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1 **Visual assessment and computer-assisted image analysis of Fusarium head blight**  
2 **in the field to predict mycotoxin accumulation in wheat grains**

3  
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25

26 **Abstract**

27

28 Phenotypic traits are regularly used to diagnose the development of Fusarium head blight (FHB) in  
29 the field, whereas mycotoxin accumulation in wheat grains can only be accurately evaluated through  
30 costly methods, such as high-performance liquid chromatography (HPLC). The aim of this study was  
31 to determine whether (i) the results provided by existing commercial decision support tools could be  
32 anticipated using phenotypic measurements, including a novel technique of computer-assisted image  
33 analysis of spikes, and (ii) these measurements could avoid using HPLC. We monitored the FHB  
34 development during two consecutive years in highly contaminated plots in the Burgundy region  
35 (France). Contamination by crop residues was simulated through a field inoculation with barley grains  
36 artificially colonized by *Fusarium graminearum*. The development of the disease on spikes and  
37 harvested grains was assessed on one tolerant and two susceptible wheat varieties. The accumulated  
38 amounts of mycotoxins were measured in harvested grains using HPLC. As expected, the measured  
39 traits revealed that (i) the inoculum responsible for infection on spikes mainly came from residues  
40 left on the soil surface, and (ii) the susceptible varieties were more diseased than the tolerant variety.  
41 Weather conditions had a strong effect on disease development. The novel computer-assisted image  
42 analysis technique had a better prediction power of deoxynivalenol accumulation, was more objective  
43 and time-saving than classical visual symptom assessments. This assessment method could be  
44 suitable to supplement the use of existing prediction tools and might avoid systematic and costly  
45 mycotoxin measurements in likely infected plots.

46

47 **Keywords**

48

49 *Fusarium graminearum*, preceding crops residues, weather conditions, computer-assisted image  
50 analysis, decision support tools.

51

## 52 **1. Introduction**

53

54 *Fusarium* diseases affect crop cultures worldwide, and more particularly wheat (*Triticum aestivum* L.  
55 *ssp aestivum* and *Triticum turgidum* L. subsp. *durum* (Desf.) Husn.), causing *Fusarium* head blight  
56 (FHB; Turkington et al. 2014; Sutton 1982). FHB affects grain quantity through substantial yield  
57 losses, and grain quality due to mycotoxins produced by the fungi. These mycotoxins are of major  
58 concern for human and animal health because they persist during storage (Joint FAO/WHO Expert  
59 Committee on Food Additives 2001). In Europe, the most frequent and abundant mycotoxin produced  
60 in wheat is deoxynivalenol (DON), which acceptable limit in grains is 1.25  $\mu\text{g g}^{-1}$  according to  
61 European regulations (Commission of the European Communities 2006). Besides, DON acetylated  
62 derivatives 3-acetyldeoxynivalenol (3-ADON) and 15-acetyldeoxynivalenol (15-ADON), as well as  
63 nivalenol (NIV) and zearalenone (ZEA) are also produced, among others. Several *Fusarium* species  
64 and *Microdochium nivale* (Fr.) Samuels & J.C. Hallet (var. *nivale* and var. *majus*) are causative agents  
65 of the FHB disease. However, *Fusarium graminearum* Schwab, the anamorph stage of *Gibberella*  
66 *zeae* (Schwein.) Petch, is one of the most prevalent species in many parts of the world (Goswami and  
67 Kistler 2004). It survives saprotrophically on crop residues which make up the primary inoculum that  
68 in turn fosters infection of wheat heads (Leplat et al. 2013). No-tillage or reduced tillage practices  
69 increase the risk of disease development and DON accumulation as compared to inversion tillage  
70 (Dill-Macky and Jones 2000). Weather conditions, among which long periods of warm temperatures  
71 and high moisture during wheat flowering, increase FHB severity (Siou et al. 2014).

72 Wheat is the most cultivated crop in France (around 5.5 million ha in 2015) as well as the most traded  
73 one (19.4 million tons exported during the 2014–15 campaign; Passion Céréales 2016). Specialized  
74 organisations are generally in charge of crop trading. For example, the union of farming cooperatives  
75 Cérévia markets the wheat production of eight cooperatives in eastern France, where this study took  
76 place. In Burgundy, the farming cooperative Dijon Céréales (a member of Cérévia), pools wheat

77 harvested from almost one thousand subscribers from four distinct pedoclimatic areas in silos. The  
78 silo contents are then pooled again to be transported by rail and waterway to Mediterranean harbours  
79 where they are finally loaded on container ships. At each of these steps, contaminated harvests can  
80 be mixed with non-contaminated ones. Therefore a main issue for the cooperative is to identify plots  
81 contaminated by mycotoxins before harvesting so as to separate their crops from healthy ones during  
82 storage and shipment.

83 Several commercial decision support tools are available to forecast the risk of mycotoxin  
84 accumulation in grains, for example Myco-LIS<sup>®</sup> (Arvalis Institut du Végétal, Paris, France; Gourdain  
85 et al. 2011), Qualimetre<sup>®</sup> (Syngenta, Basel, Switzerland; Froment et al. 2011), or Bay+ DONcast<sup>®</sup>  
86 (Bayer CropScience, Monheim am Rhein, Germany; Pitblado et al. 2007). They are based on the  
87 statistical analysis of agronomical hazards (preceding crops, soil tillage, varietal susceptibility to  
88 DON accumulation in grains) coupled to a local climatic hazard analysis. The efficiency of such tools  
89 has been proved, but the main drawback of statistical models remains that they do not take into  
90 account the actual health status of the plants. Moreover, results are only available just before harvest  
91 time, so that farming cooperatives only have a very short time to perform DON analyses using ELISA  
92 tests on grains from suspected plots. Finally, high-pressure liquid chromatography (HPLC) controls  
93 are systematically performed on plots which showed a positive reaction for DON with ELISA tests.  
94 This raises an economical concern to cooperatives because such analyses are costly. Therefore,  
95 investigating indicators free from mycotoxin analysis and allowing for early diagnosis is still relevant  
96 today. Visual disease indicators are traditionally used to try to predict mycotoxin accumulation in  
97 harvested grains, but they are not always relevant (Paul et al. 2005). They have to combine  
98 observations by several assessors to provide a reliable and objective measurement of disease  
99 development, which is time-consuming. This is why image analysis of representative samples of the  
100 plots could be an interesting alternative to identify appropriate indicators.

101 The aim of this study was (i) to evaluate the involvement of agronomical factors in the development  
102 of FHB under the Burgundian pedoclimatic context, (ii) to determine whether visual assessment of

103 the symptoms could supplement or improve the use of existing decision support tools, and (iii) to  
104 evaluate the efficiency of a disease assessment technique based on computer-assisted image analysis.

