Aggressiveness Changes in Populations of Didymella pinodes over Winter and Spring Pea Cropping Seasons

Gaëlle Laloi, Josselin Montarry, M. Guibert, Didier Andrivon, Didier Michot, Christophe Le May

Aggressiveness changes over time in populations of *Didymella pinodes* over winter and spring pea cropping seasons

Laloi G.[1, 4], Montarry J. [1], Guibert M.[1], Andrivon D. [1], Michot D. [2, 3], Le May C.[1, 2, *]

[1] INRA, UMR1349 Institut de Génétique, Environnement et Protection des Plantes (IGEPP), Domaine de la Motte, 35653 Le Rheu, France.

[2] Agrocampus-Ouest, Laboratoire Ecologie et Santé des Plantes (ESP), 65 rue de Saint Brieuc, 35042 Rennes, France.

[3] INRA, UMR1069 Sol Agro et Hydrosystème Spatialisation (SAS), 65 rue de Saint Brieuc, 35042 Rennes, France.

[4] Present address : INRA, UMR1345 Institut de Recherche en Horticulture et Semences (IRHS), 42 rue Georges Morel BP60057, 49071 Beaucouzé France.

*** Corresponding author**

Christophe Le May

E-mail: lemay@agrocampus-ouest.fr

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ABSTRACT

Ascochyta blight, caused by the necrotrophic ascomycete *Didymella pinodes,* is responsible for severe losses in winter and spring pea crops. Despite different climatic conditions, epidemics on winter and spring crops are due to a single population of *D. pinodes,* suggesting gene flow either between the two crops or from reservoir sources during the cropping season. This should lead to similar pathogenicity characteristics in isolates sampled from both crops. However, these hypotheses have never been formally tested. We thus sampled a total of 520 *D. pinodes* strains throughout a growing season from winter and spring pea plots (WP and SP, respectively) and from trap plants (TWP and TSP). AFLP markers revealed a high genetic diversity within sub-populations, whereas pathogenicity tests showed that mean aggressiveness increases over the course of epidemics. These results support the idea that allo-inoculum contributes to the carry-over of epidemics between winter and spring crops, and that the most aggressive isolates are selected as the epidemics progress.

IMPORTANCE

Ascochyta blight, caused *Didymella pinodes,* is responsible for severe losses in pea crops. While previous study have showed that Ascochyta blight epidemics on winter and spring crops are due to a single population of *D. pinodes,* suggesting that isolates from both crops present similar pathogenicity characteristics, that hypothesis have never been tested. Genetic analysis of sub-populations sampled throughout a growing season from winter and spring pea plots revealed a high genetic diversity within sub-populations, whereas pathogenicity tests showed that mean aggressiveness increases over the course of epidemics.

Keywords: Ascochyta blight, genetic structure, gene flow, *Didymella pinodes,* AFLP, pea, aggressiveness

Introduction

The spatio-temporal dynamics of crop diseases are simultaneously impacted by pathogens, host plants, environment and human activities (**1, 2**). Indeed, whether or not hosts and pathogens interact is largely determined by spatial and temporal components of host and pathogen life-history traits largely (**3, 4**). These interactions can thus be conceptualized as a continuous sequence of biological cycles including dormancy, growth, reproduction, dispersal, and pathogenesis (**5**).

Gene flow, resulting from pathogen reproduction and dispersal, can drastically increase the extent to which pathogen epidemics spread across a landscape (**6**). As such, it is therefore a main factor in the transmission of disease to previously uninfected areas, and drives the spatial structure of pathogen populations in fragmented landscapes by influencing the long-term survival and genetic composition of populations (**7, 8, and 9**). Individual dispersal events, occurring over periods of days or weeks during both the cropping and intercropping seasons (**2**), originate from a large number of potential inoculum sources: resting structures in soil (mycelium, oospores, chlamydospores or sclerotia), infested stubble left on the soil surface, infested seed, and alternative hosts (wild or cultivated plants, including volunteers). The degree of connectivity among host populations is thus likely to influence spatial patterns of disease occurrence and persistence (**10, 11, 12, 13, and 14**). Dispersal events also shape the structure and changes of population genetic variability. The role of gene flow within and among plant pathogen populations is still insufficiently characterized, but is crucial to understand the distribution of alleles conferring virulence or fungicide resistance within populations. Disease dispersal can favor the contact between wild-cultivated or cultivated-cultivated areas during the cropping and the intercropping season, and thus gene flow between these compartments can influence the genetic structure of populations. By mixing populations submitted initially to different selection pressures, it can also negate the effect of local selection on adaptation and result in local or general maladaptation (**15**).

Reproduction is the other important factor which impacts the genetic structure of plant pathogens (**16, 17**). The life cycle of many fungal plant pathogens alternates asexual multiplication with

episodes of sexual reproduction, but their relative importance varies both within and among species (**18**). Asexual multiplication rapidly increases the size of populations. Strains with the best reproductive success are amplified and tend to decrease the overall population genotypic diversity in time (**19, 9**). For instance, in the case of the grapevine powdery mildew, a positive relationship between spatial and genetic distances shows that epidemics result from the spread of clones within a crop (**20**). Besides, the genotypic diversity may be increased with a genetic mixing by sexual reproduction (**21, 18**). For the potato late blight pathogen *Phytophthora infestans*, clonal reproduction has been reported in populations with restricted levels of genotypic diversity from UK (**22**) and France (**23, 15**), whereas sexual reproduction has been reported in populations with high genotypic diversity from Nordic countries (**21, 24**).

