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Fashionably late partners have more fruitful encounters: Impact of the timing of co-infection and pathogenicity on sexual reproduction in *Zymoseptoria tritici*

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ABSTRACT

The wheat pathogen *Zymoseptoria tritici* is a relevant fungal model organism for investigations of the epidemiological determinants of sexual reproduction. The objective of this experimental study was to determine which intrinsic factors, including parental fitness and timing conditions of infection, affect the numbers of ascospores produced. We first performed 28 crosses on adult wheat plants in semi-controlled conditions, with 10 isolates characterized for their fitness traits. We validated the efficiency of the crossing method, opening up new perspectives for epidemiological studies. We found that the ability to reproduce sexually was determined, at least partly, by the parental genotypes. We also found that the number of ascospores released was correlated with the mean size of the sporulating lesions of the parental isolates on the one hand, and the absolute difference in the latent periods of these isolates on the other. No functional trade-off between the two modes of reproduction in *Z. tritici* was revealed: there was no adaptive compromise between pathogenicity (asexual multiplication on leaves) and transmission (intensity of sexual reproduction on wheat debris). Moreover, a few days' difference in the latent periods of the two parental isolates, such that one progressed more rapidly in the host tissue than the other, seemed to be slightly beneficial to ascosporogenesis. This may be because the first parental isolate breaks down host defenses, thereby facilitating infection for the other parental isolate. However, a larger difference (a few weeks), generated by leaving two to three weeks between the inoculations of the plant with the parental isolates, was clearly detrimental to ascosporogenesis. In this case, the host tissues were likely colonized by the first isolate, leaving less host resources available for the second, consistent with a competition effect during the asexual stage.

1. Introduction

The heterothallic ascomycete *Zymoseptoria tritici* causes Septoria tritici blotch, a foliar disease of wheat (*Triticum aestivum*) found in most wheat-growing areas worldwide. During the growing season, the disease is propagated clonally between wheat plants by asexual pycnidiospores, which are splash-dispersed over short distances and act as the main secondary inoculum. The rate of development of the epidemic, which progress vertically in the canopy, is determined by the number of asexual multiplication cycles completed by the pathogen, which depends principally on host susceptibility, temperature and rain events. Septoria tritici blotch epidemics are initiated by airborne sexual ascospores mostly produced on infested wheat debris left in the field between consecutive crops (Scott et al., 1988; Eriksen and Munk, 2003; Suffert et al., 2011; Morais et al., 2016). Thus, ascospores and pycnidiospores may both be involved in the same stages of epidemic development: in a wheat monoculture system in which infested debris is not completely buried, pycnidiospores can theoretically act as primary inoculum (Suffert and Sache, 2011). Similarly, ascospores, discharged either from old debris on distant plots or from nearby infected wheat plants, can act as secondary inoculum (Clinckemaillie et al., 2010; Duvivier et al., 2013; Duvivier, 2015).

Z. tritici is considered to be a rapidly evolving pathogen with high genetic diversity and genomic plasticity, partly due to its highly active sexual reproduction cycle (e.g., Linde et al., 2002; Wittenberg et al., 2009). A reciprocal cross-infection experiment

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recently showed that seasonality can affect local adaptation patterns in a *Z. tritici* population (Suffert et al., 2015). Indeed, isolates obtained from upper leaves at the end of a field epidemic appeared to be more adapted to winter conditions for fitness components relating to sporulation intensity and more adapted to spring conditions for fitness traits relating to the latent period (generation time) than isolates obtained from the basal leaves at the beginning of the epidemic. This was interpreted as the result of short-term seasonal selection driven by a combination of temperature and host stage. Comparisons of these findings with evolutionary ecology concepts suggest that there are probably counterselection mechanisms at work during the period between epidemics (van den Berg et al., 2011). A functional trade-off between the two modes of reproduction in epidemic and inter-epidemic periods is entirely plausible, as shown experimentally (e.g., Susi and Laine, 2013; Pasco et al., 2015) and described in reviews (Laine and Barrès, 2013). Life-history trade-offs in pathogens are of particular interest because they may constrain epidemiological dynamics at various spatiotemporal scales. Such trade-offs would be expressed as a phenotypic compromise between fitness traits representative of the two modes of reproduction in *Z. tritici*. Ascospores are the main form of primary inoculum in this species. There may, therefore, be a trade-off between asexual and sexual reproduction in green wheat tissues gradually becoming senescent and in debris. We tested this hypothesis indirectly in this study.

Sexual reproduction requires a physical encounter between two compatible strains (Mat1-1 and Mat1-2; Waalwijk et al., 2002). The leaf lesions caused by the two parental strains must therefore either coalesce or be located very close together. The severity of foliar disease, characterized by the density of lesions, is, thus, a crucial determinant (Suffert et al., unpubl.). This has been confirmed at field scale: more severe epidemics are associated with higher levels of ascospore production during epidemics occurring in the following year (Cowger et al., 2002). However, it remains unclear how close together infections need to be for effective mating to occur. Metcalfe (1998) found internal hyphae spanning mean distances of no more than 11 mm in a susceptible cultivar just before the production of *Z. tritici* pycnidia, suggesting that infection foci located more than 20 mm apart may be isolated. The limits to the time period between infections with the two parental strains for sexual reproduction to occur have never been studied in *Z. tritici* or any other heterothallic fungal plant pathogen.

