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Population structure of the faba bean blight pathogen *Ascochyta fabae* (teleomorph, *Didymella fabae*) in Tunisia

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Abstract. Ascochyta blight, caused by *Ascochyta fabae* (teleomorph: *Didymella fabae*) has decreased faba bean production in Tunisia and worldwide. The teleomorph has recently been observed in Tunisia, raising new questions of how to control this major disease. Isolates (317) of the pathogen were obtained between 2011 and 2013, from four geographical regions in Tunisia (Beja, Bizerte, Jendouba and Tunis). The 240 isolates obtained in 2012 were compared for mating type frequency and genetic variation by using ten polymorphic SSR markers. Of these isolates, MAT1-2 was more common (2:1) in Tunisia than MAT1-1, but this ratio can change according to population. Low to high genetic variation was detected between locations and among pathogen populations. Beja showed the greatest genotypic richness (R = 0.42), followed by Tunis (R = 0.25), Bizerte (R = 0.13), and Jendouba (R = 0.11). Indices of association (I_A) and *Rd* were significantly different from 0 in all the populations, suggesting high multilocus linkage disequilibrium and confirming clonal populations. Population structure of the isolates was inferred using Bayesian analyses, Principal Component Analysis (PCA), and Minimum Spanning Networks, which all revealed that the populations from each location were not distinct. Evaluating changes in seasonal genetic diversity showed low to high variances of F_{ST} values between the two cropping seasons in all regions. However, the PCA analysis failed to separate the *A. fabae* isolates sampled during the two successive seasons into two groups, indicating that these populations did not constitute distinct genetic groups. These results suggest that gene flow was limited among populations, even those separated by short geographic distances. Future studies should enlarge the number of samples of representative populations, to overcome the limitations of a small sample size and to provide a more accurate assessment of *A. fabae* population structure.

Keywords. Ascochyta blight, *Ascochyta fabae*, faba bean, genetic differentiation, population genetics.

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INTRODUCTION

The faba bean disease, Ascochyta blight, caused by *Ascochyta fabae* Speg. (anamorph) and its teleomorph *Didymella fabae* Jellis & Punith, is a widespread disease, particularly in temperate regions, including Canada, Argentina, Europe, the Mediterranean region, the Middle East, Australia, New Zealand, Japan, Korea and China (Maurin *et al*., 1990). This is also one of the most important foliar diseases affecting faba beans in Tunisia (Kharrat *et al*., 2006). The fungus attacks all aerial parts of host plant (leaves, stems, pods and seeds) causing necrotic lesions, and leads to faba bean yield and quality damage. In Western Europe, depending on weather conditions during the cropping seasons, Ascochyta blight causes averages of 10 to 30% yield losses (Hanounik and Robertson, 1989). In Tunisia, yield losses are estimated to 15%, and may reach 95% in disease favourable conditions (Kharrat *et al*., 2006).

Ascochyta fabae is an haploid heterothallic pathogen that can survive during intercropping seasons on infested seeds and plant debris. Seeds constitute the major source of primary inoculum, and seed exchanges between and within countries are considered the main sources of introduction and mechanism of disease expansion or dispersal (Kaiser *et al.* 1997). *Ascochyta fabae* is maintained on crop stubble as asexual and sexual forms for at least one season (Rubiales and Trapero-Casas, 2002; Omri Ben Youssef *et al.,* 2012). Due to the stage of maturation of pseudothecia, the release of ascospores may overlap with the vegetative development of faba bean (Rubiales and Trapero-Casas, 2002).

Although applying fungicides to control *A. fabae* on seeds and faba bean plants gives satisfactory results, plant resistance remains the best way to control this disease. Considerable efforts have been made to identify sources of resistance in faba bean germplasm, particularly in Syria, Egypt, Canada, Morocco, France and Tunisia (Hanounik and Robertson, 1989; Rashid *et al.* 1991; Maurin and Tivoli, 1992; Kharrat *et al*., 2006). Partial host resistance has contributed to reducing chemical intervention to control the disease (Avila *et al*., 2004; Kharrat *et al*., 2006).

Pathogen variability represents a major challenge for the development and use of resistant cultivars. In general, new virulent isolates of pathogens emerge through selection imposed by their hosts. These shifts in virulence necessitate the adoption of disease control strategies that target pathogen populations rather than individuals (McDonald and Linde, 2002). Studying plant pathogen diversity principally aims to identify which forces will heavily influence the evolution of pathogen populations, and to assess their ability to evolve (Milgroom and Peever, 2003; de Meeûs *et al*., 2007). Several studies have evaluated population diversity of some *Ascochyta* spp. using different molecular tools, in particular for *A. rabiei* (Bayraktar *et al*., 2007; Ali *et al*., 2012; Atik *et al*., 2013) and *A. pinodes* (Le May *et al*., 2012; Padder *et al*., 2012; Laloi *et al*., 2016; Le May *et al*., 2017). Studies conducted on populations of *A. rabiei* collected in several countries showed high diversity levels that were attributed to genetic flow provided by the exchange of seeds between farmers (Nourollahi *et al*., 2011). For *A. fabae* populations, very few studies evaluated the genetic diversity of this fungus. A study using Random Amplified Polymorphic DNA (RAPD) markers, which included 36 isolates taken from different countries (Tunisia, Morocco, Italy, France, Spain, Australia and Algeria), showed high genotypic diversity, with 26 different genotypes observed within these isolates (Rouaissi *et al*., 2001). More recently, Ozkilink *et al.* (2011, 2015), using a set of 18 Simple Sequence Repeat (SSR) markers, showed that *A. fabae* populations from Syria displayed a high level of polymorphism. They showed high diversity but no differentiation, and rejected a null hypothesis of random mating in the studied population, which is indicative of predominating asexual reproduction.

Ascochyta blight has decreased faba bean production in Tunisia, a country characterized by variable weather conditions, diverse crop areas, various seed origins (produced by farmers or otherwise), and cultural practices. Since *D. fabae* has been observed recently in Tunisia (Omri Ben Youssef *et al*., 2012), it has become especially important to study the genetic diversity of the pathogen populations in the main faba bean growing areas affected by Ascochyta blight. The present study was undertaken to: (i) collect information about the genetic diversity of *A. fabae* populations in the various areas of faba bean production in Tunisia; (ii) assess the mating type distribution in this country using mating-type (MAT) specific primers; and (iii) determine whether the diversity of *A. fabae* populations evolves from one cropping season to another.

MATERIALS AND METHODS

Fungal isolates

A total of 317 *A. fabae* isolates were used in this study to analyze genetic variability. All isolates were collected between 2011 and 2013, from several faba bean fields in four different geographical locations of Tunisia, including Beja (three to nine fields, 122 isolates), Bizerte (two to seven fields, 87 isolates), Jendouba (three to eight

Table 1. Origin of *Ascochyta fabae* isolates (location, sampling date) used in the study.

