Comparative pathogenicity of sexual and asexual spores of *Zymoseptoria tritici* (septoria tritici blotch) on wheat leaves

D. Morais[a], V. Laval[a], I. Sache[b] and F. Suffert[a]*

[a]*INRA, UMR1290 BIOGER, F-78850 Thiverval-Grignon, and* [b]*AgroParisTech, UMR1290 BIOGER, AgroParisTech, F-78850 Thiverval-Grignon, France*

Zymoseptoria tritici ascospores and pycnidiospores are considered the main forms of primary and secondary inoculum, respectively, in septoria tritici blotch epidemics. The pathogenicity of the two types of spores of the same genotypic origin were compared through a two-stage inoculation procedure in controlled conditions. Adult wheat leaves were inoculated with ascospores collected from field sources, yielding 119 lesions; pycnidiospores collected from 12 lesions resulting from these ascospore infections were then used for inoculation. Lesion development was assessed for 5 weeks; latent period, lesion size, and pycnidium density were estimated for different isolates. The latent period was calculated as the maximum likely time elapsed between inoculation and either the appearance of the majority of the sporulating lesions (leaf scale) or the appearance of the first pycnidia (lesion scale). The latent period was significantly longer (*c.* 60 degree-days, i.e. 3–4 days) after infection with ascospores than with pycnidiospores. No difference was established for lesion size and density of pycnidia. A comparison with other ascomycete fungi suggested that the difference in latent period might be related to the volume of spores and their ability to cause infection. Fungal growth before the appearance of lesions may be slower after inoculation with an ascospore than with a pycnidiospore. The mean latent period during the very beginning of epidemics, when first lesions are mainly caused by ascospores, may be longer than during spring, when secondary infections are caused by pycnidiospores. Disease models would be improved if these differences were considered.

Keywords: ascospore, Gompertz function, latent period, *Mycosphaerella graminicola*, pycnidiospore, wheat

Introduction

The ascomycete fungus *Zymoseptoria tritici* (formerly known as *Mycosphaerella graminicola*, anamorph *Septoria tritici*; Quaedvlieg *et al.*, 2011) causes septoria tritici blotch, a foliar disease of wheat (*Triticum aestivum*) found in most wheat-growing areas worldwide. In western Europe, the disease was reported to induce up to 30–40% crop loss when the upper leaves are severely infected (Eyal *et al.*, 1987). The difference between treated (with azole fungicides) and untreated yields was recently estimated as 10% on average for varieties moderately susceptible to septoria tritici blotch in UK conditions (HGCA, 2012). Disease epidemics are initiated by airborne sexual ascospores, mainly produced during the intercrop period (after harvest in summer and autumn) on infested wheat debris, which are considered the main primary inoculum (Shaw & Royle, 1989; Hunter *et al.*, 1999; Suffert *et al.*, 2011). The disease is clonally propagated among wheat plants during the growing season by pycnidiospores, which are splash-dispersed over short distances and act as the main secondary inoculum. How-

ever, ascospores and pycnidiospores can be jointly involved in the same stages of the epidemic development. Indeed, in a wheat monoculture system, when infested debris is not completely buried, both pycnidiospores and ascospores can initiate the epidemic (Suffert & Sache, 2011; Suffert *et al.*, 2011). Moreover, in spring, secondary infections can be induced by ascospores (Clinckemaillie *et al.*, 2010; Duvivier *et al.*, 2013) released either from old, still infectious debris located in distant plots or from infected wheat plants located in the same plot. Field reports of infection of the uppermost (F1) and lowermost green leaves (F3–F4), but healthy middle leaves (F2) (C. Maumené, Arvalis-Institut du Végétal, Boigneville, France, personal communication), could be explained by a greater susceptibility of expanding leaves and also by the aforementioned secondary infection by ascospores. The pre-eminence of one type of spore has been explained by contrasting distances of dispersal (Shaw, 1987; Shaw & Royle, 1989) and by different amounts of the spores during the epidemic build-up (Eriksen *et al.*, 2001; Suffert & Sache, 2011), but rarely by differences in pathogenicity between sexual and asexual spores, as measured, for example, by the number of sporulating lesions they induce (Karolewski *et al.*, 2002; Trapero-Casas & Kaiser, 2007).

In fungi with an alternating reproduction regime, the comparison of the pathogenicity of spore types is experimentally challenging. In *Z. tritici* the production

*E-mail: frederic.suffert@versailles.inra.fr

and handling of large amounts of ascospores is cumbersome in artificial conditions (Kema *et al.*, 1996). Asexual spores (conidia) produced from yeast-like mycelium grown in liquid or solid culture media were used to inoculate wheat plants (e.g. Cowger & Mundt, 2002; Leyva-Mir *et al.*, 2008; Suffert *et al.*, 2013) rather than spores produced on infected leaves. Whether conidia produced *in vitro* and conidia produced *in planta* (hereafter called pycnidiospores for the sake of clarity) have the same pathogenicity has not yet been tested. Finally, ascospores and pycnidiospores of the same genotype are required to perform a valid comparison, due to the high genetic diversity of *Z. tritici* populations (Linde *et al.*, 2002).

A first important component of pathogenicity of *Z. tritici* is sporulation (Suffert *et al.*, 2013), assessed through the measurement of maximum sporulating area (e.g. Arraiano & Brown, 2006), pycnidium density (e.g. Simon & Cordo, 1997) and sporulation capacity (Eyal, 1971). A second important component of pathogenicity in *Z. tritici* is latent period (Suffert *et al.*, 2013), which reflects the pathogen's generation time. Latent period is usually defined as the time interval between infection and the onset of sporulation from that infection (Pariaud *et al.*, 2009); in the case of septoria tritici blotch, it is the time elapsed between infection and the appearance of first pycnidia (Shearer & Zadoks, 1972). When a population of lesions rather than a single lesion is considered, as usual in experimental studies, the latent period is estimated, at the scale of a leaf, as the maximum likely time elapsed between inoculation and the appearance of the majority of the sporulating lesions (Shaw, 1990; Lovell *et al.*, 2004; Zearfoss *et al.*, 2011), or, at the scale of a lesion, as the maximum likely time elapsed between inoculation and the appearance of the first pycnidia (Armour *et al.*, 2004; Suffert *et al.*, 2013).

