Evolution within a given virulence phenotype (pathotype) is driven by changes in aggressiveness: a case study of French wheat leaf rust populations

Cécilia Fontyn, Kevin JG Meyer, Anne-Lise Boixel, Ghislain Delestre, Emma Piaget, Corentin Picard, Frédéric Suffert, Thierry C Marcel*, Henriette Goyeau*

Université Paris-Saclay, INRAE, UR BIOGER, 91120 Palaiseau, France

*corresponding authors: thierry.marcel@inrae.fr, henriette.goyeau@inrae.fr

ABSTRACT

Plant pathogens are constantly evolving and adapting to their environment, including their host. Virulence alleles emerge, and then increase, and sometimes decrease in frequency within pathogen populations in response to the fluctuating selection pressures imposed by the deployment of resistance genes. In some cases, these strong selection pressures cannot fully explain the evolution observed in pathogen populations. A previous study on the French population of *Puccinia triticina*, the causal agent of wheat leaf rust, showed that two major pathotypes — groups of isolates with a particular combination of virulences — predominated but then declined over the 2005-2016 period. The relative dynamics and the domination of these two pathotypes — 166 317 0 and 106 314 0 —, relative to the other pathotypes present in the population at a low frequency although compatible, i.e. virulent on several varieties deployed, could not be explained solely by the frequency of *Lr* genes in the landscape. Within these two pathotypes, we identified two main genotypes that emerged in succession. We

assessed three components of aggressiveness — infection efficiency, latency period and sporulation capacity — for 44 isolates representative of the four *P. triticina* pathotype-genotype combinations. We showed, for both pathotypes, that the more recent genotypes were more aggressive than the older ones. Our findings were highly consistent for the various components of aggressiveness for pathotype 166 317 0 grown on Michigan Amber — a 'naive' cultivar never grown in the landscape — or on Apache — a 'neutral' cultivar, which does not affect the pathotype frequency in the landscape and therefore was postulated to have no or minor selection effect on the population composition. For pathotype 106 314 0, the most recent genotype had a shorter latency period on several of the cultivars most frequently grown in the landscape, but not on 'neutral' and 'naive' cultivars. We conclude that the quantitative components of aggressiveness can be significant drivers of evolution in pathogen populations. A gain in aggressiveness stopped the decline in frequency of a pathotype, and subsequently allowed an increase in frequency of this pathotype in the pathogen population, providing evidence that adaptation to a changing varietal landscape not only affects virulence but can also lead to changes in aggressiveness.

Keywords

Aggressiveness, quantitative phenotyping, genotype evolution, host adaptation, *Puccinia triticina*

INTRODUCTION

Plant diseases and pests cause crop damage accounting for up to 40% of yield losses (Boonekamp, 2012). Pathogenicity, or the ability of plant pathogens, especially fungi, to cause

48 disease, is generally broken down into a qualitative term, 'virulence', and a quantitative term,
49 'aggressiveness' (Lannou, 2012). Virulence is defined as the capacity of the pathogen to infect
50 its host (compatible interaction), as opposed to avirulence, which expresses the resistance of
51 the host (incompatible interaction), according to the gene-for-gene model (Flor, 1971). A
52 virulence phenotype, also known as a pathotype or race, is defined by a virulence profile: two
53 pathogenic isolates are considered to belong to the same pathotype if they have the same
54 combination of virulences. Aggressiveness, the quantitative variation of pathogenicity on a
55 compatible host (Pariaud *et al.*, 2009a), can be viewed as the detrimental impact of a
56 pathogen on its host, leading to damage to the crop plant and, thus, yield losses (Shaner *et*
57 *al.*, 1992; Pariaud *et al.*, 2009a; Lannou, 2012). Aggressiveness also determines the rate at
58 which a given disease intensity is reached. Its assessment is intrinsically complex because it is
59 related to various life-history traits of the pathogen specific to its biology and the nature of
60 the symptoms that it produces. The different components of aggressiveness can be measured
61 by evaluating several of these complementary quantitative traits expressed during the host–
62 pathogen interaction. The most widely assessed aggressiveness components for rust
63 pathogens are infection efficiency, latency period and sporulation capacity (Pariaud *et al.*,
64 2009a; Lannou, 2012; Azzimonti *et al.*, 2013). A higher infection efficiency will directly cause
65 more host damage, while latency period and sporulation capacity will also increase the
66 parasitic fitness of the pathogen (Shaner *et al.*, 1992) by favoring its transmission before
67 damaging the host . However, the relationship between these aggressiveness components of
68 the pathogen and the reduction in crop yield and biomass remains a theoretical assumption
69 that is rarely verified experimentally (Van Roermund and Spitters, 1990).
70

Infection efficiency is calculated by determining the proportion of unit of inoculum, i.e. spores, that cause a new infection when deposited on compatible host plant tissues (Sache, 1997). The estimation of this component is complex due to technical issues, particularly the need for great precision in the spore deposition process (Lehman & Shaner, 1997), which involves placing a fixed and known number of spores — ideally one by one — on the leaf.

The latency period is the length of time between "the start of the infection process by a unit of inoculum", i.e. the deposition of a spore on plant tissues, and "the start of production of infectious units", i.e. first sporulation (Parlevliet, 1979; Madden *et al.*, 2007). In rusts, this component is often defined as the length of time between inoculation and the appearance of 50% of the sporulating structures, also known as 'uredinia' (Parlevliet, 1975; Johnson, 1980; Pfender, 2001). Latency period estimations therefore require counts of uredinia on at least a daily basis. The latency period is highly temperature-dependent, and its expression in thermal time is therefore recommended, to allow comparisons between trials (Lovell *et al.*, 2004).

Sporulation capacity is assessed as the number of spores produced per individual sporulating structure and per unit time (Sache, 1997; Pariaud *et al.*, 2009a). Spores can be collected and counted directly (e.g. with a cell counter) or indirectly (e.g. weighed) (Imhoff, 1982; Robert *et al.*, 2004; Delmotte *et al.*, 2014). However, in rusts, uredinium density affects the number of spores produced (Robert *et al.*, 2004) and must, therefore, be taken into account in some analysis (Lannou & Soubeyrand, 2017). Moreover, sporulation is a continuous process, so sporulation capacity is time-dependent. Thus, this component, like several others, is dependent on latency period. The interdependence of traits can be reduced by measuring sporulation capacity at a normalized time point.

95

96 Leaf rust caused by *Puccinia triticina* is one of the most damaging wheat diseases, causing high
97 yield losses worldwide (Huerta-Espino *et al.*, 2011; Savary *et al.*, 2019). Qualitative resistance
98 is the easiest and most effective means of limiting leaf rust epidemics. Eighty-two *Lr* genes
99 have been identified in wheat cultivars, most displaying qualitative interactions (Bariana *et*
100 *al.*, 2022). It has been shown that the deployment of qualitative resistance genes in the
101 landscape exerts a strong selective pressure, acting as an important driver of evolution in *P.*
102 *triticina* populations (Goyeau *et al.*, 2006). This effect was highlighted by surveys of virulence
103 phenotypes (pathotypes), which showed that the corresponding virulence can emerge rapidly
104 after the introduction of a new *Lr* gene into cultivars. For example, in France, virulence against
105 *Lr*28 appeared within only two years of the release of cultivars carrying *Lr28* (Fontyn *et al.*,
106 2022). Adaptation to qualitative resistances occurs rapidly, despite the clonality of the
107 population, through 'boom-and-bust' cycles of resistance (McDonald & Linde 2002).

108

109 However, qualitative resistance alone cannot fully explain the evolution of the composition of
110 the pathogen population. The occurrence of selection for greater aggressiveness has already
111 been established for various pathogens (Delmas *et al.*, 2016; Frézal *et al.*, 2018).Milus *et al.*
112 (2009) showed that the replacement of an 'old' *Puccinia striiformis* f. sp. *tritici* population by
113 a 'new' population could be explained by the greater aggressiveness of isolates from this new
114 population, in addition to a change in its composition in pathotypes. A survey of the French *P.*
115 *triticina* population from 1999-2002 (Goyeau *et al.*, 2006) revealed the domination of a single
116 pathotype (073 100 0), coinciding with a period in which the cultivar landscape was dominated
117 by the cultivar Soissons. Pathotype 073 100 0 was found to be more aggressive on this cultivar
118 than other virulent pathotypes present in the *P. triticina* population during this period

