# 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, 78850 Thiverval-Grignon, 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 the same combinations of virulences — predominated but then declined over the 2005-2016 period. The relative dynamics of these two pathotypes — 166 317 0 and 106 314 0 — relative to the others present in the population 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 most 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, with no selection effect on the landscape-pathotype pattern. For pathotype 106 314 0, the most recent genotype was more aggressive on several of the cultivars most frequently grown in the landscape, but not on 'neutral' and 'naive' cultivars, and only in terms of its latency period. We conclude that the quantitative components of aggressiveness can be significant drivers of evolution in pathogen populations. A gain in aggressiveness allowed the maintenance of a declining pathotype, and even further expansion of that pathotype, in the pathogen population, providing evidence that virulence alone is not sufficient, aggressiveness also being required for the adaptation of a pathogen to a changing varietal landscape.

## Keywords

Aggressiveness, quantitative phenotyping, genotype evolution, host adaptation, leaf rust

## 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 disease, is generally broken down into a qualitative term, 'virulence', and a quantitative term, 'aggressiveness' (Lannou, 2012). Virulence is defined as the capacity of the pathogen to infect its host (compatible interaction), as opposed to avirulence, which expresses the resistance of

48 the host (incompatible interaction), according to the gene-for-gene model (Flor, 1971). A
49 virulence phenotype, also known as a pathotype or race, is defined by a virulence profile: two
50 pathogenic isolates are considered to belong to the same pathotype if they have the same
51 combination of virulence genes. Aggressiveness, the quantitative variation of pathogenicity
52 on a compatible host (Pariaud *et al.*, 2009a), can be viewed as the detrimental impact of a
53 pathogen on its host, leading to damage to the crop plant and, thus, yield losses (Shaner *et*
54 *al.*, 1992; Pariaud *et al.*, 2009a; Lannou, 2012). Aggressiveness also determines the rate at
55 which a given disease intensity is reached. Its assessment is intrinsically complex because it is
56 related to various life-history traits of the pathogen specific to its biology and the nature of
57 the symptoms that it produces. The different components of aggressiveness can be measured
58 by evaluating several of these complementary quantitative traits expressed during the host–
59 pathogen interaction. The most widely assessed aggressiveness components for rust
60 pathogens are infection efficiency, latency period and sporulation capacity (Pariaud *et al.*,
61 2009a; Lannou, 2012; Azzimonti *et al.*, 2013).
62
63 Infection efficiency is calculated by determining the proportion of spores able to cause a new
64 infection when deposited on compatible host plant tissues (Sache, 1997). The estimation of
65 this component is complex due to technical issues, particularly the need for great precision in
66 the spore deposition process (Lehman & Shaner, 1997), which involves placing a fixed and
67 known number of spores — ideally one by one — on the leaf.
68
69 The latency period is the length of time between the initiation of the infection process by a
70 unit of inoculum and the start of infectious unit production (Parlevliet, 1979; Madden *et al.*,
71 2007). In rusts, it is often defined as the length of time between inoculation and the

appearance of 50% of the sporulating structures (e.g. uredinia), also known as 'pustules' (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, pustule 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. This trait, like several others, is, thus, dependent on latency period. The interdependence of traits can be reduced by measuring sporulation capacity at a normalized time point.

Leaf rust caused by *Puccinia triticina* is one of the most damaging wheat diseases, causing high yield losses worldwide (Huerta-Espino *et al.*, 2011; Savary *et al.*, 2019). Qualitative resistance is the easiest and most effective means of limiting leaf rust epidemics. Eighty-two *Lr* genes have been identified in wheat cultivars, most displaying qualitative interactions (Bariana *et al.*, 2022). It has been shown that the deployment of qualitative resistance genes in the landscape exerts a strong selective pressure, acting as an essential driver of evolution in *P. triticina* populations (Goyeau *et al.*, 2006). This effect was highlighted by surveys of virulence phenotypes (pathotypes), which showed that the corresponding virulence can emerge rapidly

96 after the introduction of a new *Lr* gene into cultivars. For example, in France, virulence against
97 *Lr*28 appeared within only two years of the release of cultivars carrying *Lr28* (Fontyn *et al.*,
98 2022). Adaptation to qualitative resistances occurs rapidly, despite the clonality of the
99 population, through 'boom-and-bust' cycles of resistance (McDonald & Linde 2002).
100
101 However, qualitative resistance alone cannot fully explain pathogen population dynamics.
102 Indeed, the quantitative resistance genes introgressed into new wheat cultivars at the same
103 time as qualitative resistance genes may also exert a selection pressure in favor of more
104 aggressive pathotypes. The occurrence of selection for greater aggressiveness has already
105 been established for various pathogens (Delmas *et al.*, 2016; Frézal *et al.*, 2018). Milus *et al.*
106 (2009) showed that the replacement of an 'old' *Puccinia striiformis* f. sp. *tritici* population by
107 a 'new' population could be explained by the greater aggressiveness of isolates from this new
108 population. A survey of the French *P. triticina* population from 1999-2002 (Goyeau *et al.*, 2006)
109 revealed the domination of a single pathotype (073 100 0), coinciding with a period in which
110 the cultivar landscape was dominated by the cultivar Soissons. Pathotype 073 100 0 was found
111 to be more aggressive on this cultivar than other virulent pathotypes present in the *P. triticina*
112 population during this period (Pariaud *et al.*, 2009b). Fontyn *et al.* (2022) recently showed that
113 the domination of the French landscape by two pathotypes — 106 314 0 and 166 317 0 —
114 during the 2005-2016 period could not be fully attributed to the deployment of *Lr* genes.
115 Indeed, several other compatible pathotypes virulent against the *Lr* genes carried by the most
116 widely grown cultivars were present in the landscape, but never reached substantial
117 frequencies. The authors suggested that aggressiveness might drive the evolution of *P.*
118 *triticina* populations, modifying pathotype frequencies at large spatiotemporal scales. The
119 variation of aggressiveness over time, during the complete life history of a pathotype,

120 corresponding to the stages of emergence, domination and replacement, has never been
121 investigated.
122
123 The objective of this study was to determine the role of aggressiveness in the changes of *P.*
124 *triticina* pathotypes frequencies over large spatiotemporal scales during their life history. To
125 this end, we focused on two major pathotypes, 166 317 0 and 106 314 0, identified as good
126 experimental case studies for this purpose on the basis of investigations of frequency
127 dynamics over the 2005-2016 period in France. We first characterized isolates of these two
128 pathotypes genotypically with microsatellite markers, to identify potential 'subpopulations'.
129 Within each of the two pathotypes, we then compared the aggressiveness components of
130 isolates from the most frequent genotypes (i) on 'neutral' cultivars, i.e. cultivars with no
131 apparent effect on the frequencies of these pathotypes (166 317 0 and 106 314 0), and, when
132 relevant, (ii) on five cultivars widely grown in France over the study period (pathotype 106 314
133 0 only).
134

135 ## MATERIALS AND METHODS

136

137 **Selection and purification of isolates**

138 Annual surveys of *P. triticina* populations have been carried out at INRAE BIOGER over the last
139 two decades. These surveys involve the collection of leaf samples from field (micro)plots sown
140 with a single variety in a network of assays and nurseries throughout the wheat-growing areas
141 of France (Goyeau *et al.*, 2006; Fontyn *et al.*, 2022). In total, 2796 leaves were collected from
142 the 10 most cultivated varieties during the 2006-2016 period in the framework of the national
143 survey. Urediniospores were bulk-harvested from each leaf and stored at -80°C. One single-