The objective of this study was to compare the pathogenicity of sexual and asexual spores of *Z. tritici* of similar genotypes, following infection of adult wheat leaves, by assessing the sporulation and latent period of the infections.

Materials and methods

Fungal material

Two plots were sown 300 m apart with wheat cv. Soissons (moderately susceptible to septoria tritici blotch; rated 5 on a 1–10 scale of decreasing susceptibility, Arvalis Institut du Végétal-CTPS) in autumn 2011 at the Grignon experimental station (France, 48°51′N, 1°58′E). Soil and weather characteristics, as well as cropping systems, have been described elsewhere (Suffert & Sache, 2011). The first plot (D+) had been grown by direct drilling and so contained wheat debris. The debris, hardly infested after a mild disease epidemic the previous season, constituted a weak source of ascospores (Suffert & Sache, 2011). The second plot (D₀), grown with oilseed rape the previous season, did not contain any wheat debris.

Airborne *Z. tritici* ascospores were caught in March and April 2012 using three 7-day recording volumetric Burkard spore traps (Burkard Manufacturing Co.) placed 0·7 m above the soil surface in plot D₀, in plot D+ (Fig. 1a) and out of the field (Fig. 1b). The traps were operated at a flow rate of 10 L min⁻¹. The collecting surface of part of the Melinex tape was covered with a thin layer of petroleum jelly (Vaseline) before sampling (Fig. 1c), while the other part of the tape was not covered (Table 1).

Wheat debris was collected on 28 March 2012 from the plot D+ and from a wheat field (cv. Soissons) grown at the Versailles experimental station (France, 48°48′N, 2°04′E); at this location, disease was more severe the previous year than in Grignon (M. Leconte, INRA, Thiverval-Grignon, France, personal communication). The debris were placed in laboratory conditions in two plastic grill boxes and sprayed with a small amount of water to induce the moisture change required to trigger the ejection of ascospores. Boxes were covered by a plastic bag connected to the trap aperture (Fig. 1d), in order to maximize the amount of collected ascospores. Different methods of trapping were used: Melinex tapes or glass slides, either covered or not with petroleum jelly, were placed in a 7-day and 24-h volumetric Burkard spore trap, respectively (Table 1). The traps were operated at a flow rate of 10 L min⁻¹.

Throughout the collection period, the Melinex tape and glass slides were kept for a few days at −20°C to limit ascospore decay or germination. Finally, 10 different samples corresponding to 10 ascospore trapping events were obtained during a 3-week period (Table 1).

Plant material

Two batches of wheat adult plants, cv. Soissons, were grown in two greenhouse compartments for subsequent inoculation. The two batches were sown in Jiffy peat pots on 7 December 2011 and 9 January 2012, respectively. Seedlings were vernalized for 7 weeks at 8°C with a 10 h light period and a 14 h dark period. Afterwards, seedlings were transferred back to the greenhouse and left to acclimatize for 1 week. Plants were then transplanted into 1·1 L pots filled with 1 L commercial compost (Peat Substrat 4; Klasmann) and fertilized with 5 g Osmocote Exact (16N:11P:11K+ 3MgO + Te). Plants were also sprayed at the seedling stage with Spiroxamine (Aquarelle SF at 2 mL L⁻¹; Bayer Crop Science) as a classical preventive measure to control powdery mildew (*Blumeria graminis*; Suffert *et al.*, 2013). The delay between treatment and inoculation was 6 weeks, for both ascospores and pycnidiospores. In addition, plants were fertilized twice with a commercial nutrient solution (1 L Hydrokani C2) diluted 1/100 and applied in the pot saucers 5 and 7 weeks after seedling transplantation and supernumerary tillers were cut from each plant to keep only 3 stems. During plant growth, natural daylight was supplemented with 400 W sodium lamps to obtain 9 h dark/15 h light, the greenhouse compartment temperature being kept under 20°C during the light period and above 12°C during the dark period. In each greenhouse compartment the air temperature was recorded every 15 min and the thermal time *t*, expressed in degree-days post inoculation (ddpi), was calculated by summing the daily mean air temperatures, starting from the inoculation dates and using 0°C as the base temperature.

Inoculation with ascospores

The day before inoculation, 10 ascospore suspensions corresponding to the 10 aforementioned ascospore trapping events were prepared, according to the type of support (Melinex tape
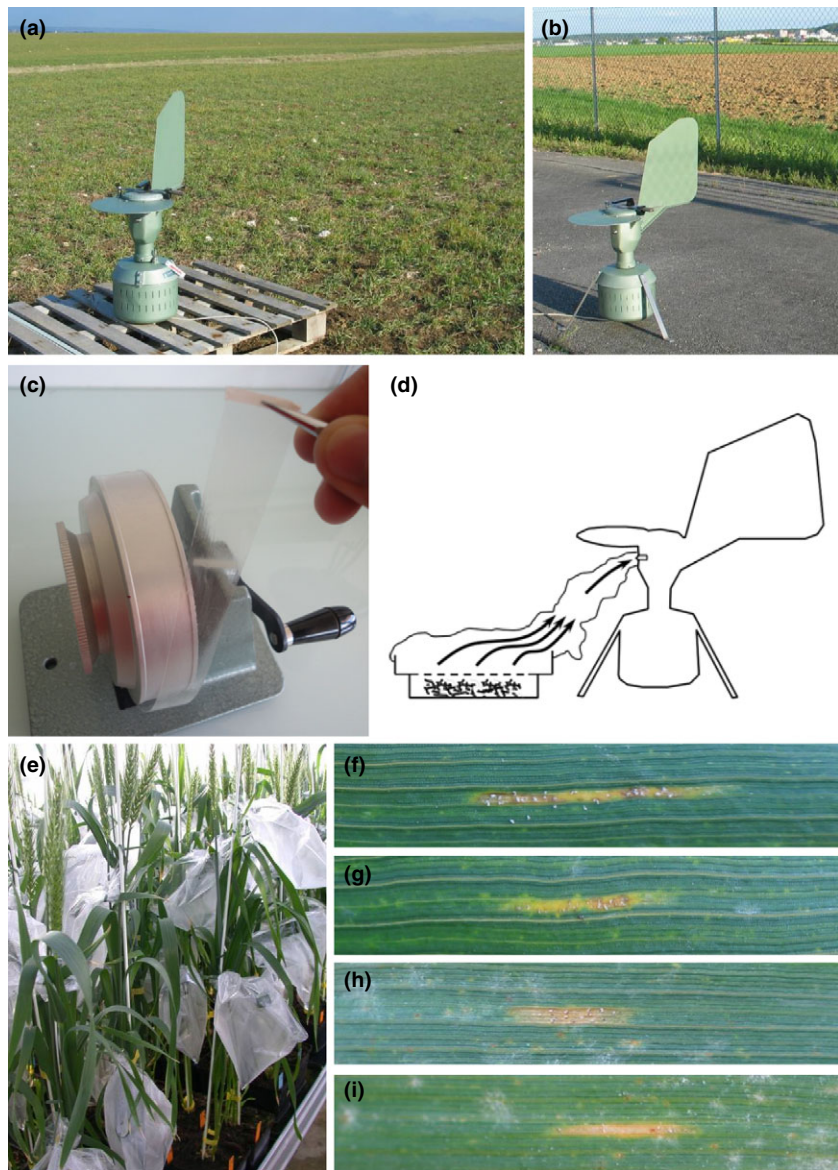
Figure 1 Experimental design used to compare the pathogenicity of ascospores and pycnidiospores of *Zymoseptoria tritici*. (a) Burkard spore trap placed in wheat monoculture plot (D+) with wheat debris (Grignon). (b) Burkard spore trap placed out of field 300 m apart (Grignon). (c) Melinex tape wound around the periphery of the rotating drum of the Burkard spore trap. (d) Collection of ascospores from wheat debris. Boxes of debris were covered by a plastic bag connected to the Burkard spore trap aperture in order to maximize the amount of collected ascospores. (e) Inoculated leaves enclosed in a bag to promote infection. (f–i) Septoria tritici blotch lesions after inoculation with ascospore suspensions.