Despite recent advances, the role of gene flow (and of its two mains triggers, reproduction and dispersal) on the local evolution of pathogen populations is still poorly assessed. This is especially the case for epidemics occurring on both winter and spring genotypes of the same host. This situation raises specific questions, particularly regarding the respective roles of auto- and allo-inoculum (Inoculum fraction produced by an exogenous source to the plot (**25**)) in the epidemic process and in the pathogenicity changes within populations. To address these questions, we analyzed the phenotypic and genotypic diversity in populations of an aggressive foliar pathogen of pea, *Didymella pinodes* along the course of an epidemic. *D. pinodes* is a necrotrophic, polyphagous, polycyclic and homothallic fungus, that causes Ascochyta blight on winter and spring pea fields worldwide (**26, 27**). This pathogen is known to present individuals varying widely in their ability to cause disease on pea (**28, 29, 30, 31, and 32**). Contrary to many necrotrophic fungi, *D. pinodes* can simultaneously develop its anamorph (asexual) and teleomorph (sexual) forms on the same plant during the growing season (**33, 32**). Moreover, due to the indeterminate growth of the pea crop, both pycnidia (asexual) and pseudothecia (sexual) can be observed simultaneously on the same plant organs. Whereas pycnidia are produced on both green and senescent plant organs, pseudothecia only appear on the senescent parts (**33**). This fungal pathogen has different ways to persist in the environment and to disperse over

more or less long distances (splashing, wind) (**27, 34, 35**). These features are more important than for pathogens where reproduction modes are dissociated, as they help to increase the population diversity and have an impact on pathogenicity. A recent study (**32**) showed that epidemics on winter and spring pea crops are due to a single *D. pinodes* population. Despite the long intercropping season, *D. pinodes* populations from winter and spring pea display a high, but similar genetic variability. Contrary to other natural plant–pathogen associations, no high annual extinction is observed (**36, 37, 38, 39, and 40**). Population turnover is thus likely to be driven by both local selection and demographic stochasticity due to seasonal population growth and decline. During spring, abundant spore production (**41**) leads to high colonization rates. The fact that winter and spring pea crops are attacked by the same populations of *D. pinodes* suggests that, as observed by Laine & Hanski (**42**) in another ascomycete plant pathogen, regional persistence of *D. pinodes* populations could occur at the scale of metapopulations, consisting of many coupled populations.

The origin and the availability of inoculum sources during the cropping season are still questionable. Do *D. pinodes* populations colonizing winter pea crops constitute the only inoculum sources for the spring pea crops? Does their apparent genetic similarity translate into similar pathogenicity levels? This study, designed to address these questions, is thus based on a collection of *D. pinodes* isolates sampled throughout a single cropping season (January to June) on both winter pea (WP) and spring pea (SP) crops, but also on trap plants (TWP and TSP) catching the allo-inoculum. The specific objectives of this work were 1) to determine if and how *D. pinodes* agressiveness evolves during the cropping season, 2) to define if *D. pinodes* populations developed on winter pea crops constitute the main inoculum source for spring pea crops, and 3) to and analyse the relationships between crop populations and allo-inoculum catched on trap plants.

Materials and methods

Field experiments. Two field experiments were carried out at the INRA experimental station of Le Rheu, western France (48°06 00 N, 1°48 00 W, 30m above mean sea level) in 2004-2005. Both fields, separated by more than three km, shared similar pedoclimatic environments and were free from soil borne inoculum, due to a rotation without pea crop during the five previous years. These fields were respectively sown with a winter (Cheyenne, GAE-Recherche, France) and a spring pea cultivar (Baccara, Florimond-Desprez, France). The winter cultivar was sown on October 25th, 2004, and the spring crop was sown on February 24th, 2005. For both crops, the experimental design consisted of a 30m^2 (3m wide x 10m long) plot divided into thirty micro-plots (1 m² each)**.**

Trap plants. Allo-inoculum, defined as the inoculum fraction produced by an exogenous source to the plot (**25**) was assessed through trap plants. Each week from mid-January to the end of June 2005, one tray containing 20 trap plants (Cheyenne or Baccara seedlings at the 5-leaf stage, respectively for the winter and spring trials) was placed at each of the four corners of the plots. After seven days exposure in the field, trap trays were brought back and incubated in a dew chamber (12h-photoperiod, 20°C night/day, 100% relative humidity) for four days. Deposition of viable ascospores on trap plants was assessed by isolating strains from the small, purple-black, irregular flecks on the five lower stipules of the plants after incubation (**41**).

Spatio-temporal disease development. Disease started in each crop without artificial supply of inoculum. Disease severity was assessed visually once a month in the different micro-plots from January to June. To determine disease development at the plot level, one plant was randomly collected from each micro-plot at the different sampling dates (five sampling dates for the winter pea crop, and three sampling dates for the spring pea crop). Disease was scored on each stipule of the sampled plants using the adapted disease scale of Tivoli (1994) (**41**), and a mean disease score (D_i) was calculated. Disease distribution maps were created for each sampling date using the Arcmap®

153 software and a deterministic method based on the estimation of the weighted inverse distance (**43**).

154

155 **Fungal strains.** A total of 520 strains of *D. pinodes* collected from infected field pea plants and trap
156 plants were used in this study. Each field was divided into 30 micro-plots where one plant was
157 randomly sampled at each sampling date. One hundred and fifty strains were sampled from winter
158 pea (WP) during the winter cropping season (February to May). Ninety strains were sampled from
159 spring pea (SP) during the spring cropping season (May and June). Two hundred and fifteen strains
160 were sampled from trap plants placed at the corner of the winter pea plots (TWP), and sixty-six
161 strains were sampled from trap plants placed at the corner of the spring pea plots (TSP). For each
162 population (WP, SP, TWP and TSP), the different sub-populations correspond to the different sampling
163 dates (**Table I**). All the strains were cultured and single-spored before being studied. For isolation,
164 approximately 5 mm^2 of diseased leaf tissue was surface-sterilized for 1 min in 70% ethanol, rinsed
165 three times in sterile water, placed on sterile filter paper to remove excess water, and plated for 14
166 days on V8 medium (99 mL V8 vegetable juice [Campbell, France], 35 g agar, and 801 mL distilled
167 water, autoclaved at 105°C for 30 min) distributed in Petri dishes. Pycnidiospores from resulting
168 cultures were spread on 2% malt agar and incubated for 12 h as described by Onfroy *et al*. (**44**). Single
169 germinating pycnidiospores were transferred to fresh V8 plates under a dissecting microscope, and
170 cultures were incubated at 20°C with a 12 h photoperiod under cool white fluorescent lamps. Single-
171 spore cultures were then maintained on malt slants and stored in the dark at 4°C.

