



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

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

3

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25 **ABSTRACT**

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

37

38 **IMPORTANCE**

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

46

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

49

## 50 Introduction

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

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

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

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

86       Despite recent advances, the role of gene flow (and of its two mains triggers, reproduction and  
87 dispersal) on the local evolution of pathogen populations is still poorly assessed. This is especially the  
88 case for epidemics occurring on both winter and spring genotypes of the same host. This situation  
89 raises specific questions, particularly regarding the respective roles of auto- and allo-inoculum (Inocu-  
90 lum fraction produced by an exogenous source to the plot (25)) in the epidemic process and in the  
91 pathogenicity changes within populations. To address these questions, we analyzed the phenotypic  
92 and genotypic diversity in populations of an aggressive foliar pathogen of pea, *Didymella pinodes*  
93 along the course of an epidemic. *D. pinodes* is a necrotrophic, polyphagous, polycyclic and homothal-  
94 lic fungus, that causes Ascochyta blight on winter and spring pea fields worldwide (26, 27). This path-  
95 ogen is known to present individuals varying widely in their ability to cause disease on pea (28, 29,  
96 30, 31, and 32). Contrary to many necrotrophic fungi, *D. pinodes* can simultaneously develop its ana-  
97 morph (asexual) and teleomorph (sexual) forms on the same plant during the growing season (33,  
98 32). Moreover, due to the indeterminate growth of the pea crop, both pycnidia (asexual) and  
99 pseudothecia (sexual) can be observed simultaneously on the same plant organs. Whereas pycnidia  
100 are produced on both green and senescent plant organs, pseudothecia only appear on the senescent  
101 parts (33). This fungal pathogen has different ways to persist in the environment and to disperse over

102 more or less long distances (splashing, wind) (**27, 34, 35**). These features are more important than for  
103 pathogens where reproduction modes are dissociated, as they help to increase the population diver-  
104 sity and have an impact on pathogenicity. A recent study (**32**) showed that epidemics on winter and  
105 spring pea crops are due to a single *D. pinodes* population. Despite the long intercropping season, *D.*  
106 *pinodes* populations from winter and spring pea display a high, but similar genetic variability. Contra-  
107 ry to other natural plant–pathogen associations, no high annual extinction is observed (**36, 37, 38,**  
108 **39, and 40**). Population turnover is thus likely to be driven by both local selection and demographic  
109 stochasticity due to seasonal population growth and decline. During spring, abundant spore produc-  
110 tion (**41**) leads to high colonization rates. The fact that winter and spring pea crops are attacked by  
111 the same populations of *D. pinodes* suggests that, as observed by Laine & Hanski (**42**) in another as-  
112 comycete plant pathogen, regional persistence of *D. pinodes* populations could occur at the scale of  
113 metapopulations, consisting of many coupled populations.

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

124

125

126

## 127 **Materials and methods**

128 **Field experiments.** Two field experiments were carried out at the INRA experimental station of Le  
129 Rheu, western France (48°06 00 N, 1°48 00 W, 30m above mean sea level) in 2004-2005. Both fields,  
130 separated by more than three km, shared similar pedoclimatic environments and were free from soil  
131 borne inoculum, due to a rotation without pea crop during the five previous years. These fields were  
132 respectively sown with a winter (Cheyenne, GAE-Recherche, France) and a spring pea cultivar  
133 (Baccara, Florimond-Desprez, France). The winter cultivar was sown on October 25<sup>th</sup>, 2004, and the  
134 spring crop was sown on February 24<sup>th</sup>, 2005. For both crops, the experimental design consisted of a  
135 30m<sup>2</sup> (3m wide x 10m long) plot divided into thirty micro-plots (1 m<sup>2</sup> each).