or glass slide) and to the presence or absence of petroleum jelly. Samples on Melinex tape were cut into sections, each matching a daily trapping. Each tape section was placed in a conical tube containing 10 mL water and 130 mg glass beads (4·5 mm diameter; Deutscher). The tubes were manually shaken for 1 min. The tape sections covered with petroleum jelly were placed in a 65°C water bath for 5 min and centrifuged at 7000 g for 5 min to remove the ascospores from the tape. Glass slides covered with petroleum jelly were placed in a conical tube containing 10 mL water then placed in a 65°C water bath for 5 min and centrifuged at 5000 g for 5 min. The contents of the tubes corresponding to the same trapping event (Table 1) were gathered and kept for a day at 4°C to limit ascospore decay or germination.

Prior to inoculation, two drops of surfactant (Tween20; Sigma) were added to the spore suspensions to ensure adequate coverage of the inoculated leaf surface. On 17 April 2012, each spore suspension was applied using a paintbrush along the adaxial face of the upper leaf (F1) of the two main tillers of five plants (10 leaves) of the first batch of plants. Each leaf was then enclosed for 72 h in a transparent bag (Fig. 1e), slightly wetted to maintain a high humidity and promote infection (Suffert *et al.*, 2013).

Inoculation with pycnidiospores

Twelve lesions were selected out of the 119 that had appeared after inoculation with ascospores (Fig. 1f–i) as a source of pycnidiospores (see Table 1 and Results). All 12 lesions originated from ascospores trapped from debris of plot D+, either on Melinex tape without petroleum jelly (8/12) or on glass slides (4/12), in order to know the origin of the inoculum (local pathogen population) and so increase the reproducibility of the experiment. On 28 May 2012, after the last assessment of lesions caused by ascospore infection (see below), pycnidiospores were collected from each of the 12 selected lesions by

Table 1 Origin of the 10 *Zymoseptoria tritici* inoculum sets used in the investigation

Location	Origin of sampling	Support	Petroleum jelly	Collect duration (days)	Period (2012)	No. asc.[a]	No. pyc.[b]	Isolates[c]
Outside[d]	Out of field (Grignon)	Melinex tape	No	7	28/03 to 03/04	14		
			Yes	14	13/03 to 26/03	12		
	Plot D$_0$ (Grignon)[e]	Melinex tape	No	7	28/03 to 03/04	7		
			Yes	14	13/03 to 26/03	16		
	Plot D+ (Grignon)[f]	Melinex tape	No	14	06/04 to 12/04 and 28/03 to 03/04	8		
			Yes	14	13/03 to 26/03	16		
Inside[g]	Debris from plot D+ (Grignon)	Melinex tape	No	10	04/04 to 09/04 and 06/04 to 09/04	15	8	FS3307, FS3309, FS3344, FS3346, FS3356, FS3357, FS3385, FS3386
		Glass slide	Yes	2	04/04 to 06/04	12	4	FS3306, FS3314, FS3327, FS3359
	Debris from wheat plot (Versailles)	Melinex tape	No	6	10/04 to 15/04	7		
		Glass slide	Yes	2	02/04 to 04/04	12		

[a]Total number of leaf lesions appeared after inoculation with the ascospore suspension.
[b]Total number of leaf lesions selected for the subsequent inoculation with the pycnidiospore suspension.
[c]Code of the 12 *Z. tritici* isolates that were inoculated both as ascospore and pycnidiospore type.
[d]Location of the Burkard spore trap.
[e]Plot without wheat debris (oilseed rape as previous crop).
[f]Plot with debris (wheat monoculture since 2007).
[g]Trapping device placed above debris (Fig. 1c,d).

placing the leaf in a 25 mm diameter tube containing 10 mL water and softly sweeping the area covered by pycnidia with a paintbrush. Each pycnidiospore suspension was then immediately applied, using a paintbrush, along the adaxial face of the upper leaf (F1) of the main tiller of four plants (12 × 4 leaves) of the second batch. Each leaf was then enclosed for 72 h in a transparent bag, as performed previously for the inoculation with ascospores.

Assessment of pathogenicity components

Disease symptoms were assessed by the same assessor twice a week from 350 to 710 ddpi, from 11 May to 28 May 2012 for ascospore inoculation (seven assessments) and from 18 June to 4 July 2012 for pycnidiospore inoculation (eight assessments). The sporulating lesions, neither spatially aggregated nor coalescent, could be counted individually (maximum three lesions per leaf after ascospore inoculation; maximum 26 lesions per leaf after pycnidiospore inoculation). The length and width of each lesion were assessed visually using a ruler graduated in increments of 0·5 mm. The number of pycnidia borne by each lesion was counted with a hand lens (×10 magnification).