105

## 106 **2. Materials and methods**

107

### 108 *2.1. Field experiment design*

109 Wheat was cropped at the INRA Experimental Unit of Epoisses (Côte-d'Or, France [5°05'E;  
110 47°14'N]) during two consecutive seasons (2008–2009 and 2009–2010). Epoisses soil has a silty–  
111 clayey texture (sand 6.1 %, silt 57.7 %, clay 36.2 %), a pH of 7.1, 2.5 % of organic matter, a C:N  
112 ratio of 9.7, and a Cation Exchange Capacity of 20.1 cmol(+) kg<sup>-1</sup> of soil.

113 The experimental setup included four blocks of twelve 9 x 1.25 m plots factorial randomized within  
114 each block. Three wheat varieties were studied: Apache (FHB-tolerant), and Charger and Maxwell  
115 (both FHB-susceptible). Sowing was performed with fungicide-free seeds, with six furrows per plot  
116 pattern, on October 24<sup>th</sup> in 2008 and on October 20<sup>th</sup> in 2009. Sowing density was 400 seeds m<sup>-2</sup>.

117 Eight grams per square meter of previously irradiated barley grains artificially colonized by *F.*  
118 *graminearum* (strain MIAE00376) were used to inoculate the soil to promote FHB development. The  
119 strain had been isolated from infested maize residues. It was morphologically characterized using  
120 phenotypic features assessed through macroscopic and microscopic observations. Its genetic  
121 identification was carried out by sequencing the ITS region (Genbank accession number KU885384;  
122 White et al. 1990; Gardes and Bruns 1993) and the *tef-1 $\alpha$*  region (KU885385; Macia-Vicente et al.  
123 2008). The strain chemotype was identified as 15-ADON type according to the study of the *Tri3* and  
124 *Tri13* trichothecene biosynthesis genes. (Chandler et al. 2003; Ward et al. 2002).

125 The plots received four different treatments: (i) non-inoculated soil; (ii) soil inoculated during sowing  
126 in the upper layer of the soil to simulate buried contaminated residues (Inoc1); (iii) soil inoculated at  
127 the soil surface when wheat reached the two-node stage (Zadoks 32; Zadoks et al. 1974) to simulate  
128 non-buried contaminated residues (Inoc2; on April 23<sup>rd</sup> in 2009 and on April 26<sup>th</sup> in 2010); (iv) soil

129 inoculated during sowing and at the two-node stage (Inoc1+Inoc2). The combinations of treatments  
130 were designed to cover the main classes of agronomical hazards defined by the decision support tools,  
131 except the effect of the preceding crop.

132 The crop was conducted under classical agricultural practices except that it was not treated against  
133 FHB. Irrigation was applied from inoculation at the two-node stage until spike emergence to promote  
134 FHB development. In parallel, a non-inoculated control trial was conducted under the same  
135 conditions as the experimental trial, apart from the fact that fungicide-treated seeds were used (0.4 L  
136 L<sup>-1</sup> Austral<sup>®</sup> [fludioxonil 10 g L<sup>-1</sup>] / 0.4 L L<sup>-1</sup> Gaucho<sup>®</sup> [bitertanol 37.5 g L<sup>-1</sup>]; 1 cL for 100 g of wheat  
137 seeds), and a treatment against FHB on spikes was applied (Abnakis<sup>®</sup> [tebuconazole 250 g L<sup>-1</sup>], 1 L  
138 ha<sup>-1</sup>).

## 140 2.2. Assessment of disease severity at the soft dough stage of wheat (Z 85)

### 141 2.2.1. Visual assessment (DS<sub>1</sub>)

142 Sampling was performed on June 16<sup>th</sup> in 2009 and on June 21<sup>st</sup> in 2010. Fifty wheat spikes were  
143 randomly sampled in each plot. Disease severity was evaluated by counting the percentages of orange  
144 spikelets because this symptom is attributed to *F. graminearum*. Some orange spikelets were  
145 incubated on Potato Dextrose Agar medium (PDA) for seven days at 25 °C to check for microbial  
146 development and identify the causative pathogens.

### 148 2.2.2. Computer-assisted image analysis (DS<sub>2</sub>; second campaign only)

149 Visual disease assessment was supplemented by disease assessment by image analysis during the  
150 second season. The same spikes were used for both assessments. The fifty spikes sampled from each  
151 plot were placed on a blue background and scanned side up and down using an A3-size scanner  
152 (Epson 10000XL, resolution: 2400 x 4800 dpi, optical density: 3.8 D). Two pictures per plot were  
153 analysed using Mesurim Pro (<http://pedagogie.ac-amiens.fr/svt/info/logiciels/Mesurim2/Index.htm>).  
154 The parameters were set to detect a range of orange-colored pixels (HSL encoding) corresponding to

155 suspected FHB symptoms. These settings were applied to each picture, leading to an estimation of  
156 the surface of FHB symptoms in mm<sup>2</sup> (Fig. 1).

157

### 158 *2.3. Assessment of Fusarium–damaged kernels (FDK; Z99)*

159 Thirty–five spikes from each plot were randomly sampled during harvest on July 25<sup>th</sup> in 2009 and on  
160 July 19<sup>th</sup> in 2010. They were threshed to obtain almost one thousand kernels. The percentage of  
161 damaged kernels was visually evaluated for each plot by counting the number of pink kernels (Beyer  
162 et al. 2007).

163

### 164 *2.4. Assessment of grain yield and quality (Z99)*

165 Yields per plot were determined after harvesting. Grain quality was evaluated through their sanitary  
166 status. The presence–absence of pathogenic agents was tested on grains of the Charger and Apache  
167 varieties from both non–inoculated and Inoc2 plots. Grains from similarly–treated plots were pooled,  
168 and one hundred grains from each modality were tested.

169 The grains were first surface–sterilized by washing in sodium hypochlorite (0.1 % of active chlorine)  
170 for 10 min, then rinsing for 3 min in a sodium chloride solution (5 g L<sup>-1</sup>) and then two more times in  
171 sterile water. They were incubated on PDA at 25 °C for 7 days. The emerging fungal colonies were  
172 morphologically and/or molecularly identified. Morphological identification was based on a  
173 reference manual (Nelson et al. 1983). Molecular identification was carried out by sequencing the  
174 ITS regions (Gardes and Bruns 1993; White et al. 1990) of the fungal strains.