172

173 **DNA extraction and AFLP typing.** Each strain was grown in 75 mL of LP liquid medium (10 g tryptone,
174 5 g extract of yeast powder, 5 g NaCl, 1 L distilled water; autoclaved at 115°C for 20 min)
175 supplemented with streptomycin (1.5 g) and penicillin (0.75 g) from four pieces (approximately 1 cm^2
176 each) cut from the margin of an actively growing culture on malt agar. Inoculated vials were
177 incubated, under agitation, for 14 days at 20°C with a 12 h photoperiod under cool white fluorescent
178 lamps. Mycelia were harvested by vacuum filtration through two layers of sterilized Miracloth

(Calbiochem CN Biosciences, Inc., La Jolla, CA), rinsed twice in sterile water, and stored at -80°C until lyophilized. DNA was extracted from lyophilized mycelium as described by Lodhi *et al.* (**45**), quantified by measuring the optical density of extracts at 260 and 280 nm with a Nanodrop 1000 spectrophotometer (Thermo Scientific), and adjusted to a final concentration of 100 $ng.L^{-1}$ for amplified fragment length polymorphism (AFLP) analysis. AFLP analysis was carried out as described by Vos *et al.* (**46**) with modifications used by Le May *et al*. (2012). AFLP reactions were performed independently three times, using the same set of primers with reference strains and a random sample of 10 isolates from the collection, and independent DNA preparations of the same strains to estimate the repeatability of fragment scoring.

AFLP analysis. The raw data were analyzed with GeneMapper (Version3.5, Applied Biosystem). The presence and absence of all fragments between 100 and 400 bp were scored in each of the 520 strains. Bands with molecular sizes exceeding 400 bp were not scored because of insufficient resolution. The dataset obtained was based on the assumption that bands of the same molecular weight were identical.

***Genotypic diversity*.** The AFLP data were used to define multilocus genotypes (MLGs) and check for repeated MLGs, , *i.e.* the strains sharing the same alleles at all loci, using the Microsoft EXCEL add-in GenAlEx version 6.5 (**47**). Genotypic diversity was calculated for each population using the Shannon & Wiener's index H' (**48, 49**). The number of different alleles (Na), Shannon's information index (I) and the unbiased diversity (uh) were computed using GenAlEx for each population and sub-population. Clonality was assessed using the index of association (I_A), a measure of the multilocus linkage disequilibrium, calculated with the Multilocus software version 3.1b (**50**). The index of association (I_A) is the traditional measure of multilocus linkage disequilibrium. The "distance" (number of loci at which they differ) between all pairs of individuals is calculated, and the variance of these distances compared to that expected if there is no linkage disequilibrium. Index of association is calculated

using the following formula:

$$I_A = \frac{V_D}{\sum var_j} - 1$$

where V_D is the variance of the distances two isolates over all loci (i.e., the number of loci at which they differ), and var_j corresponds to the variance of the mean distance (either 0 or 1) between all $n(n\text{-}1)/2$ possible pairs of isolates. The higher the I_A, the more clonal the population. Departure from the null hypothesis, *i.e.* complete panmixia, was checked by permuting alleles between individuals independently for each locus (500 permutations).

***Population differentiation*.** The analysis of molecular variance (AMOVA; online as Arlequin version 3.1 software, hosted by the Department of Anthropology, University of Geneva, Switzerland; (**51**) was used to partition molecular variance between populations, between sub-populations within populations and within sub-populations. Pairwise F_{ST} values were calculated with Arlequin for each pair of sub-populations of *D. pinodes* and compared within plots (SP-SP and WP-WP) or trap plants (TSP-TSP and TWP-TWP), between plots (SP-WP) and between plots and related trap plants (SP-TSP and WP-TWP).

***Genetic structure*.** A principal component analysis (PCA) was performed using the procedure available in the package adegenet (**52**) for the statistical freeware R version 3.1.1 (©2014, The R Foundation for Statistical Computing). PCA has an important advantage over other methods, such as the Bayesian clustering algorithm implemented in STRUCTURE (**53**), 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 (**52**).

Aggressiveness of *D. pinodes* strains. Aggressiveness level was evaluated for a random sample of *D. pinodes* strains sampled on winter (sampling dates: WP07, WP10, WP12, WP14) and spring (sampling dates: SP01, SP03) pea plots, and of *D. pinodes* strains sampled on winter trap (sampling dates:

TWP20, TWP24, TWP30, TWP34) and spring trap plants (sampling dates: TSP02, TSP03, TSP06). Five *D. pinodes* strains were randomly chosen within each sub-population and their aggressiveness was evaluated on three pea genotypes: the winter cultivar Enduro (Florimond-Desprez, France), the spring cultivar Lumina (Nickerson, France), and the spring breeding line DP (**54**). Enduro and Lumina were chosen to replace Cheyenne and Baccara, respectively, for which seeds were no longer available at the time of the pathogenicity tests. They presented a similar and high susceptibility level towards the disease, while DP has a higher level of quantitative resistance. Plants were grown at 18 to 20°C for three weeks, until they reached the 5- to 6-leaf stage, before inoculation. Plant preparation and experimental design were as described by (**44**). The inoculation method was based on that proposed by Onfroy *et al.* (**56**). Briefly, strains were grown for 10 days on V8 medium under white light with a 12 h photoperiod at 20°C before pycnidiospores suspensions were prepared by flooding the surface of cultures with sterile distilled water, gently scraping with a glass rod, and filtering the suspension through two layers of sterile cheesecloth. The spore concentration was adjusted to 5.10^4 spores.mL^{-1}, and Tween 20 (VWR International SAS, Strasbourg, France) was added as a wetting agent (2 drops per 500 mL spore suspension). Inoculation consisted of depositing a 10 µL drop of the spore suspension on the upper surface of freshly detached stipules floated, lower surface down, on tap water in a compartmentalized square Petri dish (12 cm side; Gosselin, France). Drops were deposited away from the main veins. To avoid drop evaporation, Petri dishes were placed into large transparent plastic boxes immediately after inoculum deposition and incubated in a climate chamber for seven days with a continuous cycle of 14 h of light at 20°C. Symptom development was assessed two, four, and seven days after inoculation, as described by Onfroy *et al.* (**55**). A 0 -3 semi-quantitative scale (0 = symptom free, 1-flecks appearing, 2 = flecks covering half of the area of drop deposition, 3 = coalescence of the flecks within the area of drop deposition) was used to score symptoms not extending past the inoculation droplet. For stipules with necrosis extending beyond the borders of inoculum drops, lesion diameter (mm) was measured with a graduated ruler. A visual assessment using a 0-7 scale adapted from Wroth (1998), where 0 = symptom free, 1 = flecks appearing, 2 = flecks covering half of

