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Sexual reproduction of *Zymoseptoria tritici* on durum wheat in Tunisia revealed by presence of airborne inoculum, fruiting bodies and high levels of genetic diversity

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ABSTRACT

Septoria tritici blotch (STB) caused by the heterothallic ascomycete *Zymoseptoria tritici* is currently one of the most devastating diseases of wheat worldwide. The extent of sexual reproduction of this pathogen is well documented on bread wheat, but not on durum wheat. The objective of the present study was to quantify the occurrence of *Z. tritici* sexual reproduction on durum wheat in the Tunisian environment. The assessment was undertaken using a triple approach combining fruiting body assessment, ascospore trapping and population genetic analyses. The results highlighted the formation of pseudothecia on leaves and stubble from the autumn until the end of the growing season. Likewise, qPCR monitoring highlighted a constant release of *Z. tritici* airborne inoculum during the wheat-growing season, with a peak of production at the end of the season. Genetic investigations using microsatellites revealed high levels of gene and genotypic diversities, an equal distribution of mating types, and a lack of genetic clustering within and between growing seasons. Taken together, these findings indicate that *Z. tritici* undergoes sexual reproduction on durum wheat in Tunisia at least to the same extent than on bread wheat in Western Europe, and that the dry and warm climate does not affect the mating process of the fungus. Frequent occurrence of sexual reproduction is a valuable knowledge to take into account in STB control strategies on durum wheat.

1. Introduction

Zymoseptoria tritici is the causal agent of Septoria tritici blotch (STB), one of the most devastating fungal diseases of wheat crops worldwide (Duveiller et al., 2007; O'Driscoll et al., 2014; Singh and Saari, 1992; Singh et al., 2016). In Tunisia, recurrent STB epidemics on durum wheat (*Triticum turgidum* subsp. *durum*) have become a common event, and yield losses can reach up to 50 %, especially when environmental conditions are suitable for disease development (Berraies et al., 2014). The introduction of shorter and susceptible high-yielding cultivars and the increase in nitrogen use have drastically aggravated the economic impact of this disease on wheat production (Bayles, 1991; Saari and Wilcoxson, 1974; Wiese, 1987). The ability of *Z. tritici* to grow at various temperatures (Zhan and McDonald, 2011; Lendenmann et al., 2016), to rapidly overcome cultivar resistance (Cowger et al., 2000; Brown et al., 2015), and to develop resistance to fungicides (Dooley et al., 2016; Leroux et al., 2007; Torriani et al., 2009) has made it one of the most problematic wheat pathogenic fungi worldwide. Those abilities are conferred by high adaptive potential of *Z. tritici*, driven by the

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interaction of several evolutionary forces, including mutation rate, population size, gene flow, sexual reproduction and selective environment (McDonald and Linde, 2002).

Z. tritici is a heterothallic fungus with a mixed reproduction system involving two types of spores: splash-dispersed asexual pycnidiospores (produced in pycnidia) and airborne sexual ascospores (formed in pseudothecia). During epidemic outbreaks, the population size increase considerably (Eyal, 1987), with a spore load in the field reaching up to 10^{11} spores/ha (Fones and Gurr, 2015). With a high mutation rate (*i.e.* 3.3×10^{-8} per site per cell, Stukenbrock et al., 2011), the number of mutations occurring at a single nucleotide position theoretically reaches up to about 20,000 in a single hectare of wheat field (considering 6-cell spores). This calculation illustrates the high potential of mutant production, possibly including strains with new adaptive traits. The proportions of new phenotypes in the population can rapidly increase if they are evolving in a very selective environment where the trait is favourable (McDonald and Mundt, 2016). Modern wheat fields sown with genetically uniform cultivars sprayed with target-specific fungicides are the perfect example of such a selective environment. Moreover, weak host diversity at the country scale also favours the directional selection of *Z. tritici* virulent strains. In Tunisia, about 60 % of the durum wheat surface in 2015 was sown with the same susceptible cultivar, Karim. Although the introduction of new alleles in populations through mutations and selection is mainly driven by productive asexual reproduction, sexual reproduction also plays a key role in the adaptive potential of *Z. tritici*. First, it allows new combinations of alleles to be created that can spread in a selective environment (McDonald and Linde, 2002). Second, the high mobility of ascospores (Duvivier et al., 2013; Fraaije et al., 2005) induces a continuous intermingling of the population on a large scale (Linde et al., 2002).

Since Kema et al. (1996) suggested that several sexual cycles of *Z. tritici* could occur during the growing season, the importance of the sexual stage of this species has been studied using different approaches, mainly on bread wheat. Spore traps have been successfully used to quantify airborne inoculum of *Z. tritici* during the entire growing season using either microscopy (Hunter et al., 1999) or, more recently, real-time quantitative PCR (Duvivier et al., 2013). These findings have shown that ascospores are frequently released throughout the growing season, with a seasonal pattern. Overall, large quantities of spores are usually trapped in late autumn and a second period of detection is observed at the end of the growing season. Studies on the release of ascospores from crop residues have shown that spore releases mainly happen in the early growth stage of wheat plants and are significantly reduced to a low level after winter (Bathgate and Loughman, 2001; Brown et al., 1978; Morais et al., 2015; Scott et al., 1988; Shaw and Royle, 1989; Suffert and Sache, 2011). On the other hand, pseudothecia have only been reported on infected wheat plants later in the growing season, generally after stem elongation (Clinckemaillie et al., 2010; Eriksen and Munk, 2003; Hunter et al., 1999). In these studies, mature pseudothecia were first visible on the lower leaves, from March–April, corresponding to the stem elongation period, and then progressively appeared on the upper leaves. Their number rose during the growing season along with the senescence of each leaf layer. In oceanic temperate climate areas where bread wheat is the most commonly grown cereal, the major source of airborne inoculum seems to change around March–April from pseudothecia developing on wheat debris to pseudothecia developing on the infected leaves of wheat crops (Duvivier, 2015).

