95	0·33	0·147	1139	3417	239	Class I
500	33·27	0·69	0·061	497	1491	104	Class II
250	33·86	0·16	0·033	290	870	61	Class II
125	35·41	0·59	0·011	127	381	27	Class II
50	37·32	0·41	<0·005	61	183	13	Class III
25	37·31	0·42	<0·005	63	189	13	Class III

Mean C_t values and mean DNA quantities were calculated using three independent qPCR replicates. A linear model ($y = 7438{\cdot}6\ x + 41{\cdot}3$; $R = 0{\cdot}999$), fitted to the amount of pycnidiospores deposited on Melinex tapes (y) and the total quantity of DNA (x), gives an estimate of the amount of pycnidiospores in relation to the quantity of DNA (ng).

[a]Calculated using the equation of the linear model.

[b]Calculated by multiplying the estimated amount of pycnidiospores by 3, taking into account the number of cells per type of spore (on average, six nuclei for a pycnidiospore and two nuclei for an ascospore; Shaner, 2010).

[c]Calculated by dividing the estimated amount of ascospores by the volume of air sucked in by the spore trap (14·3 m^3 day^{-1}).

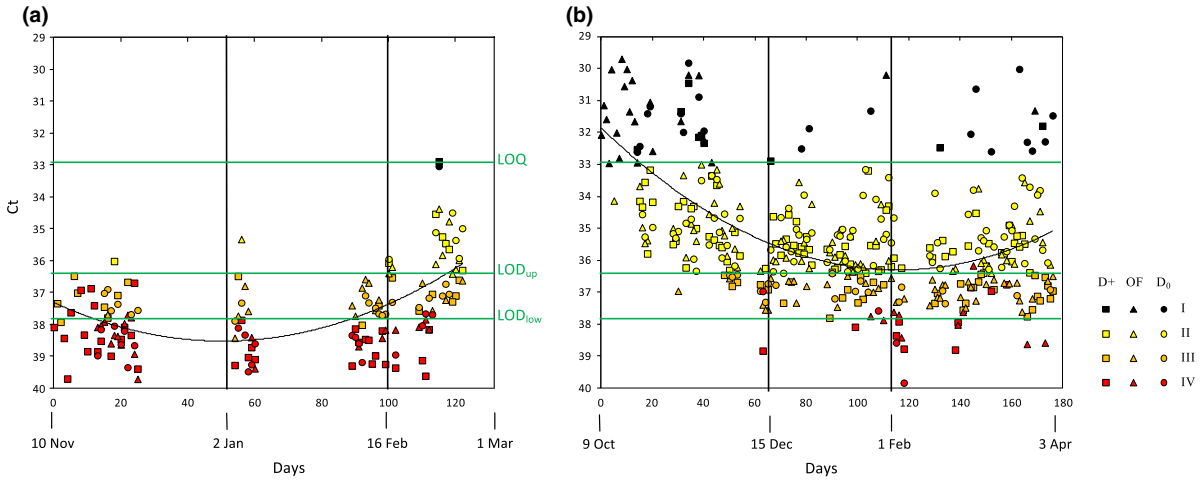

Figure 5 Cycle threshold (C_t) values for all samples (1-day tape sections) analysed by qPCR for the first (2011/12; a) and second (2012/13; b) seasons, from spore traps situated in a plot with wheat debris (D+), a plot without wheat debris (D_0) and out of field (OF). Each C_t value was calculated using three independent qPCR replicates. Samples obtained from the three different spore traps (O: D_0; △: OF; □: D+) were assigned to four classes (black, class I; yellow, class II; orange, class III; red: class IV) according to their C_t value. Class V, to which only two samples were assigned, is not presented. The limit of quantification (LOQ) and the upper and lower limits of detection (LOD_{up} and LOD_{low}) are indicated by horizontal green lines. The sampling period was split into three periods (P1, P2, P3), as defined in the main text. A quadratic curve ($y = ax^2 + b$) was fitted to the complete data sets.