175

### 176 *2.5. Mycotoxin extraction and quantification (DON; Z99)*

177 A sample of 15 g of harvested grains from each plot was milled and sieved to 0.5 mm. Two grams of  
178 that meal were used to determine the moisture content of the samples. Mycotoxins were extracted by  
179 shaking 2 g of meal in 10 mL of acetonitrile/H<sub>2</sub>O (84/16) for 3 h at 250 rpm and 25 °C. The  
180 supernatant was recovered after centrifugation at 2,000 g for 3 min and purified through a

181 Trichothecene P column (R-Biopharm Rhône Ltd., Saint-Didier-au-Mont-d'Or, France). Two  
182 millilitres of filtrate were evaporated to dryness at room temperature. The residue was dissolved in  
183 200  $\mu\text{L}$  of methanol/ $\text{H}_2\text{O}$  (1/1) and was filtered through a 0.45- $\mu\text{m}$  membrane (Merck Millipore,  
184 Billerica, USA). The samples were analysed by HPLC in a Beckman Gold<sup>®</sup> HPLC system equipped  
185 with a programmable pump module 126 coupled to a 168-nm photodiode array detector (Beckman  
186 Coulter, Fullerton, USA). HPLC conditions were adapted from Bily et al. (2004). Twenty microlitres  
187 of sample were injected into an Ultrasphere<sup>®</sup> 5 Octyl column equipped with a Pre-Column  
188 Ultrasphere Octyl Guard<sup>®</sup> (Hichrom, Reading, U.K.). A gradient elution was performed by varying  
189 the proportion of solvent A (ultra-pure water – orthophosphoric acid pH 2.6, 99.99:0.01 v/v) to  
190 solvent B (acetonitrile) with a flow rate of 1  $\text{mL min}^{-1}$ . The gradient started at 5 % B and went up  
191 linearly to 30 % B 14 min post injection (MPI), then to 90 % B 24 MPI, and remained isocratic for 5  
192 min before returning to initial conditions 31 MPI. Mycotoxins were detected at 230 nm with a 10-  
193 nm bandwidth. Mycotoxin quantification was performed using external standards (1  $\mu\text{g mL}^{-1}$  to 50  
194  $\mu\text{g mL}^{-1}$ ) prepared from commercial solutions (n<sup>o</sup> TOX, Saint-Jean-d'Ilac, France) in methanol/ $\text{H}_2\text{O}$   
195 (1/1). The retention times of the standard solutions were 5.3 min for NIV, 7.6 min for DON, between  
196 13.3 and 13.6 min for 3-ADON and 15-ADON, and 22.4 min for ZEA. The detection limit was  
197 measured at 0.16  $\mu\text{g}$  of NIV  $\text{g}^{-1}$  of wheat, 0.11  $\mu\text{g}$  of DON and 0.07  $\mu\text{g}$  of ZEA. 3-ADON and 15-  
198 ADON were not separated by this technique.

199

200

201

## 202 2.6. Meteorological data

203 Meteorological data were provided by the Climatik database managed by the Agroclim INRA Unit  
204 (Avignon, France). The meteorological station was located in the experimental unit close to the  
205 experimental fields. The cumulated hours with both a humidity rate above 80 % and temperatures

206 above 15 °C or 20 °C were chosen to represent the effect of humidity and temperature over the period  
207 from inoculation at the two–node stage of wheat until harvest.

208

## 209 2.7. Statistical analyses

210 Statistical analyses were carried out using R 3.2.5 with  $\alpha = 5\%$  (R Core Team 2014). The generalized  
211 linear mixed models (GLMMs) are commonly used to analyse data from field experiments, especially  
212 in case of disease assessment (Madden et al. 2002). They have the advantage of being compatible  
213 with complex designs involving random and fixed effects.

214 The GLMM for this study was:

215

$$216 \text{Link}(p_{ijklmn}) = \eta_{ijklmn} = \theta + \alpha_i + \beta_j + \gamma_k + \delta_l + \lambda_m + \mu_{ijklm} + \varepsilon_{ijklmn}$$

217

218 where  $\theta$  is the general effect,  $\alpha_i$  is the effect of *Inoc1*,  $\beta_j$  is the effect of *Inoc2*,  $\gamma_k$  is the varietal effect,  
219  $\delta_l$  is the effect of replicated blocks,  $\lambda_m$  is the effect of the year of experiment,  $\mu_{ijklm}$  is the experimental  
220 error in the *ijklm*-th plot and  $\varepsilon_{ijklmn}$  is the residual error.  $\alpha$ ,  $\beta$ ,  $\gamma$  and  $\lambda$  were considered as fixed effects  
221 when  $\delta$  and  $\mu$  were considered as random effects.  $p_{ijklmn}$  was the variable to be explained (i.e. *DS<sub>1</sub>*,  
222 *DS<sub>2</sub>*, *FDK*, *Yield* and *DON*). The term  $\eta_{ijklm}$  was the linear predictor. This term was obtained by  
223 submitting the variable  $p_{ijklm}$  to the link function of the model. The choice of the link function depends  
224 on the distribution law of  $p_{ijklm}$ . Binomial distribution is generally well adapted to evaluate disease  
225 incidence (i.e. *DS<sub>1</sub>* and *FDK*; Hughes and Madden 1992). The shapes of the distributions of the other  
226 variables were checked before applying the GLMM. Then the normal link was chosen to study *Yield*,  
227 the log-normal link was chosen to study *DON*, and the gamma link was chosen to study *DS<sub>2</sub>*. GLMM  
228 parameters were estimated using the Penalized Quasi-Likelihood technique (package MASS;  
229 function `glmmPQL`; Wolfinger and O'Connell 1993). GLMMs were complemented with Tukey's  
230 post-hoc test to check the significance of differences between means (package `multcomp`; function  
231 `glht`; Bretz et al. 2011).

232 The power of the relation between the different disease assessments (*i.e.*  $DS_1$ ,  $DS_2$  and  $FDK$ ) and the  
233 quality markers of the harvest (*Yield* and *DON*) was assessed through linear regressions (package  
234 stats; function lm; Chambers and Hastie 1992). Pearson's' correlation coefficient (package stats;  
235 function cor; Becker et al. 1988) and Root Mean Squared Errors (RMSEs, package nsRFA; function  
236 RMSE; Armstrong and Collopy 1992) were chosen to illustrate the power of the regressions. RMSE  
237 represents the average distance between the regression curve and the observations. The significance  
238 levels of the differences between obtained correlations were estimated using the correlation  
239 comparison test between two dependent correlations sharing one variable (William's test; package  
240 psych; function r.test; Steiger 1980). The significance levels of the differences between obtained  
241 RMSE were estimated by bootstrapping the RMSEs from original data (1,000 replicates; package  
242 boot; function boot; Davison and Hinkley 1997). Then, the intervals containing 95 % of the obtained  
243 values were compared and were considered as significantly different when they did not overlap.

244

### 245 **3. Results**

246

247 *3.1 Effects of agronomical factors on disease development, grain yield and DON accumulation in*  
248 *harvested wheat grains – Generalized linear mixed models*

249 Generalized linear mixed models yielded the same results for all variables (*i.e.*  $DS_1$ ,  $DS_2$ ,  $FDK$ , *Yield*  
250 and *DON*): Inoc1 (sowing) had no effect on the variables, while Inoc2 (at the soil surface), wheat  
251 variety and year had an effect on all variables (Table 1).