136

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

145

146 **Spatio-temporal disease development.** [Disease started](#) in each crop without artificial supply of  
147 inoculum. Disease severity was assessed visually once a month in the different micro-plots from  
148 January to June. To determine disease development at the plot level, one plant was randomly  
149 collected from each micro-plot at the different sampling dates (five sampling dates for the winter pea  
150 crop, and three sampling dates for the spring pea crop). Disease was scored on each stipule of the  
151 sampled plants using the adapted disease scale of Tivoli (1994) (41), and a mean disease score ( $D_i$ )  
152 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<sup>2</sup> 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<sup>2</sup>  
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



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

188

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

194

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

205 using the following formula:

$$206 \quad I_A = \frac{V_D}{\sum var_j} - 1$$

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

212

213 **Population differentiation.** The analysis of molecular variance (AMOVA; online as Arlequin version  
214 3.1 software, hosted by the Department of Anthropology, University of Geneva, Switzerland; (51) was  
215 used to partition molecular variance between populations, between sub-populations within  
216 populations and within sub-populations. Pairwise  $F_{ST}$  values were calculated with Arlequin for each  
217 pair of sub-populations of *D. pinodes* and compared within plots (SP-SP and WP-WP) or trap plants  
218 (TSP-TSP and TWP-TWP), between plots (SP-WP) and between plots and related trap plants (SP-TSP  
219 and WP-TWP).

220

221 **Genetic structure.** A principal component analysis (PCA) was performed using the procedure available  
222 in the package adegenet (52) for the statistical freeware R version 3.1.1 (©2014, The R Foundation for  
223 Statistical Computing). PCA has an important advantage over other methods, such as the Bayesian  
224 clustering algorithm implemented in STRUCTURE (53), because it does not require strong  
225 assumptions about an underlying genetic model, such as the Hardy-Weinberg equilibrium or the  
226 absence of linkage disequilibrium between loci (52).

227

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

231 TWP20, TWP24, TWP30, TWP34) and spring trap plants (sampling dates: TSP02, TSP03, TSP06). Five  
232 *D. pinodes* strains were randomly chosen within each sub-population and their aggressiveness was  
233 evaluated on three pea genotypes: the winter cultivar Enduro (Florimond-Desprez, France), the  
234 spring cultivar Lumina (Nickerson, France), and the spring breeding line DP (54). Enduro and Lumina  
235 were chosen to replace Cheyenne and Baccara, respectively, for which seeds were no longer available  
236 at the time of the pathogenicity tests. They presented a similar and high susceptibility level towards  
237 the disease, while DP has a higher level of quantitative resistance. Plants were grown at 18 to 20°C  
238 for three weeks, until they reached the 5- to 6-leaf stage, before inoculation. Plant preparation and  
239 experimental design were as described by (44). The inoculation method was based on that proposed  
240 by Onfroy *et al.* (56). Briefly, strains were grown for 10 days on V8 medium under white light with a  
241 12 h photoperiod at 20°C before pycnidiospores suspensions were prepared by flooding the surface  
242 of cultures with sterile distilled water, gently scraping with a glass rod, and filtering the suspension  
243 through two layers of sterile cheesecloth. The spore concentration was adjusted to  $5.10^4$  spores.mL<sup>-1</sup>,  
244 and Tween 20 (VWR International SAS, Strasbourg, France) was added as a wetting agent (2 drops per  
245 500 mL spore suspension). Inoculation consisted of depositing a 10 µL drop of the spore suspension  
246 on the upper surface of freshly detached stipules floated, lower surface down, on tap water in a  
247 compartmentalized square Petri dish (12 cm side; Gosselin, France). Drops were deposited away from  
248 the main veins. To avoid drop evaporation, Petri dishes were placed into large transparent plastic  
249 boxes immediately after inoculum deposition and incubated in a climate chamber for seven days with  
250 a continuous cycle of 14 h of light at 20°C. Symptom development was assessed two, four, and seven  
251 days after inoculation, as described by Onfroy *et al.* (55). A 0-3 semi-quantitative scale (0 = symptom  
252 free, 1=flecks appearing, 2 = flecks covering half of the area of drop deposition, 3 = coalescence of the  
253 flecks within the area of drop deposition) was used to score symptoms not extending past the  
254 inoculation droplet. For stipules with necrosis extending beyond the borders of inoculum drops,  
255 lesion diameter (mm) was measured with a graduated ruler. A visual assessment using a 0-7 scale  
256 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.