Location	Sampling date	Number of populations	Number of isolates collected
Beja	2011	3	13
	2012	9	109
Bizerte	2012	2	71
	2013	7	16
Jendouba	2011	8	28
	2012	3	39
Tunis	2012	2	21
	2013	2	20

fields, 67 isolates), and Tunis (two fields, 41 isolates) (Table 1). All isolates were collected from infected host leaves, and as single-conidium isolates. For each isolation, approx. 5 mm² of diseased leaf tissue was surface sterilized for 1 min in 70% ethanol, rinsed three times in sterile water, placed on sterile filter paper to remove excess water. The tissue piece was then cultured on a V8 medium (99 mL V8 vegetable juice (Campbell), 35 g agar, and 901 mL distilled water, autoclaved at 105°C for 30 min) in Petri dishes for 14 d. Pycnidiospores from resulting cultures were spread on 2% malt agar and incubated for 12h, as described by Onfroy *et al.* (1999). Single germinating pycnidiospores were transferred (with dissecting microscope magnification) to fresh potato dextrose agar (PDA) plates, and cultures were incubated at 20°C with a 12h photoperiod under cool white fluorescent lamps. These single-spore cultures were then maintained on malt agar slants and stored in the dark at 4°C.

DNA extraction

Each isolate was grown in 75 mL of Tripton (LT) liquid medium supplemented with streptomycin (1.5 g) and penicillin (0.75 g). Each culture was raised from four pieces (each approx. 1 cm²) cut from the margin of an actively growing culture on malt agar. Inoculated vials were incubated, under agitation, for 14 d at 20°C, with a 12h photoperiod under cool white fluorescent lamps. Mycelia were harvested by vacuum filtration through two layers of sterilized Miracloth (Calbiochem CN Biosciences, Inc.), rinsed twice in sterile water, and then stored at -80°C until lyophilized. DNA was extracted from mycelium and isolated using Nucleospin Plant II Kit (Macherey-Nagel) according to the manufacturer's instructions.

Mating type assays

The mating type of all the 240 *A. fabae* isolates collected in 2012 was determined using the multiplex MAT-specific PCR assay (Cherif *et al.,* 2006). Primer combinations AL2p2SeqF4 (5'GCAACATCCTAGCATGATG3') specific to MAT1-1, AL1p1SeqF5 (5'CTGTCTCACCCAAGGCAAAC3') specific to MAT1-2, and ACom1A1AvAfAp (5'CACATCACCCCACAAGTCAG3') were used, specific to an aligning flanking 3′ region of *A. lentis, A. viciae-villosae, A. fabae* and *A. pisi*. Single PCR was carried out in 25 µL containing 10 ng of genomic DNA, 1× PCR buffer (containing 1.5 mM $MgCl_2$), 0.2 mM dNTPs, 1 unit of *Taq* DNA polymerase (Promega) and 0.2 µM each of the primers. Amplification was performed in a BioRad Cycler thermal cycler (Bio-Rad Laboratories), and cycling conditions consisted of an initial denaturation at 95°C for 3 min followed by 35 cycles, each of 94°C for 20 sec, 58°C for 20 sec, and 72°C for 40 sec, with a final extension at 72°C for 10 min. DNA amplicons were separated in 1.5% ethidium bromide-stained agarose gels, and were visualized under UV light on a gel documentation system ChemiDOCTM XRS (Bio-Rad). Amplicon size was estimated using a DNA ladder (Hyperladder II).

SSR amplification and analyses

Ten SSR loci and their primer sequences (Table 2) were obtained from a published report on *A. fabae* (Ozkilink *et al.*, 2011). The M13-tailed primer was used to detect genetic diversity of all *A. fabae* isolates of the collection. The M13 sequence (CACGACGTTGTAAAACGAC) was added to the 5′ end of the forward primers, which were synthesized by Sigma-Genosys Ltd. PCR was performed in a 12 µL reaction mixture containing 0.2 mM dNTP, 1× PCR buffer, 1.5 mM $MgCl_2$, and 1 unit of *Taq* DNA polymerase (Promega), with the addition of 1ng genomic DNA, 0.24 µL (10 nM) of the forward primer labeled at the 5′ end with M13 sequence, 0.3 µL (10 nM) of the reverse primer and 0.06 µL (10 nM) of the fluorescent dye FAM (Applied Biosystems). Following an initial denaturation step of 2 min at 94°C, PCR was performed for a total of 50 cycles, each of 20 sec at 94°C, 25 sec at 58°C, and 23 sec at 67°C, with a final extension for 10 min at 72°C. For analysis on the genetic analyzer, 3 µL of the 25 µL diluted and pooled PCR products were mixed with 9.9 µL of formamide and 0.1 µL of LIZ-445 size standard (Applied Biosystem) in a 96 well PCR plate (GeneMate). The mixture was heated for 5 min at 95°C then chilled on ice and analyzed with the DNA analyzer.

Table 2. Primer sequences of the ten SSRs markers used for genetic study of *Ascochyta fabae* isolates.

Locus	Repeating motif	Primer sequences (5' to 3')
CAA46	F: CTA CAT TTC CCG TGC CTG AC R: GGC AGC CAG AGT TTG AGA AC	(CAA)17
CAA28	F: GAG TCA GTG GCG AGT GTG G R: GTC CGT TGC CCG TCT TTC	(GTGTAGT)3N(94) (TGT)8
AFCAA12	F: TCT TGG ACG CGT CTC TCT TG R: GCC AGT CTG GTT CAT CTA CC	(GTT)8N(32) (CACTG)6
AFCAA13	F: TTC GGC AGC ACA TCC TTC AG R: TGA GCA ATC TGA GCG GTT GG	(GCA)6N(13) (CAGCAACAA)3N(27) (CAA)4N(15)(CAA)(16)
AFCAA5	F: ATC ATC GCG TAC GTC GAC AC R: AAG ATG CTG GAG GGT GTC AG	(CTGCCACTGACACAGCTA)6
CA31	F: GAGCGTACCCAAACGCTATC R: GCTTCTTCGGCCTCAGTATG	(TGTGAGCG)11
AFCAA1	F: TCT ACT GAC GAT GCA TAG CG R: TAC CCA AAC GCT ATC GAA GC	(TTG)8
CA10	F: GCT TGT GCT TGT GCT TGT TC R: ACA TTC GTC CAT TGC ACC TT	(GTGTTGTGC)3N(103) (TGTGT)3
CA3	F: AGC AAC AAC AAG ACG CAG TG R: AGC TTG GGA TCT GCT TCC TT	(GTGTGCAGTGTGTA)7
CAA57	F: GAG CGT ACC CAA ACG CTA TC R: GCT TCT TCG GCC TCA GTA TG	(TGTGAGCG)9

Data analyses

As indicated in Table 1, isolates from the various locations were sampled over several seasons. Two different analyses were performed on the collection of *A. fabae* isolates. These were: i) gene diversity, genetic differentiation and genetic diversity, carried out for 240 isolates obtained in 2012; and ii) seasonal population differentiation, carried out for the isolates (317) sampled during the following seasons in the four locations (Beja, Bizerte, Jendouba and Tunis).