252

#### 253 3.1.1. Disease assessments ( $DS_1$ , $DS_2$ , $FDK$ )

254 The proportion of visually assessed orange spikelets ( $DS_1$ ) ranged from 0 % to 9.01 % (Table 2; Fig.  
255 2); the surface of orange spikelets assessed by image analysis ( $DS_2$ ) ranged from 0.04 mm<sup>2</sup> to 34.48  
256 mm<sup>2</sup>; finally, the proportion of damaged kernels ( $FDK$ ) ranged from 0 % to 28.51 %. GLMM yielded  
257 the same results for all disease assessment parameters. Wheat from Inoc2 plots was more diseased

258 than wheat from non-inoculated plots. Both susceptible varieties (Maxwell and Charger) were  
259 significantly more infested than the tolerant variety (Apache). Maxwell was also significantly more  
260 infested than Charger in the case of  $DS_1$  and  $FDK$ , whereas the difference between these two varieties  
261 was not significant in the case of  $DS_2$ . The incidence of *F. graminearum* pathogenic activity was  
262 significantly more pronounced in 2009 than in 2010 shown by both  $DS_1$  and  $FDK$  scores ( $DS_2$  was  
263 only assessed in 2010).

264 All isolations from orange spikelets confirmed the presence of *F. graminearum* (Fig. 3) but proof was  
265 not made that the species was responsible for the visible symptoms since Koch's postulates were not  
266 checked.

267

### 268 3.1.2. Grain yield and quality

269 Yields ranged from 5414 kg ha<sup>-1</sup> to 9252 kg ha<sup>-1</sup> (Table 2, Fig. 2). Yields from Inoc2 plots and from  
270 the plots grown with susceptible varieties were lower than those from the non-inoculated plots and  
271 from the plots grown with the tolerant variety. In the control trial (non-inoculated), there was no  
272 significant difference in yield among varieties (data not shown). Yields were significantly higher in  
273 2010 than in 2009.

274 In 2009, the grains from Inoc2 plots were more infested than those from non-inoculated plots.  
275 Moreover, the proportion of infested grains was higher in the Charger variety than in the Apache  
276 variety (Table 3). The difference between inoculated and non-inoculated plots was less significant in  
277 2010. Moreover, Charger grains variety less infested than Apache grains in the inoculated plots that  
278 year. *Fusarium graminearum* was the dominant species recorded in most of the plots of the inoculated  
279 trial. However, several other fungal species involved in the fungal complex that causes Fusarium head  
280 blight were also isolated in 2009 and in 2010, both in the experimental trial and in the control trial.  
281 The main species isolated from the control trial were *F. tricinctum* in 2009 and *Microdochium nivale*  
282 (var. *nivale* and var. *majus*) in 2010 from both wheat varieties.

283

284

285

### 286 3.1.3. Mycotoxin assessment

287 DON was the only mycotoxin quantitatively detected during both campaigns. ZEA was detected in a  
288 few plots, but was below the detection limit in most of the plots. DON concentrations in grains ranged  
289 from 0 to 19.41  $\mu\text{g g}^{-1}$  of dry meal (Table 2; Fig. 2). DON concentrations were higher in the grains  
290 harvested from the Inoc2 plots than from the non-inoculated plots. DON concentrations were the  
291 highest in the Maxwell susceptible variety, and the lowest in the Apache tolerant variety. Finally,  
292 DON accumulation was significantly higher in 2009 than in 2010.

293

### 294 *3.2. Correlations between the various assessments*

295 The correlation coefficients of  $DS_1$  and  $DS_2$  versus  $FDK$  were not statistically different, and nor were  
296 the correlation coefficients of  $DS_1$ ,  $DS_2$  and  $FDK$  versus *Yield* (Table 4). In contrast, the correlation  
297 coefficient of  $DS_2$  and  $FDK$  versus  $DON$  were statistically better than the correlation coefficient of  
298  $DS_1$  versus  $DON$ .  $DS_2$  RMSE of was the lowest in all regressions, and  $DS_1$  RMSE of was the highest.  
299 The difference obtained between these two RMSE was significant for all regressions.  $FDK$  RMSE of  
300 had an intermediate value in all regressions and the difference with the two others was not significant.  
301 The prediction interval of  $DON$  associated to  $DS_2$  was therefore the finest (Fig. 4). The lower  
302 prediction curves intercepted the European regulation curve at 5.53 % ( $DS_1$ ), 13.06 % ( $FDK$ ) and  
303 20.61  $\text{mm}^2$  ( $DS_2$ ); they represented the minimal values above which the legal limit was definitely  
304 exceeded.

### 305 *3.3. Meteorological data*

306 The cumulated hours with a high temperature and high humidity was higher in 2009 than in 2010,  
307 notably during the flowering time of wheat (Fig. 5). In 2009, 85 h with humidity rates above 80 %  
308 and temperatures above 15 °C were recorded between April 23<sup>rd</sup> and May 25<sup>th</sup>, while only 1 h was  
309 recorded in 2010. From May 25<sup>th</sup>, the 2010 curve started to catch up with the 2009 curve without

310 overlapping it. Moreover, there was no period with a humidity rate above 80 % and temperatures  
311 above 20 °C until the 3<sup>rd</sup> of July in 2010, while 34 hours were recorded in 2009.

312

## 313 **4. Discussion**

314

315 *4.1. Consistency between the results and the risk factors proposed by commercial decision support*  
316 *tools*

317 The first purpose of this study was to check if the effect of agronomical factors on disease  
318 development was in accordance with the prediction of commercial decision support tools under the  
319 Burgundian pedoclimatic context. All checked parameters, i.e. soil tillage, varietal effect and climatic  
320 context, actually led to expected effects on disease development.

321

### 322 4.1.1. Effect of soil tillage on FHB development

323 Inoc2 (i.e. at the soil surface) significantly affected disease development and mycotoxin production,  
324 while Inoc1 (i.e. in the first layer of soil) did not. This is consistent with previous results, which  
325 showed that non-buried infested residues provided the inoculum that fostered FHB rather than  
326 infested residues buried in the first layer of soil (Leplat et al. 2013; Maiorano et al. 2008). The  
327 presence of strongly colonized residues at the soil surface promotes inoculum dispersal through  
328 ascospore discharge and rain-splash (Osborne and Stein 2007; Manstretta and Rossi 2015). On the  
329 other hand, inverse tillage is known to reduce disease development as compared to non-inverse tillage  
330 which promotes it (Dill-Macky and Jones 2000). Finally, non-buried residues provide a longer-  
331 lasting availability of nutrients on which *F. graminearum* can grow saprotrophically in comparison  
332 with buried residues which are decomposed faster, especially as *F. graminearum* is a poor competitor  
333 in the soil over time (Pereyra et al. 2004).