144 pustule isolate was selected from each bulk and its pathotype was determined as described
145 by Goyeau *et al.* (2006). In total, 932 isolates were identified as pathotype 106 314 0 and 473
146 isolates were identified as pathotype 166 317 0. For the purposes of this study, we selected
147 286 urediniospore bulks of pathotype 106 314 0 and 115 from pathotype 166 317 0, as
148 identified during the national survey (Table S1 and S2). The bulks were defrosted and
149 repurified by the re-inoculation of seven-day-old cv. Michigan Amber wheat seedlings, to
150 obtain 401 new single-pustule isolates. Before inoculation, the plants were grown in cabinets
151 with air filters in a greenhouse at temperatures between 15 and 20°C, under a 14-h
152 photoperiod (daylight supplemented with 400 W sodium lamps). Plants were treated with 15
153 mL maleic hydrazide solution (0.25 g maleic hydrazide per liter of $H_2O$) to prevent the
154 emergence of secondary leaves and to increase spore production. Inoculated seedlings were
155 placed in a dew chamber at 15°C for 24 h and were then transferred to the greenhouse. One
156 week after inoculation, the seedlings were trimmed such that only one plant with one
157 uredinium remained in each pot. Before sporulation, cellophane bags were placed over the
158 pots to prevent contamination between isolates. Ten days after inoculation, 401 leaf
159 segments, each carrying only one uredinium, were collected for DNA extraction and
160 genotyping with microsatellite markers. Other single-pustule isolates were also selected from
161 28 and 16 bulks initially identified as pathotypes 106 314 0 and 166 317 0, respectively. Each
162 of these isolates was pathotyped (Goyeau *et al.*, 2006) and one of these isolates, after
163 confirmation that its virulence phenotype was as expected, was selected for DNA extraction
164 and genotyping with microsatellite markers. Spores from these 44 isolates were stored at -
165 80°C for further assessments of aggressiveness (Table S3). The various stages in the
166 purification, selection, pathotyping and genotyping of *P. triticina* isolates for which
167 aggressiveness components were evaluated are summarized in Figure 1.

168

169 **Genotyping of isolates with microsatellite markers**

170 DNA extraction

171 DNA was extracted from all the purified isolates in 96-well extraction plates, with Qiagen
172 DNeasy® Plant Mini Kit buffers (Qiagen, Hilden, Germany). To this end, each leaf segment
173 carrying a single uredinium was placed in a Qiagen collection microtube with a tungsten bead
174 and 100 μL of hot AP1 buffer (65°C). Leaf segments were ground by shaking the microtubes in
175 a Retsch® MM400 ball mill twice, for 30 seconds each, at 25 Hz. The tubes were then
176 centrifuged for 1 minute at 3000 x *g*. AP1 buffer supplemented with RNase A and Reagent DX
177 was then added to each tube (300 μL). After mixing, we added 130 μL P3 buffer to the tube,
178 which was then incubated for 10 minutes at -20°C and centrifuged at 4°C for 10 minutes at
179 20 000 x *g*. The supernatant (200 μL) was transferred to a new tube, to which we added 1
180 volume of sodium acetate (3 M pH 5) and 3 volumes of isopropanol (100%). After mixing, the
181 tubes were placed at -20°C for 30 minutes and then centrifuged for 20 minutes at 6000 x *g*.
182 The supernatant was removed and 3 volumes of 70% ethanol were added. The tube was
183 placed at -20°C for 5 minutes and was then centrifuged at 4°C for 15 minutes at 6000 x *g*. The
184 pellet containing the DNA was allowed to dry overnight, and was ten resuspended in 100 μL
185 ultra-purified water. We transferred 20 μL of the resulting suspension to Qiagen elution
186 microtubes RS in a 96-tube rack, which was sent to Eurofins (Eurofins, Luxembourg) for
187 genotyping. The DNA suspensions had concentrations of between 1 and 25 ng/μL.

188

189 Microsatellite genotyping and analyses

190 The 401 and 44 single-pustule isolates were genotyped for 19 microsatellite markers: RB8,
191 RB11, RB12, RB17, RB25, RB26, PtSSR13, PtSSR50, PtSSR55, PtSSR61, PtSSR68, PtSSR91,

192 PtSSR92, PtSSR152, PtSSR154, PtSSR158, PtSSR164, PtSSR173, and PtSSR186 (Duan *et al.*,
193 2003; Szabo & Kolmer, 2007). The microsatellite markers were assembled into two
194 multiplexes of 9 and 10 markers and labeled with four fluorochromes (Table S4) to prevent
195 overlaps between markers with the same range of allele sizes. PCR amplification was
196 performed by Eurofins (Eurofins, Luxembourg), with the following amplification program:
197 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
198 min. Each reaction contained 5 µL DNA solution and PCR mixture with Taq Type-it (Qiagen®).
199 PCR products were analyzed by capillarity electrophoresis on an ABI3130xl sequencer. The
200 two-multiplex PCR system was tested and validated on eight reference *P. triticina* DNA
201 extracts from isolates obtained from bread and durum wheat. Chromatograms were visually
202 inspected for all markers and for all individuals with Peak Scanner software version 2.0, before
203 the final assignment of SSR alleles. The chromosome position of SSR markers was determined
204 by performing a blastn analysis (with default parameter values) of the sequence of each
205 primer against the two genome sequences of the Australian isolate Pt76 (Duan *et al.*, 2021)
206 with the NCBI blast tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi).
207
208 The genotyping of the 401 isolates revealed two major genotypes within each pathotype: 106
209 314 0-G1 and 106 314 0-G2 for pathotype 106 314 0, and 166 317 0-G1 and 166 317 0-G2 for
210 pathotype 166 317 0. These genotypes were taken into account in the design of subsequent
211 experiments.
212
213 **Evaluation of aggressiveness components**
214 Experimental design

215 Three components of aggressiveness were assessed for 28 isolates of pathotype 106 314 0
216 and 16 isolates of pathotype 166 317 0 that had been purified, pathotyped and genotyped
217 (Table S3): latency period, infection efficiency and sporulation capacity. These aggressiveness
218 components were measured in the greenhouse on seedlings of two wheat varieties: (i)
219 Apache, a commercial French cultivar characterized as 'neutral' in a previous study (Fontyn *et*
220 *al.*, 2022), i.e. with no selection effect on the landscape-pathotype pattern, and (ii) Michigan
221 Amber, considered intrinsically 'naive' or 'neutral', both because it carries no known leaf rust
222 resistance gene and because it has never been cultivated in France and cannot, therefore,
223 have played any significant role in the evolutionary trajectory of *P. triticina* in France.
224
225 It was not possible to perform a large single trial at our facilities, so the 44 isolates were
226 characterized in five successive series, according to the same protocol and under the same
227 experimental conditions. In series 1 and 2, we investigated the variation of aggressiveness in
228 pathotypes 106 314 0 and 166 317 0, distinguishing the two genotypes of each pathotype (106
229 314 0-G1 and 106 314 0-G2, 166 317 0-G1 and 166 317 0-G2, respectively) on Apache and
230 Michigan Amber. The isolates used for these two series were collected between 2005 and
231 2016 on Apache (Table 1). Series 3, 4 and 5 were performed to investigate the variation of
232 aggressiveness in 106 314 0 only. Again, the two genotypes (106 314 0-G1 and 106 314 0-G2)
233 were distinguished, this time on three additional cultivars from the following list — Aubusson,
234 Premio, Michigan Amber, Sankara, Expert and Bermude — all of which, except Michigan
235 Amber, were among the 35 most frequently grown cultivars in the French landscape during
236 the 2006-2016 period. The isolates used in these three series were collected in 2012 and 2013,
237 on Aubusson, Premio and Apache (Table 2). The isolates used in series 3 were collected on
238 cultivars Aubusson and Premio and were tested on these two cultivars and Michigan Amber.