The maximum size of a lesion ($SIZE_{max}$, mm^2) was calculated as the product of its length and width.

A Gompertz function (Berger, 1981) was fitted to the number of pycnidia PYC(t) recorded on each lesion (Suffert *et al.*, 2013):

$$PYC(t) = PYC_{max} \exp(-B_{PYC} e^{-r_{PYC} \cdot t}) \quad (1)$$

where PYC_{max} is the maximum number of pycnidia reached at the end of the assessment period, r_{PYC} is a rate parameter, B_{PYC} is a position parameter, and t is the time of observation expressed in degree-days post inoculation (ddpi). The density of pycnidia (PYCdens) was then calculated as:

$$PYC_{dens} = \frac{PYC_{max}}{SIZE_{max}} \quad (2)$$

The latent period was assessed using two complementary methods. First, $LatPYC_5$, the time elapsed from inoculation to the appearance of 5% of the maximum number of pycnidia

Table 2 Analysis of variance to test the effect of the origin of the inoculum (Grignon or Versailles) and of the presence or absence of petroleum jelly on the trapping support on the latent period ($LatPYC_5$) assessed for 119 *Zymoseptoria tritici* isolates inoculated as ascospores on wheat cv. Soissons

Factor	d.f.	Sum of squares	Mean square	F	P
Origin	1	3879·8	3879·8	0·503	0·48
Petroleum jelly	1	271·9	271·9	0·035	0·85
Origin × petroleum jelly	1	9323·4	9323·4	1·209	0·27
Residual	115	886 976·2	7712·8		

Table 3 Analysis of variance to test the effect of spore type (ascospores vs pycnidiospores) on lesion size ($SIZE_{max}$), pycnidium density (PYCdens), and latent period ($LatPYC_5$) of 12 *Zymoseptoria tritici* isolates inoculated on wheat cv. Soissons

Variable	Factor	d.f.	Sum of squares	Mean square	F	P
$SIZE_{max}$	Spore type	1	73·5	73·5	2·58	0·12
	Residual	22	627·8	28·5		
PYCdens	Spore type	1	0·90	0·90	1·67	0·21
	Residual	22	11·90	0·54		
$LatPYC_5$	Spore type	1	15 975·9	15 975·9	4·70	0·04
	Residual	22	74 816·1	3400·7		

(PYC_{max}), was calculated using Eqn (1) for each lesion induced either by ascospores or pycnidiospores of each isolate. PYC_{max} ranged from 20 to 80, so $LatPYC_5$ was considered a good indicator of the time elapsed between inoculation and the appearance of the 1–4 earliest pycnidia. Secondly, a Gompertz growth curve was fitted to the total number of sporulating lesions LES (t) (i.e. lesions for which $PYC(t) \geq 0.05 \times PYC_{max}$) assessed on several leaves:

$$LES(t) = LES_{max} \exp(-B_{LES}e^{-r_{LES}.t}) \qquad (3)$$

where LES_{max} is the maximum number of lesions, r_{LES} is a rate parameter, and B_{LES} is a position parameter.

$LatLES_{37}$, the time elapsed from inoculation to $1/e \approx 36.8\%$ of LES_{max} (inflexion point of a Gompertz function), was calculated using Eqn (3). This method could not be used after asco-spore inoculation because each individual was unique and caused only one lesion.

Data analysis

$SIZE_{max}$, PYCdens and $LatPYC_5$ were estimated after ascospore inoculation for each of the 119 isolates, based on the assessment of a single lesion (no replication), and after pycnidiospore inoculation by mean values calculated from the assessment of all the lesions induced by each of the 12 selected isolates (Table 1).

For the ascospore inoculation, $LatLES_{37}$, calculated using the 119 lesions, was an average estimate of the latent period for 119 different isolates. For the pycnidiospore inoculation, $LatLES_{37}$ was estimated first for a given isolate (among the 12 selected isolates), and secondly for the whole population using all the data corresponding to the 12 isolates.
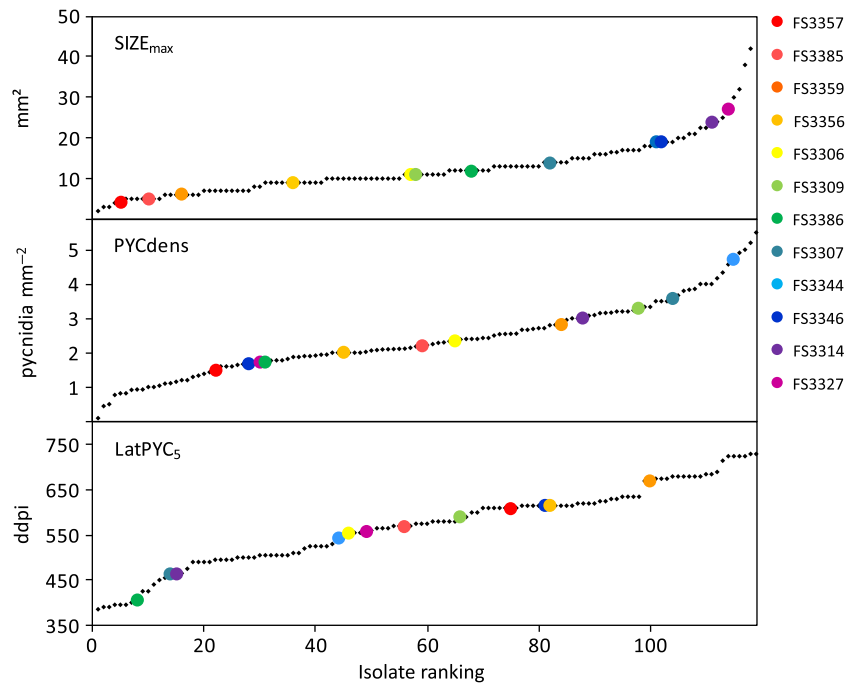