334 The effect of soil tillage on FHB development observed in the present study is consistent with the  
335 prevision of commercial decision support tools which point to minimal tillage as the first risk factor  
336 of FHB development.

337

338

#### 339 4.1.2. Effect of the wheat variety on FHB development

340 Susceptible varieties were most affected by FHB and their grains contained higher DON amounts  
341 than the tolerant variety, so the choice of the cultivated wheat variety was determining. The wheat  
342 variety had no effect on yield in the non-inoculated trial, which demonstrates that the yield decrease  
343 observed in the inoculated trial was mainly due to *Fusarium graminearum* infections and FHB. This  
344 result is consistent with previously observed behaviours of the Apache and Charger varieties  
345 (Champeil et al. 2004). Moreover, we measured significant differences between the two susceptible  
346 varieties: Maxwell was in general more affected than Charger. This difference could be explained by  
347 the different types of resistance to FHB among wheat cultivars (Mesterhazy 1995). As a result, similar  
348 levels of disease on spikes can lead to different amounts of *Fusarium*-damaged kernels and of  
349 accumulated mycotoxins in grains.

350 The varietal effect observed in this experiment was consistent with the prediction of commercial  
351 decision support tools which point to the choice of a susceptible wheat variety as the second risk  
352 factor in FHB development.

353

#### 354 4.1.3. Effect of the climatic context on FHB development

355 Disease severity and DON accumulation were significantly higher in 2009 than in 2010. This result  
356 can be explained by the important role played by weather conditions in FHB development. *Fusarium*  
357 *graminearum* development is promoted by warm, wet conditions (Xu et al. 2007). Weather conditions  
358 were warmer and wetter in 2009 than in 2010, as demonstrated by the results, and especially during  
359 wheat flowering (second half of May), which is the main step in FHB development (Schaafsma and

360 Hooker 2007; Siou et al. 2014). The sanitary status of the harvested grains showed that *F.*  
361 *graminearum* was not the only isolated species. This result probably reflected the expression of the  
362 indigenous flora. *Fusarium tricinctum* and *Microdochium nivale* were also isolated, and so were *F.*  
363 *poae* and *F. avenaceum* in smaller amounts. These species have been the predominant ones in Europe  
364 for several years (Nielsen et al. 2011; Bottalico and Perrone 2002), especially in France (Dubournet  
365 et al. 2008). Moreover, the sanitary status of the harvested grains from the non-inoculated trial  
366 showed that *F. graminearum* was not the dominant species among the indigenous mycoflora.  
367 *Fusarium tricinctum* was the dominant species in 2009, and *M. nivale* was the dominant species in  
368 2010. This result is consistent with a survey by Ios et al. (2004) which showed that *F. graminearum*  
369 was not the most frequent species on wheat in eastern France. The difference between years could be  
370 attributed to climatic factors (Birzele et al. 2002). 2010 was much cooler and drier than 2009 during  
371 the wheat flowering period, so that the development of *M. nivale* may have been enhanced (Doohan  
372 et al. 2003).

373 The effect of weather conditions observed in this experiment is consistent with the prediction of  
374 commercial decision support tools and also with the fungal species associated to FHB previously  
375 recorded in the study area. Among the three risk factors checked in this study (i.e. soil tillage, varietal  
376 choice and weather conditions), Savary et al. (2016) estimated that the climatic factor was the main  
377 factor defining the epidemic risk in the multiple wheat-disease pathosystem, followed in decreasing  
378 order by varietal choice and crop management.

379

#### 380 4.1.4. Reliability of the measured DON amounts

381 Strains of the species *Fusarium graminearum* are divided into two groups for mycotoxin production:  
382 the DON-chemotype and the NIV-chemotype (Seo et al. 1996). In this study, strain MIAE00376 was  
383 used for inoculations as a DON-chemotype, a representative of the main chemotype in the area  
384 (Boutigny et al. 2014). Strains of the DON-chemotype are supposed to be able to produce DON but  
385 also its derivative compounds (3-ADON and 15-ADON) and/or ZEA. However, DON was the only

386 mycotoxin quantitatively detected in this study. The maximum amount of DON authorized in wheat  
387 grains is 1.25  $\mu\text{g}$  of DON  $\text{g}^{-1}$  of grain in Europe (Commission of the European Communities 2006).  
388 The quantities we measured were generally above the limit except for the Apache variety in 2010.  
389 The highest average value was reached by the Maxwell variety in 2009 (11.3  $\mu\text{g}$   $\text{g}^{-1}$ ). These high  
390 amounts can be partly explained by the way plots were inoculated since DON amounts in the non-  
391 inoculated trial were much lower (data not shown). However, similar or higher DON amounts have  
392 already been recorded in Germany, Poland, Japan, the USA or Argentina in non-inoculated fields  
393 (Placinta et al. 1999; Hietaniemi et al. 2016). Therefore, inoculating *F. graminearum* on the soil  
394 surface could mimic conditions encountered during a year of severe infection.

#### 396 4.2. Using disease assessment bioindicators to improve DON management through commercial 397 decision support tools

398 The main purpose of this study was to check if disease assessments could be used to improve DON  
399 management through commercial decision support tools, and especially with a novel computer-  
400 assisted image analysis.

##### 402 4.2.1. Power of computer-assisted image analysis in the determination of DON accumulation 403 as compared to visual assessments

404 We found the highest correlation between *Fusarium*-damaged kernels and DON accumulation, and  
405 the lowest between FHB severity assessed by visual measurements and DON accumulation. The  
406 correlation between computer-assisted assessment of severity at the soft dough stage of wheat and  
407 DON accumulation had an intermediate value between  $DS_1$  and  $FDK$  but was not significantly  
408 different from the correlation obtained with  $FDK$ . This result is consistent with a study by Paul et al.  
409 (2005) who used meta-analyses of published and unpublished studies to conclude that the percentage  
410 of *Fusarium*-damaged kernels is the most strongly DON-correlated parameter in harvested grains,  
411 while disease severity assessed on spikes is the least correlated one. In contrast, the RMSEs of the

412 regressions between computer-assisted FHB assessment at the soft dough stage of wheat and DON  
413 accumulation were lower than the RMSEs obtained in both cases of visual assessment at the soft  
414 dough stage and *FDK* assessment. Thus, the predictive power of computer-assisted image analysis  
415 for this assessment was much better than that of the other two pre-existing assessments.