239 The isolates used in series 4 were collected on cultivar Apache and were tested on Apache
240 and Michigan Amber. The isolates used in series 5 were collected on cultivar Apache and were
241 tested on cultivars Sankara, Expert and Bermude. In all five series, each isolate × cultivar
242 interaction was replicated eight times, on eight wheat seedlings. Three replicates of the
243 experimental design, including the five series, were established.
244
245 Assessment of aggressiveness
246 All the phenotyping tests were performed in a greenhouse on eight-day-old wheat seedlings
247 grown in a 21 x 15 x 6 cm plastic box containing potting soil placed under standardized
248 conditions (18°C at night and 22°C during the day, with a 16-h photoperiod). The second leaf
249 of each seedling was maintained, with double-sided tape, on a rigid plate coated with
250 aluminum foil and a 3 cm-long segment of this leaf was inoculated with the fungus. Inoculation
251 was performed with 10 fresh (two-week-old) spores, picked one by one with a human eyelash
252 under binocular magnifier and deposited on the leaf (Figure 2A and 2B). The plants were then
253 placed in a dew chamber, in the dark, at 15°C, for 18 to 24 h. They were then returned to the
254 greenhouse and the rigid plate was removed. When the first uredinia (pustules) began to
255 break through the leaf epidermis, generally six days after inoculation, they were counted at
256 10- to 14-hour intervals (twice daily) until no new pustules appeared (Figure 2C). Infection
257 efficiency (IE) was estimated for each leaf as the ratio between the final number of uredinia
258 and the number of spores deposited (10 spores). Latency period (LP), estimated as the time
259 between inoculation and the appearance of 50% of the total number of uredinia, was
260 expressed in degree-days, based on the air temperature measured in the greenhouse every
261 ten minutes. Sporulation capacity (SP) was estimated, once the number of uredinia had
262 stabilized, as the number of spores produced per uredinium over a four-day period. Once the

263 final number of uredinia had been reached (i.e. 9 days after inoculation) the spores that had
264 already been produced were removed from the leaf with a small brush. Slightly incurved
265 aluminum gutters (2 x 7cm) made from blind slats were then positioned under each inoculated
266 leaf, which was attached to the gutter with clips (Figure 2D). After four days, all the newly
267 produced spores (Figure 2E) were removed by suction with a cyclone collector into a portion
268 of plastic straw sealed at one end (Figure 2F). Each portion of straw was weighed before and
269 after spore harvesting. SP was calculated by dividing the total weight of the spores collected
270 from a single leaf by the number of uredinia on that leaf.
271
272 **Statistical analyses**
273 For each of the three aggressiveness traits (the response variable Y), two ANOVA models were
274 used: model (1) for series 1 and 2; model (2) for pooled data from series 3 and 4 (without a
275 series factor, as a preliminary analysis showed that the interactions of the series factor with
276 other factors were never significant) and for series 5, for each cultivar, as the cultivars tested
277 were not the same in the three series:
278 $$(1)\quad Y_{ijklm} = \mu + R_i + G_j + C_k + G_j/I_l + \varepsilon_{ijklm}$$
279 $$(2)\quad Y_{ijkl} = \mu + R_i + G_j + G_j/I_k + \varepsilon_{ijkl}$$
280 where $Y_{ijklm}$ and $Y_{ijkl}$ are the values of the studied trait in replicate (R) *i*, of genotype (G) *j* and
281 isolate (I) *l* or *k*, nested within genotype, on cultivar (C) *k* (when this occurred). $\mu$ is the overall
282 mean value for this trait and $\varepsilon$ is the residual, representing the measurement error, with
283 $\varepsilon \sim N(o, \sigma 2)$
284
285 Log, √ or 1/x transformation was applied to IE, LP and SP when necessary, to obtain a
286 normalized distribution of residuals. When the distribution of residuals could not be

287 normalized by any transformation, a non-parametric Kruskal-Wallis test was performed to
288 analyze the effect of genotype on the aggressiveness components.
289 All the analyses were performed with R software version 4.1.0.
290

291 **RESULTS**

292

293 **Frequency dynamics of two major *P. triticina* pathotypes in the French landscape in the**
294 **2006-2020 period**

295 The frequency of pathotype 106 314 0 in the French *P. triticina* population increased from 30%
296 in 2006 to 51% in 2009 (Figure 3). After a plateau at 30-33% from 2011 to 2014, the frequency
297 of this pathotype decreased strongly, to less than 1% in 2018. Within pathotype 106 314 0,
298 two main genotypes, G1 and G2, were identified and were considered to be dominant as their
299 cumulative frequency ranged from 40% to 65% during the 2006-2016 period (Figure 3). These
300 two genotypes differed at five of the 19 SSR loci studied (RB8, RB11, PtSSR68, PtSSR92 and
301 PtSSR164; Table 3). Genotype 106 314 0-G1 was the most frequent from 2006 to 2012, but it
302 decreased in frequency thereafter (Figure 4). Genotype 106 314 0-G2 was present at a very
303 low frequency from 2007 to 2011. In 2012, the frequencies of the two genotypes were fairly
304 similar, at 23% for 106 314 0-G1 and 30% for 106 314 0-G2. Genotype 106 314 0-G1 then
305 continued to decrease in frequency, eventually disappearing completely from the sampled
306 population in 2014. Conversely, genotype 106 314 0-G2 continued to increase in frequency. It
307 accounted for 42% of the total 106 314 0 pathotype population in 2013 and 66% in 2016. Over
308 the entire period, genotypes other than G1 and G2 were also identified (detailed data not
309 shown) within pathotype 106 314 0. Their cumulative frequency ranged from 40% to 60%
310 from 2006 to 2016.

311

312 Pathotype 166 317 0 first appeared in 2007, initially at a very low frequency (less than 2%). Its
313 temporal evolution in the French *P. triticina* population was characterized by two peaks, at
314 32% in 2012 and 41% in 2018, with a decrease in frequency to 13% in 2015 (Figure 3). The
315 frequency of this pathotype decreased after 2018 reaching 27% in 2020. Within pathotype
316 166 317 0, two genotypes, differing by only one of the 19 SSR loci used (RB8; Table 3), were
317 identified and considered to be predominant during the 2013-2016 period. In 2013 and 2014,
318 genotype 166 317 0-G1 dominated, as it accounted for more than 65% of the total 166 317 0
319 pathotype population (Figure 5). In 2015, the 166 317 0-G2 genotype emerged and became
320 co-dominant with 166 317 0-G1. In 2016, 166 317 0-G2 sharply increased in frequency, to 62%,
321 whereas the frequency of 166 317 0-G1 decreased to 7%. Over the four-year period
322 considered, several other genotypes were identified (detailed data not shown). Their
323 cumulative frequencies reached 46% during the co-dominance of the two major genotypes,
324 and 30% when a single genotype dominated.

325

326 **Differences in aggressiveness between genotypes of the two major *P. triticina* pathotypes**
327 **expressed on 'neutral' and 'naive' cultivars**

328 Genotype 166 317 0-G2 was more aggressive than genotype 166 317 0-G1 in assessments of
329 all three aggressiveness components on a 'neutral' cultivar, Apache, and a 'naive' cultivar
330 Michigan Amber (in analyses of the pooled dataset for the two cultivars; Figure 6A, B and C).
331 The data for assessments of aggressiveness components on both cultivars are provided in the
332 supplementary data (Figure S1). Infection efficiency (IE) was higher for genotype 166 317 0-
333 G2 (53.1 %) than for genotype 166 317 0-G1 (47.6%). Sporulation capacity (SP) was also higher
334 for genotype 166 317 0-G2, with 0.120 mg/lesion versus 0.114 mg/lesion for genotype 166