119 (Pariaud *et al.*, 2009b). Fontyn *et al.* (2022) recently showed that the domination of the French
120 landscape by two pathotypes — 106 314 0 and 166 317 0 — during the 2005-2016 period
121 could not be fully attributed to the deployment of *Lr* genes. Indeed, several other compatible
122 pathotypes virulent against the *Lr* genes carried by the most widely grown cultivars were
123 present in the landscape, but never reached substantial frequencies. The authors suggested
124 that aggressiveness might drive the evolution of *P. triticina* populations, modifying pathotype
125 frequencies at large spatiotemporal scales. The variation in aggressiveness over time, during
126 the complete lifespan of a pathotype, including its emergence, domination and replacement,
127 has never been investigated.
128
129 The objective of this study was to determine if an evolution of aggressiveness can occur within
130 a pathotype of *P. triticina* over a large temporal scale and whether information on
131 aggressiveness allows us to explain retrospectively or even predict changes in pathotypes
132 frequency in the landscape. To this end, we focused on two major pathotypes, 166 317 0 and
133 106 314 0, identified as good experimental case studies for this purpose, because of their long
134 lifespan and high frequency in the French landscape over the 2005-2016 period. We first
135 characterized isolates of these two pathotypes using microsatellite markers, to identify their
136 genotypic diversity. Within each of the two pathotypes, we then compared the aggressiveness
137 components of isolates from the most frequent genotypes (i) on 'neutral' cultivars, i.e.
138 cultivars with no apparent effect on the frequencies of these pathotypes, and, for pathotype
139 106 314 0 only, (ii) on five cultivars widely grown in France over the study period.
140

141 **MATERIALS AND METHODS**

142

143 **Selection and purification of isolates**

144 Annual surveys of *P. triticina* populations were carried out at INRAE BIOGER over the last two
145 decades. These surveys involve the collection of leaf samples from field (micro)plots sown
146 with a single variety in a network of field trials and nurseries throughout the wheat-growing
147 areas of France (Goyeau *et al.*, 2006; Fontyn *et al.*, 2022). In total, 2796 leaves were collected
148 from the 10 most cultivated varieties during the 2006-2016 period in the framework of the
149 national survey. Urediniospores were bulk-harvested from each leaf and stored at -80°C. One
150 single-uredinium isolate was selected from each bulk and its pathotype was determined as
151 described by Goyeau *et al.* (2006). In total, 932 isolates were identified as pathotype 106 314
152 0 and 473 isolates were identified as pathotype 166 317 0 during the national survey. For the
153 purposes of this study, we selected 286 urediniospore bulks of pathotype 106 314 0 collected
154 between 2006 and 2016, and 115 from pathotype 166 317 0 collected between 2013 and 2016
155 (Table S1 and S2). No bulk were selected for pathotype 166 317 0 before 2013 because the
156 frequency of this pathotype in the landscape was too low. The bulks were defrosted and
157 repurified by the re-inoculation of seven-day-old cv. Michigan Amber wheat seedlings, to
158 obtain 401 new single-uredinium isolates. Before inoculation, the plants were grown in
159 cabinets with air filters in a greenhouse at temperatures between 15 and 20°C, under a 14-h
160 photoperiod (daylight supplemented with 400 W sodium lamps). Plants were treated with 15
161 mL maleic hydrazide solution (0.25 g maleic hydrazide per liter of H_2O) to prevent the
162 emergence of secondary leaves and to increase spore production. Inoculated seedlings were
163 placed in a dew chamber at 15°C for 24 h and were then transferred to the greenhouse. One
164 week after inoculation, the seedlings were trimmed such that only one plant with one
165 uredinium remained in each pot. Before sporulation, cellophane bags were placed over the
166 pots to prevent contamination between isolates. Ten days after inoculation, 401 leaf

167 segments, each carrying only one uredinium, were collected for DNA extraction and
168 genotyping with microsatellite markers. Other single-uredinium isolates were also selected
169 from 28 and 16 bulks initially identified as pathotypes 106 314 0 and 166 317 0, respectively.
170 Each of these 44 isolates was pathotyped (Goyeau *et al.*, 2006) and, after confirmation that
171 their virulence phenotypes were as expected, genotyped with microsatellite markers. Spores
172 from these 44 isolates were stored at -80°C for further assessments of aggressiveness (Table
173 S3). The various stages in the purification, selection, pathotyping and genotyping of *P. triticina*
174 isolates for which aggressiveness components were evaluated are summarized in Figure 1.
175
176 **Genotyping of isolates with microsatellite markers**
177 DNA extraction
178 DNA was extracted from all the purified isolates in 96-well extraction plates, with Qiagen
179 DNeasy® Plant Mini Kit buffers (Qiagen, Hilden, Germany). To this end, each leaf segment
180 carrying a single uredinium was placed in a Qiagen collection microtube with a tungsten bead
181 and 100 μL of hot AP1 buffer (65°C). Leaf segments were ground by shaking the microtubes in
182 a Retsch® MM400 ball mill twice, for 30 seconds each, at 25 Hz. The tubes were then
183 centrifuged for 1 minute at 3000 x *g*. AP1 buffer supplemented with RNase A and Reagent DX
184 was then added to each tube (300 μL). After mixing, we added 130 μL P3 buffer to the tube,
185 which was then incubated for 10 minutes at -20°C and centrifuged at 4°C for 10 minutes at
186 20 000 x *g*. The supernatant (200 μL) was transferred to a new tube, to which we added 1
187 volume of sodium acetate (3 M pH 5) and 3 volumes of isopropanol (100%). After mixing, the
188 tubes were placed at -20°C for 30 minutes and then centrifuged for 20 minutes at 6000 x *g*.
189 The supernatant was removed and 3 volumes of 70% ethanol were added. The tube was
190 placed at -20°C for 5 minutes and was then centrifuged at 4°C for 15 minutes at 6000 x *g*. The

191 pellet containing the DNA was allowed to dry overnight, and was then resuspended in 100 µL
192 ultra-purified water. We transferred 20 µL of the resulting suspension to Qiagen elution
193 microtubes RS in a 96-tube rack, which was sent to Eurofins (Eurofins, Luxembourg) for
194 genotyping. The DNA suspensions concentrations were between 1 and 25 ng/µL.
195
196 Microsatellite genotyping and analyses
197 The 401 and 44 single-uredinium isolates were genotyped for 19 microsatellite markers: RB8,
198 RB11, RB12, RB17, RB25, RB26, PtSSR13, PtSSR50, PtSSR55, PtSSR61, PtSSR68, PtSSR91,
199 PtSSR92, PtSSR152, PtSSR154, PtSSR158, PtSSR164, PtSSR173, and PtSSR186 (Duan *et al.*,
200 2003; Szabo & Kolmer, 2007). The microsatellite markers were assembled into two
201 multiplexes of 9 and 10 markers and labeled with four fluorochromes (Table S4) to prevent
202 overlaps between markers with the same range of allele sizes. PCR amplification was
203 performed by Eurofins (Eurofins, Luxembourg), with the following amplification program:
204 95°C for 5 min and 35 cycles of 95°C for 30 s, 58°C for 30 s, 72°C for 30 s, and then 60°C for 30
205 min. Each reaction contained 5 µL DNA solution and PCR mixture with Taq Type-it (Qiagen®).
206 PCR products were analyzed by capillarity electrophoresis on an ABI3130xl sequencer. The
207 two-multiplex PCR system was tested and validated on eight reference *P. triticina* DNA
208 extracts from isolates obtained from bread and durum wheat. Chromatograms were visually
209 inspected for all markers and for all individuals with Peak Scanner software version 2.0, before
210 the final assignment of SSR alleles. The chromosome position of SSR markers was determined
211 by performing a blastn analysis (with default parameter values) of the sequence of each
212 primer against the two genome sequences of the Australian isolate Pt76 (Duan *et al.*, 2022)
213 with the NCBI blast tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi).
214

215 The genotyping of the 401 isolates revealed two major genotypes within each pathotype: 106
216 314 0-G1 and 106 314 0-G2 for pathotype 106 314 0, and 166 317 0-G1 and 166 317 0-G2 for
217 pathotype 166 317 0. These genotypes were taken into account in the design of subsequent
218 experiments.
219
220 **Evaluation of aggressiveness components**
221 Experimental design
222 Three components of aggressiveness, infection efficiency, latency period and sporulation
223 capacity, were assessed for 28 isolates of pathotype 106 314 0 and 16 isolates of pathotype
224 166 317 0 that had been purified, pathotyped and genotyped (Table S3). These aggressiveness
225 components were measured in the greenhouse on seedlings of two wheat varieties: (i)
226 Apache, a commercial French cultivar characterized as 'neutral' in a previous study (Fontyn *et*
227 *al.*, 2022), i.e. with no selection effect on the landscape-pathotype pattern, and (ii) Michigan
228 Amber, considered intrinsically 'naive' or 'neutral', both because it carries no known leaf rust
229 resistance gene and because it has never been cultivated in France and cannot, therefore,
230 have played any significant role in the evolutionary trajectory of *P. triticina* in France.
231
232 It was not possible to perform a large single trial at our facilities, so the 44 isolates were
233 characterized in five successive series, according to the same protocol and under the same
234 experimental conditions. In series 1 (performed in March-April 2021) and 2 (August-
235 September 2021), we tested whether the two genotypes within each *P. triticina* pathotype
236 differed in aggressiveness on Apache and Michigan Amber. The isolates used for these two
237 series were collected between 2005 and 2016 on Apache (Table 1). In series 3 (July-august
238 2020), 4 (April-May 2021) and 5 (May-June 2021), we tested the difference in aggressiveness