the drop deposit, 3 = coalescence of the flecks in the area of the drop deposit, 4 = 3-to-6mm lesion diameter, 5 = 6-to-9mm lesion diameter, 6 = 9-to-12mm lesion diameter, 7= >12mm diameter, was also performed. Two stipules from each of four different plants per genotype were inoculated. For each genotype, the area under the disease progress curve (AUDPC) was calculated as

$$AUDPC_i = \sum_{j=1}^{m} \frac{(D_{i,j} + D_{i,j+1})}{2} \times (t_{j+1} - t_j)$$

where $D_{i,j}$ and $D_{i,j+1}$ correspond to disease scores at two consecutive dates, t_j and t_{j+1} (**56**). Statistical analysis of the data was performed with the R statistical software, version 3.1.1 (©2014, The R Foundation for Statistical Computing). Normality and homogeneity of variances were checked by the Shapiro-Wilk and the Leven tests, respectively. The effects of population (WP, SP, TWP and TSP), pea genotype (DP, Lumina and Enduro) and their interaction on AUDPC values were tested through a multi-way ANOVA. The effects of sub-populations, pea genotypes and their interaction were tested on AUDPC values using a second ANOVA model. When significant effects were detected, mean values were compared with Tukey tests (alpha = 0.05).

Results

D. pinodes displayed a homogeneous spatial development during the cropping season

Disease layout maps in winter and spring pea crops, as determined with the Arcmap® software, were spatially similar for each set of maps in both pea crops (**Fig. 1**). Disease severity increased with time, but was spatially homogeneously distributed through the plots, with no strong foci. The mean disease scores were higher in the winter pea crop than in the spring pea crop, essentially because of the longer growing season and the more conducive climatic conditions in the late winter and early spring.

Genotypic flow acts on the genetic structure of *D. pinodes* population during the cropping season

The three AFLP primer sets allowed the detection of 646 loci, 388 of them were polymorphic over all tested populations. The percentage of polymorphic markers in each sub-population ranged from 29.90% in TWP21 to 100% in WP8. No repeated MLG was found among the 520 strains, indicating a very low incidence of clonality. However, the overall I_A value significantly differed from zero (I_A = 1.97), thus rejecting the null hypothesis of recombination (P = 0.001) (**Fig. 2**). Number of alleles (Na), Shannon's Information index (I) and unbiased diversity (uh) showed that the highest genetic diversity within sub-populations was for WP12 (I=0.61, uh=0.43) and the least for TWP21 (I=0.176, uh=0.151). The low genetic diversity observed for the sub-population TWP21 may be due to the low number of isolates within this sub-population. At the scale of the populations (WP, SP, TWP and TSP), number of alleles, Shannon's Information index and unbiased diversity showed that the genetic diversity was higher for populations sampled into the plots (SP and WP) than for populations sampled on trap plants (TWP and TSP) (**Table I**).

AMOVA revealed that 80.35 % of the total genetic variance was partitioned within sub-populations (**Table II**). A relatively low proportion of genetic variability was attributable to differences between populations (9.62 %) and between sub-populations within populations (10.03 %). High variances of F_{ST} values were detected, ranging from -0.055 to 0.586. Pairwise F_{ST} were higher between plots and trap plants (WP-TWP and SP-TSP) than within plots (WP-WP or SP-SP) (**Fig. 3**), quite low between plots (SP-WP) and within spring trap plants (TSP-TSP), but quite high within winter trap plants (TWP-TWP).

Principal Component Analysis (PCA) failed to separate into different groups the strains of the sub-populations sampled on WP, SP, TWP and TSP (**Fig. 4**). Moreover, the low percentage of genetic diversity explained by the two principal axis of the PCA (11% and 9.95%, respectively) suggested that these different sub-populations did not constitute distinct genetic groups and that all the strains belonged to a single population.

Aggressiveness changes during the cropping season differed between winter and spring pea populations

The aggressiveness level differed according to test host genotypes (pea genotype effect, **Table III**): as expected, AUDPC values were significantly lower for the resistant pea genotype DP than for the pea genotype Enduro, and intermediate (and not significantly different from the two other genotype) for the pea genotype Lumina. However, the lack of significant interaction between populations or sub-populations and pea genotypes (**Table III**) showed that all populations shared the same ranking across hosts.

Overall, strains sampled on winter plots (WP) and on trap plants (TWP and TSP) were significantly more aggressive than strains sampled on spring plots (SP) (**Fig. 5A**). Moreover, at the sub-population level, the aggressiveness of SP strains increased strongly during the growing season, whereas it remained stable over time in the other three populations (**Fig. 5B**).

Allo-inoculum sampled on TWP or TSP showed a similar aggressiveness level than strains sampled from the winter plot (**Fig. 5A**, **4B**). Altogether, these results tended to show that trap plants were always infected from the winter populations, and that the spring plots initially select against aggressive isolates.