Only 7·7% of the samples were considered as neither quantifiable nor detectable. Only one sample (0·2%) was ascospore-free. The class distribution of the samples was significantly different between the two seasons (Kruskal–Wallis test, $H = 155{\cdot}6$, $P < 0{\cdot}001$).

The mean quantity of DNA of the samples considered as quantified (classes I, II and V) was 0·017 ng for the first season (26 samples) and 0·063 ng for the second season (290 samples; Table 3). On average, the amount of ascospore DNA was nearly four times higher in the second than in the first season.

When considering the time periods of trapping, most quantifiable samples of the first season (23 out of 35) were obtained during period P3, while only one quantifiable sample was obtained in each of the P1 and P2 periods. In the second season, the quantity of DNA in the samples significantly differed between the three time periods ($P < 0{\cdot}001$; Table 5). The quantity of DNA was significantly higher in period P1 (0·105 ng) than in periods P2 and P3 (0·026 ng and 0·046 ng, respectively; Fig. 6d); the difference in the quantity of DNA between periods P2 and P3 was, however, not significant.

In the first season, spore trap location had no significant effect on the number of samples assigned to each class (Table 6). No significant difference was found either in the pairwise comparison of the spore traps. In

Table 4 Number of samples (each from a 1-day tape section) assigned to each class (I, II, III, IV, V) for three spore traps and two seasons.

	Class					
	I	II	III	IV	V	
Trap location	Quantified	Quantified	Detected	Not detected	Absent	Total
First season (2011/12)						
D+[a]	1	7	17	38	0	63
OF[b]	0	10	16	22	0	48
D_0[c]	1	6	20	21	1	49
Total	2	23	53	81	1	160
Percentage per class	1·3	14·4	33·1	50·6	0·6	
Second season (2012/13)						
D+[a]	9	79	39	13	0	140
OF[b]	23	72	44	14	1	154
D_0[c]	21	85	21	6	0	133
Total	53	236	104	33	1	427
Percentage per class	12·4	55·3	24·4	7·7	0·2	

[a]D+: plot with wheat debris.
[b]OF: out of field.
[c]D_0: plot without wheat debris.

the second season, the effect of spore trap location was significant; the class distribution of samples in trap D_0 was significantly different from the other traps. However, fewer samples were quantifiable in D+.

In the first season, the quantity of DNA of the samples considered as quantified did not significantly differ between spore traps (data not shown). In the second season, significant differences were found (Table 5). Pairwise comparison of spore traps showed a significantly higher quantity of DNA in OF than in D+ trap (0·093 ng and 0·035 ng, respectively; Fig. 6c), while the quantity of DNA on D_0 trap was not significantly different from either D+ or OF trap.

Table 5 Effect of spore trap and trapping period on the amount of *Zymoseptoria tritici* DNA[a] during the second season (2012/13)

	df	Mean Square	*F*	*P*
Trap	2	0·08	6·58	0·002
Period	2	0·15	12·69	<0·001
Trap × Period	4	0·03	2·41	0·049
Residual	281	0·01		

[a]Only samples assigned to classes for which DNA was considered as quantifiable (I, II and V) were analysed.

Discussion

In this two-year study performed at a small spatiotemporal scale, the onset of disease epidemics was concomitantly surveyed with the variation in the airborne concentration of spores purportedly acting as the initial inoculum. The assessment of initial, low levels of disease severity and incidence was performed as described earlier (Suffert & Sache, 2011). For the sake of reliability and comparison with other studies, a Burkard spore trap was used, which is the standard device for the capture of airborne microorganisms (Jackson & Bayliss, 2011). The main technical challenge in this study was the detection and quantification of small amounts of airborne spores. Reports of detection and quantification of one or two spores (Carisse *et al.*, 2009; Rogers *et al.*, 2009; Parker *et al.*, 2014) using a serial dilution of DNA of a spore suspension are questionable, because of loss of DNA during extraction steps. To validate the qPCR assay in the present study, both its limit of detection (LOD) and its limit of quantification (LOQ) were assessed. Those two limits are often not explicitly stated in the literature (e.g. Haegi *et al.*, 2013; Parker *et al.*, 2014). The 'detection threshold for quantification' reported for *Z. tritici* ascospores (Fraaije *et al.*, 2005) is equally ambiguous.