239 between the two genotypes within pathotype 106 314 0 (106 314 0-G1 and 106 314 0-G2) on
240 some or all of the wheat cultivars Aubusson, Premio, Michigan Amber, Sankara, Expert and
241 Bermude. Except Michigan Amber, all were among the 35 most frequently grown cultivars in
242 the French landscape during the 2006-2016 period. The isolates used in these three series
243 were collected in 2012 and 2013, on Aubusson, Premio and Apache (Table 2). The isolates
244 used in series 3 were collected on cultivars Aubusson and Premio and were tested on these
245 two cultivars and Michigan Amber. The isolates used in series 4 were collected on cultivar
246 Apache and were tested on Apache and Michigan Amber. The isolates used in series 5 were
247 collected on cultivar Apache and were tested on cultivars Sankara, Expert and Bermude. In all
248 five series, each isolate × cultivar interaction was replicated eight times, on eight wheat
249 seedlings. Three replicates of the experimental design, including the five series, were
250 established.
251
252 Assessment of aggressiveness
253 All the phenotyping tests were performed in a greenhouse on eight-day-old wheat seedlings
254 grown in a 21 x 15 x 6 cm plastic box containing potting soil placed under standardized
255 conditions (18°C at night and 22°C during the day, with a 16-h photoperiod). The second leaf
256 of each seedling was maintained, with double-sided tape, on a rigid plate coated with
257 aluminum foil and a 3 cm-long segment of this leaf was inoculated with the fungus. Inoculation
258 was performed with 10 fresh (two-week-old) spores, picked one by one with a human eyelash
259 under a dissecting microscope and deposited on the leaf (Figure 2A and 2B). The plants were
260 then placed in a dew chamber, in the dark, at 15°C, for 18 to 24 h. They were then returned
261 to the greenhouse and the rigid plate was removed. When the first uredinia began to break
262 through the leaf epidermis, generally six days after inoculation, they were counted at 10- to

263 14-hour intervals (twice daily) until no new uredinia appeared (Figure 2C). Infection efficiency
264 (IE) was estimated for each leaf as the ratio between the final number of uredinia and the
265 number of spores deposited (10 spores). Latency period (LP), estimated as the time between
266 inoculation and the appearance of 50% of the total number of uredinia, was expressed in
267 degree-days, based on the air temperature measured in the greenhouse every ten minutes.
268 Sporulation capacity (SP) was estimated, once the number of uredinia had stabilized, as the
269 number of spores produced per uredinium over a four-day period. Once the final number of
270 uredinia had been reached (i.e. 9 days after inoculation) the spores that had already been
271 produced were removed from the leaf with a small brush. Slightly incurved aluminum gutters
272 (2 x 7cm) made from blind slats were then positioned under each inoculated leaf, which was
273 attached to the gutter with clips (Figure 2D). After four days, all the newly produced spores
274 (Figure 2E) were removed by suction with a cyclone collector into a portion of plastic straw
275 sealed at one end (Figure 2F). Each portion of straw was weighed before and after spore
276 harvesting. SP was calculated by dividing the total weight of the spores collected from a single
277 leaf by the number of uredinia on that leaf.
278
279 **Statistical analyses**
280 For each of the three aggressiveness traits (the response variable Y), two ANOVA models were
281 used: model (1) for series 1 and 2; model (2) for pooled data from series 3 and 4 (without a
282 series factor, as a preliminary analysis showed that the interactions of the series factor with
283 other factors were never significant), and for series 5. For series 3, 4 and 5, the analysis was
284 performed independently for each cultivar, as the cultivars tested were not the same.

285 $$(1)\quad Y_{ijklm} = \mu + R_i + G_j + C_k + G_j/I_l + \varepsilon_{ijklm}$$

286 $$(2)\quad Y_{ijkl} = \mu + R_i + G_j + G_j/I_k + \varepsilon_{ijkl}$$

287 where Y_{ijklm} and Y_{ijkl} are the values of the studied trait in replicate (R) *i*, of genotype (G) *j* and
288 isolate (I) *l* or *k* (isolate being nested within genotype), on cultivar (C) *k* (when this occurred).
289 μ is the overall mean value for this trait and ε is the residual, representing the measurement
290 error, with $\varepsilon \sim N(o, \sigma 2)$
291
292 Log, √ or 1/x transformation was applied to IE, LP and SP when necessary, to obtain a
293 normalized distribution of residuals. When the distribution of residuals could not be
294 normalized by any transformation, a non-parametric Kruskal-Wallis test was performed to
295 analyze the effect of genotype on the aggressiveness components, and also the cultivar effect
296 in series 1 and 2 only. Nevertheless, the Kruskal-Wallis analysis always gave the same results
297 as the ANOVA analysis, even if the data could not be normalized.
298 All the analyses were performed with R software version 4.1.0.
299

300 **RESULTS**

301

302 **Frequency dynamics of two major *P. triticina* pathotypes in the French landscape in the**
303 **2006-2020 period**

304 The frequency of pathotype 106 314 0 in the French *P. triticina* population increased from 30%
305 in 2006 to 48% in 2008 and 51% in 2009 (data from Fontyn *et al.*, 2022), the maximum
306 frequency in the landscape for this pathotype (Figure 3). After a plateau at 30-33% from 2011
307 to 2014, the frequency of this pathotype decreased strongly, to 18% in 2015, 5% in 2016 and
308 less than 1% in 2018. Within pathotype 106 314 0, two main genotypes, 106 314 0-G1 and 106
309 314 0-G2, were identified and were considered to be dominant as their cumulative frequency
310 ranged from 40% to 65% during the 2006-2016 period (Figure 4). These two genotypes

311 differed at five of the 19 SSR loci studied (RB8, RB11, PtSSR68, PtSSR92 and PtSSR164; Table
312 3). Genotype 106 314 0-G1 was the most frequent from 2006 to 2012, but it decreased in
313 frequency thereafter (Figure 4). Genotype 106 314 0-G2 was present at a very low frequency
314 from 2007 to 2011. In 2012, the frequencies of the two genotypes were fairly similar, at 23%
315 for 106 314 0-G1 and 30% for 106 314 0-G2. Genotype 106 314 0-G1 then continued to
316 decrease in frequency, eventually disappearing completely from the sampled population in
317 2014. Conversely, genotype 106 314 0-G2 continued to increase in frequency. It accounted for
318 42% of the total 106 314 0 pathotype population in 2013 and 66% in 2016. Over the entire
319 period, genotypes other than 106 314 0-G1 and 106 314 0-G2 were also identified (Figure 4;
320 detailed data not shown), which could correspond to other genotypes within pathotype
321 106 314 0, or to other pathotypes mixed with pathotype 106 314 0 in the original
322 urediniospore bulks.
323
324 Pathotype 166 317 0 first appeared in 2007, initially at a very low frequency (less than 2%). Its
325 temporal evolution in the French *P. triticina* population was characterized by two peaks (data
326 from Fontyn *et al*., 2022). The first peak occurred after a gradual increase of its frequency
327 between 2007 and 2012, reaching 32% of the *P. triticina* population in 2012. Then, it
328 decreased until 2015 to a frequency of 13%, before increasing again to reach a second peak
329 in 2018 at 41% of frequency in the landscape (Figure 3). The frequency of this pathotype
330 finally decreased after 2018 reaching 27% in 2020. Within pathotype 166 317 0, two
331 genotypes, differing by only one of the 19 SSR loci used (RB8; Table 3), were identified and
332 considered to be predominant during the 2013-2016 period. In 2013 and 2014, genotype 166
333 317 0-G1 dominated, as it accounted for more than 65% of the total 166 317 0 pathotype
334 population (Figure 5). In 2015, genotype 166 317 0-G2 emerged and became co-dominant