335 317 0-G1. Latency period (LP) differed significantly between the two genotypes; it was shorter
336 for genotype 166 317 0-G2 (132.6 vs 135.0 degree-days). Significant differences were found
337 between isolates of the two genotypes.
338
339 Infection efficiency (IE), latency period (LP) and sporulation capacity (SP) did not differ
340 between genotypes 106 314 0-G1 and 106 314 0-G2 assessed on cultivars Apache and
341 Michigan Amber (in analyses of the pooled dataset for the two cultivars; Figure 6D, E and F).
342 The data for assessments of aggressiveness components on both cultivars are provided in the
343 supplementary data (Figure S2). The IE for genotype 106 314 0-G1 was 43.8%, which is not
344 significantly different from the value of 43.4% obtained for genotype 106 314 0-G2. SP was
345 0.128 mg/lesion for genotype 106 314 0-G1, which is not significantly different from the value
346 of 0.124 mg/lesion obtained for genotype G2. Finally, LP differed by less than one degree-day
347 between the two genotypes, at 133.1 degree-days for genotype 106 314 0-G1 and 133.3
348 degree-days for genotype 106 314 0-G2. By contrast to our findings for pathotype 166 317 0,
349 we observed no significant difference between genotypes of the same pathotype for 106 314
350 0. This singularity justified our decision to perform phenotypic characterization by comparing
351 the aggressiveness of isolates of pathotype 106 314 0 on other cultivar sets with a view to
352 detecting potential differences.
353
354 **Difference in aggressiveness between the two major genotypes of pathotype 106 314 0 on**
355 **Michigan Amber and major cultivars present in the French varietal landscape**
356 The two major genotypes of pathotype 106 314 0 had significantly different latency periods
357 (LP) on five of the six cultivars tested (Table 4). These differences were significant on the
358 cultivar Michigan Amber, whereas no difference was found in analyses of the data for series

359 1, as described above. The infection efficiency (IE) of genotype 106 314 0-G2 was higher than
360 that of 106 314 0-G1 (48.7% versus 39.1%) on cultivar Expert, but no differences were found
361 on the other cultivars. Genotype 106 314 0-G2 had a shorter LP than 106 314 0-G1 on five of
362 the six cultivars tested. This difference in LP ranged from 2.1 degree-days on Premio to 4.8
363 degree-days on Expert. A difference of 5 degree-days is equivalent to 8 h at 15°C. Bermude
364 was the only cultivar on which LP did not differ significantly between the two pathogen
365 genotypes. No significant differences in sporulation capacity (SP) were observed between the
366 two genotypes on any of the cultivars tested. SP varied between cultivars, ranging from 0.099
367 mg on Michigan Amber to 0.134 mg on Sankara, with slightly higher values for 106 314 0-G2
368 than for 106 314 0-G1. It was not possible to detect significant differences between cultivars
369 because different cultivars were analyzed in different series.
370
371 Within genotypes, there was a significant isolate effect for LP, but no significant isolate effect
372 for IE or SP, on Aubusson, Premio and Expert.
373
374 For LP, IE and SP, the replication factor (x3) was almost always significant. The interaction
375 between replication factor and the three aggressiveness components was significant for some
376 aggressiveness component measurements (Table 4).
377

378 **DISCUSSION**

379 Focusing on two major pathotypes from the French *P. triticina* population, we found that
380 several genotypes were present within each pathotype, and that the frequency of the most
381 common genotypes changed over time. The initially dominant genotypes representative of
382 pathotypes 166 317 0 and 106 314 0 were replaced, in each pathotype, by another genotype

383 over the period 2006-2016. The most recent dominant genotype was more aggressive than
384 the older one in both pathotypes.
385
386 **Methodological aspects of aggressiveness measurement at the wheat seedling stage**
387 The mean values used to characterize the aggressiveness of different *P. triticina* isolates on
388 wheat seedlings revealed significant variation for each of the three aggressiveness
389 components measured — infection efficiency, latency period and sporulation capacity — at
390 the seedling stage. Aggressiveness and its variation can be measured on seedlings (Milus *et*
391 *al.*, 2006; de Vallavieille-Pope *et al.*, 2018) or on adult plants (Lehman & Shaner, 1997; Pariaud
392 *et al.*, 2009b; Azzimonti *et al.*, 2013). Plant stage is known to affect the aggressiveness
393 components of *P. triticina* (Pariaud *et al.*, 2009b). We decided to assess aggressiveness
394 components on wheat seedlings in this study, because the use of seedlings in a semi-
395 controlled environment results in more homogeneous physiological properties of the plant at
396 inoculation and because this approach requires less space and time than studies on adult
397 plants.
398
399 With the phenotyping method developed and used in this study, we were able to estimate the
400 three aggressiveness components simultaneously on the same inoculated plant. We
401 measured infection efficiency (IE) more precisely here than in previous studies, in which the
402 variability was sometimes very high. For instance, Pariaud *et al.* (2009b) applied a 1:10 mixture
403 of *P. triticina* urediniospores and *Lycopodium* spores to the leaf surface with a soft brush; they
404 reported IE values ranging from 18% to 80% for the same genotype. This high level of variation
405 resulted from difficulty controlling the number of urediniospores deposited with this method,
406 resulting in differences between experimental series. Although fastidious, the deposition of

407 urediniospores one-by-one on the leaf surface resulted in a much more accurate estimation
408 of IE than other methods based on the dilution of *P. triticina* urediniospores among
409 *Lycopodium* spores or in liquids, such as mineral oils (Pariaud *et al.*, 2009; Sørensen *et al.*,
410 2016). Nevertheless, the interaction between IE and the replication factor was almost always
411 significant, indicating an impact of environmental conditions on this component of
412 aggressiveness. This interaction reflects the difficulty ensuring uniform dew quality in the dew
413 chamber just after inoculation, as the success of urediniospore germination and penetration
414 depends on the presence of a water film on the leaf surface (Bolton *et al.*, 2008).
415
416 Sporulation capacity (SP) was measured by collecting urediniospores produced between 9 and
417 12 days after inoculation, and differences in SP between genotypes were detected for only
418 one experimental series. Pariaud *et al.* (2009b) found differences in SP between pathotypes
419 of *P. triticina* in a study in which urediniospores were collected 15 to 23 days after the
420 inoculation of adult plants. They also found that the difference in SP between isolates
421 increased with the regular collection of urediniospores until 59 days after inoculation. The
422 collection of urediniospores more than 13 days after inoculation is of potential relevance for
423 future experiments with our method, to maximize differences and reveal small differences in
424 this component of aggressiveness between isolates.
425
426 Latency period (LP) was the component that differed most between pathogen genotypes and
427 was the least affected by the replication factor. The calculation of LP in degree-days made it
428 possible to take the temperature-dependence of both infection processes and pathogen
429 development in the leaf into account (Lovell *et al.*, 2004). In most pathosystems, latency
430 period is a component of choice for studies of quantitative interactions (aggressiveness,

431 partial resistance) because time is a variable that is much easier to fractionate and, therefore,
432 to quantify precisely, than any biological trait, making it possible to highlight extremely small
433 differences between isolates repeatedly. This is almost an epistemological issue for
434 experimental epidemiology and phytopathometry (Suffert & Thompson, 2018; Bock *et al.*,
435 2022).
436
437 **Evolution of greater aggressiveness in two *P. triticina* pathotypes**
438 In the case of pathotype 166 317 0, genotype G2, which replaced genotype G1, was more
439 aggressive than its predecessor, with highly consistent results obtained for the different
440 components of aggressiveness. Following this switch between genotypes, the frequency of
441 pathotype 166 317 0 increased considerably over a period of three years, whereas the
442 proportion of compatible host cultivars in the landscape decreased (data not shown). Severe
443 *P. triticina* epidemics have been shown to be associated with a high sporulation capacity, a
444 high infection efficiency and a short latency period (Azzimonti *et al.*, 2022). In pathotype 106
445 314 0, comparisons of aggressiveness on a 'neutral' cultivar revealed no significant difference
446 between the most recent and oldest genotypes. A comparison of the aggressiveness of these
447 two genotypes on some of the cultivars mostly frequently grown in the landscape revealed
448 that 106 314 0-G2 was more aggressive than 106 314 0-G1 only in terms of its latency period.
449 This change in predominant genotype coincided with a halt in the decline of pathotype 106
450 314 0 frequency in the landscape. The shorter latency period of genotype 106 314 0-G2 seems
451 to have an effect on the frequency of this pathotype in the landscape, consistent with the
452 assertions of several studies that latency period is the aggressiveness component with the
453 largest effect on pathogen dynamics in field conditions (Lannou, 2012). In modeling studies,
454 this also appeared to be the trait with the largest impact on the intensity of *P. triticina*