Discussion

One of the objectives of this study was to determine how *D. pinodes* population structures change during a growing season. The amount of genetic variation was similar within *D. pinodes* sub-populations sampled from winter or spring pea plots (H' = 0.83, and 0.86 respectively). The genetic variability observed at the beginning of the season remained roughly the same throughout the growing season. The main part of this genetic variability was observed within sub-populations (80%), which confirms the PCA result of an absence of a clear genetic structure over time, and suggests that significant gene flow occurs between sub-populations. The low pairwise F_{ST} values observed within

(SP-SP and WP-WP) and between plots (SP-WP) suggest also that sub-populations were not genetically differentiated. Previous works released on *D. pinodes*/pea pathosystems showed that *D. pinodes* populations from Canada, France, and Algeria displayed a high degree of genetic variability (**32, 57**). This variation estimated to a different scale than in our study, is illustrated by the high value of Shannon's information index and Nei's gene diversity, and by the high number of distinct haplotypes. Our study showed that population diversity estimated at a field level, and during the growing season is changing, but slightly higher to the diversity estimated among countries or locations within a country. Indeed, H' value in our study ranged from 0.70 to 0.83, whereas it ranged from 0.39 to 0.47 to *D. pinodes* populations collected from different locations in Algeria (**57**). Moreover, H' value was closely similar to those obtained for fungi showing great genetic diversity, as in *A. rabiei* [H'=0.58 (**58**)] *Mycosphaerella fijiensis* [H'=0.58 (**59**)] or *Botrytis cinerea* [H' ranging from 0.401 to 0.518 (**60**)]. This change of diversity level observed during the growing season reinforced the fact that sexual reproduction was important in the life cycle of *D. pinodes* and the predominance of this mode of reproduction seemed to affect earlier the population structure.

On trap plants, pairwise F_{ST} were higher within winter than within spring sub-populations, suggesting that the same allo-inoculum sources must be active during all the whole spring growing season, whereas other allo-inoculum sources must be active during the winter cropping season. This result suggests that epidemics on winter crops are initiated from a large number of sources producing pseudothecia and releasing ascospores, whereas the release of ascospores, probably from the infected winter crops, fuel the spring crop epidemics with a steadier and genetically similar source of inoculum. It also suggests that asexual inoculum is not an important component of epidemic extension, although it might play a major role in local disease intensification. The spatially homogeneous disease severity observed throughout the winter and spring plots sampled here also point to an homogeneous distribution of initial inoculum consistent with ascospore showers from external sources rather than with short distance splashes of asexual pycniospores. If ascospore release indeed constitute the main mechanism fueling the epidemic development and spread, it

would provide also a good explanation for the connected genetic structures of pathogen populations sampled in different plots. As indicated in the material and methods section, our experiment was realized only once, but we have assessed disease development and genetic diversity evolution on two different crops. Past study showed that *D. pinodes* disease developed on winter and spring pea was initiated by a single population, whose pathogenicity is a plastic trait modulated by the physiological status of the host plant. The fact that only a single year was conducted suggests different limitations. A second year of experiment will help us to see if a similar pool of allo-inoculum would be mobilized during the cropping season. Indeed a second year of field experiments exposed us to different constraints: *i)* climatic change that will modify plant development, plant receptivity, availability of different sources of inoculum, *ii)* a second year of field experiment will also suggest that diversity of agrosystem will change (change in prevalence of legume species, diversity of cultivated species in a neighboring environment). As described by Plantegenest *et al*. (**62**) the composition of landscape determines the local abundance of potential reservoirs of inoculum that may obviously influence the global propagule pressure and hence the risk of infection of a plant. Those reservoirs may, in particular, consist of diseased individuals of the same host species or of alternative, either cultivated or wild, hosts. Moreover, change in landscape composition and diversity will change dynamics of pathogen.

The initial, natural hypothesis is that each type of pea crop would serve as the main inoculum source for the other, via exchanges between plots. However, since ascopores are most likely the main inoculum form starting and fueling the epidemics, and given the large dispersal capabilities of wind-borne ascospores, sources other than plants growing in adjacent or neighboring plots need to be considered as inoculum reservoirs. Indeed, our results suggest that various parts of the metapop-ulation are mobilized during the growing season, particularly during winter growing season. Two further sources of inoculum could be involved: alternative host and chlamydospores (**35, 61**).

Compared to infected pea debris, alternative hosts are generally considered of minor importance in the epidemiology of Ascochyta blight (**63**). Recently, Trapero-Casas & Kaiser (**64**) showed that *A.*

382 *rabiei* is capable of infecting different plant species, suggesting that these naturally infected alterna-
383 tive hosts could serve as significant sources of inoculum to initiate disease epidemics on cultivated
384 chickpea. In a recent work, Le May *et al*. (**61**) showed that the asexual stage of *D. pinodes* could be
385 observed on pea, common vetch and clover, fuelling the suggestion that other legume species may
386 also act as inoculum sources for epidemics on peas (**65, 63, 66, 67**). Legume species like vetch or clo-
387 ver generally sympatric to pea crops during the growing season, but also persist during the intercrop-
388 ping season. As shown by Savage *et al*. (**6**), long distance dispersal generally results in rapid transmis-
389 sion of disease to previously uninfected areas, and can facilitate genetic interaction between spatially
390 separated populations, resulting in the introduction of new virulent alleles into existing populations
391 (**68**). Thus, a more extensive knowledge of the host range of *D. pinodes* and of the relatedness be-
392 tween populations from peas and other hosts could help to estimate the risk of Ascochyta blight epi-
393 demics of pea arising from alternate hosts, as in other pathosystems where cultivated and wild hosts
394 grow sympatrically *(***69,70**).

395 *D. pinodes* can also survive in soil as mycelium or chlamydospores (**71, 72**). Davidson *et al*. (**35**)
396 investigated the survival of ascochyta blight pathogens in soils of commercial pea-cropping paddocks,
397 and showed that the level of pathogen populations in the soil was related to the severity of the epi-
398 demic in the last pea crop grown. Although airborne ascospores of *D. pinodes* appear to be the pri-
399 mary inoculum during establishment of field pea crops, soil borne inoculum has also been associated
400 with disease (**35**). To evaluate the impact of this inoculum source on the genetic structure of *D. pi-*
401 *nodes* populations, it would be interesting to develop a genetic approach to characterize *D. pinodes*
402 genetic population variability before and after harvest. It would be particularly interesting to define
403 which level of diversity is maintained between two growing season.