LODs and LOQ were derived from standard curves established with gDNA extracted from *Z. tritici* pycnidiospores. The use of gDNA (Lees *et al.*, 2012) rather

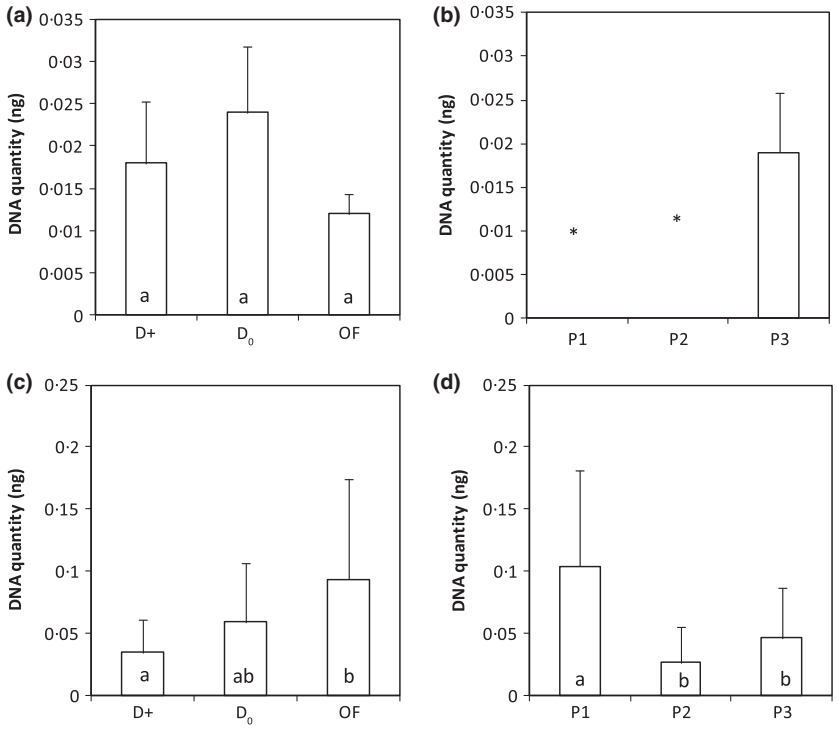

Figure 6 Amount of *Zymoseptoria tritici* DNA for samples belonging to classes I, II and V, according to the spore trap location (D+, plot with wheat debris; D_0, plot without wheat debris; OF, out of field) and the time period (P1, P2 and P3), as defined in the main text, for the first (2011/12; a, b) and the second season (2012/13; c, d). Significant differences, revealed by the Student–Newman–Keuls test ($P < 0·05$), are indicated by different letters. Note the difference in the scale of the vertical axis between the two years. In (b), * corresponds to a single sample.

Table 6 Comparison of class distribution of samples (I, II, III, IV, V) between spore trap locations (D+, D_0 and OF)[a] for two seasons.

	df	N[b]	H[c]	P
First season (2011/12)				
All traps	2	160	2·85	0·241
D+ vs OF	1	111	2·41	0·121
D+ vs D_0	1	112	1·67	0·197
D_0 vs OF	1	97	0·09	0·761
Second season (2012/13)				
All traps	2	427	12·54	0·002
D+ vs OF	1	294	0·44	0·504
D+ vs D_0	1	273	12·63	<0·001
D_0 vs OF	1	287	6·76	0·009

[a]D+, plot with wheat debris; D_0, plot without wheat debris; OF, out of field.
[b]Number of analysed samples.
[c]Kruskal–Wallis statistic.

than plasmid cDNA (e.g. Duvivier *et al.*, 2013) was as efficient as the use of ascospore DNA. The assignment of samples to the classes established using LOD_{up}, LOD_{low} and LOQ allowed the exploitation of the full data set and the performance of statistical analyses. The definition of 'detectable samples' allowed an overall comparison between traps, while 'quantifiable samples' were used to compare daily patterns of ascospore releases.