455 epidemics, as it determined the number of reproductive cycles (i.e. from inoculation to the
456 spore dispersion) of the pathogen possible in a single season (Rimbaud *et al.*, 2018). Although
457 only latency period differed significantly between 106 314 0-G2 and 106 314 0-G1, the data
458 for the other aggressiveness components also supported the notion that 106 314 0-G2 was
459 more aggressive, for every component, on all cultivars, except for infection efficiency on
460 Premio. Measurements of aggressiveness components on seedlings in controlled conditions
461 (only one reproductive cycle of the pathogen) have a limited capacity for the detection of
462 small phenotypic variations.
463
464 The competitive advantage of one particular genotype within a pathotype, expressing a
465 difference in fitness for the same virulence profile, may account for the replacement of one
466 genotype with another. This hypothesis is particularly realistic here as the emergence of the
467 more aggressive genotype in each pathotype, 106 314 0 and 166 317 0, coincided with a short-
468 term change in the mid-term trend in frequency changes: (i) the decrease in the frequency of
469 pathotype 106 314 0 in the landscape observed from 2009 to 2011 was temporarily halted
470 during the 2011-2014 period, when the frequency of this pathotype reached a plateau (Figure
471 2), coinciding with the replacement of the less aggressive genotype 106 314 0-G1 with the
472 more aggressive genotype 106 314 0-G2 in 2012 and 2013 (Figure 3); (ii) the decrease in the
473 frequency of pathotype 166 317 0 in the landscape observed from 2012 to 2015 was halted
474 over the 2015-2018 period, resulting in a new peak frequency (Figure 2) coinciding with the
475 replacement of the less aggressive 166 317 0-G1 genotype with the more aggressive 166 317
476 0-G2 genotype in 2012 and 2013 (Figure 3).
477

478 These results suggest that the replacement of some pathotypes by others, driven by changes
479 in the frequencies of resistance genes in the varietal landscape, may be slowed, to various
480 extents, by increases in the aggressiveness of certain genotypes or 'lineages' (defined as
481 genetically related genotypes of the same group of pathotypes, i.e. with the same close
482 ancestor), consistent with the recent analysis by Fontyn *et al.* (2022). It is even possible, as for
483 pathotype 166 317 0, for the pathotype to gain a 'second life' — for a period of three to four
484 years — due to an increase in the aggressiveness of a new genotype, as long as it remains
485 adapted (or at least not maladapted) to the resistance genes present in the varieties deployed
486 in the landscape. This empirical study shows that differences in aggressiveness can be
487 expressed on a 'neutral' cultivar (for pathotype 166 317 0), but sometimes (for pathotype 106
488 314 0) only on 'non-neutral' cultivars representative of a varietal landscape. This finding
489 clearly complicates the experimental approach (difficulty determining the most appropriate
490 experimental design without making assumptions), but also the data analysis. The magnitude
491 of the differences in aggressiveness probably depends on the type of cultivars tested ('neutral'
492 or 'non-neutral'). Indeed, we found that the intensity of the mid-term trend in the change in
493 frequencies highlighted above was variable, with either a simple slowing of a trend towards a
494 decrease (plateau, as for pathotype 106 314 0), or a change in direction, with a new increase
495 (peak, as for pathotype 166 317 0).
496
497 **Greater aggressiveness as a selective advantage within plant pathogen populations**
498 Increases in aggressiveness have already been proposed as an explanation for the shifts
499 observed in rust populations. Milus *et al.* (2009) measured five aggressiveness components
500 for *P. striiformis* f. sp. *tritici* isolates with similar virulence profiles collected in the U.S. before
501 or after 2000. Isolate aggressiveness was assessed on a susceptible cultivar, and comparisons

502 revealed that the most recent isolates were more aggressive than the older ones (Milus *et al.*,
503 2009). Studies on host quantitative resistance effects on pathogen populations have revealed
504 directional selection toward an increase in pathogen aggressiveness on host cultivars
505 (Andrivon *et al.*, 2007; Frézal *et al.*, 2018). Isolates of *Phytophthora infestans* collected on a
506 partially resistant potato cultivar at the end of the epidemic produced larger lesions and had
507 higher sporulation rates than those collected at the beginning of the epidemic (Andrivon *et*
508 *al.*, 2007). *Z. tritici* isolates collected from a partially resistant wheat cultivar were found to be
509 more aggressive than isolates collected from a susceptible wheat cultivar (Cowger & Mundt,
510 2002). Isolates of *Venturia inaequalis* collected from an apple cultivar carrying a QTL for
511 resistance had higher lesion densities and sporulation rates than isolates collected from a
512 susceptible variety (Caffier *et al.*, 2016). Isolates of *Plasmopara viticola* collected from partially
513 resistant varieties were found to have shorter latency periods and higher rates of spore
514 production than isolates collected from susceptible cultivars (Delmas *et al.*, 2016). Many
515 studies have shown that the use of quantitative sources of resistance affects the evolution of
516 pathogen population by leading to the selection of more aggressive isolates. Quantitative
517 interactions based on partial resistance in the host and aggressiveness in the pathogen are a
518 significant driver of evolution in pathogen populations. We show here that increases in
519 aggressiveness were associated with genotype replacement in two major *P. triticina*
520 pathotypes, consistent with all the examples of selection based on quantitative plant-
521 pathogen interactions cited above.
522
523 **Putative origin of the more aggressive genotypes**
524 All surveys in wheat-growing areas to date have provided evidence for continual evolution of
525 *P. triticina* populations, with rapid changes in pathotype frequencies (Goyeau *et al.*, 2012;

526 Kosman *et al.*, 2019; Zhang *et al.*, 2020; Fontyn *et al.*, 2022; Kolmer & Fajolu, 2022). These
527 changes can be explained intrinsically by the acquisition of new virulence genes, by mutations,
528 somatic exchanges or, more rarely, genetic recombination. A study on the Australian *P.*
529 *striiformis* f. sp. *tritici* population showed that it had evolved due to mutation events (Steele
530 *et al.*, 2001). Whole-genome sequencing has detected similar recurrent mutations in *P.*
531 *triticina*, suggesting that such events play a major role in genetic variability within clonal
532 lineages (Fellers *et al.*, 2021). Somatic exchange may also have played a role, as in the
533 emergence of the Ug99 lineage of *Puccinia graminis* f. sp. *tritici,* which had one identical
534 haploid nucleus in common with an old African lineage, with no sign of recombination (Li *et*
535 *al.*, 2019). A similar situation has been described in *P. triticina*, with the Australian isolate Pt64
536 resulting from somatic exchange between two parental isolates (Wu *et al.*, 2019). More rarely,
537 genetic recombination may also underlie the emergence of new genotypes with new virulence
538 genes. Indeed, *P. triticina* is a heteroecious fungus that requires an alternative host,
539 *Thalictrum speciosissimum* (not naturally present in most places worldwide, including France),
540 for sexual reproduction. The high proportion of repeated genotypes and the heterozygosity
541 rates of European and French leaf rust populations confirmed the very dominant role of clonal
542 reproduction (Goyeau *et al.*, 2007; Kolmer *et al.*, 2013). Genetic changes do not necessarily
543 result in different virulence profiles, so isolates with identical or very similar pathotypes may
544 have different origins. In a previous study on *P. triticina* nine microsatellite markers were used
545 to analyze the genotypes of 33 pathotypes (Goyeau *et al.*, 2007). This analysis highlighted the
546 presence of different genotypes, differing by only one of the nine SSRs tested, in five of the
547 pathotypes. Another study on 121 European *P. triticina* isolates with 21 SSR markers revealed
548 a significant correlation between phenotype and genotype, but the same phenotype could,
549 nevertheless, be associated with several genotypes (Kolmer *et al.*, 2013). The results of our