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

281 The three AFLP primer sets allowed the detection of 646 loci, 388 of them were polymorphic over all  
282 tested populations. The percentage of polymorphic markers in each sub-population ranged from  
283 29.90% in TWP21 to 100% in WP8. No repeated MLG was found among the 520 strains, indicating a  
284 very low incidence of clonality. However, the overall  $I_A$  value significantly differed from zero ( $I_A =$   
285 1.97), thus rejecting the null hypothesis of recombination ( $P = 0.001$ ) (**Fig. 2**). Number of alleles ( $N_a$ ),  
286 Shannon's Information index ( $I$ ) and unbiased diversity ( $u_h$ ) showed that the highest genetic diversity  
287 within sub-populations was for WP12 ( $I=0.61$ ,  $u_h=0.43$ ) and the least for TWP21 ( $I=0.176$ ,  $u_h=0.151$ ).  
288 The low genetic diversity observed for the sub-population TWP21 may be due to the low number of  
289 isolates within this sub-population. At the scale of the populations (WP, SP, TWP and TSP), number of  
290 alleles, Shannon's Information index and unbiased diversity showed that the genetic diversity was  
291 higher for populations sampled into the plots (SP and WP) than for populations sampled on trap  
292 plants (TWP and TSP) (**Table I**).

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

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

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

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

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

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

321

322 **Discussion**

323 One of the objectives of this study was to determine how *D. pinodes* population structures change  
324 during a growing season. The amount of genetic variation was similar within *D. pinodes* sub-  
325 populations sampled from winter or spring pea plots ( $H' = 0.83$ , and  $0.86$  respectively). The genetic  
326 variability observed at the beginning of the season remained roughly the same throughout the  
327 growing season. The main part of this genetic variability was observed within sub-populations (80%),  
328 which confirms the PCA result of an absence of a clear genetic structure over time, and suggests that  
329 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

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

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

380 Compared to infected pea debris, alternative hosts are generally considered of minor importance  
381 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 alternative 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

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

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

462

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467

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636

**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 ( $= -1 \sum (p \cdot \ln(p) + q \cdot \ln(q))$ ), h = Diversity ( $= 1 - (p^2 + q^2)$ ), uh = Unbiased diversity ( $= (N/(N-1)) h$ ), where p = Band freq., and q = 1 - p (GenAlEx 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	

8 **Table II:** Analysis of molecular variance (AMOVA) for *D. pinodes* strains sampled from different plots  
 9 (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