Gene diversity and genetic differentiation

SSR data were used to define MultiLocus Genotypes (MLGs), and were checked for repeated MLGs. The number of repeated MLGs (G) was identified using GENCLONE 2.0 (Arnaud-Haond and Belkir, 2007). Genotypic evenness was evaluated using the index R= (G-1)/ (N-1), with G as the number of distinct multilocus genotypes and N the number of isolates (Grünwald *et al*., 2003). Genetic diversity was estimated by allelic richness (*Ar*) using POPULATIONS 1.2.32 software (Langella, 1999). *Ar* was corrected for unequal sample size in each datasetby standardizing allelic richness to the smallest sample size, set at six individuals. Expected heterozygosity (H′) was computed using Arlequin software 3.1 (Excoffier *et al*., 2005). Clonality was assessed with the index of association (I_A) and the R_d statistic, a measure of the multilocus linkage disequilibrium, and was calculated using Multilocus software version 3.1b (Agapow and Burst, 2001). The association between the scored alleles was estimated by comparing the variance of the genetic distances in the data set to the mean variance of 1,000 artificial re-sampled datasets. The R_d statistic is much less dependent on the number of loci than the index of association (Montarry *et al*., 2010). I_A and R_d values are low for recombining population, and high for clonal or selfed populations (Burt *et al*., 1996).

Population structure analyses

Partition of molecular diversity among and within regions, as well as among and within populations, was studied for the 240 isolates collected in 2012, using an analysis of molecular variance (AMOVA) (Excoffier *et al*., 1992; Lynch and Milligan, 1994). AMOVA was performed using Arlequin 3.1 (Excoffier *et al*., 2005). Pairwise F_{ST} values were calculated with Arlequin software, for sub-populations of *A. fabae* sampled in the different locations. A Bonferroni correction (adjusted alpha = 0.00316; 15 comparisons) was applied to take into account multiple testing.

A principal component analysis (PCA) was performed using the procedure available in the package ADEGENET (Jombart *et al*., 2008) for the statistical

freeware R version 3.1.1. PCA has an important advantage over other methods, such as the Bayesian clustering algorithm implemented in STRUCTURE (Pritchard *et al*., 2000), because it does not require strong assumptions about an underlying genetic model, such as the Hardy-Weinberg equilibrium or the absence of linkage disequilibrium between loci (Jombart *et al*., 2008).

STRUCTURE software version 2.2 was also used without any assumptions about population structure or assigning individuals to populations. The analysis was performed using 5×10^5 burn-in replicates and a run length of 1×10^6 Markov chain Monte Carlo (MCMC) replicates, adopting the admixed model and the correlated allele frequencies option. The number of genetic groups (K value) was estimated using the model developed by Evanno *et al.* (2005), which provides an estimate of the posterior probability of the data for a given K, Pr(X/K) (Perrier and Jacquemoud-Collet, 2006). The height of the modal value of the distribution was used as an indicator of the strength of the signal detected by STRUCTURE software. Five independent runs were performed for each analysis in order to verify the convergence of parameter estimates, and isolates were classified by location in order to determine if the same genotypes were present in the different populations and locations.

A Minimum Spanning Network (MSN) is an excellent way to visualize relationships among individuals. By using the package Poppr from R statistical software, version 3.1.2 (©2014, The R Foundation for Statistical Computing), MSN was calculated using Nei's distance for the different *A. fabae* populations. Multilocus genotypes (MLGs) were collapsed to multilocus haplotypes, represented by circles each containing the number of associated isolates and sized in proportion to haplotype frequency. Haplotype information could lead to more powerful tests of genetic association than single-locus analyses, but it is not easy to estimate haplotype frequencies from genotype data due to phase ambiguity. The challenge is compounded when individuals are pooled to save costs or to increase sample size. By collapsing the total allele frequencies of each pool suitably, the maximum likelihood estimates of haplotype frequencies based on the collapsed data can be calculated very quickly regardless of pool size and haplotype length (Kuk *et al*., 2013).

Seasonal population differentiation

Pairwise comparisons were carried out between populations collected over two successive seasons, independently for each location (Beja, Bizerte, Jendouba, and Tunis). Multilocus genotype variability (N_T) and allelic richness (R) were estimated (described above), and pairwise F_{ST} values were calculated using Arlequin software. APCA was also performed to detect the distributions of isolates collected in the two successive growing seasons.

Mating type frequency analyses

Chi-square was used to test mating type ratios for all the locations sampled in the 2012 growing season. The ratios were expected to be 1:1 for randomly mating populations (Milgroom, 1996). Mating type ratios were considered significantly different from 1:1 at $P < 0.01$.

RESULTS

Population diversity of Ascochyta fabae *in Tunisia*

The genetic diversity and structure of Tunisian *A. fabae* populations were investigated using ten SSRs and mating type markers. The multiplex PCR easily differentiated two mating types, and the isolates were unambiguously assigned to one of those types. Of the 240 Tunisian isolates sampled in 2012, MAT1-2 was more common in Tunisia than MAT1-1 with a ratio of 2:1 (Table 3). At Beja and Tunis, the two mating types occurred in a 1:1 ratio ($P = 0.925$). For the other two locations, all the populations showed skewed mating type distributions, indicating that MAT1-2 was more common in all these populations (Table 3). The overall distribution showed statistically significant deviation from the 1:1 ratio in only two locations: Bizerte and Jendouba ($P < 0.001$).

All ten microsatellite loci were found to be polymorphic for the 240 isolates. The greatest heterozygosity were obtained for AFCAA13 and CAA28. Low levels of genetic diversity were detected among and within the locations, as populations from different locations shared many common alleles at all the SSR loci (percentage of common alleles greater than 35%) (Table 4). Narrow genetic diversity was found within each of the sixteen

Table 3. Isolates of *Ascochyta fabae* collected from different locations of Tunisia in the 2012 cropping season, and their mating types (MAT 1-1 and MAT 1-2) as determined by PCR.

Location	Number of isolates	*MAT 1-1*	*MAT 1-2*	c^{2e}	P^f
Beja	109	55	54	0.008	0.925
Bizerte	71	15	56	28.928	< 0.001
Jendouba	39	1	38	38.348	< 0.001
Tunis	21	10	11	0.154	0.694

Table 4. Distribution of private (P) and common (C) alleles among populations of *Ascochyta fabae* sampled in 2012 from four main locations of faba bean production in Tunisia (Beja, Bizerte, Jendouba, Tunis).