335 with 166 317 0-G1. In 2016, 166 317 0-G2 sharply increased in frequency, to 62%, whereas
336 the frequency of 166 317 0-G1 decreased to 7%. Over the four-year period considered, several
337 other genotypes were identified (Figure 5; detailed data not shown), which could correspond
338 to other genotypes within pathotype 166 317 0 or to other pathotypes mixed in the original
339 urediniospore bulks.
340
341 **Differences in aggressiveness between genotypes of the two major *P. triticina* pathotypes**
342 **expressed on 'neutral' and 'naive' cultivars**
343 Genotype 166 317 0-G2 was more aggressive than genotype 166 317 0-G1 in assessments of
344 all three aggressiveness components on a 'neutral' cultivar, Apache, and a 'naive' cultivar
345 Michigan Amber (in analyses of the pooled dataset for the two cultivars; Figure 6A, B and C;
346 Table S5). A comparison of aggressiveness components between the two cultivars is provided
347 in the supplementary data (Figure S1). Infection efficiency (IE) was higher for genotype 166
348 317 0-G2 (53.1 %) than for genotype 166 317 0-G1 (47.6%). Sporulation capacity (SP) was also
349 higher for genotype 166 317 0-G2, with 0.120 mg/lesion versus 0.114 mg/lesion for genotype
350 166 317 0-G1. Latency period (LP) was significantly shorter for genotype 166 317 0-G2 (132.6
351 vs 135.0 degree-days). Significant differences were found between isolates of the two
352 genotypes.
353
354 IE, LP and SP did not differ between genotypes 106 314 0-G1 and 106 314 0-G2 assessed on
355 cultivars Apache and Michigan Amber (in analyses of the pooled dataset for the two cultivars;
356 Figure 6D, E and F; Table S5). A comparison of aggressiveness components between the two
357 cultivars is provided in the supplementary data (Figure S2). IE for genotype 106 314 0-G1 was
358 43.8%, which is not significantly different from the value of 43.4% obtained for genotype 106

359 314 0-G2. SP was 0.128 mg/lesion for genotype 106 314 0-G1, which is not significantly
360 different from the value of 0.124 mg/lesion obtained for genotype G2. Finally, LP differed by
361 less than one degree-day between the two genotypes, at 133.1 degree-days for genotype 106
362 314 0-G1 and 133.3 degree-days for genotype 106 314 0-G2. By contrast to our findings for
363 pathotype 166 317 0, we observed no significant difference between genotypes of the same
364 pathotype for 106 314 0. This singularity justified our decision to perform phenotypic
365 characterization by comparing the aggressiveness of isolates of pathotype 106 314 0 on other
366 cultivar sets with a view to detecting potential differences.
367
368 **Difference in aggressiveness between the two major genotypes of pathotype 106 314 0 on**
369 **Michigan Amber and major cultivars present in the French varietal landscape**
370 The two major genotypes of pathotype 106 314 0 had significantly different LP on five of the
371 six cultivars tested (Table 4; Table S6). These differences were significant on the cultivar
372 Michigan Amber, whereas no difference was found in analyses of the data for series 1, as
373 described above. IE of genotype 106 314 0-G2 was higher than that of 106 314 0-G1 (48.7%
374 versus 39.1%) on cultivar Expert, but no differences were found on the other cultivars.
375 Genotype 106 314 0-G2 had a shorter LP than 106 314 0-G1 on five of the six cultivars tested.
376 This difference in LP ranged from 2.1 degree-days on Premio to 4.8 degree-days on Expert. A
377 difference of 5 degree-days is equivalent to 8 h at 15°C. Bermude was the only cultivar on
378 which LP did not differ significantly between the two pathogen genotypes. No significant
379 differences in SP were observed between the two genotypes on any of the cultivars tested. SP
380 varied between cultivars, ranging from 0.099 mg on Michigan Amber to 0.134 mg on Sankara,
381 with slightly higher values for 106 314 0-G2 than for 106 314 0-G1. It was not possible to look

382 for significant differences between cultivars, as different cultivars were analyzed in different
383 series.
384
385 Within genotypes, there was a significant isolate effect for LP, but no significant isolate effect
386 for IE or SP, on Aubusson, Premio and Expert.
387
388 For LP, IE and SP, the replication factor (x3) was almost always significant. The interaction
389 between replication factor and the three aggressiveness components was significant for some
390 aggressiveness component measurements (Table 4; Table S6).
391

392 **DISCUSSION**

393 Focusing on two major pathotypes from the French *P. triticina* population, we found that
394 several genotypes were present within each pathotype, and that the frequency of the most
395 common genotypes changed over time. The initially dominant genotypes representative of
396 pathotypes 166 317 0 and 106 314 0 were replaced, in each pathotype, by another genotype
397 over the period 2006-2016. The most recent dominant genotype was more aggressive than
398 the older one in both pathotypes.
399

400 **Methodological aspects of aggressiveness measurement at the wheat seedling stage**

401 The mean values used to characterize the aggressiveness of different *P. triticina* isolates on
402 wheat seedlings revealed significant variation for each of the three aggressiveness
403 components measured — infection efficiency, latency period and sporulation capacity — at
404 the seedling stage. Aggressiveness and its variation can be measured on seedlings (Milus *et*
405 *al.*, 2006; de Vallavieille-Pope *et al.*, 2018) or on adult plants (Lehman & Shaner, 1997; Pariaud

406 *et al.*, 2009b; Azzimonti *et al.*, 2013). We decided to assess aggressiveness components on
407 wheat seedlings in this study, because the use of seedlings in a semi-controlled environment
408 results in more homogeneous physiological properties of the plant at inoculation and because
409 this approach requires less space and time than studies on adult plants. However, we should
410 be aware that plant stage is known to affect the estimation of the aggressiveness components
411 of *P. triticina* (Pariaud *et al.*, 2009b).
412
413 With the phenotyping method developed and used in this study, we were able to estimate the
414 three aggressiveness components simultaneously on the same inoculated plant. We
415 measured infection efficiency (IE) more precisely here than in previous studies, in which the
416 variability was sometimes very high. For instance, Pariaud *et al.* (2009b) applied a 1:10 mixture
417 of *P. triticina* urediniospores and *Lycopodium* spores to the leaf surface with a soft brush; they
418 reported IE values ranging from 18% to 80% for the same genotype. This high level of variation
419 resulted from difficulty controlling the number of urediniospores deposited with this method,
420 resulting in differences between experimental series. Although fastidious, the deposition of
421 urediniospores one-by-one on the leaf surface resulted in a much more accurate estimation
422 of IE than other methods based on the dilution of *P. triticina* urediniospores among
423 *Lycopodium* spores or in liquids, such as mineral oils (Pariaud *et al.*, 2009b; Sørensen *et al.*,
424 2016). Nevertheless, the interaction between IE and the replication factor was almost always
425 significant, indicating an impact of environmental conditions on this component of
426 aggressiveness. This interaction reflects the difficulty ensuring uniform dew quality in the dew
427 chamber just after inoculation, as the success of urediniospore germination and penetration
428 depends on the presence of a water film on the leaf surface (Bolton *et al.*, 2008).
429

430 Sporulation capacity (SP) was measured by collecting urediniospores produced between 9 and
431 12 days after inoculation, and differences in SP between genotypes were detected for only
432 one experimental series. Pariaud *et al.* (2009b) found differences in SP between pathotypes
433 of *P. triticina* in a study in which urediniospores were collected 15 to 23 days after the
434 inoculation of adult plants. They also found that the difference in SP between isolates
435 increased with the regular collection of urediniospores until 59 days after inoculation. The
436 collection of urediniospores more than 13 days after inoculation is of potential relevance for
437 future experiments with our method, to maximize differences and reveal small differences in
438 this component of aggressiveness between isolates.
439
440 Latency period (LP) was the component that differed most between pathogen genotypes and
441 was the least affected by the replication factor. The calculation of LP in degree-days made it
442 possible to take the temperature-dependence of both infection processes and pathogen
443 development in the leaf into account (Lovell *et al.*, 2004). In most pathosystems, LP is a
444 component of choice for studies of quantitative interactions (aggressiveness, partial
445 resistance) because time is a variable that is much easier to fractionate and, therefore, to
446 quantify precisely, than any biological trait, making it possible to highlight extremely small
447 differences between isolates repeatedly. This is almost an epistemological issue for
448 experimental epidemiology and phytopathometry (Suffert & Thompson, 2018; Bock *et al.*,
449 2022).
450
451 **Evolution of greater aggressiveness in two *P. triticina* pathotypes**
452 In the case of pathotype 166 317 0, genotype 166 317 0-G2 was more aggressive than the one
453 it replaced, 166 317 0-G1, with highly consistent results obtained for the different components

454 of aggressiveness. Following this switch between genotypes, the frequency of pathotype 166
455 317 0 increased considerably over a period of three years, whereas the proportion of
456 compatible host cultivars in the landscape decreased (data not shown). Severe *P. triticina*
457 epidemics have been shown to be associated with a high SP, a high IE and a short LP (Azzimonti
458 *et al.*, 2022). In pathotype 106 314 0, comparisons of aggressiveness on a 'neutral' cultivar
459 revealed no significant difference between the most recent and oldest genotypes. A
460 comparison of the aggressiveness of these two genotypes on some of the cultivars most
461 frequently grown in the landscape revealed that 106 314 0-G2 was more aggressive than 106
462 314 0-G1 only for LP. This change in predominant genotype coincided with a halt in the decline
463 of pathotype 106 314 0 frequency in the landscape. The shorter LP of genotype 106 314 0-G2
464 seems to have an effect on the frequency of this pathotype in the landscape, consistent with
465 the assertions of several studies that LP is the aggressiveness component with the largest
466 effect on pathogen dynamics in field conditions (Lannou, 2012). In modeling studies, this also
467 appeared to be the trait with the largest impact on the intensity of *P. triticina* epidemics, as it
468 determined the number of reproductive cycles (i.e. from inoculation to spore dispersion) of
469 the pathogen possible in a single season (Rimbaud *et al.*, 2018). Although only LP differed
470 significantly between 106 314 0-G2 and 106 314 0-G1, the data for the other aggressiveness
471 components also supported the notion that 106 314 0-G2 was more aggressive, for every
472 component, on all cultivars, except for IE on Premio. Measurements of aggressiveness
473 components on seedlings in controlled conditions (only one reproductive cycle of the
474 pathogen) have a limited capacity for the detection of small phenotypic variations.
475
476 The competitive advantage of one particular genotype within a pathotype, expressing a
477 difference in parasitic fitness for the same virulence profile, may account for the replacement