Z. tritici has been well studied for genetic diversity and population structure in recent years, and the findings have provided important insights into the biology, epidemiology and evolutionary history of the pathogen (McDonald and Mundt, 2016). Overall, these studies have reported high levels of genetic diversity and rates of population structure and gene flow that vary depending on the sampled geographical area and/or the marker system used on bread wheat (e.g. Abrinbana et al., 2010; El Chartouni et al., 2011; Gurung et al., 2011; Linde et al., 2002; Siah et al., 2018). On durum wheat also, for instance in Tunisia, *Z. tritici* has been shown to be highly diverse and a part of a single panmictic population, according to both mitochondrial and nuclear markers (Berraies et al. 2013; Boukef et al., 2012; Naouari et al., 2016). The high diversity of *Z. tritici* even at fine spatial scales such as field, plant and leaf scales suggests a constant mating in the populations, is consistent with the fact that ascospores could provide primary as well as secondary inoculum (Linde et al., 2002; Morais et al., 2019; Siah et al., 2018).

In Tunisia for instance, *Z. tritici* has been shown to be highly diverse and a part of a single panmictic population, according to both mitochondrial and nuclear markers. The high diversity of *Z. tritici* even at fine spatial scales such as field, plant and leaf scales suggests a constant mating in the populations, indicating that ascospores could provide primary as well as secondary inoculum (Linde et al., 2002; Siah et al., 2018). Ascospores may be involved in the infection of the upper leaves in the spring and summer, accelerating the upward progression of the disease on the plants, as suggested in several studies performed using genetic approaches (Zhan et al., 1998) or a mechanistic model (Duvivier, 2015).

Quantification of sexual reproduction and ascospore production under local agro-environmental conditions could provide valuable information to better understand the epidemiology of *Z. tritici* in North Africa. However, most quantitative studies on *Z. tritici* sexual reproduction have been conducted in Western Europe in an oceanic temperate climate, in countries where bread wheat (*Triticum aestivum* L.) is the predominant cereal crop. By contrast, in Tunisia, durum wheat is the most cultivated wheat (80 % of wheat-cultivated land), with 617,000 ha cultivated during the 2017/2018 season (http://gain.fas.usda.gov). The Mediterranean climate as well as the historical dominance of durum wheat production in Tunisia offer an ideal environment to measure the rate of sexual reproduction in *Z. tritici* under field conditions. Evidence of the occurrence of *Z. tritici* sexual reproduction has been reported in many North African countries, including Morocco (Elbekali et al., 2012), Algeria (Allioui et al., 2014; Harrat et al., 2017; Meamiche Neddaf et al., 2017) and Tunisia (Berraies et al. 2013; Boukef et al., 2012; Ben Hassine and Hamada, 2014; Naouari et al., 2016). Nevertheless, no quantification has been performed to evaluate the importance of sexual reproduction of *Z. tritici* over the growing season in this Mediterranean area characterised by a warm and dry climate. The objective of the present study was therefore to quantify *Z. tritici* sexual reproduction throughout the growing season of durum wheat in the Tunisian environment. This characterisation was undertaken using a multi-faceted approach, combining fruiting body identification, spore trapping and population genetic analyses.

2. Material and methods

2.1. Field trials

Experimental fields of durum wheat measuring 2.5 ha were established during two consecutive growing seasons 2015–2016 and 2016–2017 at exactly the same location at the Oued-Beja experimental station of the Regional Field Crop Center (CRRGC-Beja) in Tunisia (GPS coordinates:36°44′05″N, 9°13′35″E). During both growing seasons, sowing was carried out in mid-November using the highly STB-susceptible durum cultivar, Karim, with a seed density of 180 seed/m^2. Since 2014, this field has been continuously cultivated with the cultivar Karim without

intermediate crop and deep tillage. Wheat growth stages (GS) were assessed on main plants according to the decimal scale of Zadoks et al. (1974). The field received two applications of nitrogen of 33.3 kg N ha^{-1} at full tillering (GS29) and at the end of anthesis (GS69) during both growing seasons, but no fungicide treatment was applied. Fields were harvested in mid-June in both years. Between the two growing seasons, straws and other crop debris were left on the soil surface. The trial assay was part of a larger experimental platform (70 ha) at CRRGC-Beja, from where experiments on durum wheat are conducted annually from November and June.

2.2. Disease severity assessment

The assessments of STB symptoms were carried out using the double-digit scale (00-99) first described by Saari and Prescott (1975) and adapted for STB by Eyal (1987). In this scoring method, a first digit (D1) indicates the relative height of disease on the plant (1 = basal leaf; 2 = leaf 7; etc.; 7 = leaf 2 and 8 = flag leaf), and a second digit (D2) refers to the degree of severity on the highest infected leaves (1 = 10 %; etc.; 9 = 90 %). Plants were marked every metre along four transect lines, as represented in Fig. 1, with 16 and 20 plants marked per transect in 2016 and 2017 respectively. Marked plants were assessed for STB severity from the beginning of March until the end of May. In order to compare the two growing seasons, a disease severity index was calculated based on the following equation (Sharma and Duveiller, 2007):

$$\text{Disease severity index}(\%) = (D1/9) \times (D2/9) \times 100$$

Disease severity index values for close dates of observation were compared between growing seasons using ANOVA performed on arcsin-root-transformed data. In this paper, the successive leaf layers are denominated as follows: flag leaf = L1, second last leaf layer = L2, third leaf layer = L3, etc.