Location		Loci										Total
		CAA46	CAA28	AFCAA13	AFCAA12	AFCAA5	CA31	AFCAA1	CA10	CA33	CAA57	
Beja	C	21	7	7	10	8	11	12	15	12	11	114
	P	3	1	0	1	0	0	2	0	3	1	11
	Total	24	8	7	11	8	11	14	15	15	12	125
	%P	*12.5*	*12.5*	*0.0*	*9.1*	*0.0*	*0.0*	*14.3*	*0.0*	*20.0*	*8.3*	8.8
Bizerte	C	4	4	4	3	3	3	3	4	4	3	35
	P	4	1	1	3	0	4	7	2	2	4	28
	Total	8	5	5	6	3	7	10	6	6	7	63
	%P	*50.0*	*20.0*	*20.0*	*50.0*	*0.0*	*57.1*	*70.0*	*33.3*	*33.3*	*57.1*	*44.4*
Jendouba	C	2	3	4	3	3	2	2	3	2	2	26
	P	5	2	0	1	0	0	1	1	4	1	15
	Total	7	5	4	4	3	2	3	4	6	3	41
	%P	*71.4*	*40.0*	*0.0*	*25.0*	*0.0*	*0.0*	*33.3*	*25.0*	*66.7*	*33.3*	*36.6*
Tunis	C	4	4	4	3	4	3	3	3	5	3	36
	P	0	0	0	0	0	0	1	0	0	1	2
	Total	4	4	4	3	4	3	4	3	5	4	38
	%P	*0.0*	*0.0*	*0.0*	*0.0*	*0.0*	*0.0*	*25.0*	*0.0*	*0.0*	*25.0*	*5.3*

populations, reflecting abundant intraregional gene flow. At a location level, the percentage of common alleles shared among populations ranged from 55.6% to 94.7% (Table 4). Isolates from Beja had four loci (AFCAA13, AFCAA5, CA31 and CA10) that were common among the isolates of the different populations sampled at this location. At Bizerte, only one locus (AFCAA5) was common between the isolates. At Jendouba, three loci were only found among the isolates sampled in the different fields at this location (AFCAA13, AFCAA5, and CA31). At Tunis, of the ten loci, only two alleles were shared among isolates of the two populations sampled (AFCAA1, CAA57) (Table 4).

Low to high genetic and genotypic diversity was detected, depending on the location and the population (Table 5). GENCLONE analysis detected 67 different multilocus genotypes (MLG) of *A. fabae* among all the isolates sampled in 2012, with only six genotypes present in more than one location. Twenty genotypes were detected only once in the overall population. No MLG was detected in all four locations (Table 5). MLGs 28, 4, 60, and 62 represented 12.4%, 7.8%, 6.6% and 5.4% of the studied isolates, respectively. MLGs 27 and 42 represented, 3.9% and 3.1% of the isolates, respectively. The other MLGs included less than 2.7% of isolates. By location, genotypic richness (R) ranged from 0.11 (Jendouba) to 0.42 (Beja). Genotypic diversity was significantly greater than 0 ($P < 0.05$) in all four locations. Isolates from Beja showed the greatest genotypic diversity (D = 0.982), followed by those from Bizerte (D = 0.809), Tunis (D = 0.803) and Jendouba (D = 0.726) (Table 5). At Beja, the frequency of the most common genotype (MLG 27) represented 8.8% of all the isolates sampled at that location. At Bizerte, the main MLG (MLG 28) represented 39.7% of all the isolates. At Jendouba and Tunis, the main MLGs (MLG 4 and MLG 42) represented 43.5 and 30.8% of all the isolates sampled at those two locations (Table 5). Different MLGs were recorded at different locations, respectively (Table 5). MLGs 13, 28, and 62 were detected at Beja and Bizerte, MLG 56 at Beja and Tunis, MLG 42 at Jendouba and Tunis, and MLG 60 was detected at Bizerte and Jendouba.

Genetic diversity indices also showed low to high variability among isolates within populations at the different locations studied (Table 6). Genotypic richness (R) ranged from 0 to 1 within the different populations studied. Isolates from Beja showed the greatest genotypic richness (R = 0.42), followed by Tunis (R = 0.25), Bizerte (R = 0.13) and Jendouba (R = 0.11). Allelic richness (*Ar*) was greatest at Beja (*Ar* = 5.84), followed by Bizerte (*Ar* = 3.95), Tunis (*Ar*= 3.35) and Jendouba (*Ar* = 1.63). As the populations studied were of unequal sample size, *Ar* was corrected for each dataset. This showed that Beja still had the greatest allelic richness (*Ar* = 5.04), followed by Tunis (*Ar* = 3.2), Bizerte (*Ar*= 3.15) and Jendouba (*Ar* = 1.49). Statistics on the Index of association (I_A) and *Rd* were significantly different from 0 at all the locations (I_A ranged from 1.827 ($P < 0.001$) to 7.223 ($P < 0.001$); *Rd* ranged from 0.182 ($P < 0.001$) to 0.642 ($P < 0.001$)). This

Table 5. Occurrence of multilocus genotypes (MLGs) among sixteen populations of *Ascochyta fabae* sampled at four locations (Beja, Bizerte, Jendouba, Tunis) in Tunisia, during the 2012 cropping season.

MLG	Beja	Bizerte	Jendouba	Tunis	Total occurence
MLG1-MLG2	X				3; 2
MLG3		X			1
MLG4-MLG5			X		20; 1
MLG6-MLG12	X				1; 2; 3; 1; 3; 2; 2
MLG13	X	X			2; 1
MLG14-MLG18	X				4; 4; 3; 2; 1
MLG19-MLG20			X		2; 1
MLG21-MLG22		X			3; 7
MLG23				X	3
MLG24		X			7
MLG25				X	2
MLG26-MLG27	X				1; 10
MLG28	X	X			3; 29
MLG29	X				1
MLG30		X			7
MLG31-MLG37	X				1; 1; 5; 3; 2; 1; 2
MLG38		X			4
MLG39-MLG40	X				4; 1
MLG41			X		1
MLG42				X	8
MLG43-MLG47	X				1; 2; 1; 4; 1
MLG48			X		4
MLG49-MLG50	X				1; 3
MLG51			X		1
MLG52-MLG55	X				2; 3; 1; 1
MLG56	X			X	1; 3
MLG57	X				3
MLG58				X	3
MLG59	X				5
MLG60		X	X		1; 16
MLG61				X	7
MLG62	X	X			1; 1"
MLG63-MLG67	X				2; 2; 4; 2; 3; 13
Ni	109	71	39	21	
Nu	48	10	8	6	
Fm	8.8%	39.7%	43.5%	30.8%	
D	0.982	0.809	0.726	0.803	

Nu = Number of unique multilocus genotypes in each population.
Fm = Frequency of the most common genotype
D = Genotypic diversity (Pielou, 1969).
Null hypothesis of no genotypic diversity was tested by comparing *D*-values obtained from 1000 randomized data sets to those estimated from the observed data set. *0.01 < *P* < 0.05, **0.001 < *P* < 0.01, ***$P < 0.001$

Table 6. Genetic diversity, multilocus gametic disequilibrium, and test for random mating within *Ascochyta fabae* populations from Tunisia sampled during the 2012 cropping season.