Figure 2 Distribution of lesion size ($SIZE_{max}$), pycnidium density (PYCdens) and latent period ($LatPYC_5$) of the 12 *Zymoseptoria tritici* isolates (●) selected from 119 isolates (♦) assessed after ascospore inoculation of adult wheat leaves; ddpi, degree days post inoculation.
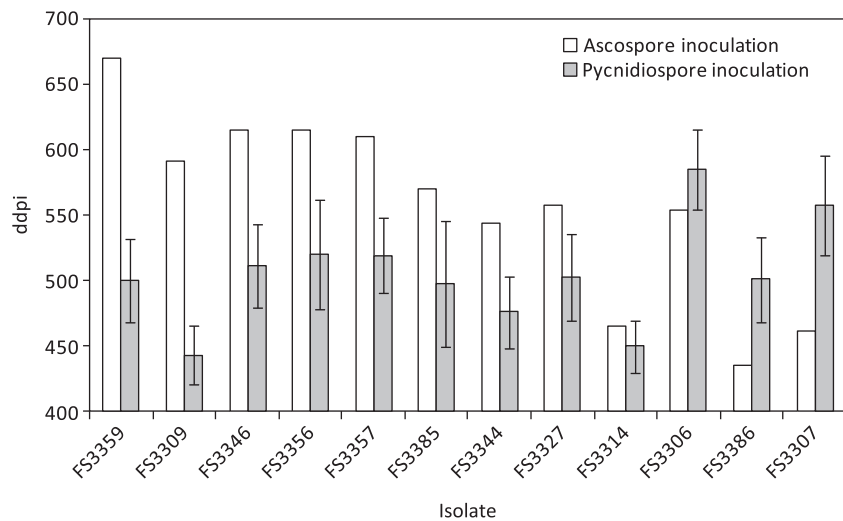

Figure 3 Latent period ($LatPYC_5$) of 12 *Zymoseptoria tritici* isolates assessed after ascospore (white columns) and pycnidiospore (grey columns) inoculations on adult wheat leaves. Isolates are ranked in descending order of difference between $LatPYC_5$ assessed for ascospore inoculation and $LatPYC_5$ assessed for pycnidiospore inoculation; vertical bars represent standard deviation; ddpi, degree days post inoculation.

The effects of petroleum jelly (with or without) and the origin of inoculum (Versailles, Grignon) on the latent period for the ascospore inoculation were tested in an analysis of variance (ANOVA; Table 2). For LatPYC$_5$ assessed on 119 isolates, the variance was partitioned into sources attributable to the presence of petroleum jelly (J) and the origin (O), according to the model:

$$Y_{joi} = M + J + O + \varepsilon \qquad (4)$$

where Y_{joi} is the value of the latent period of isolate i, for the presence or not of petroleum jelly j, for the origin o, and ε is the error term.

The effect of the type of spore on the three pathogenicity components (SIZE$_{max}$, PYCdens, and LatPYC$_5$) was tested in an ANOVA (Table 3). For each component assessed on 12 isolates, the variance was partitioned into sources attributable to the type of spore (S), according to the model:

$$Y_{si} = M + S + \varepsilon \qquad (5)$$

where Y_{si} is the value of the pathogenicity component Y of isolate i, for type of spore s, and ε is the error term.

Results

The inoculation of 100 wheat leaves with 10 ascospore suspensions yielded 119 lesions (Fig. 1f–i). One hundred lesions were induced by ascospores trapped from wheat debris at Grignon (against 19 from wheat debris at Versailles) and 68 were trapped using petroleum jelly (against 51 without) (Table 1). The inoculation of 12×4 wheat leaves with the 12 pycnidiospore suspensions yielded 425 lesions; each of the 12 isolates induced between 12 and 26 lesions. The distribution of SIZE$_{max}$, PYCdens, and LatPYC$_5$ for the 119 lesions obtained after ascospore inoculation is shown in Figure 2; pathogenicity components of the 12 isolates selected from the 119 assessed after ascospore inoculation, fell quite evenly within that distribution.

LatPYC$_5$ calculated for the 119 lesions obtained after ascospore inoculation was not significantly affected either by the use of petroleum jelly (567·2 ddpi without petroleum jelly [68 lesions] versus 570·8 ddpi with petroleum jelly [51 lesions]), or the origin of the isolates (567·2 ddpi for isolates originating from Versailles [19 lesions] versus 566·8 ddpi for isolates originating from Grignon [100 lesions]) (Table 2).

For each of the 12 selected isolates, the latent period LatPYC$_5$ (Fig. 3) was statistically different after ascospore and pycnidiospore inoculation ($P = 0.04$; Table 3); LatPYC$_5$ was significantly longer after ascospore (557·4 ddpi) than pycnidiospore inoculation (501·9 ddpi). There was no difference either for lesion size SIZE$_{max}$ ($P = 0.12$) or pycnidium density PYCdens ($P = 0.21$). Means of SIZE$_{max}$ (13·4 mm^2 after ascospore inoculation vs 11·4 mm^2 after pycnidiospore inoculation) and of PYCdens (2·55 pycnidia mm^{-2} vs 2·22 pycnidia mm^{-2}) were not significantly different.

The mean latent period LatLES$_{37}$, calculated from the cumulative number of sporulating lesions appeared after

inoculation (when PYC(t) $\geq 0.05 \times$ PYC$_{max}$), was 536·2 ddpi for ascospore inoculation and 472·7 ddpi for pycnidiospore inoculation (Fig. 4). LatLES$_{37}$, which corresponds to the maximal rate of lesion appearance (time at which the mean number of new sporulating lesions appeared during one degree-day, ΔLES(t)/Δt, is maximum), was reached, on average, 63·5 ddpi (3–4 days) later for ascospore than for pycnidiospore inoculation. The significance of the difference could not be tested statistically because of the heterogeneity of the two data sets used to calculate LatLES$_{37}$: in one set, 119 lesions
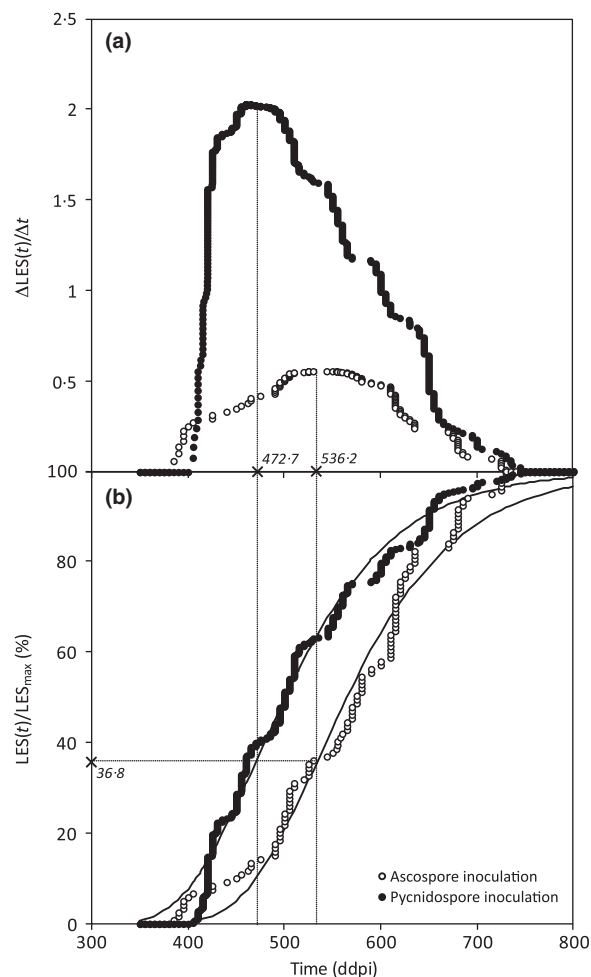