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

16

17 **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		

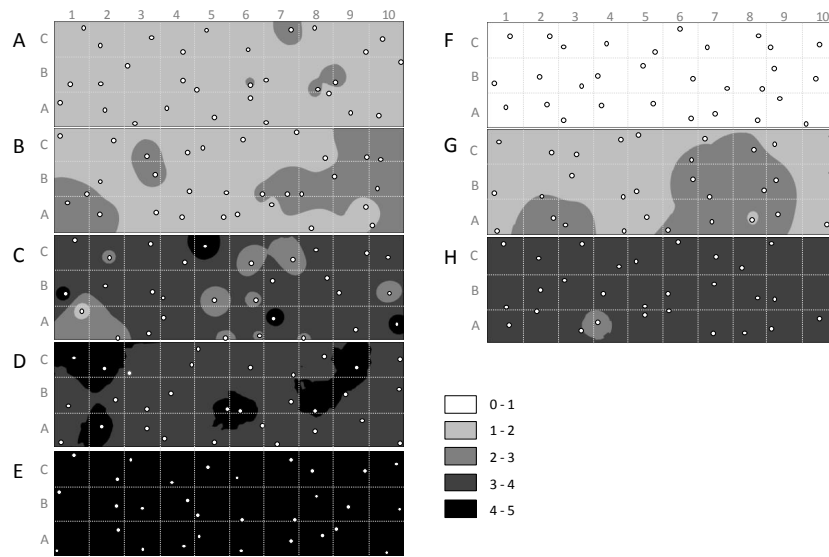
19

20 **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		

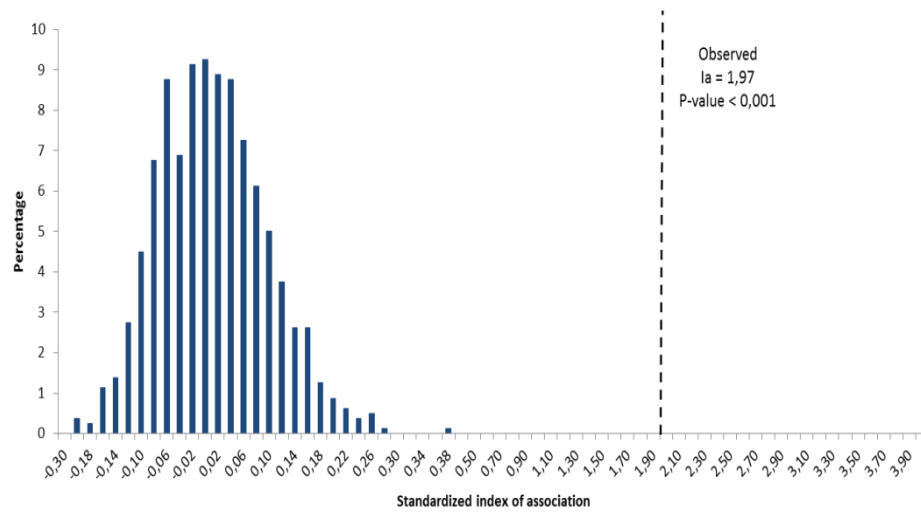
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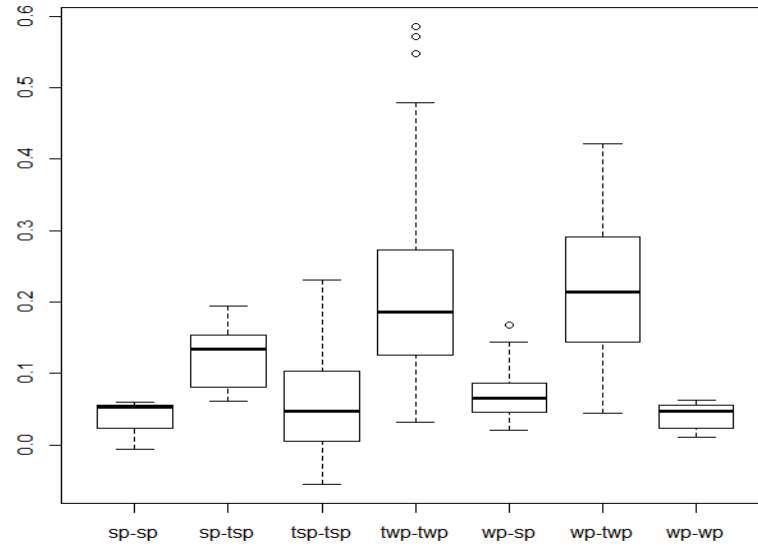


**Fig. 1:** Maps representing the evolution of disease severity due to ascochyta blight within winter and spring pea plots and for different sampling dates (**Winter sampling dates:** **A:** 17 February, **B:** 28 February, **C:** 29 March, **D:** 25 April, **E:** 23 May; **Spring sampling dates:** **F:** 30 May, **G:** 13 June, **H:** 27 June). Each plot was divided into 1m<sup>2</sup> 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 ( $D_j$ ) was calculated. Disease distribution maps were created for each sampling date using the Arcmap® software.