Location	Populations	N	G	R	*Ar*	*Ar* (n=6)	H'	I_A	R_d
Beja	Bej1	8	8	1	5.5	5.3	0.886	0.241	0.029
	Bej2	7	6	0.83	4.1	4.1	0.742	1.531	0.174
	Bej3	12	11	0.91	6.0	5.3	0.850	1.464	0.171
	Bej4	12	10	0.82	6.7	5.7	0.841	2.971	0.349
	Bej5	16	11	0.67	6.5	5.1	0.800	2.677	0.304
	Bej6	6	6	1	4.4	4.4	0.867	1.360	0.175
	Bej7	14	12	0.85	6.8	5.4	0.809	2.192	0.252
	Bej8	25	19	0.75	7.7	5.4	0.806	1.735	0.198
	Bej9	9	8	0.86	4.9	4.7	0.792	2.274	0.271
	Total	*109*	*48*	*0.42*	*5.84*	*5.04*	*0.821*	*1.827*	*0.182*
Bizerte	Biz1	16	4	0.20	3.5	2.9	0.495	7.376	0.820
	Biz2	55	6	0.10	4.4	3.4	0.589	7.071	0.786
	Total	*71*	*10*	*0.13*	*3.95*	*3.15*	*0.542*	*7.223*	*0.641*
Jendouba	Jen1	6	2	0.20	1.9	1.9	0.300	8.000	1.000
	Jen2	22	3	0.10	1.1	1.1	0.089	-	0.511
	Jen3	11	1	0	2.0	1.5	0.018	2.996	-
	Total	*39*	*5*	*0.11*	*1.63*	*1.49*	*0.135*	*5.498*	*0.603*
Tunis	Tun1	15	6	0.36	3.7	3.4	0.480	4.789	0.534
	Tun2	6	4	0.60	3.0	3.0	0.613	4.251	0.476
	Total	*21*	*6*	*0.25*	*3.35*	*3.2*	*0.630*	*4.520*	*0.483*

N = number of isolates.
G = number of distinct multilocus genotypes.
R = genotypic richness.
Ar=, allelic richness
Ar (n=6), allelic richness corrected for samples size
H': expected heterozygosity without bias (Nei, 1978).
I_A = association index.
Rd statistic is a measure of the multilocus linkage disequilibrium (Agapow and Burt, 2001)

indicates high multilocus linkage disequilibrium and clonal populations (Table 6).

Structure of Ascochyta fabae *populations in Tunisia*

Partition of molecular diversity among and within locations, as well as among and within populations, was studied using an analysis of molecular variance (AMOVA) (Excoffier *et al*., 1992; Lynch and Milligan, 1994). AMOVA revealed that 74.34% ($P < 0.001$) of the total genetic variance was partitioned within populations. A relatively low proportion of genetic variability was attributable to differences among populations within locations (14.52 %, $P < 0.001$)) and among locations (11.14 %, $P = 0.0146$).

High variances of F_{ST} values were detected, ranging from -0.055 to 0.982. Pairwise F_{ST} s were greater between

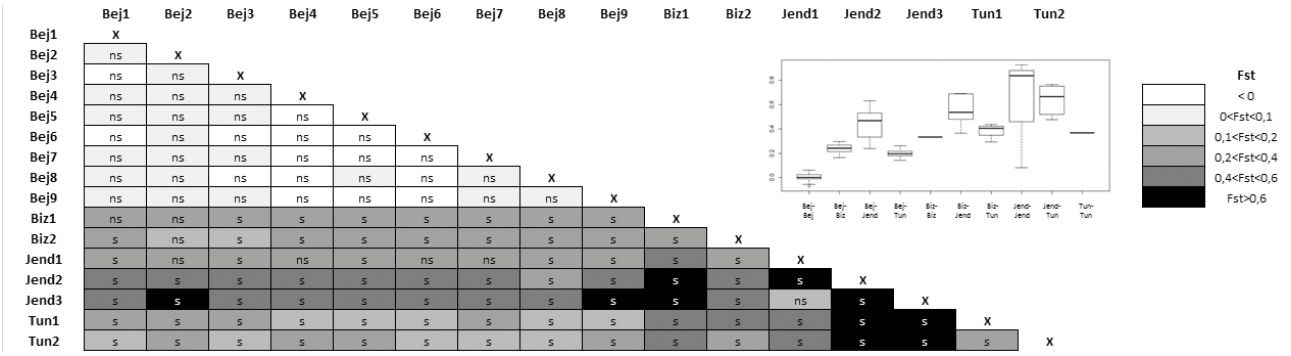

Figure 1. Matrix of pairwise F_{ST} among *Ascochyta fabae* sub-populations sampled in 2012. "ns" indicates non-significant differentiation between two populations (p: sub-population) from different locations (Bej = Beja, Biz = Bizerte, Jend = Jendouba, Tun =: Tunis). The distribution of pairwise F_{ST}s among each sub-population is indicated by the box plots.

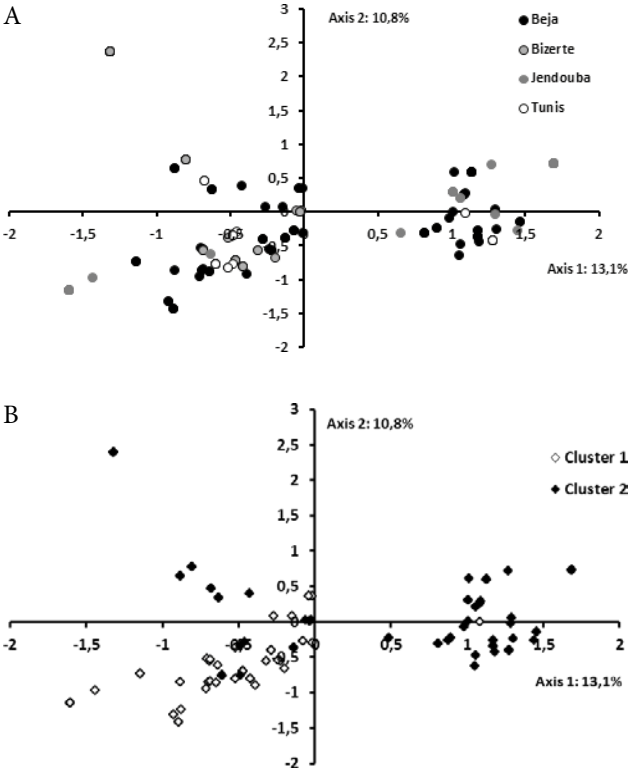

Figure 2. Principal component analysis (PCA) realized on the *Ascochyta fabae* sub-populations sampled from the five locations in Tunisia in 2012. **A**: isolates were assigned to their respective location (Beja, Bizerte, Jendouba, Kef, or Tunis), **B**: isolates were assigned to their respective clusters (C1, C2, or C1/C2) estimated with STRUCTURE (k = 2, deltak = 10).

locations than between populations within specific locations (Figure 1). The isolation for distance test did not show significant correlation (data not shown). The F_{ST} value was not correlated to the geographic distance separating two populations.