478 of one genotype with another. This hypothesis is particularly realistic here as the emergence
479 of the more aggressive genotype in each pathotype, 106 314 0 and 166 317 0, coincided with
480 a short-term change in pathotype frequency: (i) the decrease in the frequency of pathotype
481 106 314 0 in the landscape observed from 2009 to 2011 was temporarily halted during the
482 2011-2014 period, when the frequency of this pathotype reached a plateau (Figure 3),
483 coinciding with the replacement of the less aggressive genotype 106 314 0-G1 with the more
484 aggressive genotype 106 314 0-G2 in 2012 and 2013 (Figure 4); (ii) the decrease in the
485 frequency of pathotype 166 317 0 in the landscape observed from 2012 to 2015 was halted
486 over the 2015-2018 period, resulting in a new peak frequency (Figure 3) coinciding with the
487 replacement of the less aggressive 166 317 0-G1 genotype with the more aggressive 166 317
488 0-G2 genotype in 2012 and 2013 (Figure 5).
489
490 These results suggest that the replacement of some pathotypes by others, driven by changes
491 in the frequencies of resistance genes in the varietal landscape, may be slowed, to various
492 extents, by increases in the aggressiveness of certain genotypes or 'lineages' (defined as
493 genetically related genotypes of the same group of pathotypes, i.e. with the same close
494 ancestor), consistent with the recent analysis by Fontyn *et al.* (2022). It is even possible, as for
495 pathotype 166 317 0, for the pathotype to gain a 'second life' — for a period of three to four
496 years — due to an increase in the aggressiveness of a new genotype, as long as it remains
497 adapted (or at least not maladapted) to the resistance genes present in the varieties deployed
498 in the landscape. This empirical study shows that differences in aggressiveness can be
499 expressed on a 'neutral' cultivar (for pathotype 166 317 0), but sometimes (for pathotype 106
500 314 0) only on 'non-neutral' cultivars representative of a varietal landscape. This finding
501 clearly complicates the experimental approach (difficulty determining the most appropriate

502 experimental design without making assumptions), but also the data analysis. The magnitude
503 of the differences in aggressiveness probably depends on the type of cultivars tested ('neutral'
504 or 'non-neutral'). Indeed, we found that the intensity of the mid-term changes in frequencies
505 highlighted above was variable, with either a simple slowing of a trend towards a decrease
506 (plateau, as for pathotype 106 314 0), or a change in direction, with a new increase (peak, as
507 for pathotype 166 317 0).
508
509 **Putative origin of the more aggressive genotypes**
510 All surveys in wheat-growing areas to date have provided evidence for continual evolution of
511 *P. triticina* populations, with rapid changes in pathotype frequencies (Goyeau *et al.*, 2012;
512 Kosman *et al.*, 2019; Zhang *et al.*, 2020; Fontyn *et al.*, 2022; Kolmer & Fajolu, 2022). These
513 changes can be explained intrinsically by the acquisition of new virulences, by mutations,
514 somatic exchanges or, more rarely, genetic recombination. A study on the Australian *P.*
515 *striiformis* f. sp. *tritici* population showed that it had evolved due to mutation events (Steele
516 *et al.*, 2001). Whole-genome sequencing has detected similar recurrent mutations in *P.*
517 *triticina*, suggesting that such events play a major role in genetic variability within clonal
518 lineages (Fellers *et al.*, 2021). Somatic exchange may also have played a role, as in the
519 emergence of the Ug99 lineage of *Puccinia graminis* f. sp. *tritici,* which had one identical
520 haploid nucleus in common with an old African lineage, with no sign of recombination (Li *et*
521 *al.*, 2019). A similar situation has been described in *P. triticina*, with the Australian isolate Pt64
522 resulting from somatic exchange between two parental isolates (Wu *et al.*, 2019). More rarely,
523 genetic recombination may also underlie the emergence of new genotypes with new
524 virulences. Indeed, *P. triticina* is a heteroecious fungus that requires an alternative host,
525 *Thalictrum speciosissimum* (not naturally present in most places worldwide, including France),

526 for sexual reproduction. The high proportion of repeated genotypes and the heterozygosity
527 rates of European and French leaf rust populations confirmed the very dominant role of clonal
528 reproduction (Goyeau *et al.*, 2007; Kolmer *et al.*, 2013). Genetic changes do not necessarily
529 result in different virulence profiles, so isolates with identical or very similar pathotypes may
530 have different origins. In a previous study on *P. triticina* nine microsatellite markers were used
531 to analyze the genotypes of 33 pathotypes (Goyeau *et al.*, 2007). This analysis highlighted the
532 presence of different genotypes, differing by only one of the nine SSRs tested, in five of the
533 pathotypes. Another study on 121 European *P. triticina* isolates with 21 SSR markers revealed
534 a significant correlation between phenotype and genotype, but the same phenotype could,
535 nevertheless, be associated with several genotypes (Kolmer *et al.*, 2013). The results of our
536 study raise questions as to the genetic relationship between the most recent genotypes, 166
537 317 0-G2 and 106 314 0-G2, and the oldest genotypes, 166 317 0-G1 and 106 314 0-G1,
538 respectively. Can the most recent and the oldest genotypes be considered to belong to the
539 same 'lineage', as previously defined? A mutation event during asexual reproduction probably
540 led to the emergence of the most recent genotype (G2) of pathotype 166 317 0, which differs
541 from the older genotype (G1) by only one SSR allele (Table 3). By contrast, genotypes G1 and
542 G2 of pathotype 106 314 0 are more differentiated, as they are differing by five SSR alleles
543 (Table 3). Genotype 106 314 0-G2 may, therefore, be the result of several mutation events
544 occurring in the same lineage and conferring greater aggressiveness than for 106 314 0-G1.
545 Alternatively, the presence of 106 314 0-G2 in France may reflect the introduction from an
546 exogenous source of a different (more aggressive) lineage that had acquired the same
547 virulences and, therefore, belonged to the same pathotype, 106 314 0. Migration events are
548 frequent in cereal rust populations, as already shown for yellow rust (Ali *et al.*, 2014; Bueno-
549 Sancho *et al.*, 2017), for which there is a famous example of migration in the form of an

550 introduction into Australia from northwest Europe in 1979 (Wellings & McIntosh, 1990).
551 Studies on *P. triticina* in wheat-growing areas worldwide have revealed a broad geographic
552 distribution of identical and highly related multilocus genotypes, highlighting the potential of
553 leaf rust for long-distance migration (Kolmer *et al.*, 2019) and the possibility of new genotypes
554 resulting from exogenous introduction.
555
556 **Additional approaches for detecting genetic variation related to aggressiveness**
557 This study was not designed to characterize overall pathotypic or genotypic changes in the *P.*
558 *triticina* population, so our data should be extrapolated with caution, as any attempt to use
559 them for such purposes would be subject to multiple sampling biases. In addition to the four
560 genotypes on which we focused here, several other genotypes (with cumulative frequencies
561 of 31% to 61%, depending on the year) were identified. This is due to the initial pathotyping
562 performed annually for the national survey and the genotyping for this study being performed
563 on different isolates, purified from a bulk of urediniospores collected from a single leaf, which
564 may well have been infected with several pathotypes and/or genotypes (Figure 1). This
565 highlights the imperfections of the protocol linked to the constraints of working on datasets
566 and biological material acquired over several decades with ever-changing techniques. It would
567 have been better to pathotype and phenotype the 401 isolates from the same purified batch
568 of urediniospores, as we did later on for the 44 isolates for which aggressiveness was
569 characterized (Figure 1). However, given the size of the sample, this limitation did not prevent
570 us from obtaining a correct overview of changes for the most common genotypes.
571 Our results revealed rapid genotype evolution within the pathotypes that was not detectable
572 if only virulence phenotypes were considered. These findings demonstrate the value of
573 analyzing population dynamics not only from the pathotype standpoint, but also with