404 Understanding how this gene flow influences the aggressiveness of pathogen populations during
405 the season is crucial for the development of sustainable strategies. In our study, two scenarios for the
406 evolution of the aggressiveness of *D. pinodes* strains installed in the crop were observed: i) an almost
407 constant level of aggressiveness maintained throughout the season in the winter pea crops, and ii) an

408 increase in the average aggressiveness of *D. pinodes* strains installed on spring peas, following initially
409 low aggressiveness levels. The 'spring peas' scenario was quite surprising, as the inoculum released
410 by the winter pea crop was rather aggressive. The low initial aggressiveness in the spring pea plots
411 might therefore sign other infection sources, such as alternate hosts and /or soil, as discussed above.
412 Moreover, the fact that *D. pinodes* population sampled at the beginning of the cropping season
413 displays a lower aggressiveness level suggests that an alterrnative hypothesis would be that spring
414 crops initially select the least aggressive components in the allo-inoculum. However, there is no
415 strong evidence or rationale to support this hypothesis. Interestingly, the fact that aggressiveness
416 levels in spring crops later increased to levels similar to those observed in trap plants and in winter
417 plots suggests either a later influx of aggressive isolates from nearby winter pea fields and/or active
418 selection of more aggressive isolates by the spring plants over the course of the epidemic – which in
419 turn would imply a polycyclic epidemic development within plots. The uniform genetic structure of *D.*
420 *pinodes* populations however does not allow to distinguish between both hypotheses, as ascospores
421 generating the late infections could come either from within or from outside the canopy.

422 This study shows that a monitoring over time allows identifying inoculums sources (auto or allo-
423 inoculums) all along the dynamic of the epidemic. Studying the temporal changes in gene and
424 genotypic diversity between the beginning and the end of an epidemic would thus be informative
425 about the different epidemiological processes involved (**73**). Many fungal plant pathogens alternate
426 rounds of asexual multiplication with a single annual episode of sexual reproduction (**74**). The
427 number of rounds of asexual multiplication has not only a demographic impact as it corresponds
428 mostly to the epidemic phase of the disease but will also have drastic consequences on the genetic
429 characteristics of pathogen populations. As a result, one would expect that both the genetic structure
430 and the gene and genotypic diversity of plant pathogens should change during the asexual phase of
431 the life cycle (**74**). Founder effects resulting from the colonization by a few sexually derived spores
432 and the subsequent asexual reproduction may result in reduced genotypic diversity at the beginning
433 of an epidemic. A few population genetic studies of airborne plant pathogens have used such a

nested hierarchical sampling strategy. Gobbin *et al*. (**75**) explained the arrival of new genotypes and the erosion of clonal structure they observed in *Plasmopara viticola* populations by the continual input of sexual spores. Examining the changes in clonal structure during the epidemic season can provide insights into the balance between auto- and allo-infection processes, thus ideally complementing direct epidemiological observations (i.e. disease monitoring) dedicated to the quantification of the auto-infection process only (**76**). Contrary to a large number of necrotrophic fungi, *D. pinodes* can simultaneously developed its anamorph and teleomorph forms on the same plant during the growing season (**32, 33**). Due to the indeterminate growth of pea plant, during crop growth, both pycnidia and pseudothecia can be observed on the same plant organs (**33**). Thus, beyond these qualitative predictions, estimating the population genetic consequences of intermediate rates of asexual or sexual reproduction remains a challenging task, especially when the organism's life cycle consists of alternate phases of sexual and asexual reproduction. In this study we particularly show that the allo-inoculum, essentially constituted by ascospores, is a driving force in the epidemic dynamics of ascochyta blight of peas. As described by Savage *et al*. (**6**), by connecting wild and cultivated compartments, this allo-inoculum allows to maintain a high level of genetic variability, and to modulate pest pressure during the growing season. Our study also shows that origin of this allo-inoculum still is a central question. Indeed, in the intensive French agriculture, and contrary to more extensive cropping like in Australia or Canada (**72, 35**), the main sources of inoculum are well managed by the growers. Hypotheses explaining the availability in ascospores throughout the cropping season of both winter and spring peas thus need to include ascospores released from stubbles or volunteers before burying, which persist for a long time in the atmosphere (**34**), or potential reservoirs of inoculum, especially in the case of winter peas. Efficient control strategies should reduce the production of allo-inoculum, and therefore to encompass all the possible alternative sources, probably dispersed through the agricultural landscape. Thus, landscape composition and characteristics are important to consider as they may influence pathogen ecology and especially the range of hosts simultaneously or successively exploited (**62**), but additional

knowledge on the possible role, dynamics and characteristics of inoculum sources other than pea (alternate hosts, soil) is also required.

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Table I: Genetic characterization of *Didymella pinodes* strains sampled on winter or spring pea plots (WP or SP) and on Trap winter plants (TWP) or Trap spring plants (TSP). Na = No. of different alleles, I = Shannon's information index (= $-1\Sigma(p.\ Ln(p) + q\ .\ Ln(q))$, h = Diversity (= $1 - (p^2 + q^2)$, uh = Unbiased diversity (= (N/(N-1)) h), where p = Band freq., and q = 1 – p (GenAIEx v6.5)

Source	Sub-populations	Sample date (d/m)	n	Na	I	uh	H'
WP	WP7	17 February	30	1.97	0.51	0.35	0.83
	WP8	28 February	30	2.00	0.58	0.41	
	WP10	29 March	30	1.96	0.49	0.34	
	WP12	25 April	30	1.99	0.61	0.43	
	WP14	23 May	30	1.99	0.56	0.39	
SP	SP1	30 May	30	1.98	0.59	0.42	0.86
	SP2	13 June	30	1.97	0.57	0.40	
	SP3	27 June	30	1.99	0.58	0.41	
TWP	TWP19	28 January	13	1.83	0.50	0.37	0.70
	TWP20	04 February	14	1.77	0.43	0.31	
	TWP21	11 February	5	1.06	0.18	0.15	
	TWP22	18 February	15	1.94	0.55	0.40	
	TWP23	25 February	13	1.81	0.44	0.31	
	TWP24	04 March	12	1.75	0.46	0.34	
	TWP28	01 April	15	1.82	0.48	0.35	
	TWP29	08 April	15	1.88	0.53	0.39	
	TWP30	15 April	14	1.82	0.48	0.35	
	TWP31	22 April	15	1.79	0.39	0.28	
	TWP32	29 April	15	1.48	0.28	0.20	
	TWP33	05 May	13	1.86	0.48	0.35	
	TWP34	16 May	12	1.49	0.31	0.23	
	TWP35	20 May	13	1.85	0.52	0.38	
	TWP36	27 May	15	1.41	0.28	0.20	
	TWP37	03 June	15	1.86	0.47	0.34	
TSP	TSP2	20 May	4	1.06	0.25	0.23	0.76
	TSP3	27 May	9	1.86	0.51	0.39	
	TSP4	03 June	10	1.84	0.49	0.37	
	TSP5	10 June	14	1.84	0.48	0.35	
	TSP6	20 June	14	1.83	0.46	0.33	
	TSP7	27 June	15	1.78	0.45	0.33	

Table II: Analysis of molecular variance (AMOVA) for *D. pinodes* strains sampled from different plots (WP, SP) and trap plants (TWP, TSP) based on AFLP markers.