Figure 4 (a) Mean number of new sporulating lesions appeared per degree-day period (ΔLES(t)/Δt) after inoculation of 100 wheat leaves with 10 *Zymoseptoria tritici* ascospore suspensions (O) and after inoculation of 4×12 wheat leaves with pycnidiospore suspensions of 12 selected isolates (●). (b) Cumulative normalized number of lesions (LES(t)/LES$_{max}$) appeared after inoculation of 100 wheat leaves with ascospore suspensions (O) and after inoculation of 4×12 wheat leaves with pycnidiospore suspensions of 12 selected isolates (●). Each point corresponds to a sporulating lesion. Lines correspond to the fitted curves (Gompertz model). Time is expressed in degree-days post-inoculation (ddpi). The mean latent period LatLES$_{37}$ (inflexion point of the fitted curves) was 536·2 ddpi for ascospore inoculation and 472·7 ddpi for pycnidiospore inoculation.
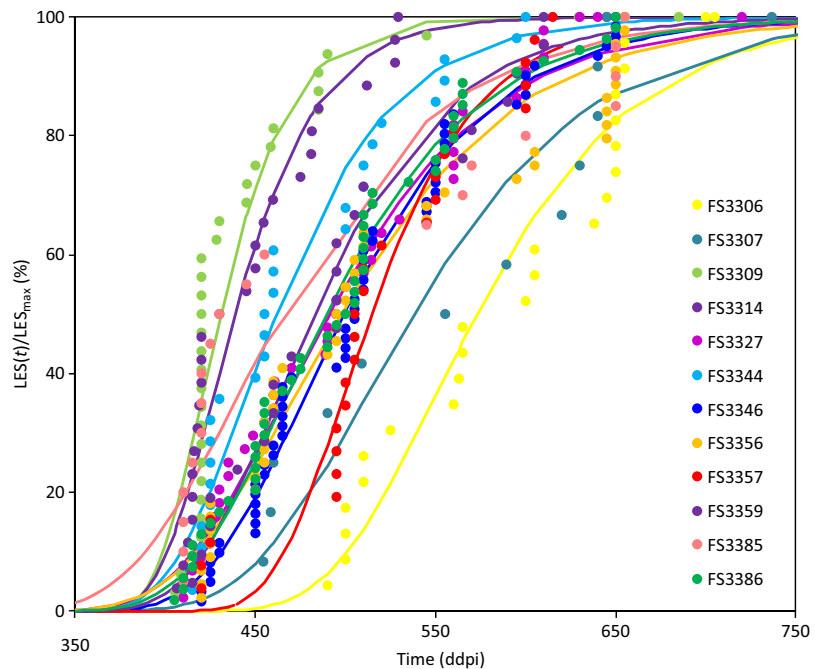
Figure 5 Cumulative normalized number of lesions (LES(*t*)/LES$_{max}$) appeared after inoculation of 4 × 12 wheat leaves with pycnidiospore suspensions of 12 *Zymoseptoria tritici* isolates. Each point corresponds to the appearance of a new sporulating lesion. Lines correspond to the fitted curves (Gompertz model). Time is expressed in degree-days post-inoculation (ddpi).

induced by 119 different isolates after ascospore inoculations, in the other set, 425 lesions induced by 12 different isolates after pycnidiospore inoculations. However, this difference in LatLES$_{37}$ was consistent with that obtained for LatPYC$_5$.

Latent period LatLES$_{37}$ after pycnidiospore inoculation was calculated for each of the 12 selected isolates (Fig. 5) and compared to their LatPYC$_5$. The two measures of latent period (LatLES$_{37}$ and LatPYC$_5$) were found to be significantly correlated (Fig. 6), with a slope close to 1. Thus, the average time of appearance of the first pycnidia is close to the average time at which the rate of sporulating lesion appearance is maximal.

The correlation between the mean values of lesion size (SIZE$_{max}$), pycnidium density (PYCdens), and latent period (LatPYC$_5$), assessed after ascospore and pycnidiospore inoculation for each of the 12 selected isolates is presented in Figure 7. For each pathogenicity component, the inter-isolate variability was higher after ascospore than pycnidiospore inoculation. The value obtained after pycnidiospore inoculation corresponds to the mean recorded on several lesions, while it corresponds to a single value recorded on one lesion after ascospore inoculation. Despite this experimental artefact, no relationship between pathogenicity components obtained after ascospore and pycnidiospore inoculation was found.