Lack of genetic differentiation of *A. fabae* populations was confirmed by the STRUCTURE and PCA analyses (Figure 2). PCA failed to separate the *A. fabae* isolates of the different populations sampled at Beja, Bizerte, Jendouba and Tunis into different groups (Figure 2A). The low percentage of genetic diversity explained by the two principal axes of the PCA (respectively, 13.1% and 10.8%) suggests that all the isolates belonged to the same population. The STRUCTURE analysis also failed to separate the isolates from the different locations into different groups (k = 2, *deltaK* = 10) (Figure 2B). The Bizerte populations were homogenic compared to the other locations.

A minimum spanning network (MSN) using Nei's distance for the different populations of *A. fabae* was calculated. As with the two other analyses, the MSN failed to clearly classify and structure the different populations (Figure 3). Except for the isolates from the population Jen 2 that constituted a single node, all others were distributed throughout the network, and the isolates were not grouped according to origin.

Seasonal genetic structure change in different Tunisian locations

Seasonal change of genetic diversity was monitored at the four locations (Beja, Bizerte, Jendouba and Tunis), and with the 317 isolates. Except for Tunis, all the locations showed different genotypic richness (R) in the two seasons. R values were greater in the second season than in the first (0.40 to 0.83 at Beja, 0.14 to 0.47 at Bizerte, and 0.16 to 0.30 at Jendouba) (Table 7). Multilocus genotypes occurred in from four to 46 isolates for the different populations. Only Beja and Jendouba showed common genotypes between the two successive seasons (Table 7).

Low to high variances of F_{ST} values were detected between the two successive seasons at the different loca-

Table 7. Genetic differentiation, test for random mating (I_A, R_d), and pairwise genetic comparison F_{ST} between two successive seasons at four Tunisian locations.

	Beja		Bizerte		Jendouba		Tunis	
	Season 1	Season 2	Season 1	Season 2	Season 1	Season 2	Season 1	Season 2
N[a]	13	109	71	16	28	39	21	20
N_T	11	46	11	8	9	8	6	4
R	0.83	0.40	0.14	0.47	0.30	0.16	0.20	0.16
N_C	2		-		3		-	
I_A	1.473***		4.797***		4.929***		4.541***	
R_d	0.167***		0.535***		0.548***		0.506***	
F_{ST}	0.0177 ns		0.1882***		0.0645*		0.3269***	

A = Number of isolates analyzed.
N_T = Number of MLG.
N_C = Number of common MLG.

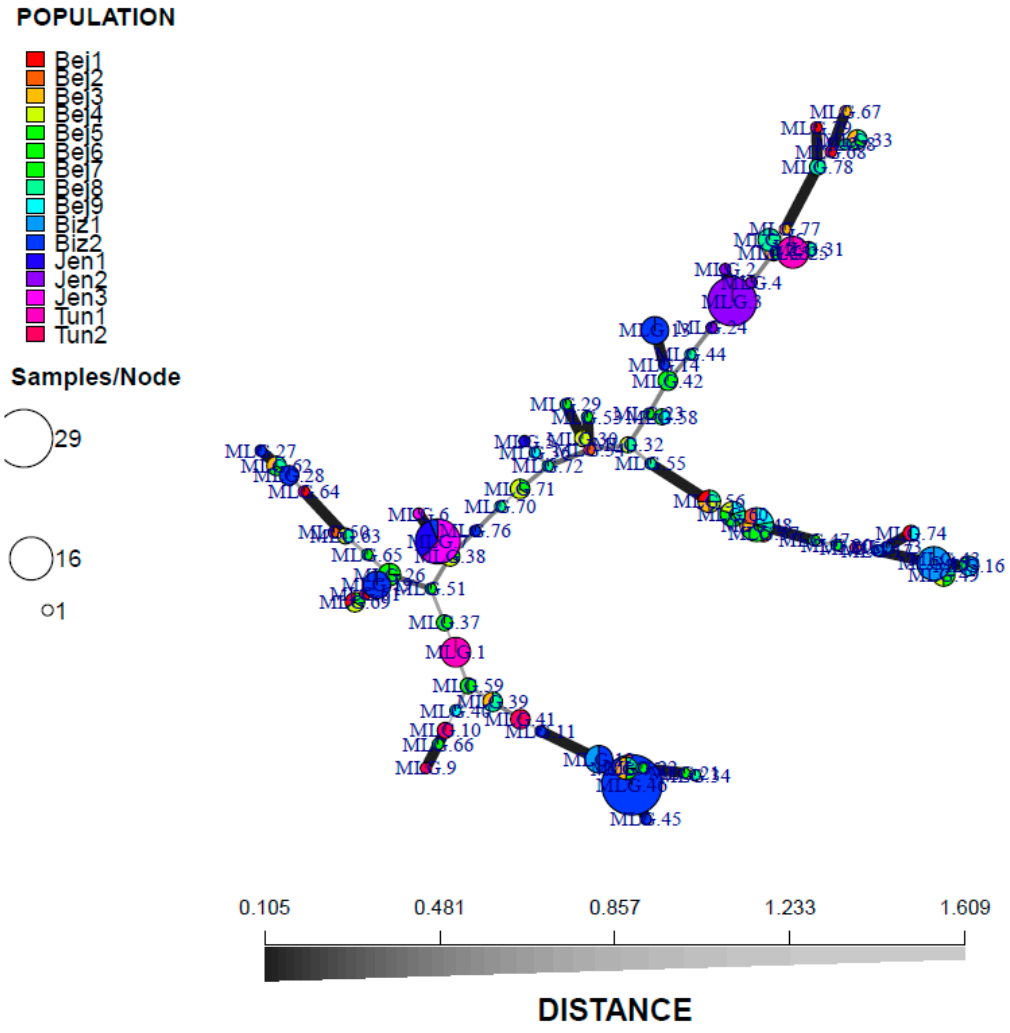

Figure 3. Minimum spanning network (MSN) of 67 haplotypes detected in the *Ascochyta fabae* collection of 2012. Each circle represents a unique haplotype and the different colours represent the sampling fields. The circle sizes represent the haplotype frequencies and the numbers of isolates is indicated in circles. Line widths and the shading represent relatedness of the haplotypes, based on Nei's genetic distance.