The overall objective of this study was to identify experimentally the factors, including fitness traits and infection conditions for the parental strains, likely to influence the intensity of sexual reproduction. We first developed a method for crossing *Z. tritici* isolates on adult wheat plants in semi-controlled conditions such that: (i) the number of progeny was maximized (number of ascospores released) to obtain different intensities of sexual reproduction and to assess their variability; (ii) contamination with immigrant strains was minimized, to obtain a pure progeny and, thus, to prevent overestimation of the intensity of sexual reproduction. Is one *Z. tritici* strain intrinsically more suitable for sexual reproduction than another? Is there a functional trade-off between asexual multiplication and sexual reproduction in *Z. tritici*? We addressed these questions by crossing simultaneously 10 selected parental isolates for which aggressiveness had already been characterized during the asexual period (Suffert et al., 2015): (i) to quantify their ability to reproduce sexually; (ii) to identify the factors influencing the intensity of sexual reproduction; (iii) to test the hypothesis of an adaptive compromise between the foliar pathogenicity of a strain (size of sporulating lesions and latent period during the asexual stage) and its ability to reproduce sexually on wheat debris. In field conditions, co-infections with two compatible strains do not necessary occur at precisely the same time on individual leaves. Moreover, even if they are concomitant, the lesions caused by the two strains may develop at different rates due to developmental differences during the infection phase. This raises questions about the impact of a time offset in the infection dynamics of the two parental strains. In practice, the offset between the two infections may be small or large, and may have two causes: (i) a delay in lesion dynamics due to differences in latent period between compatible strains, despite the occurrence of concomitant infections or (ii) a time interval between the infections with the two compatible strains. We addressed these questions by assessing the impact on ascospore production of co-inoculation with parental isolates with different latent periods, using the results obtained in the previous experiment testing the trade-off hypothesis. We used a small number of isolates for inoculation experiments, inoculating each plant with two isolates, two or three weeks apart. We then assessed the effects on ascospore production.

2. Materials and methods

2.1. Fungal material

Ten *Z. tritici* isolates differing in aggressiveness and mating type were selected for this study (Table 1; Fig. 1). They were sampled during the 2009–2010 cropping season from a field of the wheat cv. Soissons (Grignon, France, 48°51′N, 1°58′E; Suffert et al., 2015). They were characterized on adult plants of wheat cv. Soissons for the size of sporulating lesions (SPO; maximum percentage of the area covered by pycnidia, expressed as a %), the latent period (LAT; the time between inoculation and the appearance of first pycnidia, expressed in degree-days), the density of pycnidia (PYCdens; number of pycnidia per unit of sporulating area), and sporulation capacity (nbSPO; number of pycnidiospores produced by a pycnidium), as described by Suffert et al. (2013) (Video S1). The mating type of the 10 isolates was determined by PCR amplification of the two mating type idiomorphs (Waalwijk et al., 2002).

For each isolate, we obtained inoculum from stock suspensions of conidia stored at −80 °C. Subcultures of each isolate were grown for five days in Petri dishes containing PDA (potato dextrose agar, 39 g L^{-1}) at 18 °C in the dark. Conidial suspensions were prepared as described by Suffert et al. (2013) and their densities were adjusted to 2×10^5 conidia mL^{-1} with a Malassez counting chamber (Video S1). Two drops of surfactant (Tween 20; Sigma, France) were added to the suspension. Biparental suspensions, containing a Mat1-1 isolate and a Mat1-2 isolate, were prepared by mixing 15 mL each of the appropriate individual parental suspensions.

In a first experiment, we used the 10 parental isolates to perform 28 crosses (Table 1). In a second experiment, four of the 10 parental isolates (I03, I04, I12, I24) were selected on the basis of their high levels of sexual reproduction (see results of the first experiment; Table 2) and were used to perform four crosses (I03 × I12, I03 × I24, I04 × I24, I04 × I12).

2.2. Plant material

Seeds of the wheat cv. Soissons (moderately susceptible to Septoria tritici blotch) were sown on December 6th 2012 and on December 4th 2013, in Jiffy peat pots kept in greenhouse conditions for two weeks. Seedlings were vernalized in a growth chamber for 8 weeks at 8 °C with a 10-h light/14-h dark photoperiod. They were then returned to the greenhouse and left to acclimate for one week before transplantation into individual pots filled with 1 L of commercial compost (Klasmann Peat Substrat 4; Klasmann France SARL, France). We added 4 g of slow-release fertilizer (Osmocote Exact 16-11-11N-P-K 3MgO Te) to each pot. The plants were also treated with Hydrokani C2 fertilizer (Hydro Agri

Table 1
Table of the 28 *Zymoseptoria tritici* crosses.

Parental isolates			Fitness traits[b]				Crosses[a]									
Name	Code	Mating type	LAT[c]	SPO[d]	PYCdens[e]	nbSPO[f]	I03	I04	I05	I07	I08	I09	I12	I24	I25	I28
INRA09-FS0802	I03	Mat1-1	509.6	55.0	9.5	1.17×10^7										
INRA09-FS0808	I04	Mat1-1	482.6	71.0	11.7	2.22×10^7										
INRA09-FS0813	I05	Mat1-1	386.7	63.3	11.9	1.40×10^7										
INRA09-FS0732	I07	Mat1-2	466.0	48.1	11.4	1.28×10^7	×	×	×							
INRA09-FS0799	I08	Mat1-2	445.4	53.8	12.3	4.27×10^7	×	×	×							
INRA09-FS0800	I09	Mat1-1	436.3	69.4	12.0	3.78×10^7				×	×					
INRA09-FS0806	I12	Mat1-2	394.8	68.1	14.7	5.43×10^7	×	×	×		×	×				
INRA09-FS1006	I24	Mat1-2	407.6	70.6	13.2	3.73×10^7	×	×	×		×	×				
INRA09-FS1008	I25	Mat1-2	396.1	37.5	13.7	2.17×10^7	×	×	×		×	×				
INRA09-FS1022	I28	Mat1-1	400.8	68.8	13.9	4.28×10^7				×	×		×	×	×	

[a] Each cross is indicated by ×.
[b] Assessed on wheat adult plants (cv. Soissons) by Suffert et al. (2015).
[c] Latent period.
[d] Size of sporulating lesions.
[e] Density of pycnidia.
[f] Number of pycnidiospores.

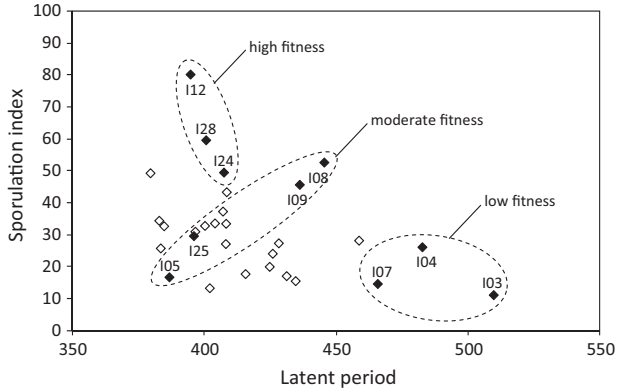

Fig. 1. Fitness of the 10 parental isolates of *Z. tritici* used in the 28 crosses. The latent period is expressed in degree-days (dd) post inoculation. The sporulation index was calculated as PYCdens × nbSPO/10^7 (Suffert et al., 2015). Fitness traits were characterized on adult plants of the wheat cv. Soissons. ◆ Represents the ten parental isolates; ◇ represents the other 20 isolates concomitantly collected in the field.