2.3. Fruiting body identification

For growing season 2067-2017 only, 20 stubble pieces and 20 leaves (five samples per transect line for both materials) per date of collection were examined for the identification of *Z. tritici* fruiting bodies (pseudothecia and pycnidia) using the method described by Eriksen and Munk (2003). For each selected stubble or leaf, a piece of about 1 cm was immersed in a solution of 70 % glycerol. Under a stereo microscope, 30 fruiting bodies were homogenously picked off along the length of the sample using a needle and scalpel. If 30 fruiting bodies could not be obtained, a second 1 cm piece was prepared from the same residue. The fruiting bodies were placed on a microscope slide in a drop of cotton blue (0.5 % in 1:1:1 mixture of lactic acid, glycerol and water), crushed and identified under a microscope based on their size and the morphology of the spore they contained. The fruiting bodies were allocated to three different classes: (i) pseudothecium with mature asci, (ii) pycnidium with visible pycnidiospores and (iii) unidentified structures. In total, 600 fruiting bodies were characterised per date for both stubble and leaf collections.

2.4. Airborne inoculum trapping and quantification

From 17 November 2016 to 13 July 2017, airborne inoculum was collected using a Burkard 7-d recording volumetric spore trap (Burkard Manufacturing Co. Ltd, UK) set up 100 m away from the trial field. The spore trap opening was placed 1 m above ground level in a wheat-free square of 7 × 7 metres, in order to avoid capturing the splash-dispersed conidia of *Z. tritici*. The spore trap was surrounded by the untreated Karim cultivar. The throughput of the spore trap was set at 10 L per minute, corresponding to 14.4 m^3 every 24 h. The trap collected airborne particles on wax-coated Melinex tape (Burkard Manufacturing Co., UK) (345 mm × 20 mm) attached to a drum that completed one rotation over a 7-d period. The spore trap tapes were covered with a thin film of Vaseline and were replaced weekly. After exposure, each tape was cut into seven segments of 48 × 20 mm. Each segment was placed in a 2 mL microtube for total DNA extraction and quantification of the *Z. tritici* DNA, using the method described by Duvivier et al. (2013). Data from real-time PCR were expressed as DNA copy per day for each DNA extract. A table for the conversion of DNA copy into the corresponding number of conidia on tape or ascospores per m^3 air is presented in Duvivier et al. (2013).

2.5. Measures of climatic conditions

Daily meteorological data were recorded at the experimental station by a weather station (Socrima, France) set up 100 m away from the trial field (next to the spore trap). Temperature (°C), precipitation (mm), wind speed (m/s) and relative humidity (%) were recorded at a height of 2 m above the ground surface.

2.6. Leaf sampling and fungal isolation

In 2016, 2017, 40 leaves with STB symptoms were collected per transect line on the 26th day of each month from February to May.

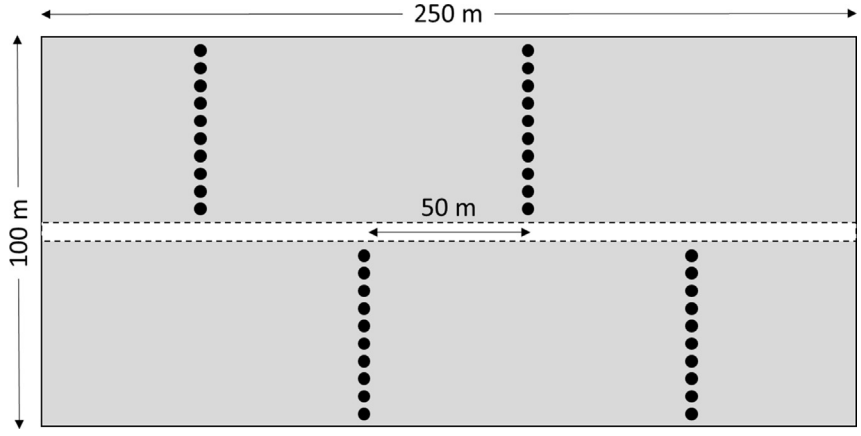

Fig. 1. Schematic illustration of the trial field and the transect lines (dotted lines) on which infected leaf samples were collected and marked plants were observed.

The leaves were collected regularly, with a spacing of at least 1 m, on the highest leaf layers showing significant symptoms. In 2016, residues consisting of wheat stubble were collected along the transect lines on the 26th day of October, November and December. All samples were air-dried and stored at 4 °C in paper envelopes.

After collection, wheat leaves were fixed to glass slides with tapes and kept under high humidity at 20–25 °C for 24 h to induce extrusion of cirrhi. One single cirrhus was collected per leaf under stereo microscope and suspended in sterile water and Tween (1 %). The resulting solution was plated onto PDA medium and incubated for 24 h at 20 °C. The plates were screened for the presence of characteristic microcolonies of *Z. tritici* using an inverted microscope. A single colony per Petri dish was then transferred onto a new PDA plate and incubated at 20 °C for a week. The obtained *Z. tritici* isolates were transferred to flasks containing yeast-sucrose broth (YES: yeast extract 10 g L^{-1}, sucrose 10 g L^{-1}) for 7 d at 18 °C under continuous light in an agitator at 100 rpm. The fungal biomass was collected by centrifugation at 5000 g for 5 min and rinsed with sterile distilled water. Approximately 300 mg of dried biomass was stored at −80 °C for further analyses. A total of 80 isolates (five per transect line per month, Table 1) were obtained throughout the 4 m of collection in 2016 and 2017. From the leaves collected in February and May 2017, 60 additional isolates (15 per transect line) were obtained. The 280 isolates sampled in both years will hereafter be referred to as collections, whereas isolates sampled within each month (February, March, April and May) will be referred to as sub-collections.