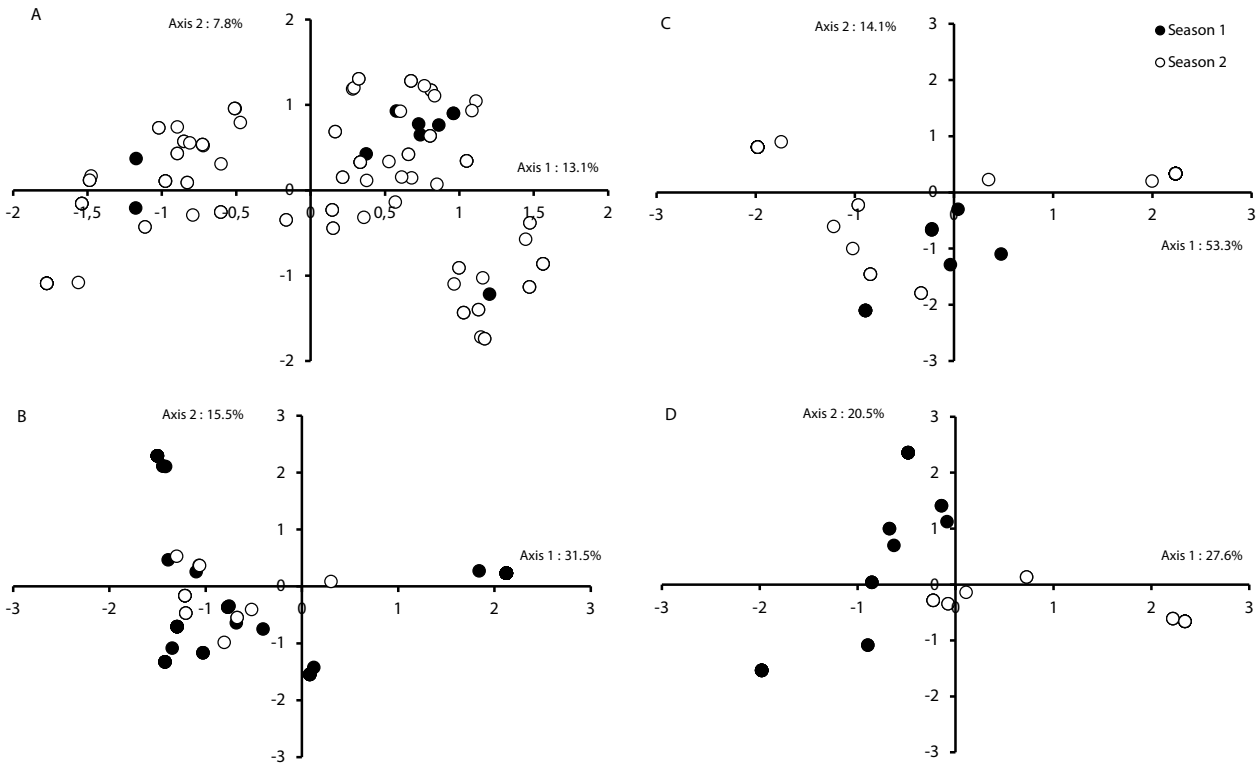

Figure 4. Principal component analysis (PCA) realized on the *Ascochyta fabae* sub-populations sampled during two successive cropping seasons at four locations in Tunisia. (A = Beja; B = Bizerte; C = Jendouba; D = Tunis).

tions. Pairwise F_{ST} were significantly different between the two seasons in Tunis (F_{ST}= 0.3269), Bizerte (F_{ST}= 0.1882) and Jendouba (F_{ST}= 0.0645) (Table 7). The PCA failed to separate the *A. fabae* isolates sampled in the two seasons from each location into two different groups, with the exception of the isolates from Tunis (Figure 4). The STRUCTURE analysis was also performed on these data, but failed to separate isolates from the different seasons into different groups (data not shown).

DISCUSSION

This study is only the second published report of examination of the diversity of *A. fabae* populations. Each of the ten pairs of SSR primers produced a single amplicon for each locus of each isolate with a high level of polymorphism, as established by Ozkilink *et al.* (2011). Among the 240 Tunisian isolates sampled in 2012, MAT1-2 was more common in Tunisia than MAT1-1, and mating type results showed that Tunisian populations have skewed distribution (1:2 ratio) for MAT1-1:MAT1-2. Of the four locations studied, two (Beja and Tunis) gave a 1:1 distribution while for the other two locations, MAT1-2 was more common than MAT1-1. This skewed distribution was confirmed by a chi-square test, suggesting that sexual reproduction may not occure at these two locations. These results were also confirmed by statistics on the Index of association (I_A) and *Rd,* both of which were significantly different from 0. This indicates that multilocus gametic disequilibrium and clonal (genotypic) diversity analyses did not provide evidence for randomly mating populations.

Our results were consistent with those of Ozkilinc *et al.* (2015) for the number of MLGs observed in *A. fabae* populations, but not for the distribution of mating types. The occurrence of sexual reproduction in the life cycle of Tunisian *A. fabae* populations is not likely to be regular, and ascospores may not be important primary inoculum, unlike for other Ascochyta blight diseases (Tivoli and Banniza, 2007). This conclusion should be confirmed, as the sample sizes of the populations in the present study were unequal , and therefore insufficient for the appropriate statistical tests. The observed disequilibrium could be due to sampling phenomena, particularly small sample sizes.

In their study, Ozkilinc *et al.* (2011) sampled *D. fabae* from chickpeas in southeastern Turkey and Israel

between 2004 and 2007. They showed high gene diversity, but with gametic disequilibrium associated with small sample sizes. This result suggests that the gametic disequilibrium observed could be caused by admixtures of genetically distinct populations and/or sampling error, as all populations were assigned to a single genetic population in the STRUCTURE analysis, PCA and MNS analyses (Milgroom, 1996).

Despite the lack of differentiation *into* populations, many rare genotypes and a significant number of private alleles were detected. Only five genotypes of the pathogen were present in more than one location. This contradiction may be explained by the characteristics of the used marker, and suggests that new introductions of the pathogen had been made, either through mutation as microsatellite loci mutate rapidly or through migration or recombination. Between 2003 and 2012, Tunisia annually imported an average of 92.6 tons of faba bean seeds, for either multiplication by seed companies or for farmer use (ONAGRI, 2015). These imports, from countries such as Syria, may be a potential source of new pathogen genotypes. Faba beans imported for consumption average approx. 392 tons annually (ONAGRI, 2015). They are less rigorously controlled for diseases and could be a potential introduction source of new genotypes.