Table 2
Ascospore production from 28 *Zymoseptoria tritici* crosses. Ascospore discharge index ADI_{ma} (mean number of ascospores discharged per g of dry wheat debris placed in a box under a moist atmosphere) and ADI_{da} (mean number of ascospores discharged per g of moistened wheat debris placed in a box under a dry atmosphere), are indicated in bold and in italics, respectively.

Isolate	I03	I04	I05	I07	I08	I09	I12	I24	I25	I28
I03										
I04										
I05										
I07	**7** *476*	**0** *9*	**98** *314*[a]							
I08	**16** *194*	**15** *29*	**22** *43*							
I09				**36** *166*	**0** *36*					
I12	**36** *967*[a]	**66** *569*[a]	**0** *181*		**3** *104*	**26** *695*				
I24	**10** *538*[a]	**29** *234*	**32** *193*		**1** *85*	**0** *42*				
I25	**3** *198*[a]	**0** *0*	**0** *67*		**0** *0*	**0** *0*				
I28				**0** *52*	**0** *22*		**8** *172*[a]	**2** *213*	**0** *132*	

[a] Including the 30 progeny isolates (from 28 to 30) genotyped with 16 SSR neutral markers (multiplex 1 and multiplex 2; Gautier et al., 2014).

Spécialités, France), diluted 1:100 and poured into the saucers under the pots four and six weeks after transplantation. Plants were sprayed once with spiroxamine (Aquarelle SF at 2 mL L^{-1}; Bayer CropScience, France) for the specific prevention of powdery mildew (*Blumeria graminis* f.sp. *tritici*), no later than six weeks before inoculation. During plant growth, the plants were illuminated with natural daylight supplemented with 400-W sodium vapor lamps between 6.00 a.m. and 9.00 p.m. The air temperature was kept below 20 °C during the 15-h light period and above 12 °C during the 9-h dark period. Plants were thinned to three stems per pot during the growth period.

2.3. Inoculation procedure

In the first experiment, which tested the hypothesis of a functional trade-off between modes of reproduction, co-inoculation with 28 biparental suspensions was carried out in the greenhouse (Table 2) on April 16th 2013, after the wheat heads had fully emerged. Each biparental suspension was applied with an atomizer (Ecospray, VWR, France) to two adult plants (six stems). The plot was turned during the 10-s spraying period, to ensure even coverage of the plants with inoculum. Infection was promoted and cross-contamination prevented by enclosing each pair of plants inoculated with the same suspension for 72 h in a sealed transparent polyethylene bag humidified with distilled water.

In the second experiment, which was performed to test the impact of a time interval between the infections with the two parental isolates, four series of inoculations were carried out. The first and second series were carried out on April 14th 2014, with the application of biparental suspensions (Δ0-mix; I03 × I12, I03 × I24, I04 × I24, I04 × I12) or of each of the two monoparental suspensions successively (Δ0-succ). The third and fourth series involved the application of two monoparental suspensions separately 15 (Δ15) and 22 (Δ22) days apart, respectively. For the third and the fourth series, the inoculations with the first monoparental suspension were carried out on April 14th 2014. The inoculations with the second monoparental suspension were carried out on April 29th 2014 for the third series and on May 6th 2014 for the fourth series. For pairs of monoparental suspensions used to inoculate the same plant, each suspension was tested as both the first and second isolate to infect the plant. Thus, each cross $I_i \times I_j$ was performed with six treatments: biparental suspension (I_i-I_j), successive inoculations not separated by a time interval (I_i and I_j), successive inoculations 15 days apart (I_i then I_j; I_j then I_i), successive inoculations 22 days apart (I_i then I_j; I_j then I_i). Each treatment

was performed on two adult plants (six stems); in total, 48 wheat plants were inoculated.

In each experiment the crosses of all isolate pairs were performed simultaneously, once. We considered that multiple crossing events occurring in several sites (leaves, stems) are biological replications. The experiment was not replicated *sensu stricto* (i.e. at different time in the year, for example when outside conditions could be different) because testing the effect of crossing regime was not the objective.

2.4. Promotion of sexual reproduction and ascosporogenesis

Co-inoculated wheat plants were kept in a greenhouse for 15 weeks. Air temperature was kept above 15 °C and reached 30 °C several times during July. During the first experiment, disease severity on leaves was assessed by eye, for each pair of co-inoculated plants, as the percentage of the leaf area displaying sporulation (mean for F1, F2, and F3 leaves) on May 10th 2013. From August 1st to October 29th 2013 and from August 7th to November 18th 2014, plants were placed outdoors, against the west side of the greenhouse, to induce the emergence and maturation of pseudothecia (Fig. 2a). Each pair of co-inoculated plants was tied up with stiff wire, with the stems and leaves clamped so as to avoid contact with other pairs of plants. On October 31st 2013 and November 19th 2014, dry leaves and stems from each pair were cut into 2-cm fragments and kept in separate open boxes in a laboratory atmosphere at 18 °C for one week, to allow them to dry out gently.

2.5. Assessment of the intensity of sexual reproduction

Ascospore production for each cross was used as a proxy for the intensity of sexual reproduction. Ascospore release from the debris of each pair of co-inoculated plants was quantified as described by Suffert et al. (2011). Forcible ascospore discharge is driven by the build-up of hydrostatic pressure within the mature ascus (Ingold, 1933) and can be induced by a rapid change in the humidity of the air (Hildebrand, 2002). We therefore considered two types of discharge event: the collection of ascospores released from dry wheat debris placed in a box under a moist atmosphere, and the collection of ascospores released from moistened wheat debris placed in a box under a dry atmosphere.