2.7. Microsatellite fingerprinting and mating type determination

DNA exaction from the 280 *Z. tritici* isolates was performed from dried biomass using the method of Lee and Taylor (1990) modified by Williams et al. (2001). All isolates were typed using eight microsatellite markers: ST1A4, ST2C10, ST1D7, ST1E3, ST2E4, ST1E7, ST1G7 (Owen et al., 1998) and AC0007 (Goodwin et al., 2007). PCR reactions and amplicon separation using capillary array electrophoresis were performed according to Siah et al. (2018). The conversion into alleles and the estimation of allele sizes were carried out using the software Genotyper™ (Applied Biosystems, California, USA). Finally, the mating types of the isolates were identified with a multiplex PCR using the primers reported by Waalwijk et al. (2002). PCR reactions, PCR thermal cycling conditions as well as amplicon separation on agarose gels were carried out using the method described by Siah et al. (2010).

2.8. Population genetics analysis

Isolates with the same combination of alleles at all loci were considered as clones and only a single representative haplotype (unique genotype) within sub-collections was used for subsequent analyses. Haplotype frequency (genotype diversity) was calculated in each collection using the XLSTAT software (Addinsoft). Gene diversity was measured by calculating Nei's index (1973), and the unbiased gene diversity (Anon, 1996) was calculated using the GenAlEx version 6.501 software (Peakall and Smouse, 2006). Genetic structure was estimated using various approaches. First, the genetic differentiation among sub-collections, according to each locus, was assessed using several fixation indices, including Wright's F index (F_{ST}) and Nei's G_{ST} index, a multi-allelic analogue of F_{ST} that is suitable for haploids as well as diploids. New standardised estimators of genetic structure, including Hedrick's standardised fixation index G''_{ST} (Meirmans and Hedrick, 2011) and Jost's standardised fixation index Jost's D_{est} (Jost, 2008), were also used as implemented in GenAlEx version 6.501. These different indices were calculated because there is currently no consensus on the best statistical test to use when measuring genetic differentiation within or between populations (e.g. Jost, 2008; Meirmans and Hedrick, 2011; Whitlock, 2011). Moreover, both F_{ST} and G_{ST} indices were calculated using GenAlEx version 6.501 to highlight the amount of population differentiation between pairs of sub-collections. Second, sources of genetic structure in the sub-collections were examined using hierarchical analysis of molecular variance (AMOVA), by estimating the degree of genetic differentiation within and between sub-collections, using 1000 permutations run with GenAlEx version 6.501. Finally, population structure was assessed using a Bayesian approach implemented in Structure version 2.3.4. This clustering model estimates the number of populations (clusters) and probabilistically assigns individuals to a population (Falush et al., 2003; Pritchard et al., 2000). The admixture model with correlated allele frequencies was applied, with 100,000 iterations of the Markov Chain Monte Carlo (MCMC) used as a 'burn-in', followed by 1,000,000 MCMC iterations. The data were analysed with K ranging from 1 to 8, with 10 repeat runs for each K. The potential for sexual reproduction was estimated by assessing whether the frequencies of the two mating types (MAT1-1 and MAT1-2) deviated significantly from the 1:1 ratio, using the χ^2 test at $P = 0.05$. The two idiomorphs are expected to occur in equal proportions under the scenario of regular cycles of sexual reproduction. In addition, the importance of sexual reproduction (random mating) was evaluated by calculating

Table 1
Genetic diversity based on eight microsatellite markers in *Zymoseptoria tritici* sub-collections sampled in 2016 and 2017 in Tunisia from a single field of durum wheat cv. Karim.

Year of sampling	Month	N[a]	H[b]	H[c]	Uh[d]	sI_A[e]	MAT[f]
2016	Feb	20	20	0.45	0.48	0.03	9:11 (0.65)
	Mar	20	20	0.46	0.49	0.02	14:6 (0.07)
	Apr	20	17	0.41	0.45	0.01	9:8 (0.81)
	May	20	20	0.46	0.49	0.04	11:9 (0.65)
	Total or overall	80	77 (96 %)	0.45	0.48	0.01	43:34 (0.30)
2017	Feb	80	77	0.48	0.49	0.01	35:42 (0.42)
	Mar	20	20	0.36	0.39	0.03	**15:5 (0.02)**
	Apr	20	20	0.48	0.51	0.04	12:8 (0.37)
	May	80	76	0.45	0.46	0.01	36:40 (0.65)
	Total or overall	200	193 (96 %)	0.45	0.46	0.01	98:95 (0.83)
2016 + 2017	Total or overall	280	270 (96 %)	0.45	0.47	0.01	141:129 (0.46)

Significant differences ($P < 0.05$) are indicated in bold.
[a] Number of isolates.
[b] Number of haplotypes per sub-collection, calculated with XSTAT. The percentage of haplotypes within year collections and within the global population is presented in brackets.
[c] Nei's gene diversity averaged over all loci, calculated with GenAlEx version 6.501.
[d] Unbiased gene diversity averaged over all loci, calculated with GenAlEx version 6.501.
[e] Standardized index of association (sIA) between pairs of loci, calculated using the program LIAN version 3.7.
[f] Mating type ratio (MAT1-1: MAT1-2). *P* values obtained for the frequencies of the two mating types using the $\chi 2$ test are presented in brackets.

the standardized index of association (sIA) using the LIAN (LInkage ANalysis) version 3.7 web interface (Haubold and Hudson, 2000), allowing to test multilocus linkage disequilibrium under the null hypothesis that alleles observed at different loci are unlinked. Significant difference from linkage equilibrium was tested using the Monte Carlo random sampling method (1000 iterations), as implemented in LIAN version 3.7.