574 genotype data, to obtain a more informative picture of pathogen diversity. For leaf rust, as for
575 many fungal pathogens that are commonly described by their virulence profile, there is no
576 univocal link between the pathotypic and the genotypic characterization. In practice,
577 pathotypic characterization and naming has been favoured because of the importance of
578 virulence in the structure of populations. Our results show that different genotypes (defined
579 here by differences in the combination of SSR markers) that have the same virulence profile
580 can nevertheless express differences in the quantitative component of pathogenicity
581 (aggressiveness). Such differences are potentially related to interactions with sources of
582 quantitative resistance. Conversely, identical genotypes may differ in one or several
583 virulences. In summary, differentiation (or not) for neutral markers is independent from
584 differentiation (or not) for functional mutations. There is currently no nomenclature for the
585 designation of a unique association between one pathotype and one genotype. Therefore, we
586 propose to define a unique association with the term 'pathogenotype', exemplified here with
587 106 314 0-G1 and 106 314 0-G2.
588
589 The use of a small number of SSR markers is not sufficient for the detection of genetic
590 variations in *P. triticina* populations. Genome-wide genotyping approaches are required to
591 characterize the genetic diversity of leaf rust populations more precisely and to explain the
592 emergence of new genotypes. As an illustration of this approach, a combined genome and
593 transcriptome analysis has been performed on 133 *P. striiformis* f. sp. *tritici* isolates collected
594 in 16 European countries. This analysis provided more precise information about the origin of
595 the new emerging races, and showed that SNP analysis was an effective approach for the
596 detection of pathogen diversity and for pathogen surveillance (Bueno-Sancho *et al.*, 2017).
597 Similarly, Fellers *et al.* (2021) genotyped 121 *P. triticina* isolates with 121 907 SNP markers,

598 and showed that recurrent mutation and selection had played a major role in differentiation
599 within clonal lineages. The results of our study highlight the importance of combining genome-
600 wide genotyping tools with precise pathotyping and aggressiveness phenotyping to detect the
601 emergence of new variants and to improve our understanding of population dynamics.
602

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616

617 **Data Availability Statement**

618 The data that support the findings of this study are openly available in the INRAE Dataverse
619 online data repository (https://data. inrae. fr/) at https://doi.org/10.57745/MZ8TDK
620

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TABLES

Table 1. Experimental design, with the allocation of isolates to series 1 and 2, for assessments of the aggressiveness of pathotypes 106 314 0 and 166 317 0. Isolates were collected during the 2005-2016 period, from cultivar Apache, and were tested on Apache and Michigan Amber.

Pathotype	Genotype[a]	Year of sampling	Number of isolates	Series
106 314 0	106 314 0-G1	2005-2006	6	1
		2012-2013	3	1
		2015-2016	0	1
	106 314 0-G2	2005-2006	0	1
		2012-2013	2	1
		2015-2016	6	1
166 317 0	166 317 0-G1	2012	6	2
		2014	6	2
		2016	0	2
	166 317 0-G2	2012	0	2
		2014	0	2
		2016	4	2

[a] Genotyping with19 SSRs specific to leaf rust (see Table 3)

Table 2. Experimental design, with the allocation of isolates to series 3, 4 and 5, for assessments of the aggressiveness of pathotype 106 314 0. Isolates were collected in 2012-2013, from Aubusson, Premio or Apache.

Genotype[a]	Number of isolates	Cultivar tested	Series
106 314 0-G1	5	Aubusson	3+4
		Premio	3+4
		Michigan Amber	3+4
	1	Sankara	5
		Expert	5
		Bermude	5
106 314 0-G2	11	Aubusson	3+4
		Premio	3+4
		Michigan Amber	3+4
	2	Sankara	5
		Expert	5
		Bermude	5

[a] Genotyping with 19 SSRs specific to leaf rust (see Table 3)

Table 3. Genotypic characteristics of the two main genotypes, G1 and G2, identified for isolates of pathotypes 166 317 0 and 106 314 0, collected from 2005-2016 for 19 SSR loci. Allele sizes differing between genotypes of the same pathotype are shown in bold typeface.

	166 317 0[a]				106 314 0[a]			
	166 317 0-G1		166 317 0-G2		106 314 0-G1		106 314 0-G2	
Locus	Allele 1	Allele 2	Allele 1	Allele 2	Allele 1	Allele 2	Allele 1	Allele 2
RB8	**143**	**146**	**146**	**152**	**143**	**143**	**143**	**152**
RB11	200	200	200	200	**200**	**204**	**175**	**204**
RB12	282	290	282	290	282	290	282	290
RB17	190	190	190	190	190	190	190	190
RB25	230	230	230	230	230	230	230	230
RB26	352	352	352	352	352	352	352	352
PtSSR13	129	131	129	131	129	131	129	131
PtSSR50	365	371	365	371	365	371	365	371
PtSSR55	308	308	308	308	308	308	308	308
PtSSR61	296	302	296	302	296	300	296	300
PtSSR68	309	311	309	311	**309**	**317**	**309**	**323**
PtSSR91	380	382	380	382	382	382	382	382
PtSSR92	246	246	246	246	**246**	**246**	**246**	**248**
PtSSR152	389	393	389	393	389	393	389	393
PtSSR154	247	267	247	267	247	267	247	267
PtSSR158	235	238	235	238	232	238	232	238
PtSSR164	219	219	219	219	**219**	**225**	**219**	**219**
PtSSR173	213	221	213	221	213	221	213	221
PtSSR186	341	341	341	341	341	341	341	341

[a] Seven-digit triplet code (Gilmour, 1973) based on a 20-*Lr* gene differential set: Thatcher lines [*Lr1*, *Lr2a*, *Lr2b*], [*Lr2c*, *Lr3*, *Lr3bg]*, [*Lr3ka*, *Lr10*, *Lr13*], [*Lr14a*, *Lr15*, *Lr16*], [*Lr17*, *Lr20*, *Lr23*], [*Lr26*, *Lr17b* (the Australian cv. 'Harrier'), *Lr37*] and [*Lr24, , Lr28* (*line* CS2A/2M)].

Table 4. Comparison of the aggressiveness of the two major genotypes of pathotype 106 314 0 on five of the most widely grown cultivars in the French landscape during the 2005-2016 period. Letters indicate a significant difference between genotypes on the same cultivar after a Tukey multiple-comparison test or a Wilcoxon-Mann-Whitney test. Numbers in bold typeface indicate a significant difference between isolates within genotypes.

		Aggressiveness components		
Cultivar	Genotype	Infection efficiency[z]	Latency period[z]	Sporulation capacity[z]
Aubusson	106 314 0-G1	48.5*	**141.0 a**	0.105
	106 314 0-G2	49.8*	**137.3 b**	0.106
Premio	106 314 0-G1	52.6*	**134.5 a**	0.102
	106 314 0-G2	49.8*	**132.4 b**	0.105
Bermude	106 314 0-G1	43.9*	143.4	0.114*
	106 314 0-G2	47.0*	142.1	0.121*
Expert	106 314 0-G1	39.1 a	**143.0 a**	0.120*
	106 314 0-G2	48.7 b	**138.2 b**	0.132*
Sankara	106 314 0-G1	45.0*	143.9 a*	0.134*
	106 314 0-G2	46.9*	140.7 b*	0.134*
Michigan Amber	106 314 0-G1	51.6*	131.7 a	0.099
	106 314 0-G2	52.2*	129.3 b	0.102

[z] Infection efficiency (IE) was measured as a %, latency period (LP) in degree-days, and sporulation capacity (SP) in mg of spores/uredinia.
* indicates a significant interaction between genotype and replication factors.