In the first experiment, each pair of discharge events was replicated once. For the first type of discharge (on November 6th and 19th 2013), dry debris fragments were weighed and spread out evenly over wet filter paper; excess water was removed and the filter paper and debris were placed in a box (24 × 36 cm) and spayed lightly with water. Eight Petri dishes (90 mm in diameter) containing PDA medium were then placed upside down 1 cm above the debris (Fig. 2b). The boxes were sealed to increase the relative humidity of the air around the debris gradually. The boxes were placed at 18 °C in the dark for 6 h. The Petri dishes were then closed and incubated in the same conditions. For the second type of discharge (on November 20th and 29th 2013), the same debris fragments were soaked in water for 20 min and spread on dry filter paper in a box, the lid of which was left half open to decrease the relative humidity of the air around the debris. Petri dishes were placed in the box and treated as described above.

In the second experiment, each pair of discharge events was replicated five times, from January to March 2015. As in the first experiment, both types of discharge were considered. For the first type of discharge, dry debris fragments were weighed, spread out evenly over wet filter paper in a box and sprayed lightly with water. For the second type of discharge, the same debris fragments were soaked in water for one hour and then spread out over dry filter paper. Debris was stored in laboratory conditions between replicates.

The ascospores released onto the Petri dishes germinated after 24 h. Six days later, yeast-like colonies resembling cream-colored convoluted heaps were observed. The colonies were counted under a microscope five and eight days after ascospore discharge (Fig. 2c). It was assumed that each colony resulted from the germination of a single ascospore, and that clusters of colonies appeared just above mature pseudothecia from which several ascospores had been discharged. Examination under a binocular microscope identified several pseudothecia on leaf sheaths above which a high density of colonies had appeared. Most of the pseudothecia were aligned along vascular bundles in the stem debris (Fig. 2d and e). Some of the vascular bundles were hollow, as if their contents had decayed in the climatic conditions outdoors.

An ascospore discharge index (ADI), defined as the number of ascospores discharged per gram of wheat debris (Suffert et al., 2011), was calculated for each discharge event, as follows:

$$\mathrm{ADI} = \frac{1}{8} \cdot \frac{x \cdot y}{\pi \cdot r^2} \cdot \frac{\mathrm{NBcol}}{\mathrm{DW}}$$

where NBcol is the total number of colonies in the eight Petri dishes, DW is the total dry weight of debris spread out in the box, r is the radius of a Petri dish (4.5 cm), x and y are the width and length of the box (24 cm and 36 cm, respectively).

2.6. Progeny analysis

In the first experiment, sets of progeny isolates from six crosses (344 isolates from I03 × I12, 157 from I03 × I24, 156 from I03 × I25, 179 from I04 × I12, 168 from I05 × I07, and 149 from I12 × I28) were collected five days after ascospore discharge onto the Petri dishes. Monospore suspensions were prepared in sterile 1:1 glycerol-water solution and stored at −80 °C. The eight parental isolates and 30 ± 1 progeny isolates from each of the six crosses were genotyped for 15 simple sequence repeat (SSR) markers (St1-E7, St2, St3B, St3C, St6, St7 and St12 in multiplex 1; St4, St5, St9, St10, St11 and St13 in multiplex 2; Gautier et al., 2014) and the mating-type marker (Waalwijk et al., 2002). The parental origin of the progeny isolates was determined, to estimate the proportion of *Z. tritici* immigrants acting as contaminants. Cross quality was also characterized by calculating the mean segregation ratio of parental alleles following sexual reproduction for each polymorphic locus for all crosses, and for each cross for all loci.

2.7. Statistical analysis

In the first experiment, we analyzed the effect of the mean aggressiveness of each pair of parental isolates on leaf disease severity, by linear regression analysis with the mean size of sporulating lesions for the two parental isolates (mSPO) and the mean latent period for the two parental isolates (mLAT) as explanatory variables (model 1). We analyzed the effect of the parental isolates on the intensity of sexual reproduction by analysis of covariance (ANCOVA) (model 2) on ascospore discharge indexes (ADI), with the parental isolates for each as a fixed factor. We included the humidity conditions during the discharge event (dry wheat debris placed in a box under a moist atmosphere or moistened wheat debris placed in a box under a dry atmosphere) and the leaf disease severity obtained after co-inoculation with the two parental isolates as fixed effects in the model, because the intensity of sexual reproduction is known to depend on both these factors (Cowger et al., 2002; Trail et al., 2002). We analyzed the effects of traits of the parental isolates on ADI by ANCOVA (model 3) with the mean size of sporulating lesions of the two parental isolates (mSPO) and the absolute difference between the latent periods of the two parental isolates (ΔLAT) as fixed effects. As in the previous model, we included humidity conditions and leaf disease severity as fixed