3. Results

3.1. Wheat-growing and disease pressure

Mild winters and very warm summers, typical of the investigated area, were recorded for the duration of the study during both growing seasons (Fig. 3A, Supplementary data Table 1). The temperature started to fall in October, reaching its lowest level in January during which daily means of less than 10 °C were generally observed. Days with temperature peaks of up to 30 °C frequently occurred from the beginning of May, while extreme temperatures of up to 40 °C were recorded in June. Due to the similar accumulation of degree-days during the two seasons, comparable plant development was also observed. The second node stage (GS32) was reached in the last week of February, flowering occurred in April, followed by grain filling and maturation in May (Fig. 3B). In 2016, frequent and intense rainfall events occurred between March and May (176 mm), leading to very conducive weather for STB development. In 2017, only 66 mm were recorded in the same period, with not a single rainfall event in May. Days with the high relative humidity were generally observed on days with rainfall events, but otherwise conditions were very dry (daily mean relative humidity <60 %).

In both seasons, the first symptoms of STB in the field were detected in mid-January. In 2016, STB reached the second leaf (L2) on 25 April and the flag leaf was infected only a few days later. In 2017, pycnidia were observed on L2 only by mid-May, but STB did not progress further (Fig. 3C). Some symptoms were sporadically detected on the flag leaf, but only by the end of May. From February until May, the disease severity indexes were always significantly higher in 2016 than in 2017 ($P < 0.05$), except for the last observation where the difference was not significant (Fig. 2).

3.2. Pseudothecia quantification on wheat leaves and stubble

In order to evaluate the production of ascospore, the debris was left on the ground surface, and stubble samples were collected every month along the four transect lines starting on 26 October 2016 (Fig. 3D). From stubble collected in October 2016, most of the fruiting bodies observed could not be identified because they were empty. Up to 20 % of the analysed bodies were pycnidia containing pycnidiospores. This proportion slightly decreased in the sample collected at the end of November 2016. The first pseudothecia with visible ascospores were observed in the samples from November, but at very low frequency (<5 %). The proportion of pseudothecia increased considerably in the samples collected at the end of December 2016, with about 35 % of the fruiting bodies identified as pseudothecia. At the end of December 2016, mature pycnidia could still be identified on the stubble, representing more than 30 % of the observed fruiting bodies.

The presence of pseudothecia with visible ascospores was also evaluated on different leaf layers collected each month from the end of February 2017 until the end of May 2017. At the end of February 2017 (appearance of the first node on wheat plants; GS31), 20 % of the fruiting bodies inspected on the basal leaves (L8–L7) were mature pseudothecia. At the end of March 2017, no pseudothecia were found on the sampled L6. On the third leaf layer, collected at the end of April 2017, a proportion of pseudothecia of more than 20 % was observed. Mature pseudothecia were also identified on the second leaf layer (L2) collected at the end of May 2017, representing around 10 % of the characterised fruiting bodies.

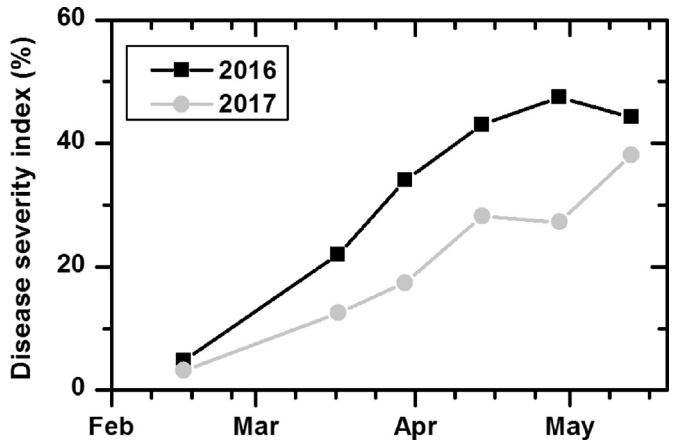

Fig. 2. Disease severity index (see M&M section for definition) evolution in a plot sown with the durum wheat cv. Karim, susceptible to STB, at Oued-Beja (Tunisia) in the growing seasons 2015–2016 and 2016–2017.

3.3. Temporal distribution of airborne inoculum

During the growing season 2016–2017, airborne inoculum was frequently measured in the field trial, with a mean daily detection of 58.3 DNA copies and a general detection frequency (or the frequency of day with positive detection) of 64 % during the surveyed period (Fig. 3D). The frequencies of detection were generally high every month, although different periods with successive high levels of daily detection were identified. The first peak was observed in mid-November 2016 (at the start of the survey and sowing period), and inoculum was afterwards detected almost continuously until early January 2017. During this period, a daily detection mean of 70.2 DNA copies was observed and the frequency of detection was the highest (85 %). From early January 2017 until early April 217, inoculum was still frequently detected (55 % of the days), but in a smaller quantity (16.1 DNA copies in average), with the exception of two separate peaks of a higher intensity recorded in March 2017 (264.7 DNA copies and 216.9 DNA copies respectively). The frequency and intensity of detections increased again around early April 2017 until the first week of May 2017. For this 30-d period, a daily detection mean of 75.3 DNA copies was found, with more than 80 % of the days with detection. Thereafter, a three-week period of low detection (mean: 9.9 DNA copies, frequency: 52 %) occurred again, corresponding to a period without a single rain event in the field. Finally, large quantities, up to 600 DNA copies, were trapped from the beginning of June 2017 until the beginning of July 2017, just after the first rainy period of the summer (mean: 152.1 DNA copies, frequency: 54 %).