The main drawback of the method used in this investigation was the lack of direct comparisons of the number of spores actually trapped and the quantity of DNA quantified using the assay. The link between the amount of ascospores and amount of genomic DNA was indirectly obtained, using suspensions of *Z. tritici* pycnidiospores. However, the limits of detection eventually calculated were in accordance with thresholds established on other pathogens (e.g. Schweigkofler *et al.*, 2004; Lees *et al.*, 2012; Klosterman *et al.*, 2014).

The effect of experimental year on the airborne ascospore concentration was consistent with the significant difference in ascospore production from contaminated debris. Such a difference, likely to occur also in other fields with wheat debris in the Grignon basin, could be related to the severity of disease on the previous year (Cowger & Mundt, 2002): the more severe the epidemics are in one season, the more frequent the sexual reproduction on debris and the higher the amount of ascospores released from debris at the beginning of the next season. Severity was high in 2011/12 (lesions covered 15–20% of the flag leaf area at mid-June 2012), while it had been very low in 2010/11 (lesions were absent from flag leaf at mid-June 2011); sexual reproduction was, most probably, more frequent during the late epidemic stages in autumn 2012, as evidenced by the high amounts of ascospores either collected from debris (Fig. 4) or caught by the spore traps (Fig. 5).

The amount of ascospores responsible for the first infections occurring immediately after seedling emergence (trapped in period P1) was about tenfold higher in late autumn 2012 than in late autumn 2011. Among the highest daily peaks of ascospores in that period, 22 daily peaks were identified in 2012/13 with more than 230 ascospores m^{-3} day^{-1} ($C_t > 32$; Table 3; Fig. 5b) from 10 October to 18 November, but none in 2011/12. Such a magnitude of airborne ascospore concentration is consistent with observations made in wheat crops in autumn: a maximum of 92 ascospores m^{-3} day^{-1} (Bathgate & Loughman, 2001), between 50 and 125 ascospores m^{-3} day^{-1} (Hunter *et al.*, 1999), and between 100 and 350 ascospores m^{-3} day^{-1} (Duvivier *et al.*, 2013). This confirmed the occurrence of peaks in ascospore production in autumn, concomitant with wheat emergence.

Peaks of ascospore concentration were also recorded in the subsequent time periods in 2012/13: one in December and two in January (period P2), four in March and one in April (period P3). Accordingly, ascospores were present in the air throughout almost the whole autumn and winter periods, as established in previous studies (Hunter *et al.*, 1999; Bathgate & Loughman, 2001).

The local presence of infected wheat debris had no obvious effect on the amount of airborne *Z. tritici* ascospores. No difference was found between the three spore traps in season 2011/12. This was not surprising because the wheat debris in D+ was not considered as a significant source of inoculum (ascospore production on this debris was undetectable). In season 2012/13 the airborne ascospore concentration was not correlated with the presence of a local source of inoculum. Other factors may explain local differences in airborne ascospore concentration, such as topography and wind currents.

The local presence of infected wheat debris also had no clear effect on the earliness of the epidemic. In plot D+, the epidemic was earlier when wheat debris was less contaminated (2011/12), that is to say when inoculum pressure was low. Conversely, no difference in earliness was found between the plot without debris (D_0) and the plot with infected debris (D+) in the season when debris was highly infected (2012/13). Despite the limited duration of the experiment (two years), these results suggest that the amount of airborne ascospores available in a wheat crop is not a limiting factor for the early stages of septoria tritici blotch epidemics, in contrast to previous studies (Holmes & Colhoun, 1975; Shaw & Royle, 1989). However, the results do indicate that, when the amount of airborne ascospores is low, the local presence of debris, even only slightly contaminated, can induce an earlier epidemic. In accordance with previous results (Suffert & Sache, 2011), the earliness of a septoria tritici blotch epidemic seems to be mostly determined by the sowing date; whatever this date, the airborne ascospore concentration is usually non-limiting so that the first infection by the fungus occurs as soon as the first leaf emerges. This implies that management of primary inoculum at a local scale by removing wheat debris from nearest fields offers little help to septoria tritici blotch control in western European conditions.

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