416 Visual assessment only reflects FHB frequency (the number of infected spikelets / damaged kernels),  
417 while image analysis is more accurate. Indeed, it takes into account not only the number of infected  
418 spikelets but also the damaged surface of these spikelets. It is therefore an improvement in the  
419 determination of fungal biomass (Paul et al. 2005). This is what certainly explains the better results  
420 obtained with this approach. Moreover, such a technique overcomes the bias related to assessors  
421 (Rasmussen et al. 2007). It ensures that results are reproducible since the settings are the same for all  
422 images. In addition, computer-assisted FHB assessment is time-saving as compared to visual  
423 assessment: visual techniques require plot per plot assessment of each plot, while the computer-  
424 assisted technique only requires adjusting the selection parameters on the first plot. The saved  
425 parameters are then applied to each following plot, and potentially year after year. Computer-assisted  
426 techniques including visible and near-infrared spectroscopy are more and more frequently used with  
427 some success for FHB assessment in the field because of the previously quoted reasons. Multispectral  
428 images have been used to detect FHB development in the field (Bauriegel et al. 2011; Dammer et al.  
429 2011), as well as to detect *Fusarium*-damaged kernels (Shahin and Symons 2011; Jaillais et al. 2015),  
430 but trying to predict DON accumulation from spike inspection is quite innovative.

431 Visual disease assessment at the soft dough stage of wheat could help to provide early confirmation  
432 of the risk encountered by plots defined by commercial prediction tools, but it is not sufficiently  
433 correlated to DON accumulation to replace HPLC measurements. The assessment of *Fusarium*-  
434 damaged kernels could be efficient enough to grade grains and avoid systematically analysing DON  
435 by HPLC (Beyer et al. 2007), but this assessment has two major drawbacks: it is time-consuming  
436 and it is performed during the harvest, so that the results of commercial decision support tools cannot  
437 be anticipated. Computer-assisted image analysis of FHB appears to be a much better indicator to

438 complete and improve the use of commercial decision support tools because it combines earliness of  
439 assessment at the soft dough stage of wheat and the relevance of *FDK* assessment. It could replace  
440 the systematic and costly use of HPLC analysis: in this study, all fifty-spike samples with a  $DS_2$  above  
441 20.61 mm<sup>2</sup> were unquestionably above the legal limit according to the prediction of the regression  
442 model. Fusarium head blight assessment by image analysis may also efficiently replace visual disease  
443 assessment on spikes in upcoming scientific studies. From a practical viewpoint, computer-assisted  
444 image analysis requires excising spikes and then scanning them, which could appear tedious. In fact  
445 it is not tedious as compared to already existing techniques of DON assessment, i.e. dipstick methods  
446 or HPLC, which also require tedious sampling effort and sample preparation (Lattanzio et al. 2013).

#### 448 4.2.2 Further needs to improve computer-assisted image analysis

449 FHB assessment by image analysis is promising but still needs to be tested and improved. First, it  
450 was only performed over one year. Therefore, the trial needs to be duplicated, even though the  
451 technique appears to be relevant since forty-eight plots were tested during that single year.  
452 Accumulating other data is necessary to reduce the size of the prediction interval associated to the  
453 method, which is already smaller than those associated to visual assessments. Moreover, the  
454 experiment was performed under high disease pressure, which could represent a year of severe  
455 infection. Thus, it also needs to be duplicated in natural conditions to check the accuracy of the  
456 method under lower disease pressure.

457 Second, the only FHB symptom we assessed in this study was orange spikelets because it was the  
458 only one that could be attributed to *F. graminearum* for sure. Nevertheless, other symptoms were  
459 noted in the field, such as necrosis on the edge of the glumes. These symptoms were caused by *F.*  
460 *graminearum* or other isolated indigenous species such as *M. nivale* and *F. tricinctum* (Christ et al.  
461 2011). They were not taken into account in statistical analysis because the real causative pathogen  
462 was difficult to identify and they may also have been linked to mycotoxin production. However, DON  
463 production was mainly attributed to *F. graminearum* since the other isolated fungal species were not

464 DON producers: *Fusarium poae* produces fusarenone and NIV among others, *F. tricinctum* and *F.*  
465 *avenaceum* produce moniliformin, and *M. nivale* is not a mycotoxin producer (Bottalico 1998;  
466 Logrieco et al. 1991). This raises the question of the performance of the technique when confronted  
467 with highly complex situations including production of multiple mycotoxins, as often happens in the  
468 field (Smith et al. 2016). Therefore, the relevance of computer–assisted image analysis to predict  
469 DON accumulation among other mycotoxins still needs to be studied, as well as its relevance to  
470 predict the accumulation of other mycotoxins. Further studies should especially focus on other  
471 mycotoxins under regulation, such as zearalenone (Commission of the European Communities 2006),  
472 but also on non–regulated emerging mycotoxins, such as beauvericin and enniatins (Fraeyman 2017,  
473 Stanciu et al. 2017). The prerequisite for studying other couples *Fusarium sp.* / mycotoxins by  
474 computer–assisted image analysis is the expression of visible symptoms on wheat leading to  
475 mycotoxins accumulation. Otherwise, multispectral image analysis should be investigated (Bauriegel  
476 et al. 2011; Dammer et al. 2011).

477 Finally, the regression models for each single wheat variety are not presented in this study because  
478 the number of observations for each single variety was too low. However, the collected data strongly  
479 suggested that varietal regression models should be more accurate than the regression model  
480 including all wheat varieties together. It probably reflects the different types of resistance to FHB  
481 among wheat cultivars. According to Mesterhazy (1995), five different mechanisms of active  
482 resistance can occur among wheat varieties and lead to a greater development of the disease between  
483 the soft dough stage and harvest for some varieties. Consequently, the computer–assisted image  
484 analysis model based on symptoms at soft dough stage to predict DON accumulation in harvested  
485 grains should be different for such varieties, as compared to tolerant varieties for example. This trend  
486 could be reinforced by weather conditions: extreme cases have been reported when late infections  
487 caused by particular weather conditions induced DON accumulation in wheat grains but no visible  
488 symptom on spikes (Del Ponte et al. 2007; Yoshida and Nakajima 2010).

489

## 490 **5. Conclusion**

491

492 Our results are consistent with the predictive models proposed by commercial decision support tools.

493 Soil tillage, varietal choice and weather conditions had an effect on *Fusarium* head blight

494 development and on DON accumulation in harvested grains. Non-buried *Fusarium*-contaminated

495 residues provided the primary inoculum responsible for FHB development on wheat spikes. The

496 susceptible wheat varieties were more diseased than the tolerant one. The study of weather conditions

497 underlined the effect of warm and wet conditions on FHB development, especially during the

498 flowering time of wheat. Conditions were therefore suitable to check whether disease assessments of

499 FHB could help to anticipate the results of commercial decision support tools and avoid systematic

500 mycotoxin measurements by HPLC. The present study highlights that visual FHB assessment at the

501 soft dough stage of wheat could supplement the use of decision support tools because visual

502 assessment gives the true health status of wheat while decision tools are only statistical models.