Source of variation	d.f.	Sum of square	Variance components	Variation (%)	Fixation Indices		P-value*
Among populations	3	3781.378	8.26273	9.62	FCT	0.09616	< 0.000001
Among sub-population within populations	26	5535.163	8.62056	10.03	FSC	0.11100	< 0.000001
Within sub-populations	490	33830.767	69.04238	80.35	FST	0.19649	< 0.000001

Table III: ANOVAs on AUDPC values at the population (A) and at the sub-population (B) levels. Sources of variation are *D. pinodes* population (or sub-population), pea genotype and the corresponding two-way interaction between these variables. Statistically significant effects are indicated by asterisks (*,P < 0.001). df: degrees of freedom.

A

Source of variation	df	F value	P>F
Sub-population	3	28.41	<0,0001*
Pea genotype	2	7.71	0.0006*
Sub-population * Pea genotype	6	0.11	0.9955
Error	180		

B

Source of variation	df	F value	P>F
Sub-population	12	11.22	<0,0001*
Pea genotype	2	8.50	0.0003*
Sub-population * Pea genotype	24	0.23	0.9999
Error	153		

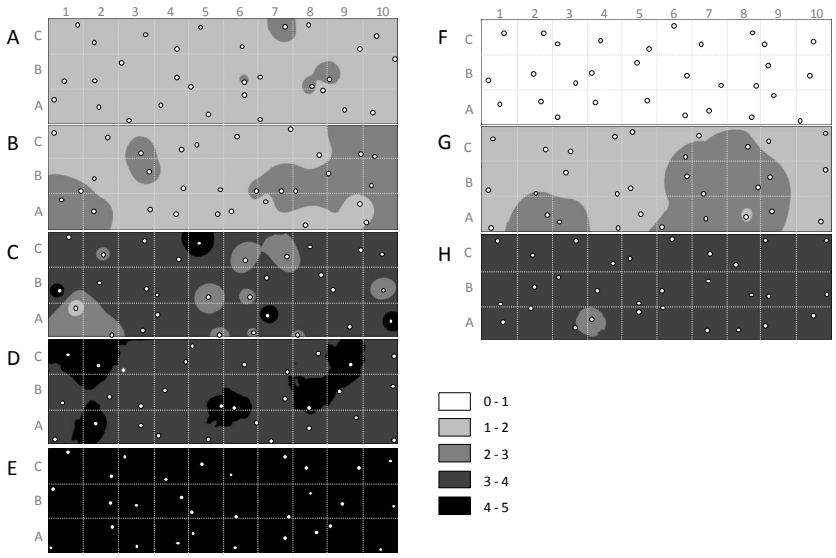

Fig. 1: Maps representing the evolution of disease severity due to ascochyta blight within winter and spring pea plots and for different sampling dates (**Winter sampling dates**: ***A***: 17 February, ***B***: 28 February, ***C***: 29 March, ***D***: 25 April, ***E***: 23 May; **Spring sampling dates**: ***F***: 30 May, ***G***: 13 June, ***H***: 27 June). Each plot was divided into 1m² micro-plots (A1 to C10) where one plant was randomly sampled at each sampling date. Disease was scored on each stipule of the sampled plants using the adapted disease scale of Tivoli (1994) (**40**), and a mean disease score (D_i) was calculated. Disease distribution maps were created for each sampling date using the Arcmap® software.

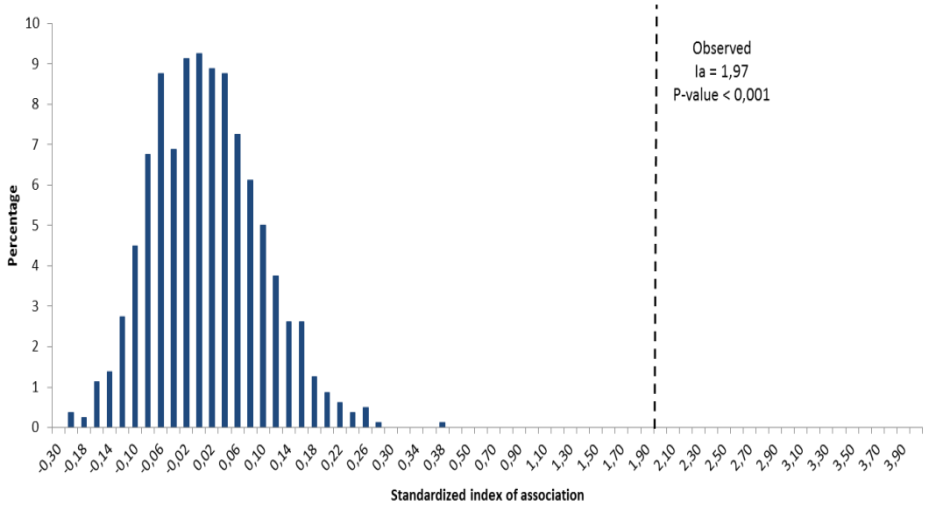

Fig. 2: Standardized index of association (I_A) calculated from the clone-corrected dataset, rejecting the absence of linkage disequilibrium among microsatellite loci (*P-value* < 0.001).