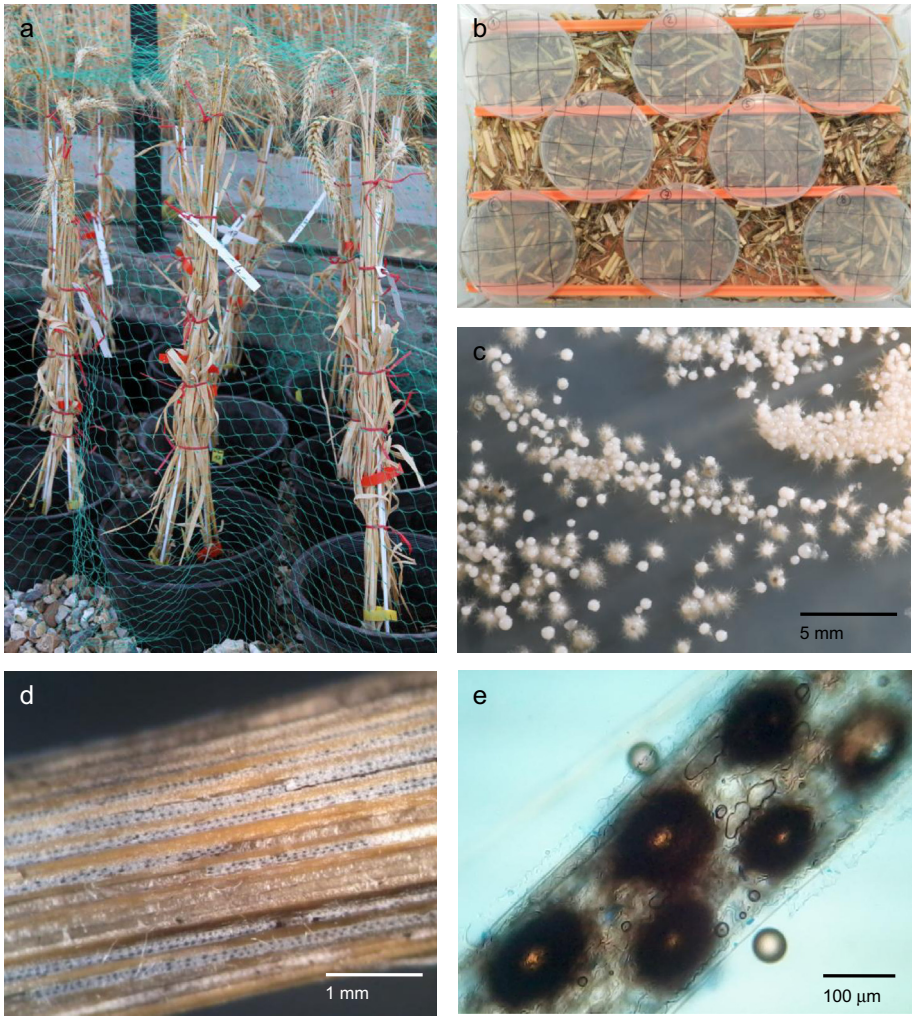

Fig. 2. (a) Wheat plants co-inoculated with *Zymoseptoria tritici* parental isolates and stored outdoors to induce the production and maturation of pseudothecia. (b) Petri dishes containing PDA medium placed upside down above wheat debris fragments to collect ascospores after their discharge. (c) Clustered yeast-like colonies of *Z. tritici* on PDA, eight days after ascospore discharge. (d and e) Pseudothecia aligned along vascular bundles on stem debris.

effects. For all analyses, a log(x + 1) transformation was applied to the ADI variable to satisfy the requirements of the statistical tests. Furthermore, data were excluded if ADI < 1 ascospore g^{-1} (37/112), as we considered sexual reproduction to have been unsuccessful in such cases due to inappropriate experimental conditions for the cross or for the maturation of pseudothecia. All analyses were performed with R software version 3.0.1 (R Core Team, 2013).

In the second experiment, we assessed the impact on ADI of the time interval between the inoculations with the two parental isolates by ANOVA (model 4). The time interval between the inoculations with the two parental isolates was included as a factorial fixed effect (Δ0-mix, Δ0-succ, Δ15 and Δ22). The humidity conditions during the discharge event (dry wheat debris placed in a box under a moist atmosphere or moistened wheat debris placed in a box under a dry atmosphere) and the cross (I03 × I12, I03 × I24, I04 × I24, I04 × I12) were included as fixed effects.

3. Results

3.1. Quality of the crossing method

The segregation ratio of each parental allele, based upon 30 ± 1 isolates per progeny, for all six crosses for which the progeny was analyzed, was close to one (0.76 for I04 × I12, 1.05 for I03 × I24, 0.98 for I03 × I25, 1.01 for I12 × I28, 1.21 for I05 × I07, 1.00 for I03 × I12; Fig. S2 and Table S3). The overall mean ratio was 1.02. The allele ratios estimated for each marker ranged from 0.90 to 1.10 for nine of the 16 markers and from 1.10 to 1.50 for seven markers (Fig. 3). Non-parental alleles were detected in only two isolates resulting from the I03 × I24 and I03 × I27 crosses. Final genotypic analysis of the 177 progeny isolates indicated a level of contamination of 0.7%. This level of contamination is very low and demonstrates the reliability of the crossing method.

3.2. Impact of the fitness traits of the parental isolates on the intensity of sexual reproduction

In model 1, the effects of the mean size of sporulating lesions (mSPO) and the mean latent period (mLAT) for the two parental isolates on leaf disease severity (SEV) were not significant ($P = 0.354$ and $P = 0.609$, respectively; Table 3).

Models 2 and 3 revealed a significant effect of discharge conditions (HUMdis; $P < 0.001$, Table 4a and b). On average, more than 10 times as many ascospores were discharged when dry debris was placed under a moist atmosphere than when moistened debris was placed under a dry atmosphere (204.7 ascospores g^{-1} of debris versus 14.6). These findings were corroborated by those of the second experiment (12.5 ascospores g^{-1} of debris versus 2.0),

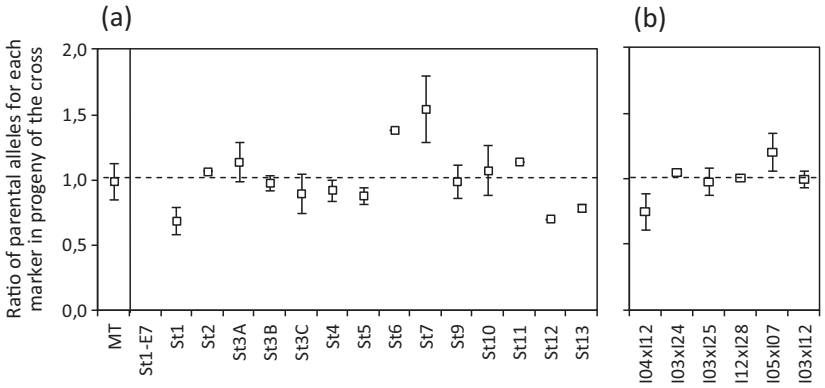

Fig. 3. Mean segregation ratios of parental alleles for the mating-type marker (MT marker) and 15 SSR markers in progeny (30 ± 1 isolates per cross) from six *Zymoseptoria tritici* crosses (I04 × I12, I03 × I24, I03 × I25, I12 × I28, I05 × I07, I03 × I12; see Table 1). (a) The mean ratios were calculated by pooling the allele ratios for each cross; (b) the mean ratios were calculated by pooling the ratios for each MT and SSR marker.

Table 3
Analysis of variance (type II tests) of disease severity (SEV) assessed on wheat plants after 28 co-inoculations with *Zymoseptoria tritici*, as a function of the mean aggressiveness of each pair of parental isolates, estimated by determining the mean size of sporulating lesions and the mean latent period for the two parental isolates.

Source of variation	df	Sum of squares	*F* statistics	*P*
Mean size of lesions (mSPO)	1	183.5	0.89	0.354
Mean latent period (mLAT)	1	55.2	0.27	0.609
mSPO × mLAT	1	642.3	3.12	0.090
Residual	24	4934.8		

confirming that the forcible discharge of ascospores from mature pseudothecia on wheat debris is promoted by a change in relative humidity.