FIGURES

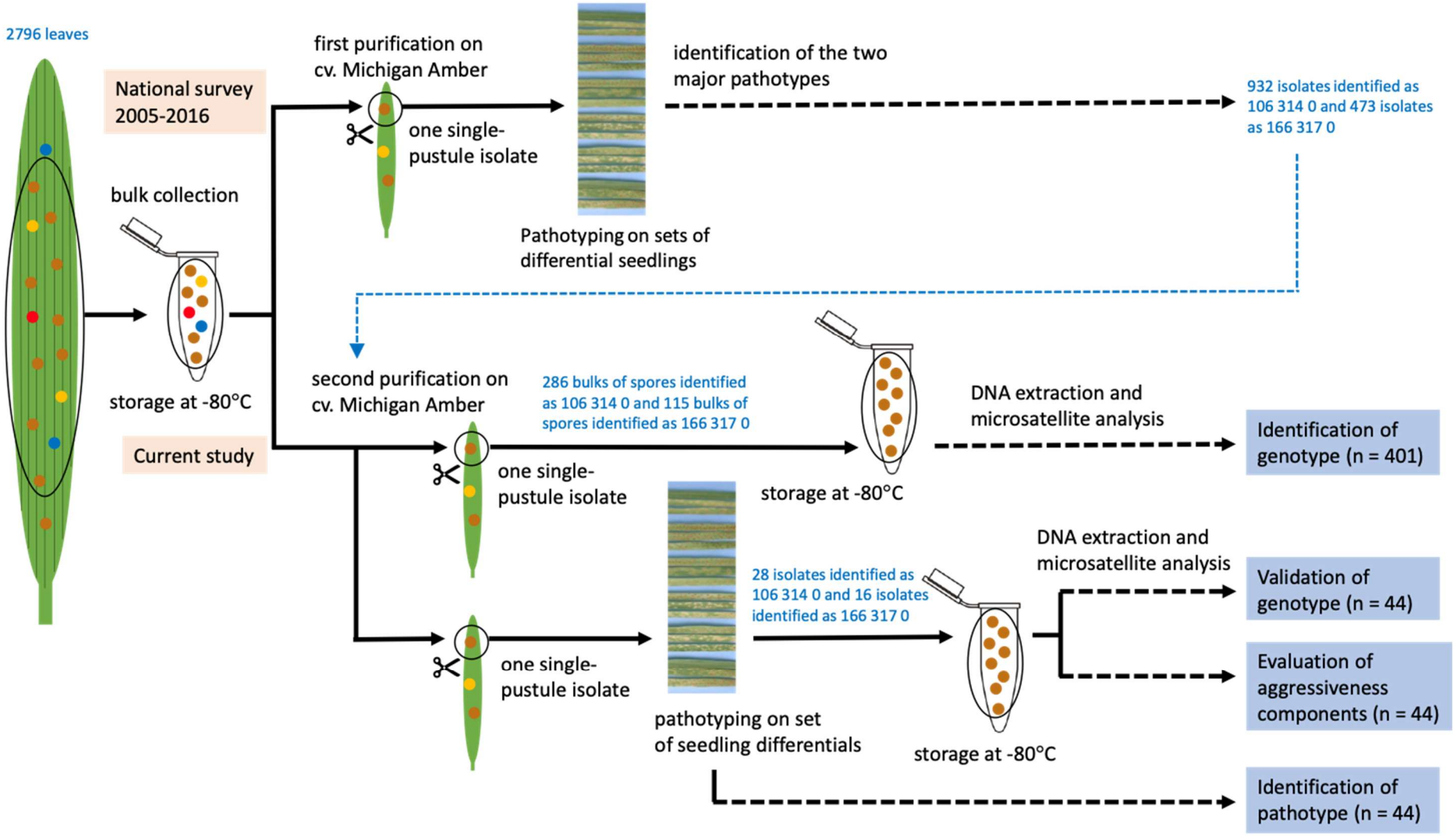

Figure 1. Overview of the purification, selection, pathotyping, and genotyping steps for the *P. triticina* isolates for which aggressiveness components were evaluated.

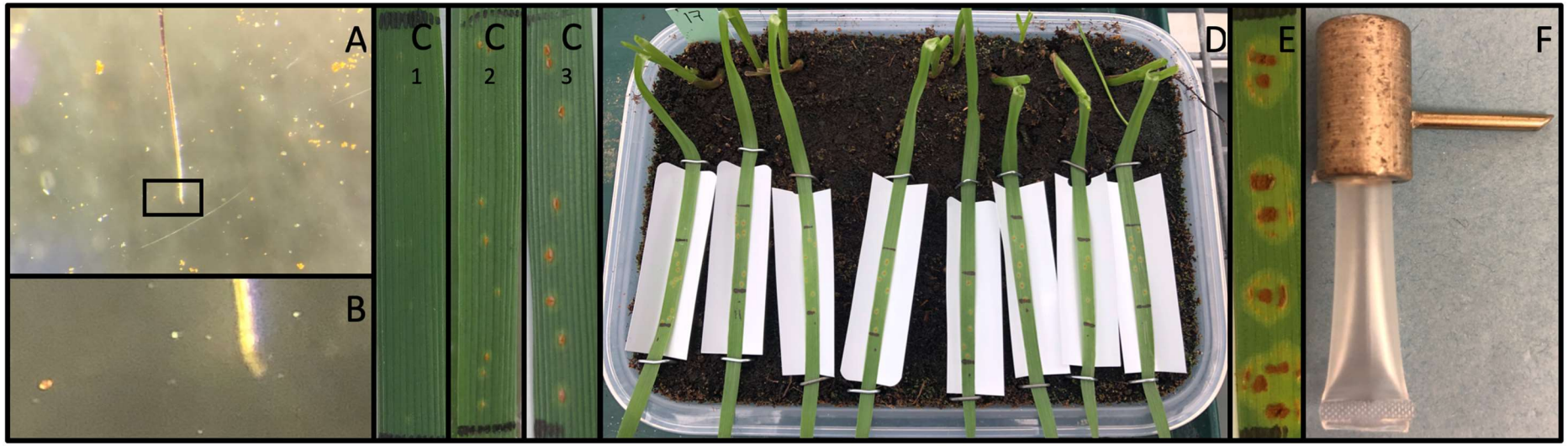

Figure 2. Experimental steps for the assessment of aggressiveness components of *Puccinia triticina* on wheat seedlings. Inoculation: (A) and (B), collection of a spore with a human eyelash and its deposition on a leaf. Latency period (C): (C1) onset of chlorosis, (C2) counting of the uredinia that have broken through the leaf, (C3) end of the latency period, when all the uredinia have emerged. Sporulation: (D) incurved aluminum gutters positioned under the leaves for spore collection, (E) end of sporulation, (F) spores retrieved with a cyclone collector into a sealed portion of plastic straw.

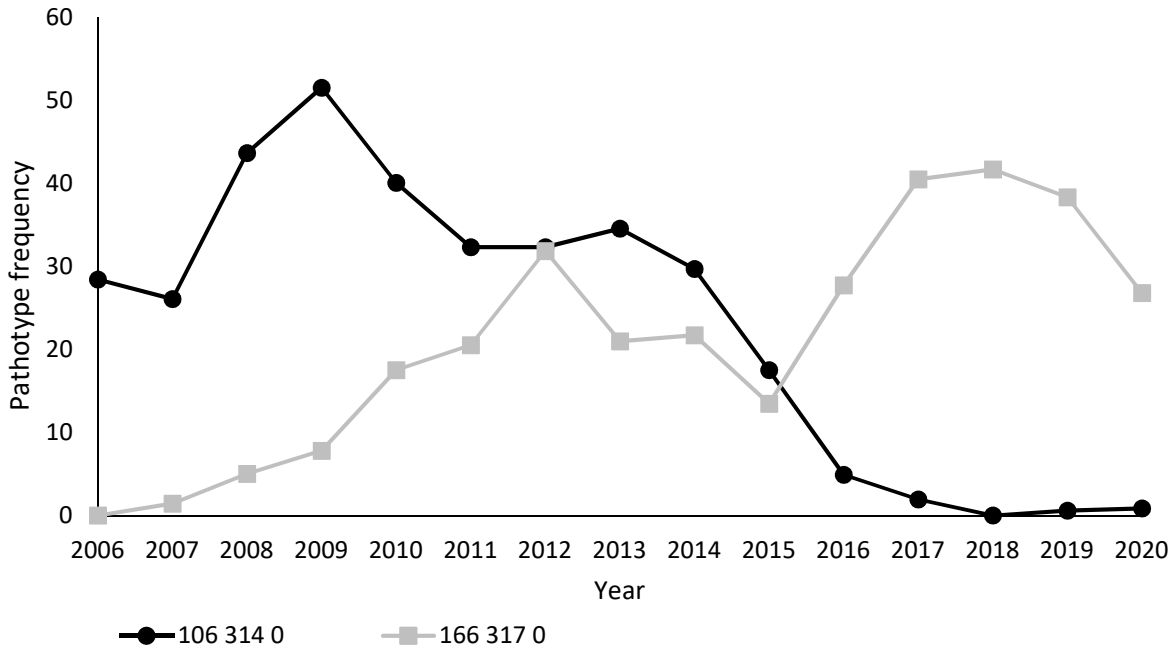

Figure 3. Frequency dynamics of two of the major *Puccinia triticina* pathotypes, 106 314 0 and 166 317 0, in the French landscape during the 2006-2020 period. Pathotype frequency was determined with data from the national survey, which returned 1025 isolates identified as 106 314 0 and 538 isolates identified as 166 317 0 among a total of 3446 pathotyped isolates (data available in Fontyn *et al.*, 2022).