The limited dispersal of *A. fabae* isolates within fields may also lead to gametic disequilibrium. Even if random mating had occurred within restricted genotype profiles, this would have resulted in population admixture, which is one of the causes of gametic disequilibrium (Milgroom, 1996). The absence of common MLGs to all four locations suggests that populations from different locations may originate from multiple population sources. This hypothesis is supported by the F_{ST} values significantly different from zero. Migration could take place through seed movement. Farmers' use of their self-produced seeds may lead to genotypic differentiation at the field scale within the same region according to the values of F_{ST} calculated for pairs of fields; this appeared particularly at Bizerte and Jendouba. The hypothesis is plausible given that agricultural systems in Tunisia are characterized by the predominance of small-holder farmers relying on subsistence farming. In addition, lack of appropriate disease management strategies can result in pathogen multiplication and spatial dispersion through rain episodes and favourable temperatures (Tivoli and Banniza, 2007).

Furthermore, the greater genetic diversity detected at Beja could also be due to climatic conditions being more conducive to the pathogen in this region. *Ascochyta fabae* is favoured by low temperatures, and severe rainfall episodes that result in splash dispersal of pycnidiospores (Maurin *et al*., 1990). The different locations had similar temperatures during the cropping season (Beja; Tmean = 14.3°C, Tmin = 8.9°C, Tmax = 19.6°C: Bizerte; Tmean = 14.9°C, Tmin = 10.5°C, Tmax = 19.3°C: Jendouba; Tmean = 14.6°C, Tmin = 8.6°C, Tmax = 20.7°C: Tunis; Tmean = 15.0°C, Tmin = 10.1°C, Tmax = 19.9°C). However, differences in rainfall were observed. Beja recorded annual rainfall of 479 mm, compared to 293 mm at Tunis, 345 mm at Jendouba, and 368 mm at Bizerte. This greater higher rainfall at Beja provided a conducive environment for *A. fabae* to spread and rapidly reproduce (Pritchard *et al*., 1989; Tivoli and Banniza, 2007).

Selection of *A. fabae* by the host may be ruled out as having any significant impact. Research was carried out in Tunisia attempting to identify sources of resistance (Kharrat *et al*., 2006). Some faba bean cultivars were released, but none are currently sold in their pure original states at seed markets. Due to the mixing of seed types, cultivars lose their distinctive characteristics after a few years, and the pathogen would face a host population characterized by high levels of genetic diversity. This would not necessarily expose the pathogen to directional selection, but would likely maintain pathogen diversity (McDonald and Linde, 2002).

Monitoring seasonal changes of the genetic structure of *A. fabae* at Beja, Bizerte, Jendouba and Tunis showed that new genotypes were detected in the second season in each region. This was unlikely to be derived from an event of sexual reproduction, since the allelic richness did not correlate with the number of MLGs identified in each region. A high level of inbreeding, revealed by significant values of F_{ST} in the two seasons, as well as the absence or scarcity of common genotypes, support the hypothesis that genotypic variation may be the result of genotype migration through seed exchange between different regions, as reported by Kaiser *et al.* (1997), and/or differential genotype adaptive potential to environmental conditions. As shown in the case study of Frenk el *et al.* (2010) from wild and domesticated *Cicer* species, a summer cropping system can act as a climatic factor for diversification of *A. rabiei* populations. These findings weaken any hypothesis of sexual reproduction in the different regions, but strengthen that of involvement of other evolutionary forces of diversity such as genotypic migration. This emphasizes the important role of seeds in disease dispersion at a regional scale (Tivoli and Banniza, 2007; Ali *et al*., 2012).

In conclusion, our study has shown that neither the genetic diversity observed within the Tunisian populations of *A. fabae*, nor multilocus gametic disequilibrium, provide evidence for randomly mating populations of

the pathogen in Tunisia. Lack of differentiation could be attributable to migration or mutation. The role of sexual reproduction should be confirmed by extending the sampling of isolates in these different locations, by calculating the P_{sex} index (GENETIX) and by evaluating the locus compatibility parameters (Arnaud-Haond *et al*., 2005). The extent of asexual compared to sexual reproduction can affect disease management.

Sexual reproduction plays a role in the overwintering/adaptability of *A. fabae* by generating pathogen variability and facilitating long-distance dispersal via airborne ascospores (Kaiser, 1992; Trapero-Casas and Kaiser, 1992; Kaiser *et al*., 1997). This can shape the structure and change genetic variability in populations. Despite recent advances, the role of gene flow (and of its two mains triggers, reproduction and dispersal) in the local evolution of pathogen populations is still poorly assessed. Many fungal plant pathogens alternate cycles of asexual multiplication with single annual episodes of sexual reproduction (Barrès *et al*., 2012). The number of cycles of asexual multiplication has demographic impact as this corresponds mostly to the epidemic phase of a disease, and will also affect the genetic characteristics of pathogen populations.

Some studies of population genetics of airborne plant pathogens have used nested hierarchical sampling strategies. Gobbin *et al*. (2005) explained the arrival of new genotypes and the erosion of clonal structure in *Plasmopara viticola* populations by the continual input of sexual spores. For *A. fabae*, no data of changes in clonal structure during epidemic seasons is available. Studying the temporal changes in genes and genotypic diversity between the beginning and the end of an epidemic would be informative for the different epidemiological processes involved (Prugnolle and De Meeûs, 2010). This could provide insights into the balance between auto- and allo-infection processes.

To fully determine the degree of variability contributed by sexual recombination in pathogen populations, sampling on small spatial, but large temporal scales would help to address this question. Furthermore, analyzing progeny from naturally occurring pseudothecia for segregation would further indicate variability due to recombination (Milgroom, 1996). Studying the biology (temperature optima) of the two mating types and their fitness may further clarify this point. Migration of clones driven by seed movement at national and international levels may play a key role in obtaining high levels of genotypic diversity (Kaiser *et al*., 1997). On the other hand, multiple cycles of asexual reproduction result in rapid increases of secondary inoculum and aggressive pathogen clones (Kaiser *et al*., 1997; Tivoli and Banniza, 2007).

Strategies to better control Ascochyta blight will prioritize a focus on seeds. Several rules will need to be developed, including: i) imposition of standards for certified faba bean seed production, and inciting farmers to use certified seeds (to reduce seed transmitted diseases), and to avoid the use of seeds sold in local markets; ii) establish a strict system to control imported faba beans for local consumption; and iii) advocating systematic fungicide seed treatments to avoid risks of disease transmission through seeds during growing seasons.

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