Discussion

In the experimental conditions of the present investigation, the latent period of *Z. tritici* on adult wheat leaves was significantly longer after infection with ascospores than with pycnidiospores, but no difference
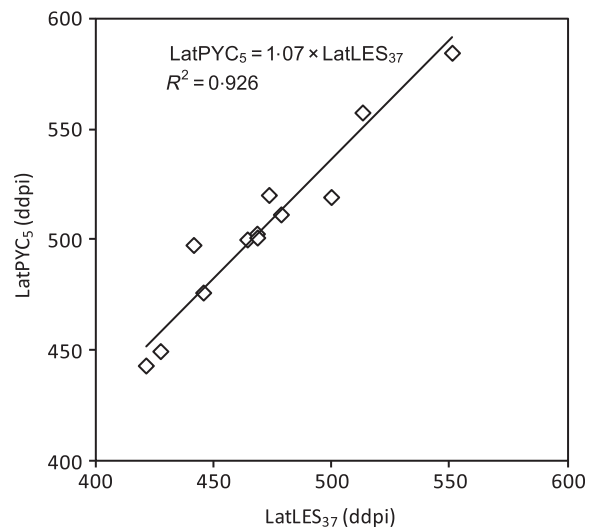

Figure 6 Assessment of the latent period (ddpi, degree-days post-inoculation) of septoria tritici blotch after inoculation of four wheat upper leaves with pycnidiospore suspensions (12 isolates, diamonds). LatLES$_{37}$ is the time elapsed from inoculation to the maximum rate of lesion appearance at the leaf scale; LatPYC$_5$ is the mean time elapsed from inoculation to the appearance of first pycnidia on the lesion.

was established for lesion size and density of pycnidia. Differences in pathogenicity between asexual and sexual spores were previously reported for only a few other ascomycete fungi (Wood & Barbetti, 1977; Gilles *et al.*, 2001; Mason & Huber, 2001; Karolewski *et al.*, 2002; Li *et al.*, 2004; Trapero-Casas & Kaiser, 2007). In contrast to those studies, the high inter-individual variation in *Z. tritici* pathogenicity (Suffert *et al.*, 2013)
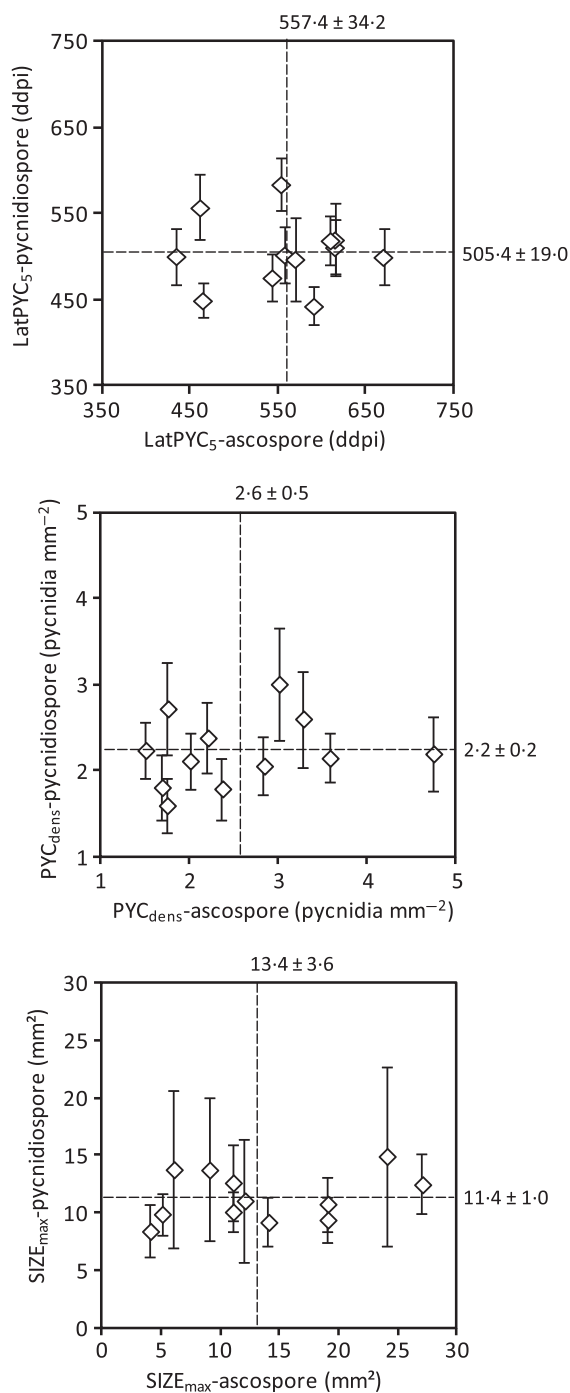
Figure 7 Latent period (LatPYC₅), pycnidium density (PYCdens) and lesion size (SIZEmax), assessed for 12 *Zymoseptoria tritici* isolates after ascospore and pycnidiospore inoculation. Each point corresponds to the mean of several values for pycnidiospore inoculation (vertical bars represent standard deviation) but to a single value for ascospore inoculation; mean values and standard deviations are given next to the dotted lines.

knowledge, this is the first report of successful infection of wheat leaves with *Z. tritici* ascospores in controlled conditions and of the subsequent development of lesions. Moreover, in all reported infection experiments, the asexual spores were conidia obtained by clonal multiplication from yeast-like mycelium grown on artificial medium, not pycnidiospores from pycnidia borne by a leaf lesion.

The difference in mean latent period between the two spore types, assessed as the maximum likely time elapsed between inoculation and the appearance of the first pycnidia (ΔLatPYC$_5$ = 55·5 ddpi), was close to the difference assessed as the maximum likely time elapsed between inoculation and the appearance of the majority of the sporulating lesions (ΔLatLES$_{37}$ = 63·5 ddpi). This consistency is supported by the significant correlation between LatLES$_{37}$ and LatPYC$_5$ assessed for the 12 selected isolates.

The longer latent period recorded after inoculation with sexual spores suggests that fungal growth that precedes the appearance of the new lesions is slower after inoculation with an ascospore than with a pycnidiospore. The early processes of infection (spore adhesion, germination, and penetration) might also be less efficient in ascospores than in pycnidiospores. However, possible differences in infection efficiency, the probability that a spore deposited on a receptive host surface produces a lesion (Pariaud *et al.*, 2009), could not be tested. Technical limitations, such as the very low amounts of collected ascospores from spore traps and the small size of both *Z. tritici* ascospores and pycnidiospores compared to spores of other fungi (e.g. *Puccinia triticina*; Azzimonti *et al.*, 2013), prevented the precise assessment of inoculum concentration in the suspensions that is required for the accurate assessment of infection efficiency.