Model 2 showed that the number of ascospores discharged decreased significantly with leaf disease severity (r = −0.02, $P = 0.047$; Table 4a) and that parental genotypes had a significant effect ($P < 0.001$; Table 4a). Pairwise comparisons between the parental isolates in model 2 (Table S4) showed that the ADI obtained with parental isolate I08 crossed with another parent was significantly lower than that obtained for crosses obtained with parental isolate I12 with another parent ($P = 0.018$, after Bonferroni correction for multiple comparisons) and tended to be lower than that for crosses involving I05 or I03 ($P = 0.061$ and $P = 0.072$, respectively). In the absence of correction for multiple comparisons, nine of the 10 parental isolates (i.e., all except I25) were significantly different from at least one other parental isolate (Fig. 4).

3.3. Impact on the intensity of sexual reproduction of a time offset in the infection dynamics of the two parental isolates

Model 3 showed that ADI increased slightly but significantly with the mean size of sporulating lesions for the two parental isolates (mSPO; r = 0.05, $P = 0.028$) and the absolute difference in latent period between the two parental isolates (ΔLAT; r = 0.10, $P = 0.014$; see Table 4b for more details). Thus, quantitative traits of pathogenicity of the pair of parental isolates had a slight, positive impact on their ability to cross.

Model 4 (Table 5) showed that the time interval between the inoculations with each of the parental isolates (ΔTinoc) had a significant effect ($P < 0.001$). The difference in the intensity of sexual reproduction between Δ0-mix (16.7 ascospores g^{-1} of debris) and Δ0-succ (11.6 ascospores g^{-1} of debris) was not statistically significant (Fig. 5). However, the difference between Δ0 and Δ15 (6.2 ascospores g^{-1} of debris) or Δ22 (1.4 ascospores g^{-1} of debris) was statistically significant: the larger the time interval between the two inoculations, the smaller the number of ascospores released. An interval of more than two weeks between the two inoculations appeared to be detrimental to sexual reproduction.

Table 4
Analysis of variance for the ascospore discharge index (ADI) assessed on wheat plants after 28 co-inoculations with *Zymoseptoria tritici*, (a) as a function of the humidity conditions during the discharge event, the parental isolates, the disease severity assessed on wheat plants after co-inoculation, and (b) as a function of the humidity conditions, the absolute difference in latent periods, the mean size of sporulating lesions for each pair of parental isolates, and disease severity. Data were excluded if ADI < 1 ascospore g^{-1}. A logarithm transformation log(ADI + 1) was applied. Bold values are used for $P < 0.05$.

Source of variation[a,b]	df	Sum of squares	*F* statistics	*P*
(a)				
Humidity conditions (HUMdis)	1	83.80	78.11	**<0.001**
Parental isolate (PAR)	9	40.11	4.15	**<0.001**
Disease severity (SEV)	1	4.42	4.12	**0.047**
Residual	62	66.52		
(b)				
Humidity conditions (HUMdis)	1	80.33	58.87	**<0.001**
Difference in latent periods (ΔLAT)	1	8.63	6.32	**0.014**
Mean size of lesions (mSPO)	1	6.83	5.00	**0.028**
Disease severity (SEV)	1	0.07	0.05	0.824
Residual	69	94.15		

[a] Interactions between factors were not significant, except for HUMdis × PAR ($P < 0.005$).
[b] Interactions between factors were not significant, except for HUMdis × SEV ($P < 0.005$).

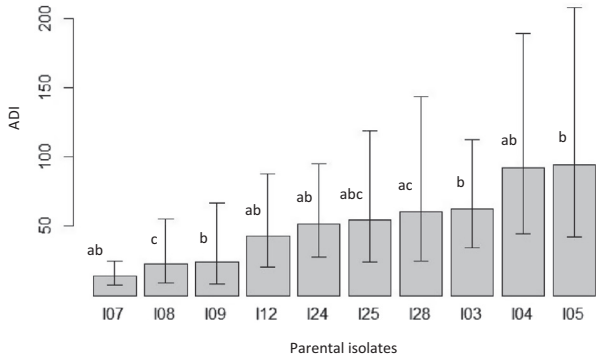

Fig. 4. Effect of genotype (parental isolates I03, I04, I05, I07, I08, I09, I12, I24, I25, I28) on the ascospore discharge index (ADI) of the 28 *Zymoseptoria tritici* crosses. Different letters indicate significant differences between means ($P < 0.05$) without multiple testing correction.

Table 5
Analysis of variance for the ascospore discharge index (ADI) assessed on wheat plants after 28 co-inoculations with *Zymoseptoria tritici*, as a function of the humidity conditions during the discharge event, the time interval between the inoculations with the parental isolates, and the parental isolate. A logarithmic transformation log (ADI + 1) was used. Bold values are used for $P < 0.05$.

Source of variation	df	Sum of squares	*F* statistics	*P*
Humidity conditions (HUMdis)	1	6.87	29.61	**<0.001**
Time between inoculations (ΔTinoc)	3	11.93	17.14	**<0.001**
Parental isolate (PAR)	3	2.80	4.02	**0.008**
ΔTinoc × PAR	9	7.10	3.40	**<0.001**
HUMdis × ΔTinoc	3	1.72	2.48	**0.06**
HUMdis × PAR	3	0.79	1.13	0.33
Residual	217	50.34		

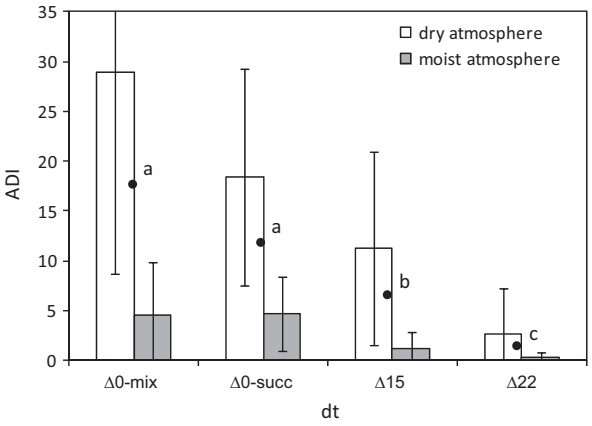

Fig. 5. Effect of a time interval between the inoculations with the two parental isolates (ΔTinoc) on the ascospore discharge index (ADI; number of ascospores per gram of debris) in four *Zymoseptoria tritici* crosses (I03 × I12, I03 × I24, I04 × I24, I04 × I12), taking into account the humidity conditions during spore discharge (dry and moist atmosphere). Co-inoculations were performed by applying a biparental suspension (Δ0-mix), by applying two monoparental suspensions successively (Δ0-succ), or by applying the first and the second monoparental suspensions separately, 15 days (Δ15) or 22 days (Δ22) apart. Vertical bars represent the standard deviation; different letters indicate significant differences ($P < 0.05$) between means (black points).