503 However, visual FHB assessment cannot replace quantitative mycotoxin analysis by HPLC because

504 it is not reliable enough. The assessment of *Fusarium*-damaged kernels was well correlated with

505 DON accumulation, but this bioindicator does not anticipate the results of decision support tools.

506 Moreover, the replacement of HPLC controls by this type of assessment does not appear to be

507 sustainable because it is too time-consuming. Finally, computer-assisted image analysis of FHB at

508 the soft dough stage of wheat is highly promising because it anticipates the results of decision support

509 tools. Moreover, it had a better prediction power of DON accumulation than visual assessment.

510 Although our results need to be duplicated, this technique appears to be promising to replace

511 systematic HPLC tests.

512

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514

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519

## 520 **Conflict of Interest**

521

522 The authors certify that this work was carried out in a public research organization and is in no way  
523 a potential source of conflict of interest with other public or private research organizations.

524

Accepted Version

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711 **Table 1** Effect of agronomical factors on disease development, grain yield and DON accumulation  
 712 in harvested wheat grains – GLMM table.  
 713

<i>Fixed effect</i>			<i>DS<sub>1</sub></i>	<i>DS<sub>2</sub></i>	<i>FDK</i>	<i>Yield</i>	<i>DON</i>	
	Reference modality	Compared modality	df (df <i>DS<sub>2</sub></i> )	p-value	p-value	p-value	p-value	
<i>Inoc1</i>	-	+	84 (40)	0.15	0.36	0.85	0.65	0.55
<i>Inoc2</i>	-	+	84 (40)	<0.001***	0.006**	<0.001***	<0.001***	<0.001***
<i>Variety</i>	Apache	Charger	84 (40)	<0.001***	0.008**	<0.001***	<0.001***	<0.001***
		Maxwell	84 (40)	<0.001***	0.004**	<0.001***	<0.001***	<0.001***
<i>Year</i>	2009	- 2010	6 (-)	0.03*	(-)	<0.001***	<0.001***	<0.001***

714 *DS<sub>1</sub>*: Disease severity assessed by visual observations; *DS<sub>2</sub>*: Disease severity assessed by image analysis; *FDK*: *Fusarium*-damaged kernels; *Yield*: grain  
 715 yield; *DON*: DON amount  
 716 *Inoc1*: inoculation in the first layer of the soil; *Inoc2*: inoculation at the soil surface  
 717 -: non-inoculated; +: inoculated  
 718 *df*: degrees of freedom  
 719 \* p < 0.05, \*\* p < 0.01, \*\*\* p < 0.001  
 720 (-): *DS<sub>2</sub>* not tested in 2009  
 721  
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736 **Table 2** Effect of agronomical factors on disease development, grain yield and DON accumulation  
 737 in harvested grains – Comparisons of the means of the different modalities.  
 738

		<i>DS<sub>1</sub></i>	<i>DS<sub>2</sub></i>	<i>FDK</i>	<i>Yield</i>	<i>DON</i>
<i>Minimum value</i>		0	0.04	0	5414	0
<i>Maximum value</i>		9.01	34.48	28.51	9252	19.41
<i>Inoc1</i>	-	2.15a	7.02a	8.45a	7310a	4.79a
	+	2.3a	9.51a	8.13a	7266a	5.05a
<i>Inoc2</i>	-	1.42a	4.4a	6.19a	7687b	3.43a
	+	3.03b	12.13b	10.39b	6890a	6.41b
<i>Variety</i>	Apache	0.9a	2.85a	2.91a	7859b	1.37a
	Charger	2.42b	9.59b	9.37b	6919a	4.62b
	Maxwell	3.36c	12.35b	12.58c	7087a	8.78c
<i>Year</i>	2009	2.78b	(-)	13.52b	6657a	6.75b
	2010	1.68a	8.26	3.05a	7920b	3.1a

739 *DS<sub>1</sub>*: Disease severity assessed by visual observations (%); *DS<sub>2</sub>*: Disease severity assessed by image analysis (mm<sup>2</sup>); *FDK*: *Fusarium*-damaged kernels  
 740 (%); *Yield*: grain yield (kg ha<sup>-1</sup>); *DON*: DON amount (µg g<sup>-1</sup>).  
 741 *Inoc1*: inoculation in the first layer of the soil; *Inoc2*: inoculation at the soil surface  
 742 -: non-inoculated; +: inoculated  
 743 Values related to a given factor in the same column are significantly different when they are followed by different letters.  
 744 (-) Computer-assisted image analysis not tested in 2009, no statistical letters could consequently be assigned to *DS<sub>2</sub>* in 2010

745  
 746 **Table 3** Proportions (percentages) of infected harvested grains and of each fungal species recovered  
 747

<i>Source of variation</i>		<i>Infected grains</i>	<i>F. graminearum</i>	<i>F. tricotinctum</i>	<i>F. poae</i>	<i>F. avenaceum</i>	<i>M. nivale</i>
2009	<i>Charger, non-inoculated</i>	76	70	20	5	1	4
	<i>Charger, Inoc2</i>	91	93	5	(-)	(-)	2
	<i>Apache, non-inoculated</i>	40	59	17	7	2	15
	<i>Apache, Inoc2</i>	70	75	15	3	(-)	7
	<i>Charger, control trial</i>	58	16	67	3	(-)	14
	<i>Apache, control trial</i>	23	17	39	17	17	26
2010	<i>Charger, non-inoculated</i>	29	83	7	(-)	(-)	10
	<i>Charger, Inoc2</i>	32	78	6	(-)	(-)	13
	<i>Apache, non-inoculated</i>	15	33	13	(-)	(-)	53
	<i>Apache, Inoc2</i>	38	79	5	(-)	(-)	16
	<i>Charger, control trial</i>	11	33	13	(-)	(-)	42
	<i>Apache, control trial</i>	22	5	5	(-)	(-)	90

748 *Inoc2*: inoculation at the soil surface

749

750 **Table 4** Parameters of the correlations between disease assessments and the harvest quality markers

751

	<i>FDK</i>		<i>Yield</i>		<i>DON</i>	
	Pearson's r	RMSE (%)	Pearson's r	RMSE (%)	Pearson's r	RMSE (q ha <sup>-1</sup> )
<i>DS<sub>1</sub></i>	0.61***a	6.2a	-0.62***a	7.61a	0.57***a	3.77a
<i>DS<sub>2</sub></i>	0.59***a	2.79b	-0.64***a	5.43b	0.70***b	2.36b
<i>FDK</i>	(-)	(-)	-0.73***a	6.67ab	0.78***b	2.87ab

752 *DS<sub>1</sub>*: Disease severity assessed by visual observations; *DS<sub>2</sub>*: Disease severity assessed by image analysis; *FDK*: *Fusarium*-damaged kernels; *Yield*: grain  
753 yield; *DON*: DON amount

754 RMSE: Root mean squared error

755 Values in the same column are significantly different when they are followed by different letters

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756 **Fig. 1** Disease assessment by image analysis at the soft dough stage of wheat (graphics program:  
757 Inkscape)

758 The same picture is shown before (top) and after (bottom) analysis. Orange pixels are selected by the  
759 computer-assisted analysis. They are transformed into yellow in the bottom-picture. The areas  
760 showing most of the yellow pixels are circled.