In contrast to the results obtained in the present study, ascospores were reported to be more infectious than asexual spores in three ascomycete fungi. In *Leptosphaeria maculans*, the cause of blackleg disease on *Brassica* crops, ascospores caused more infections than pycnidiospores (Mason & Huber, 2001) and the latent period was shorter after infection with ascospores (Brunin & Lacoste, 1970; Alabouvette *et al.*, 1974). Moreover, germination and penetration, as well as the subsequent development of lesions, were much earlier with ascospores than with pycnidiospores (Li *et al.*, 2004). In *Pyrenopeziza brassicae*, the cause of light leaf spot of oilseed rape, about 25 ascospores or about 700 pycnidiospores per leaf were required for successful infection (Karolewski *et al.*, 2002), confirming that ascospores of *P. brassicae* are more infective than conidia (Gilles *et al.*, 2001). These investigations did not test for a difference in latent period between the two spore types used for inoculation. In *Didymella rabiei*, the cause of ascochyta blight of chickpea, inoculation of plants with ascospores resulted in a significantly higher disease severity than conidia, which had a slower germination process than ascospores (Trapero-Casas & Kaiser, 2007). The differences among

prompted the use of asexual and sexual spores of the same genotypic origin, produced through a two-stage inoculation procedure. To the best of the authors'

Table 4 Number of cells and volume of ascospores and pycnidiospores/conidia in four ascomycete fungi

Fungus	Number of cells		Volume (μm^3)[a]	
	Ascospore	Pycnidiospore or conidia	Ascospore	Pycnidiospore or conidia
Zymoseptoria tritici[b]	2	4–8	50	210
Pyrenopeziza brassicae[c]	1–2	1	70	80
Leptosphaeria maculans[d]	6	1	1100	7
Didymella rabiei[e]	2	1–2	330	70

[a]Volume was approximated as $\pi/6 \times a \times b^2$ (ellipsoid) with mean length *a* and mean width *b*, whatever the shape of the spore.
[b]$a = 12.5$, $b = 2.8$ μm for ascospore (range 10–15 × 2.5–3.0 μm; Shaner, 2010); $a = 59.0$, $b = 2.6$ μm for pycnidiospore (range 20–98 × 1.4–3.8 μm; Shaner, 2010).
[c]$a = 14.5$, $b = 3.0$ μm for ascospore (range 13.5–15.5 × 2.5–3.0 μm; Rawlinson *et al.*, 1978); $a = 13.0$, $b = 3.5$ μm for pycnidiospore (range 10–16 × 3.0–4.0 μm; Rawlinson *et al.*, 1978).
[d]$a = 50$, $b = 6.5$ μm for ascospore (range 30–70 × 4.0–9.0 μm; Smith & Sutton, 1964); $a = 4.0$, $b = 1.8$ μm for pycnidiospore (range 3.0–5.0 × 1.5–2.0 μm; Smith & Sutton, 1964).
[e]$a = 15.4$, $b = 6.4$ μm for ascospore (range 13.5–17.3 × 6.0–6.8 μm; Nene, 1982); $a = 13$, $b = 3.3$ μm for pycnidiospore (range, 10–16 × 3.0–5.0 μm; Punithalingam & Holliday, 1972).

these fungi might be due to differences in the size of the spores (Table 4). In *Z. tritici*, the spore volume is four times bigger for a pycnidiospore than for an ascospore. The volumes of the two types of spores are nearly equivalent in *P. brassicae*. In contrast, the spore volume is much bigger for an ascospore than for a pycnidiospore in *D. rabiei* (5×) and *L. maculans* (150×). The difference in latent period observed between the two spore types in those three fungi may be related to a differential ability to cause infection: smaller spores, which also contain a smaller number of cells (Table 4), are likely to contain less nutrient reserves (Garrett, 1973); consequently, the time required for germ tube growth and penetration into host tissues may be longer. In the case of *Z. tritici*, it may be that the smaller ascospores germinated less rapidly than the bigger pycnidiospores, and so took longer to enter the host tissue. It may also be possible that a longer latent period was due to a slower growth period after infection. Both assumptions should be tested using microscopic observations.

Differences in latent period for ascospore and pycnidiospore infections could be related to different microclimatic requirements. Temperature and wetness requirements for infection by two spore types have rarely been compared in ascomycete fungi (Karolewski *et al.*, 2002; Trapero-Casas & Kaiser, 2007). However, in the present investigation, it is unlikely that wetness duration was a limiting factor as both spore types were kept in a humid environment for a long period (72 h). The average temperature during the 3 weeks after inoculation was slightly higher for ascospores (18.9°C) than pycnidiospores (17.2°C). However, these temperatures are quite close to the thermal optimum of *Z. tritici* after conidial inoculation (*c.* 18°C; Bernard *et al.*, 2013); therefore, it is unlikely that temperature was a limiting factor for any of the spore types.

Models of the development of septoria tritici blotch that explicitly consider both ascospores and pycnidiospores, either all through the crop season (Eriksen *et al.*, 2001) or only at the onset of the epidemic (Robert *et al.*, 2008), assume that the infection process after spore deposition is the same for both spore types. The results of the present study suggest that the mean latent period during the very beginning of an epidemic, when first lesions are mainly caused by wind-dispersed ascospores (Suffert *et al.*, 2011), is longer than during the spring epidemic stage, when secondary infections are caused by splash-dispersed pycnidiospores. This difference might be increased by winter cold temperatures and the non-linearity of the thermal performance curve of *Z. tritici* (Shaw, 1990; Bernard *et al.*, 2013). Although spring infections are mainly caused by pycnidiospores splash-dispersed upwards in the wheat canopy, infections by airborne ascospores released by pseudothecia developing on wheat plants cannot be excluded (Duvivier *et al.*, 2013). The pycnidiospore surface density is likely to be high in a wheat crop from first node visible Z31 to flag leaf visible Z37 (Lovell *et al.*, 1997; Suffert & Sache, 2011) and the ascospore surface density is much lower (Hunter *et al.*, 1999; Suffert & Sache, 2011). However, environmental conditions (wind versus rainfall) could affect the relative availability of ascospores versus pycnidiospores, so inducing infections on upper leaves by ascospores rather than by pycnidiospores; this has been sporadically suspected in field observations (C. Maumené, Arvalis-Institut du Végétal, Boigneville, personal communication). A 60-dd difference in latent period seems to be minor at the epidemic scale, but this delay should be incorporated into epidemiological models. Moreover, forecasting models used for timing fungicide sprays might be improved if the difference in latent period between the two spore types were considered.

Acknowledgements

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