The mean number of ascospores collected differed significantly between crosses (i.e. pairs of isolates). For example, crosses including the parental isolate I24 resulted in a higher intensity of sexual reproduction (13.1 or 6.6 ascospores g^{-1} of debris, when the second parent was I03 or I04, respectively; data not shown) than crosses including the parental isolate I12 (5.6 and 3.7 ascospores g^{-1} of debris, when the second parent was I04 or I03, respectively).

4. Discussion

4.1. An efficient crossing method

We developed a method for crossing *Z. tritici* isolates on adult wheat plants in semi-controlled conditions. Current methods are based exclusively on the simultaneous co-inoculation of wheat seedlings (Kema et al., 1996) and are therefore less time-consuming than the method described here. However, this method based on the co-inoculation of adult plants not only provides progeny for genetic studies, but can also be used to assess the intensity of sexual reproduction, a crucial fitness trait for quantitative epidemiological studies based on evolutionary ecology concepts (e.g., the trade-off between pathogenicity and transmission in polycyclic plant pathogens; van den Berg et al., 2011; Laine and Barrès, 2013).

Progeny analysis revealed that the SSR markers displayed Mendelian inheritance, with segregation of the parental alleles in a 1:1 ratio. The rate of contamination with immigrant ascospore isolates was extremely low probably because the plants were already totally senescent when they were placed outdoors and they were not, therefore susceptible to additional infections. However, accurate assessment of the rate of contamination was necessary because sexual reproduction can occur despite the lack of asexual propagation of one of the parental isolates (e.g., an avirulent strain; Kema et al., 2010; Ware, 2006) within the host tissues.

Only three of the 28 crosses failed (I04 × I25, I08 × I25 and I09 × I25). The cause of this failure was not mating-type incompatibility between isolates, as the crosses between I25 and other Mat1-1 isolates were successful (I03, I05 and I28). A lower capacity for sexual reproduction of isolate I25 is one possible explanation. This variability illustrates that each isolate can be classified according to its ability to reproduce sexually, which may be determined genetically. Finally, our results demonstrate that the crossing method was efficient and reliable, despite being only semi-controlled. They also confirm that the outdoor environment is favorable for pseudothecium formation and ascosporogenesis, given that no successful crosses have yet been achieved with plants maintained exclusively in greenhouse conditions.

Suffert and Sache (2011) previously used a transition from dry to moist conditions to promote ascospore release for the quantification of ascospore production on debris collected from a wheat field. Transition from moist to dry conditions was also used by Kema et al. (1996) for progeny collection. This second method is more efficient if only small numbers of pseudothecia are present on plant tissues.

4.2. Encounters between Z. tritici strains are more likely to be fruitful if one of the strains is "fashionably late"

The first experiment showed that a difference in the latent period (Suffert et al., 2013; Morais et al., 2015) of the two *Z. tritici* parents, leading a difference in the early infection dynamics of the two isolates after concomitant leaf inoculations, resulted in slightly but significantly higher levels of sexual reproduction. It therefore seems to be beneficial for one of the two parental isolates to progress slightly more rapidly in host tissues (a few days ahead) than the other isolate during the biotrophic stage, mimicking a synergistic effect.

The short time offset in the early infection dynamics of the two parental isolates may have a positive direct effect on physiological processes involved in sexual reproduction, or an indirect effect on crossing ability exerted through processes involved in the asexual multiplication preceding sexual reproduction. Hypothetically, the first isolate may break down host defenses before the second isolate, thereby accelerating its infection dynamics and increasing subsequently its ability to reproduce both sexually. This hypothetical synergistic effect may favor "double-site infections with multiple genotypes" (Clément et al., 2012) having a quantitative difference in aggressiveness. However, as far as asexual multiplication is concerned, this hypothesis is not supported by the experimental results obtained for co-inoculation with virulent and avirulent strains of *Leptosphaeria maculans*, a pathogen of *Brassica napus* (Li et al., 2006a). One possible explanation for this finding is that the sensitization of a plant with an avirulent strain may lead to a more rapid response to infection, potentially resulting in stronger protection against virulent strains. Moreover, co-inoculation with *L. maculans* ascospores increased the ability of pycnidiospores to cause disease, but it was established that the

time interval between the two infections (from 0 to 2 days) had no effect on the pathogenicity of pycnidiospores (Li et al., 2006b).

The second experiment clearly showed that longer intervals between inoculations (2 or 3 weeks) were detrimental to sexual reproduction. The negative impact of a long time offset in the early infection dynamics of the two parental isolates may be due to competition between the two strains for host resources for asexual multiplication. Such a competitive effect has been demonstrated in the oomycete *Phytophthora infestans*. By contrast to our approach, the effect of co-infection was investigated by inoculation with only non-compatible strains, to prevent sexual reproduction (Clément et al., 2012). This approach made it possible for the authors to eliminate sexual reproduction as an extraneous factor likely to mask the real effects of multiple infections on asexual reproductive fitness. It can be hypothesized that larger time offsets in early infection are associated with larger effects of such competition. The 'advance' of the first strain thus prevents the growth of the second. The infection dynamics of the second isolate are decreased simply because the host tissues are already colonized by the first isolate, which has used up the host resources. The consequent imbalance in the proportions of intercellular hyphae between the two parental isolates may therefore have decreased the probability of sexual reproduction on stems and leaves.