3.4. Genetic structure evolution

A set of 280 *Z. tritici* isolates was typed using microsatellite markers to examine the evolution of fungal genetic diversity and structure of the populations within and between the two cropping seasons. In total, 77 different alleles were recorded over the eight analysed loci, with an average of 9.62 alleles per locus. A high rate of genotype diversity was highlighted within all tested sub-collections, with an overall number of 270 (96 %) haplotypes detected among the 280 sampled isolates (Table 1). The haplotype frequency was high overall and stable throughout the sampled

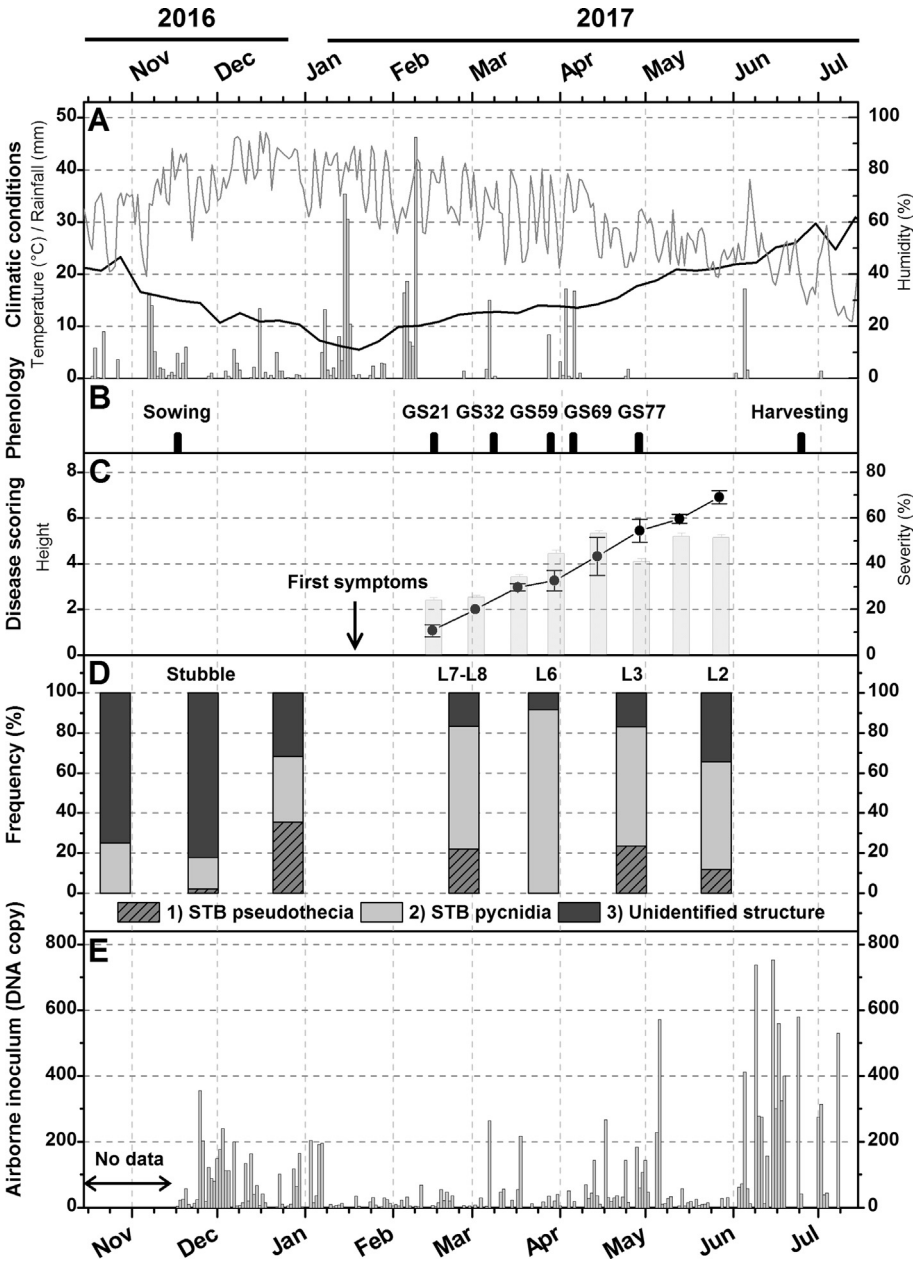

Fig. 3. Meteorological conditions, wheat phenology and temporal evolution of the presence of *Zymoseptoria tritici* (on wheat leaves, on residues and in the air) in the field in the 2016–2017 growing season. (A) daily mean relative humidity (grey line), daily precipitation (grey bar) and weekly mean temperature (black line) observed in the trial field. (B) phenology of the wheat in the field sown with the durum wheat cultivar Karim. (C) evolution of the disease on leaves in the trial field using the double-digit scoring method describing the height of the disease in the canopy (black line) and the severity on the highest leaf layer showing symptoms (grey bar). (D) proportion of the different types of fruiting bodies observed on stubble and leaf samples collected in the trial fields (hatched grey: pseudothecia with ascospores; light grey: pycnidia with pycnidiospores; dark grey: unidentified fruiting body). (E) daily quantities of *Z. tritici* trapped with Burkard 7-d recording spore traps placed near the trial field. Inoculum data are expressed in DNA copy in the real-time PCR (see Materials and Methods section).

period (within and between 2016 and 2017 collections). High and close levels of gene diversity were also found within all studied sub-collections, with an overall average of 0.45 for Nei's index and 0.47 for the unbiased gene diversity index (standardised for sample size) for the whole population (Table 1). The values of the unbiased gene diversity index were slightly higher overall than those of Nei's index, but both indices displayed similar overall levels of diversity (Table 1).