550 study raise questions as to the genetic relationship between the most recent genotypes, 166
551 317 0-G2 and 106 314 0-G2, and the oldest genotypes, 166 317 0-G1 and 106 314 0-G1,
552 respectively. Can the most recent and the oldest genotypes be considered to belong to the
553 same 'lineage', as previously defined? A mutation event during asexual reproduction probably
554 led to the emergence of the most recent genotype (G2) of pathotype 166 317 0, which differs
555 from the older genotype (G1) by only one SSR allele (Table 3). By contrast, genotypes G1 and
556 G2 of pathotype 106 314 0 displayed a higher degree of genetic diversity, differing by five SSR
557 alleles (Table 3). Genotype 106 314 0-G2 may, therefore, be the result of several mutation
558 events occurring in the same lineage and conferring greater aggressiveness than for 106 314
559 0-G1. Alternatively, the presence of 106 314 0-G2 in France may reflect the introduction from
560 an exotic source of a different (more aggressive) lineage that had acquired the same virulence
561 genes and, therefore, belonged to the same pathotype, 106 314 0. Migration events are
562 frequent in cereal rust populations, as already shown for yellow rust (Ali *et al.*, 2014; Bueno-
563 Sancho *et al.*, 2017), for which there is a famous example of migration in the form of an
564 introduction into Australia from northwest Europe in 1979 (Wellings & McIntosh, 1990).
565 Studies on *P. triticina* in wheat-growing areas worldwide have revealed a broad geographic
566 distribution of identical and highly related multilocus genotypes, highlighting the potential of
567 leaf rust for long-distance migration (Kolmer *et al.*, 2019) and the possibility of new genotypes
568 resulting from exotic introduction.
569
570 **Additional approaches for detecting genetic variation related to aggressiveness**
571 Pathotypes of *P. triticina*, defined on the basis of their virulence profiles, appear to be
572 represented by several genotypes, the distribution of which changes over time. An analysis of
573 genotypes within the two major pathotypes collected in France in 2006-2020, 166 317 0 and

574 106 314 0, revealed the presence in each pathotype of two main genotypes, one of which
575 replaced the other over time. This study was not designed to characterize overall pathotypic
576 or genotypic changes in the *P. triticina* population, so our data should be extrapolated with
577 caution, as any attempt to use them for such purposes would be subject to multiple sampling
578 biases. In addition to the four genotypes on which we focused here, several other genotypes
579 (with cumulative frequencies of 31% to 61%, depending on the year) were identified. This is
580 due to the initial pathotyping performed annually for the national survey and the genotyping
581 for this study being performed on different isolates, purified from a bulk of urediniospores
582 collected from a single leaf, which may well have been infected with several pathotypes
583 and/or genotypes. This highlights the imperfections of the protocol linked to the constraints
584 of working on datasets and biological material acquired over several decades with ever-
585 changing techniques. It would have been better to pathotype and phenotype the 401 isolates
586 from the same purified batch of urediniospores, as we did later on for the 44 isolates for which
587 aggressiveness was characterized (Figure 1). However, given the size of the sample, this
588 limitation did not prevent us from obtaining a correct overview of changes for the most
589 common genotypes.
590
591 Our results revealed rapid genotype evolution within the pathotypes that was not detectable
592 if only virulence phenotypes were considered. These findings demonstrate the value of
593 analyzing population dynamics not only from the pathotype standpoint, but also with
594 genotype data, to obtain a more informative picture of pathogen diversity. The use of a small
595 number of SSR markers is not sufficient for the detection of genetic variations in *P. triticina*
596 populations. Genome-wide genotyping approaches are required to characterize the genetic
597 diversity of leaf rust populations more precisely and to explain the emergence of new

genotypes. As an illustration of this approach, a combined genome and transcriptome analysis has been performed on 133 *P. striiformis* f. sp. *tritici* isolates collected in 16 European countries. This analysis provided more precise information about the origin of the new emerging races, and showed that SNP analysis was an effective approach for the detection of pathogen diversity and for pathogen surveillance (Bueno-Sancho *et al.*, 2017). Similarly, Fellers *et al.* (2021) genotyped 121 *P. triticina* isolates with 121 907 SNP markers, and showed that recurrent mutation and selection had played a major role in differentiation within clonal lineages. The results of our study highlight the importance of combining genome-wide genotyping tools with precise pathotyping and aggressiveness phenotyping to detect the emergence of new variants and to improve our understanding of population dynamics.

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## Data Availability Statement

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

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813

## 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 wirh19 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 mostly 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