It is not possible to distinguish between these two hypotheses based on the results of this study. Both effects probably occurred and further experiments would be required to estimate the best time offset in the early infection dynamics of the parental dynamics for ensuring that sexual reproduction occurs. The impact of concomitant mixed-genotype inoculation on the course of asexual multiplication in *Z. tritici* has been investigated in previous experimental studies (Zelikovitch and Eyal, 1991; Halperin et al., 1996; Schürch and Roy, 2004). A decrease in pycnidial coverage was established, suggesting that inter-strain competition during mixed infections decreases pathogen fitness. Halperin et al. (1996) specifically demonstrated the detrimental effects of sequential inoculations, two, five and 10 days apart, with two isolates, whereas Eyal (1992) showed that the suppressive effect of a mixture of isolates was almost negligible under environmental conditions conducive to the development of blotch Septoria tritici blotch epidemics. Halperin et al. (1996) suggested that the suppression of pycnidia coverage was driven by two different mechanisms: cross-protection and competition. Competition, which can explain the result of our second experiment, may have similar consequences for subsequent sexual reproduction. However, cross-protection is likely to have a lesser effect on sexual reproduction. Indeed, various crossing experiments have demonstrated that host resistance hinders the asexual development of Septoria tritici blotch, but does not prevent sexual reproduction: in the extreme case, an avirulent strain that did not establish a compatible interaction with the host plant is perfectly capable of reproducing sexually (Kema et al., 2010; Ware, 2006).

4.3. Consequences of the possible absence of a functional trade-off between the two modes of reproduction in Z. tritici

We found that the intensity of sexual reproduction was positively correlated with the mean sporulating area for the two parental *Z. tritici* isolates and the difference in their latent periods. These findings tend to invalidate the hypothesis of the existence of a functional trade-off between asexual multiplication in *Z. tritici* (foliar aggressiveness expressed as the size of the sporulating lesions or the latent period) and sexual reproduction on wheat debris. There have been reports of negative correlations between two foliar aggressiveness components (latent period and asexual spore production) in *Puccinia triticina* (Pariaud et al., 2013), and between foliar aggressiveness and asexual transmission over seasons in *P. infestans* (Pasco et al., 2015). Trade-offs between asexual multiplication and sexual reproduction are still poorly understood in fungal plant pathogens with a heterothallic life cycle. To our knowledge, this is the first time that the hypothesis of such a trade-off has been invalidated experimentally.

Clément et al. (2010) reported that sexual reproduction rates in the oomycete *P. infestans* were highest on hosts favoring asexual multiplication (with higher rates of sporangium production), suggesting that there may be similar nutritional requirements for both sexual and asexual sporulation. Sexual reproduction rates were also highest on hosts favoring a shorter latent period. Clément et al. assessed the correlation between sexual reproduction and host resistance components (i.e. between pathogen-related and host-related life history traits). The impact of the mean aggressiveness of each parental isolate (assessed for each isolate separately) and the impact of the difference in their latent periods (i.e. the difference in their early infection dynamics) on crossing ability were not assessed. We therefore believe that the results obtained for *P. infestans* (Clément et al., 2010) should not be interpreted as the effect of a trade-off *sensu stricto* between asexual multiplication (growth and sporulation) and sexual reproduction. Finally, our failure to detect an aggressiveness cost in this study suggests that there is no strong adaptive compromise between pathogenicity (asexual multiplication) and inter-annual transmission (sexual reproduction). Such a compromise is often put forward as an explanation for the persistence of pathogens over time.

In the experimental conditions of this study, the mean aggressiveness of the various pairs of parental isolates could not account for differences in leaf disease severity reached after co-inoculation. Moreover, the negative correlation between leaf disease severity and the intensity of sexual reproduction was weak and inconclusive. These results, contrary to those of previous studies, provide no firm evidence for or against the existence of a trade-off between the two modes of reproduction. The experiment was not set up so as to obtain large variations in disease severity. Further experiments, based on a larger range of inoculum concentrations for the same parental isolates, for example, would be useful, to assess more specifically the effect of disease severity (i.e. lesion density) on the intensity of sexual reproduction.

The lack of counterselection against the most aggressive strains during the survival phase of the life cycle of a plant pathogen would imply a gradual increase in the mean aggressiveness level of pathogen populations with time. An increase of this kind, over the course of several years, was reported for *P. infestans* by Day and Shattock (1997), whereas Pasco et al. (2015) recently demonstrated a trade-off between aggressiveness on leaves and asexual transmission. In a previous experimental study, Suffert et al. (2015) found any analogous functional trade-off between asexual multiplication on leaves during the epidemic phase and residual asexual spore production on debris during the saprophytic survival phase. The absence of such a trade-off would amplify the long-term consequences of short-term selection processes during epidemic phases, as illustrated by the increase in aggressiveness of *Z. tritici* populations from the beginning to the end of an annual epidemic (Cowger and Mundt, 2002; Suffert et al., 2015). However, Suffert et al. suggested that there is an adaptive compromise, due to an Allee effect (disproportionate decrease in reproductive success at low population densities), interpreted as difficulty finding mates at low lesion densities (Castel et al., 2013). At the end of epidemics of moderate intensity, a low pathogen density on the upper leaves would lead to a lower likelihood of strains with compatible mating types encountering each other and mating, thus resulting in a low rate of sexual reproduction and low transmissibility of the strains selected during the season. Moreover, the crop debris left in the field comes from the very bottom parts of the plants, with the upper parts generally removed from the field.

According to life-history theory, evolution is constrained by adaptive compromises between fitness traits. Several evolutionary processes influence aggressiveness, and there may be trade-offs between transmission and pathogenicity. However, in many cases, no measurable fitness costs or trade-offs have been detected (Laine and Barrès, 2013). Previous experiments in *Z. tritici* have demonstrated an absence of correlation between reproductive fitness and virulence (Zhan et al., 2002). Experimental findings in other cereal pathogens (*Parastagonospora nodorum*, Sommerhalder et al., 2011 and *Rhynchosporium commune*, Abang et al., 2006) suggested differences in selection between biotrophic and necrotrophic stages of the life cycle, consistent with trade-offs between parasitic competitive ability and saprophytic competitive ability (McDonald and Mundt, 2016). However, experimental studies demonstrating firmly a functional trade-off between sexual multiplication and asexual reproduction in plant pathogens are uncommon, probably due to methodological difficulties. The method developed here will make it possible to investigate the genetic and epidemiological determinism of sexual reproduction and its consequences for survival during the interepidemic phase.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.fgb.2016.05.004.

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