All measures of genetic structure showed a lack of genetic clustering among all sampled collections and sub-collections. The values of the different fixation indices (F_{ST}, G_{ST}, G''_{ST}, and D_{est}) were low overall at all loci, except for ST1E7, ST1D7 and ST1E3 which displayed moderate to high values for G''_{ST} index (Table 2). Pairwise F_{ST} and G_{ST} comparisons among sub-collections revealed a lack of population differentiation between all sampled sub-collections, with F_{ST} and G_{ST} values among sub-collections ranging from 0 to 0.06 and from 0.01 to 0.09 respectively (Table 3). AMOVA analyses confirmed these findings and showed that only 4 % of the genetic variation could be attributed to differences between sub-collections (months of sampling) and 96 % to differences within sub-collections (Table 4). Additional analyses using the Bayesian statistical approach implemented in Structure version 2.3.4

Table 2
Genetic structure at eight microsatellite markers in a *Zymoseptoria tritici* population sampled in 2016 and 2017 in Tunisia from a single field of durum wheat cv. Karim.

Locus	F_{ST}	G_{ST}	G''_{ST}	D_{est}
ST2C10	0.05	0.01	0.01	0.00
ST2E4	0.08	0.04	0.07	0.02
AC0007	0.08	0.04	0.06	0.02
ST1E7	0.12	0.07	0.17	0.10
ST1D7	0.11	0.07	0.27	0.22
ST1A4	0.06	0.02	0.07	0.05
ST1G7	0.07	0.03	0.04	0.00
ST1E3	0.07	0.03	0.25	0.23
Mean	0.08	0.04	0.09	0.04

F_{ST} = Wright's F index providing a measure of genetic differentiation among populations, calculated with GenAlEx version 6.501.
G_{ST} = Nei's fixation index estimating genetic differentiation among subpopulations, calculated with POPGENE version 1.32.
G''_{ST} = Hedrick's standardised G_{ST}, adjusted for small population size, calculated with GenAlEx version 6.501.
D_{est} = Jost'sD_{est} standardised fixation index, implemented in GenAlEx version 6.501.

supported the lack of population structure and showed an overall stability of the genetic structure throughout the sampled period (*data not shown*).

3.5. Mating type frequency and gametic equilibrium

Both fungal mating types (MAT1-1 and MAT1-2) were found to co-occur in all sampled sub-collections (Table 1). Statistical analyses using the $\chi 2$ test applied to examine deviation from the 1:1 ratio null hypothesis expected for random-mating populations revealed non-significant difference between the idiomorph frequencies in all sub-collections, except in the sub-collection sampled in March 2017, where the mating type proportions departed significantly ($P = 0.02$) from the 1:1 ratio (Table 1). The proportion of the two mating types was not significantly different at the whole season level (2016 and 2017) and at the overall population scale (Table 1). Values of the multilocus standardized index of association (sIA) were overall very low in all sampled sub-collections as well as the whole year scale (Table 1), indicating no departure from gametic equilibrium and fungal random mating in both growing seasons.

4. Discussion

During the first growing season (2015–2016), disease development on the STB-susceptible cultivar Karim was typical for the investigated area, with disease severity reaching around 50 % on the second-last leaf layer (L2) by the end of May. By contrast, the disease pressure during the second growing season (2016–2017) was quite weak, as a consequence of a very dry spring. During this second growing season, the frequency of *Z. tritici* airborne inoculum was high, but varies depending on the months. The high quantity of airborne inoculum detected from the end of November until the beginning of January was certainly the result of pseudothecia production recorded on wheat stubble. Indeed, mature pseudothecia or unidentified structures (potentially discharged pseudothecia) were identified in high proportions in all stubbles collected in the autumn. Screening of fruiting bodies on collected stubbles showed that the production and discharge of both types of spores occurred continuously until December. There was then a drastic decrease at the beginning of January in the quantity of inoculum detected, before the quantity began to rise again in early April. This 3-m gap may have been due to the fade-out of residues in the field. From January, it was not possible to find stubbles anymore, due likely to natural destruction or moving by wind. An occurrence of pseudothecia with mature ascospores was highlighted on basal leaves at the end of February, although the intensity of detection of airborne inoculum at this period was still low. From April, the quantity of airborne inoculum considerably increased, before a marked decrease in May due likely to the severe drought recorded at that time, although small and frequent detections of airborne inoculum were still occurred. This last finding agrees with previous observations in Belgium during the 2016–2017 growing season, where small quantities of airborne inoculum were noticed despite a low STB pressure and a very dry spring (Duvivier M., *unpublished data*).

Overall, the pattern of airborne inoculum obtained with the spore trap assay was quite similar to those obtained in Europe (Duvivier et al., 2013; Hunter et al., 1999; Morais et al., 2016). This cyclic pattern in inoculum quantity may therefore be the result of a change in the main source of ascospore production during the growing season, as observed in Belgium. Indeed, Duvivier (2015) found a correlation between the quantity of trapped inoculum and the disease pressure observed in a given field and during the previous year. In Tunisia, the degradation of debris in the field corresponded exactly to a decrease of the trapped inoculum. Moreover, the real increase of the surface area occupied by leaves with symptoms in the canopy around April corresponds to renewed intensity of detection of airborne inoculum. Comparison of fruiting bodies on samples from stubble and leaves suggests that the major source of airborne inoculum in Tunisia changes between January and March from pseudothecia developing on wheat debris to pseudothecia developing on the infected wheat leaves.

Table 4
Hierarchic analysis of molecular variance at three levels in *Zymoseptoria tritici* collections sampled in 2016 and 2017 in Tunisia from a single field of durum wheat cv. Karim.

Source of variation	df	Sum of squares	Est. var.	Variation (%)
Between sub-collections	7	36	0.10	4
Within sub-collections	262	545	2.08	96
Total	269	581	2.18	100

Table 3
Population differentiation measured by G_{ST} (below the diagonal) and F_{ST} (above the diagonal) among eight *Zymoseptoria tritici* sub-collections sampled in 2016 and 2017 in Tunisia from a single field of durum wheat cv. Karim.