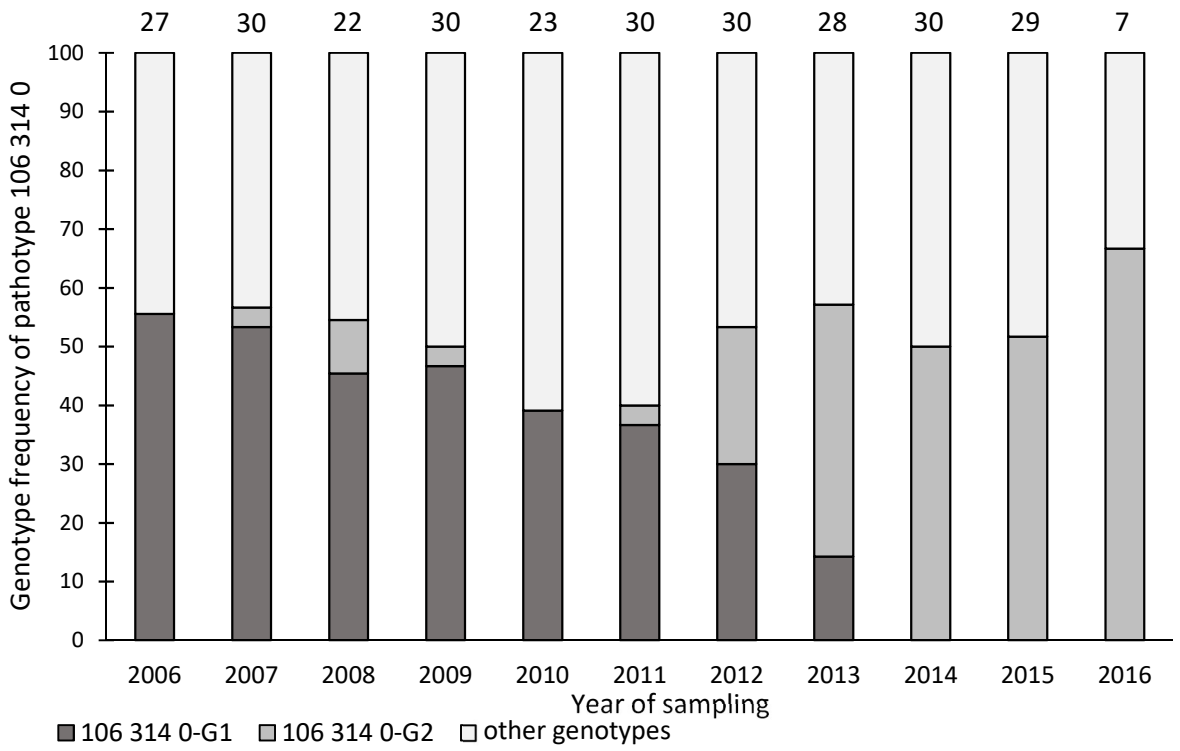

Figure 4. Changes in genotype frequencies within pathotype 106 314 0 in the French landscape during the 2006-2016 period, as determined with 19 SSR markers. The numbers on top of the bars are the numbers of isolates genotyped (286 in total).

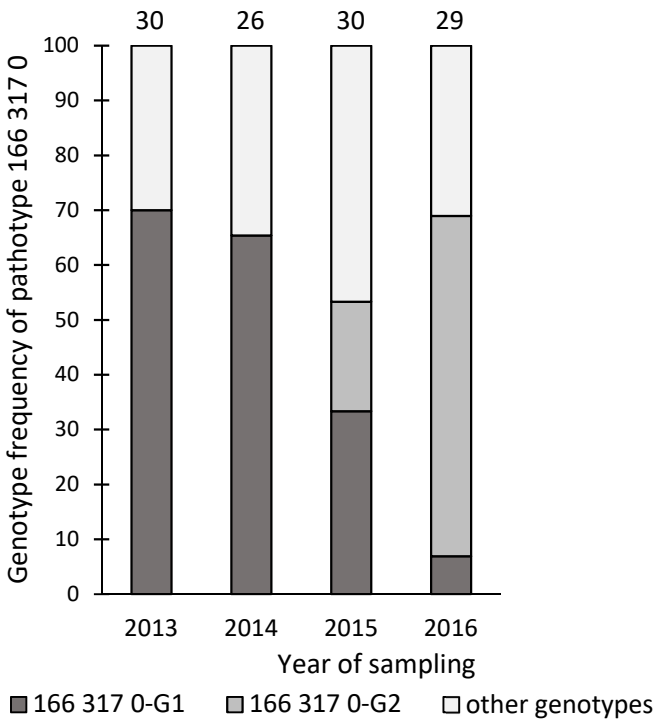

Figure 5. Changes in genotype frequencies within pathotype 166 317 0 in the French landscape during the 2013-2016 period, as determined with 19 SSR markers. Numbers on top of the bars represent the numbers of isolates genotyped (115 in total).

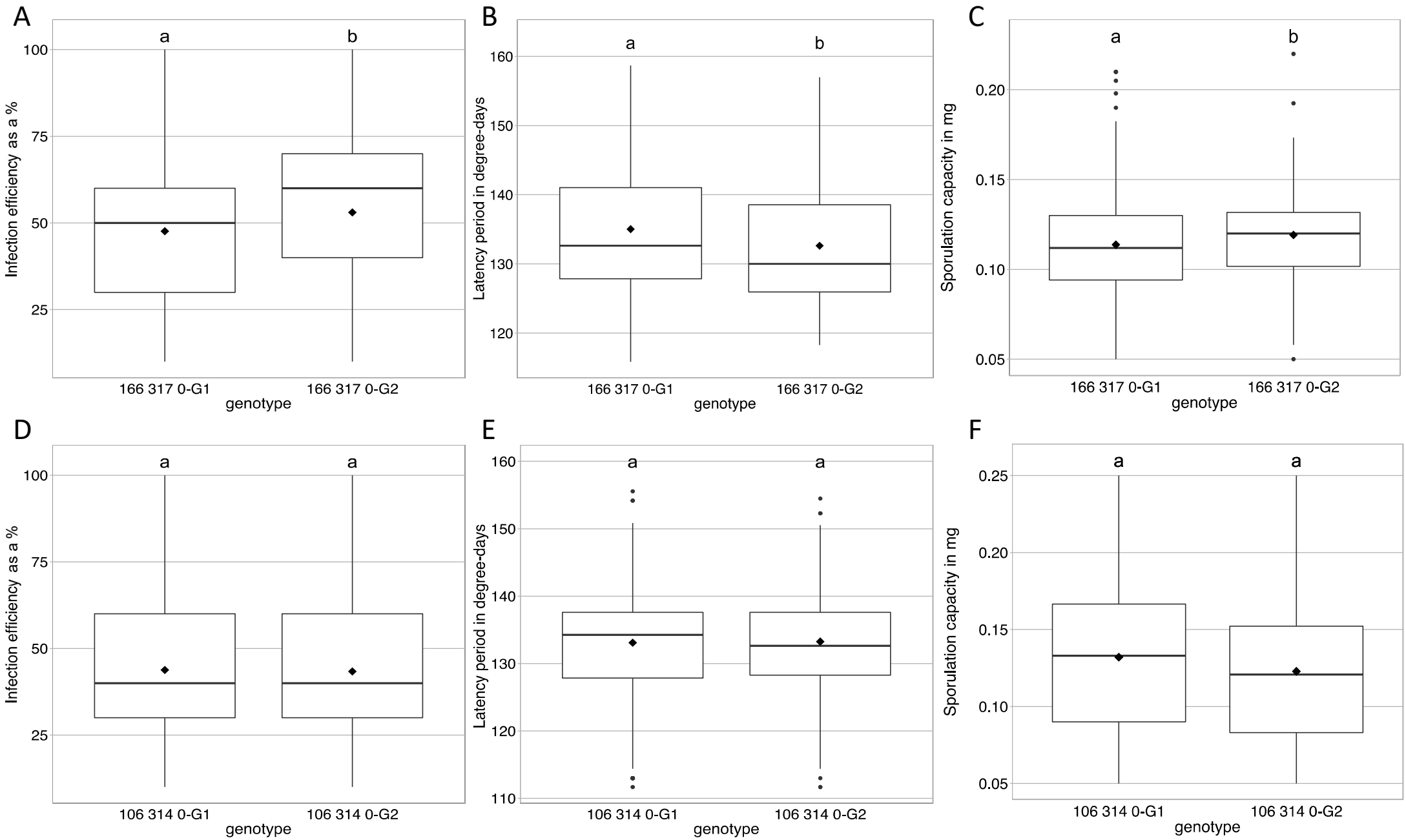

Figure 6. Comparison of genotype aggressiveness within the two major *Puccinia triticina* pathotypes. Infection efficiency (IE) as a %, latency period (LP) in degree-days and sporulation capacity (SP) in mg for pathotypes 166 317 0 (A, B, C) and 106 314 0 (D, E, F) were assessed on cultivars Apache and Michigan (pooled data, see Figures S1 and S2 for results by cultivar). Isolates were sampled from Apache in 2012, 2014, and 2016 for pathotype 166 317 0, and in 2005-2006, 2012-2013 and 2015-2016 for pathotype 106 314 0. Within a box plot, the black diamond represents the mean value and the bar indicates the median value. Letters indicate significant differences between genotypes in Kruskal-Wallis tests (A, C, E, F) or ANOVA (B and D).