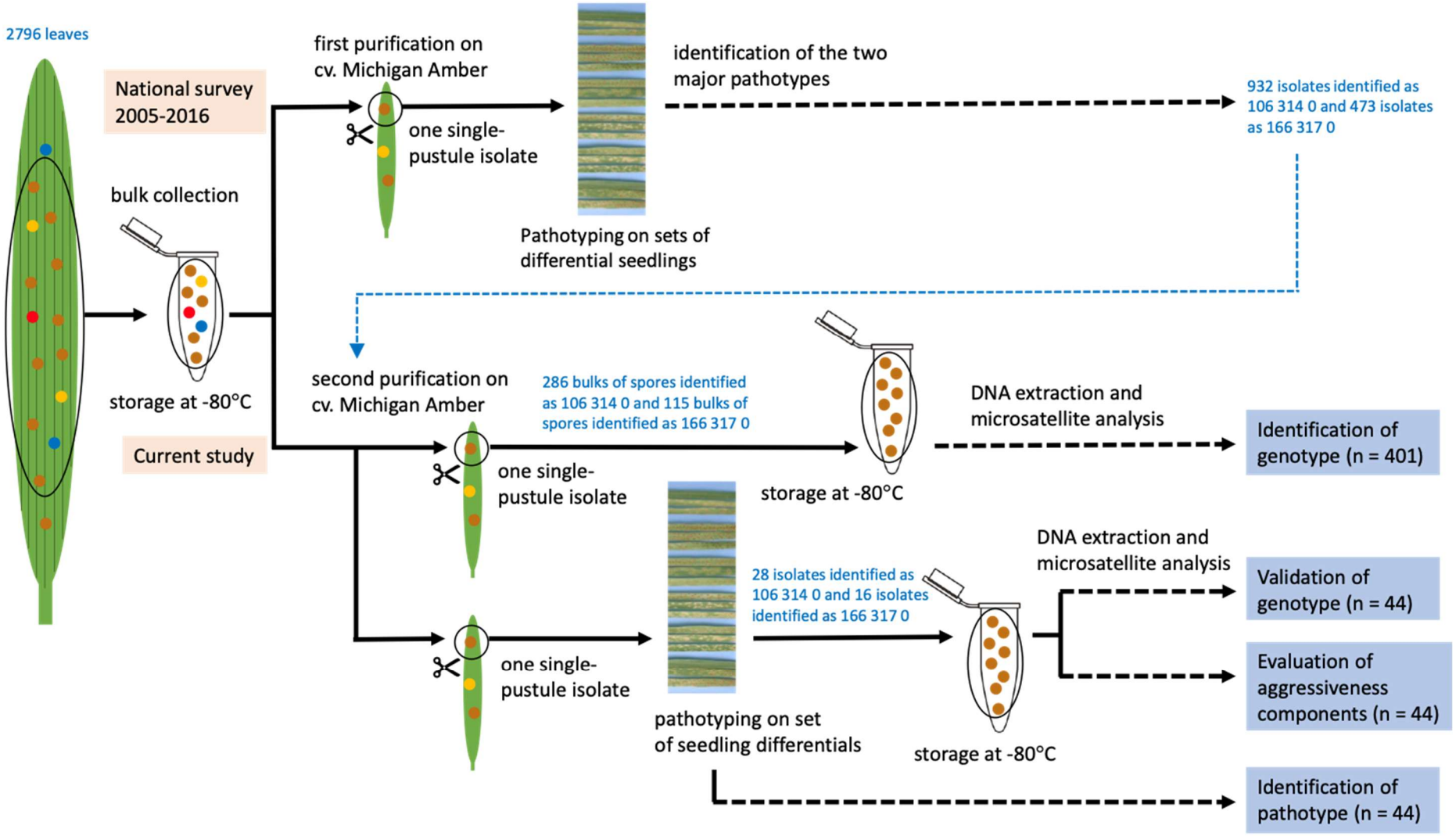


**Figure 1.** Overview of the purification, selection, pathotyping, and genotyping steps for the *P. triticina* isolates for which aggressiveness components were evaluated.

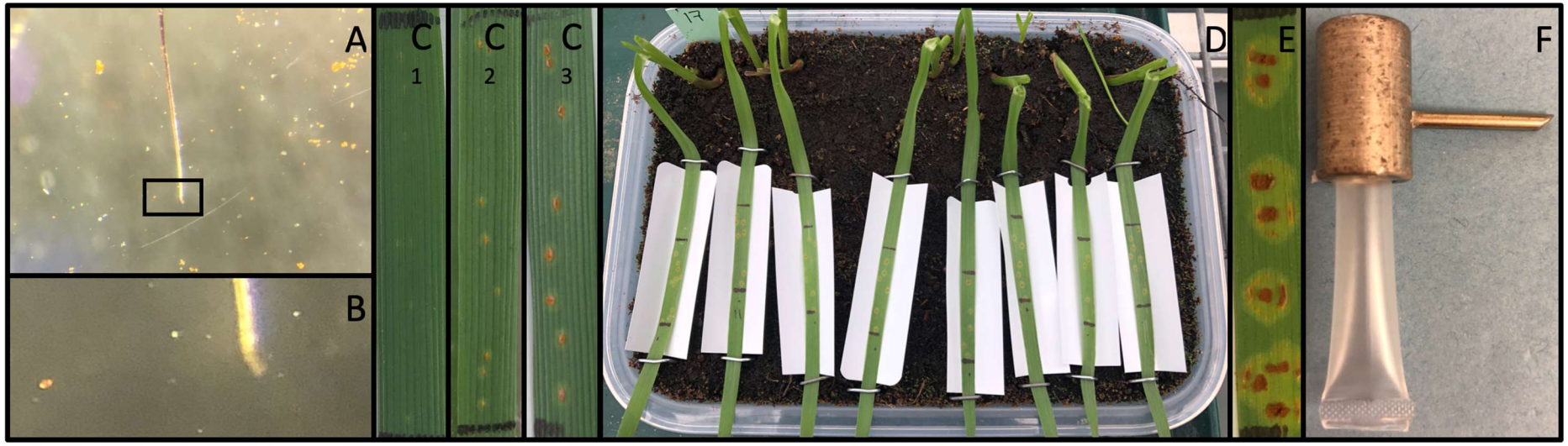


**Figure 2.** Experimental steps for the assessment of aggressiveness components of *Puccinia triticina* on wheat seedlings. Inoculation: (A) and (B), collection of a spore with a human eyelash and its deposition on a leaf. Latency period (C): (C1) onset of chlorosis, (C2) counting of the uredinia (pustules) that have broken through the leaf, (C3) end of the latency period, when all the pustules 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.

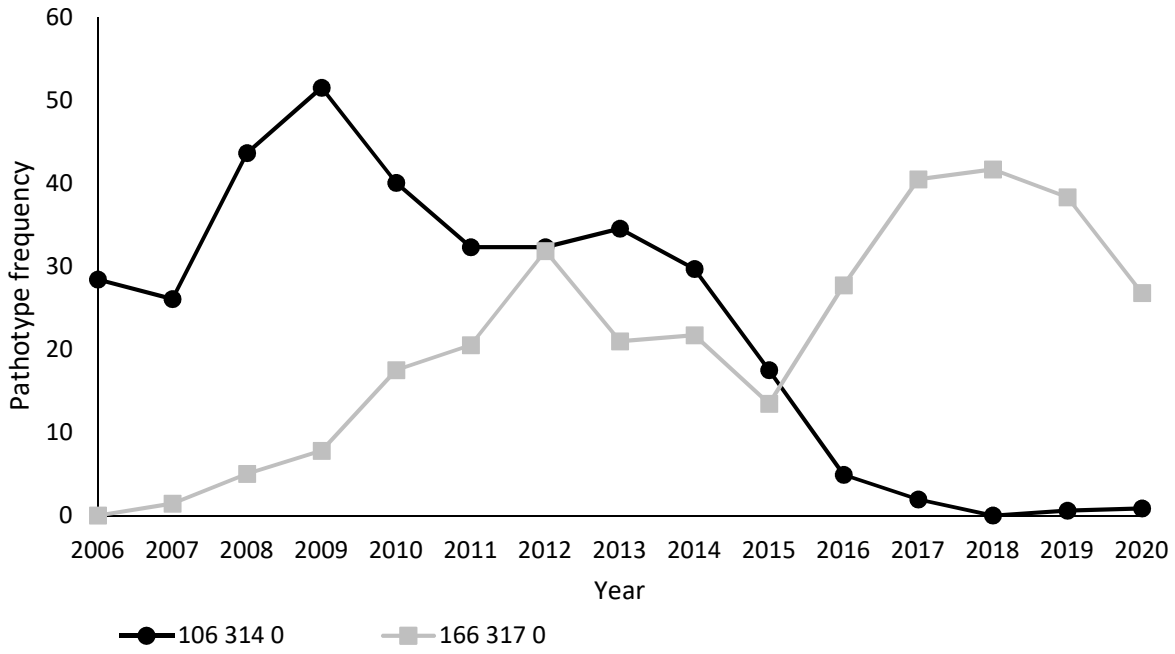


**Figure 3.** Frequency dynamics of two of the major *Puccinia triticina* pathotypes, 106 314 0 and 166 317 0, in the French landscape during the 2006-2020 period. Pathotype frequency was determined with data from the national survey (see Fontyn *et al.*, 2022).