		2016				2017			
		Feb	Mar	Apr	May	Feb	Mar	Apr	May
2016	Feb	…	0.03	0.07	0.08	0.03	0.05	0.08	0.04
	Mar	0.00	…	0.05	0.04	0.03	0.03	0.06	0.03
	Apr	0.04	0.02	…	0.04	0.05	0.06	0.09	0.05
	May	0.05	0.01	0.01	…	0.05	0.07	0.09	0.05
2017	Feb	0.02	0.01	0.03	0.03	…	0.02	0.04	0.01
	Mar	0.02	0.00	0.03	0.04	0.01	…	0.07	0.03
	Apr	0.05	0.03	0.06	0.06	0.02	0.04	…	0.03
	May	0.03	0.01	0.03	0.03	0.01	0.01	0.01	…

In the United Kingdom (Hunter et al., 1999), Belgium (Clinckemaillie et al., 2010) and Denmark (Eriksen and Munk, 2003), first pseudothecia on the newly sown crop were generally detected around GS30 in very low proportions (<5 %) on the basal leaves of bread wheat. These proportions usually rise with the senescence of the leaves. At the same wheat growth stage in Tunisia, more than 20 % of the fruiting bodies inspected on basal leaves were mature pseudothecia. Such a high proportion suggests that pseudothecia formation occurs even earlier than GS30. From the results based on two growing seasons in Denmark, the wheat flowering was reached before the same proportion of pseudothecia was observed on the lower leaf layers (L7 or L6). Moreover, a significant proportion of pseudothecia on the uppermost leaf layers (L3–L1) was only reported as the harvest period approached. Here, more than 20 % of the fruiting bodies counted on L3 at GS77 (*i.e.* 2 m before harvesting) were mature pseudothecia. A significant proportion of mature pseudothecia was also observed on L2 at the end of May. Eriksen and Munk (2003) showed that pseudothecia generally appeared a long time (29–55 d) after the pycnidia on the same leaf layers. In the United Kingdom, the interval between the appearance of first pycnidia and pseudothecia detection was also long and estimated at between 61 d and 95 d (Hunter et al., 1999).

On L3 and L2 investigated in the present study, high proportions of mature pseudothecia were scored around two weeks after the first pycnidia are detected on those leaf layers. This suggests that the sexual cycle of STB in Tunisia occurs rapidly during the epidemics. The high proportion of ascospores on L3, already observed at GS77, corroborated the results obtained by Suffert et al. (2018). In this study, the number of ascospores collected per gram of plant debris depends on the vertical position of the host tissues in the field and was the highest between 25 and 35 cm above the ground, corresponding roughly to the L3.

Overall, the frequencies of detection and the quantities of trapped spores in Tunisia under low STB pressure were comparable to those obtained under severe STB pressure in Belgium, *i.e.* 2012 growing season (Duvivier, 2015; Duvivier et al., 2013). This means that sexual reproduction under conditions favourable to STB in Tunisia is able to generate a huge quantity of ascospores. Likewise, the quantities of spores trapped between the beginning of the season and January, following a season of normal STB pressure, were higher than the quantity of spores trapped in Belgium during the autumn, regardless of the year. This finding suggests that *Z. tritici* undergoes sexual reproduction in the Tunisian environment to the same extent as in Western Europe and that the dry and warm climate does not affect the mating process in *Z. tritici*.

The study results showed a high level of genetic diversity during the two wheat-growing seasons. Clone analysis revealed that 96 % of the assessed isolates were unique haplotypes. Comparable levels of genotypic (96 %) and gene (0.44) diversities were recently reported in Europe (France) on bread wheat at the field level (Siah et al., 2018), thus corroborating the findings of spore trapping and pseudothecia identification. High rates of genetic diversity have also been reported on larger scales in several areas around the world, including Europe (El Chartouni et al., 2011; Drabešová et al., 2013; Linde et al., 2002; Siah et al., 2018; Welch et al., 2018), America (Gurung et al., 2011; Zhan et al., 2003), Asia (Abrinbana et al., 2010; Linde et al., 2002), Australia (Linde et al., 2002) and Northern Africa (Boukef et al., 2012; Naouari et al., 2016). Sexual reproduction, allowing for the generation of new genotypes and new combinations of alleles, is the reason for the genetic diversification of the pathogen in the field. The frequent occurrence of *Z. tritici* sexual reproduction on durum wheat was also confirmed by the equal frequencies of the two mating types and the low values of sIA observed during the two studied seasons, in line with previous results in Tunisia and Europe (Boukef et al., 2012; Siah et al., 2018). Our findings revealed a lack of fungal genetic clustering using both non-Bayesian and Bayesian approaches as well as AMOVA. These results indicate that the population is not structured at the field level and that genetic diversity of the fungus is distributed homogenously at this small spatial scale. An overall stability in the genetic structure of a *Z. tritici* population from a small spatial scale (nearby fields), within and among three growing seasons, was recently reported in France (Morais et al., 2019). Airborne ascospores released during sexual reproduction probably participate strongly in this homogenisation and significantly increase gene flow within the field (Linde et al., 2002; Siah et al., 2018).

In conclusion, the present study provides evidence for active sexual reproduction of *Z. tritici* on durum wheat and demonstrates that this fungus can engage in sexual mating in a dry and warm climate (northern Africa) to the same extent as on bread wheat in a more humid and colder climate (north-western Europe). Such findings could explain the recurrence of STB epidemics in Tunisia and could be a valuable factor to take into consideration in STB management programmes on durum wheat.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.funbio.2019.06.006.

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