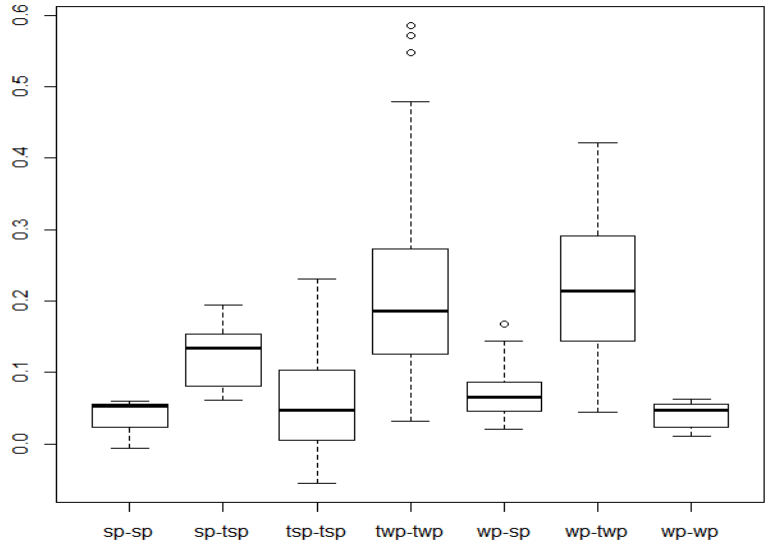

Fig. 3: Box plots representing the distribution of pairwise F_{ST} within sub-population (sp-sp, wp-wp, twp-twp, tsp-tsp) and among each sub-population (wp-sp, wp-twp, sp-tsp).

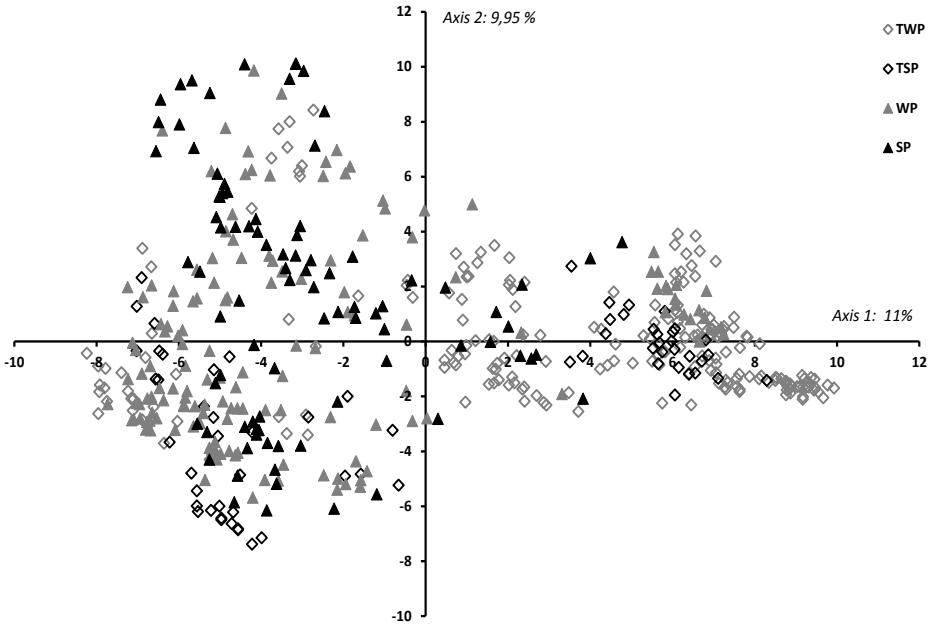

Fig. 4: Principal component analysis (PCA) realized on the *D. pinodes* sub-populations respectively sampled within winter pea plot (WP, 150 strains), spring pea plot (SP, 90 strains), trap winter pea (TWP, 214 strains), and trap spring plant (TSP, 66 strains).

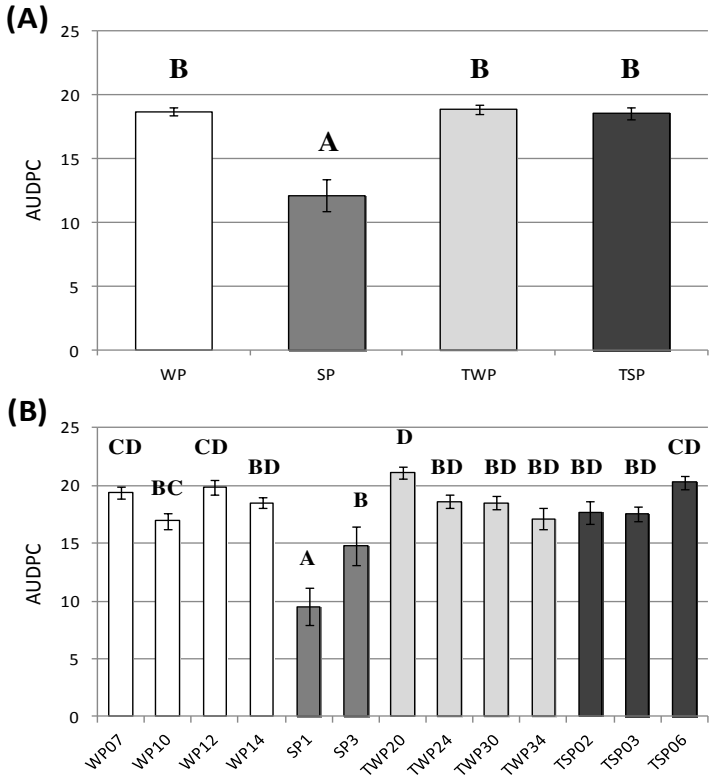

Fig. 5: Mean aggressiveness level (AUDPC) for each *D. pinodes* population **(A)** and for each *D. pinodes* sub-population **(B)**. AUDPC was measured on detached stipules of three pea genotypes (Lumina, Enduro and DP) for *D. pinodes* strains sampled at the different sampling dates on winter (WP), spring (SP) pea plots, and for *D. pinodes* strains sampled on winter (TWP) and spring (TSP) trap plants. AUDPC was calculated a described by Shaner & Finney (**55**) by estimating the integral of the disease progress curve including assessment of the disease at 2, 4 and 7 days after inoculation.