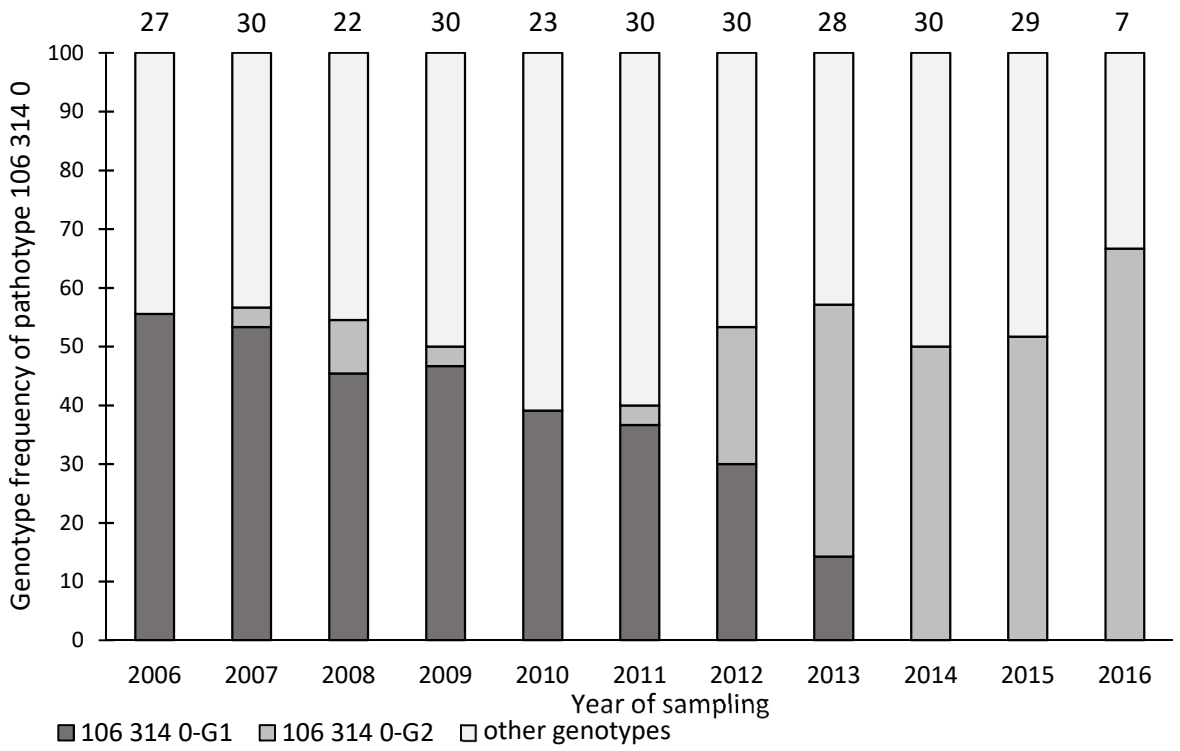


**Figure 4.** Changes in genotype frequencies within pathotype 106 314 0 in the French landscape during the 2006-2016 period, as determined with 19 SSR markers. The numbers on top of the bars are the numbers of isolates genotyped.

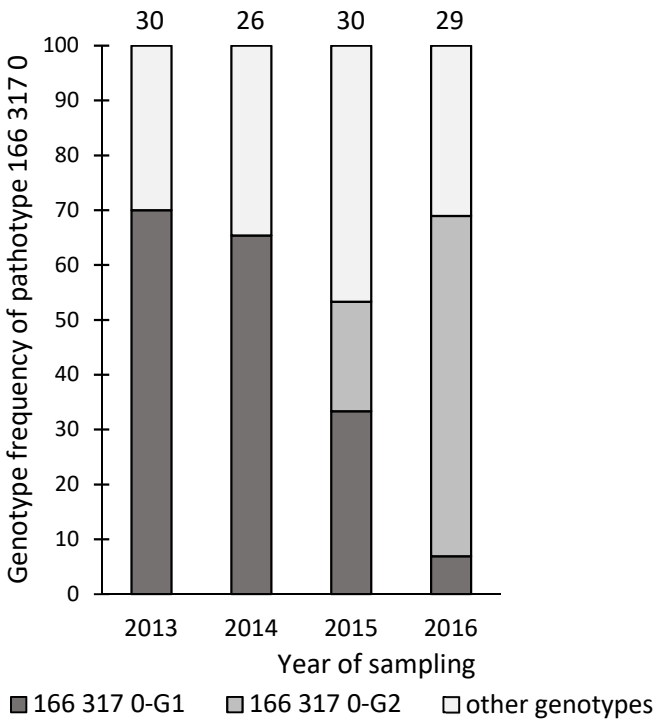


**Figure 5.** Changes in genotype frequencies within pathotype 166 317 0 in the French landscape during the 2013-2016 period, as determined with 19 SSR markers. Numbers on top of the bars represent the numbers of isolates genotyped.

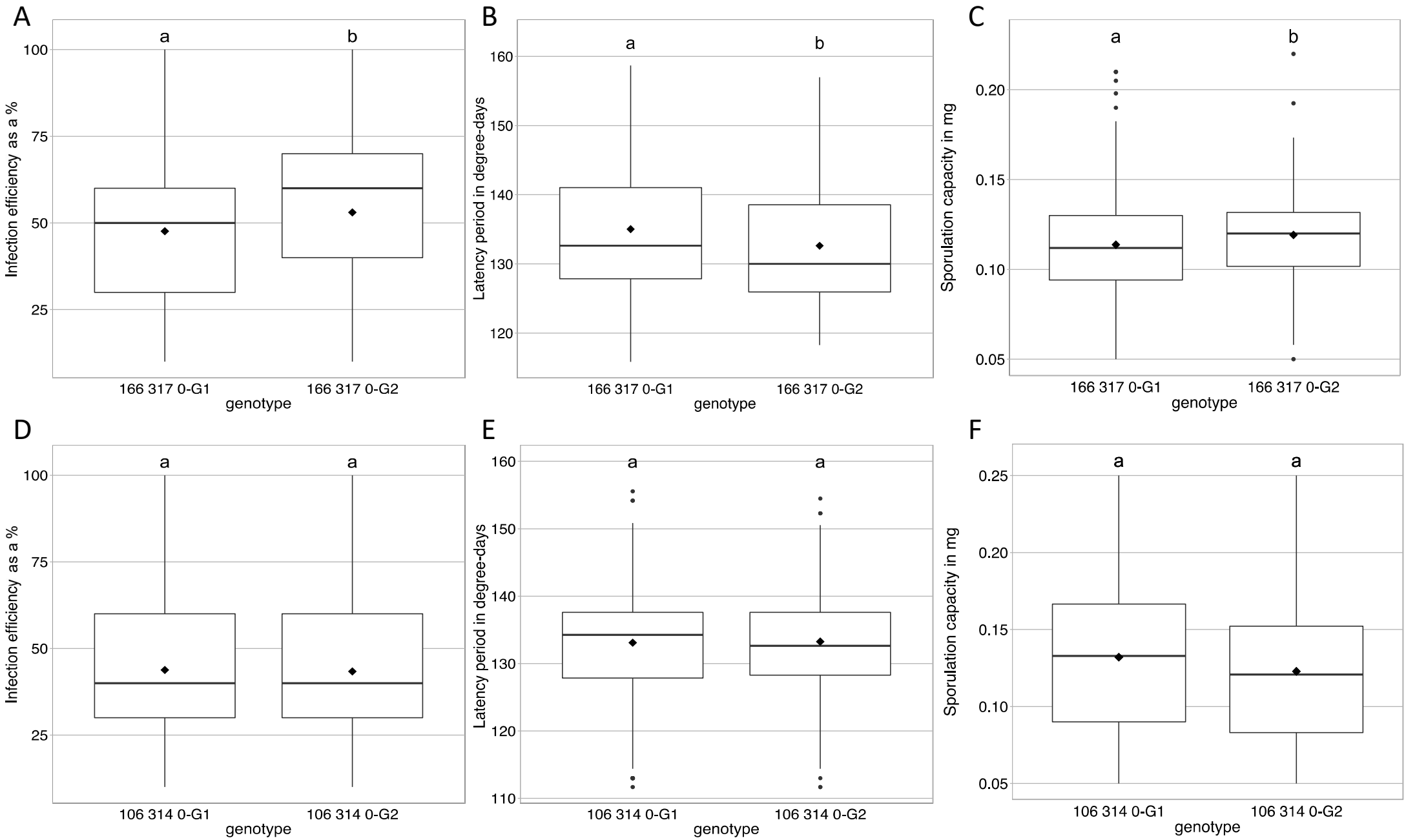


**Figure 6.** Comparison of genotype aggressiveness within the two major *Puccinia triticina* pathotypes. Infection efficiency (IE) as a %, latency period (LP) in degree-days and sporulation capacity (SP) in mg for pathotypes 166 317 0 (A, B, C) and 106 314 0 (D, E, F) were assessed on cultivars Apache 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).