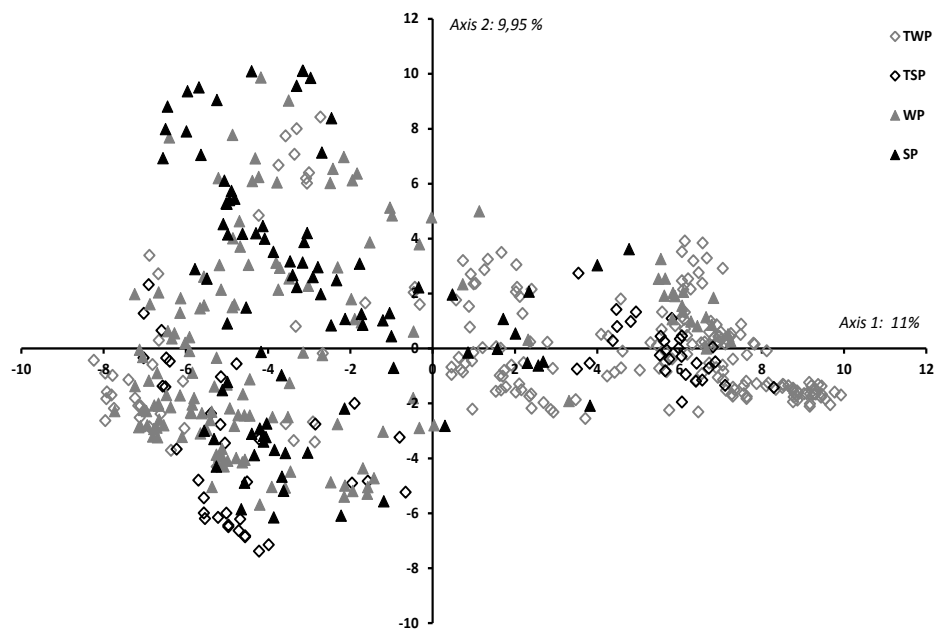




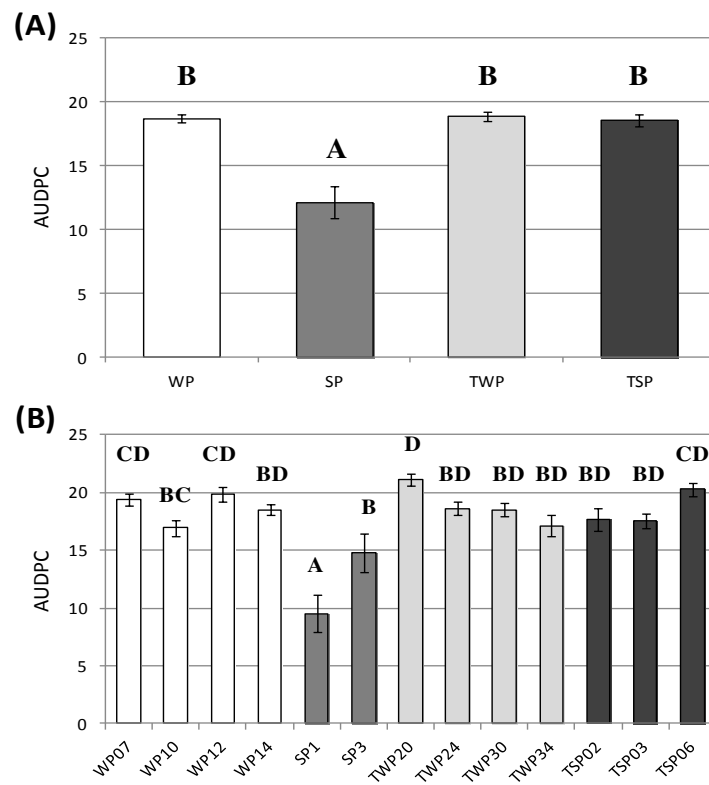
**Fig. 2:** Standardized index of association ( $I_A$ ) calculated from the clone-corrected dataset, rejecting the absence of linkage disequilibrium among microsatellite loci ( $P\text{-value} < 0.001$ ).



**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 as 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.