761

762 **Fig. 2** Effect of agronomical factors on disease development, grain yield and DON accumulation in  
763 harvested grains – Boxplot representation (graphics program: R + Inkscape)

764 The figure represent the effect of significant agronomical factors, *i.e.* Inoc2 and wheat variety, as well  
765 as the effect of the year on disease development, grain yield and DON accumulation in harvested  
766 grains.  $DS_2$  has not been tested in 2009.

767 Wheat from plots inoculated at the soil surface (Inoc2 +) was more diseased than wheat from non-  
768 inoculated plots. Both susceptible varieties (Maxwell and Charger) were more infested than the  
769 tolerant variety (Apache). Finally, the disease development was more pronounced in 2009 than in  
770 2010.

771

772 **Fig. 3** Isolation of the agent associated with the development of orange spikelets on Potato Dextrose  
773 Agar (PDA)

774 The colony exhibited the typical large amount of dense mycelium that characterizes *F. graminearum*  
775 cultures.

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781 **Fig. 4** Correlation curves between disease assessments and DON accumulation (graphics programs:  
782 R + Inkscape)

783 The figure presents the linear regressions of the visual disease assessment at the soft dough stage  
784 ( $DS_1$ ), computer-assisted disease assessment at the soft dough stage ( $DS_2$ ) and assessment of  
785 *Fusarium*-damaged kernels ( $FDK$ ) versus DON accumulation. The finest prediction interval was  
786 clearly obtained with computer-assisted image analysis. According to this prediction interval, a batch  
787 with  $DS_2$  above 20.61 mm<sup>2</sup> exceeded the DON legal limit.

788

789 **Fig. 5** Weather conditions between soil surface inoculation (Inoc2) time and harvest time in 2009 and  
790 2010 (Graphics program: Excel)

791 Cumulative hours with a humidity rate above 80 % and a temperature above 15 °C in 2009 (— — — —

792 ); Cumulative hours with a humidity rate above 80 % and a temperature above 15 °C in 2010 (— — — —

793 •); Cumulative hours with a humidity rate above 80 % and a temperature above 20 °C in 2009 (•••••);

794 Cumulative hours with a humidity rate above 80 % and a temperature above 20 °C in 2010 (— — — —)

795 Wheat flowering occurred in the second half of May. The role played by wet and warm weather

796 conditions in the FHB development is the most critical during this period.

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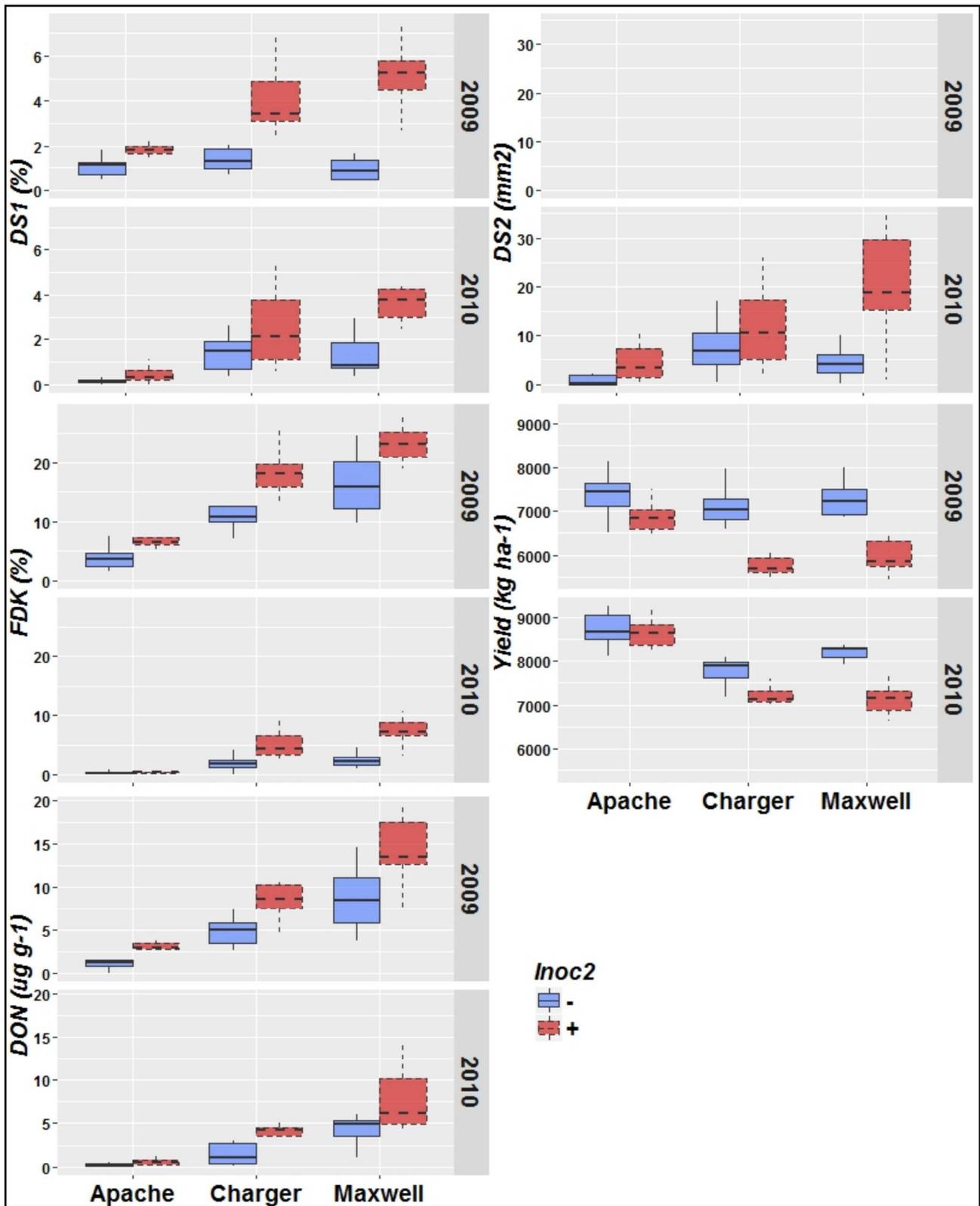
797 **Fig. 1**

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801 **Fig. 3**

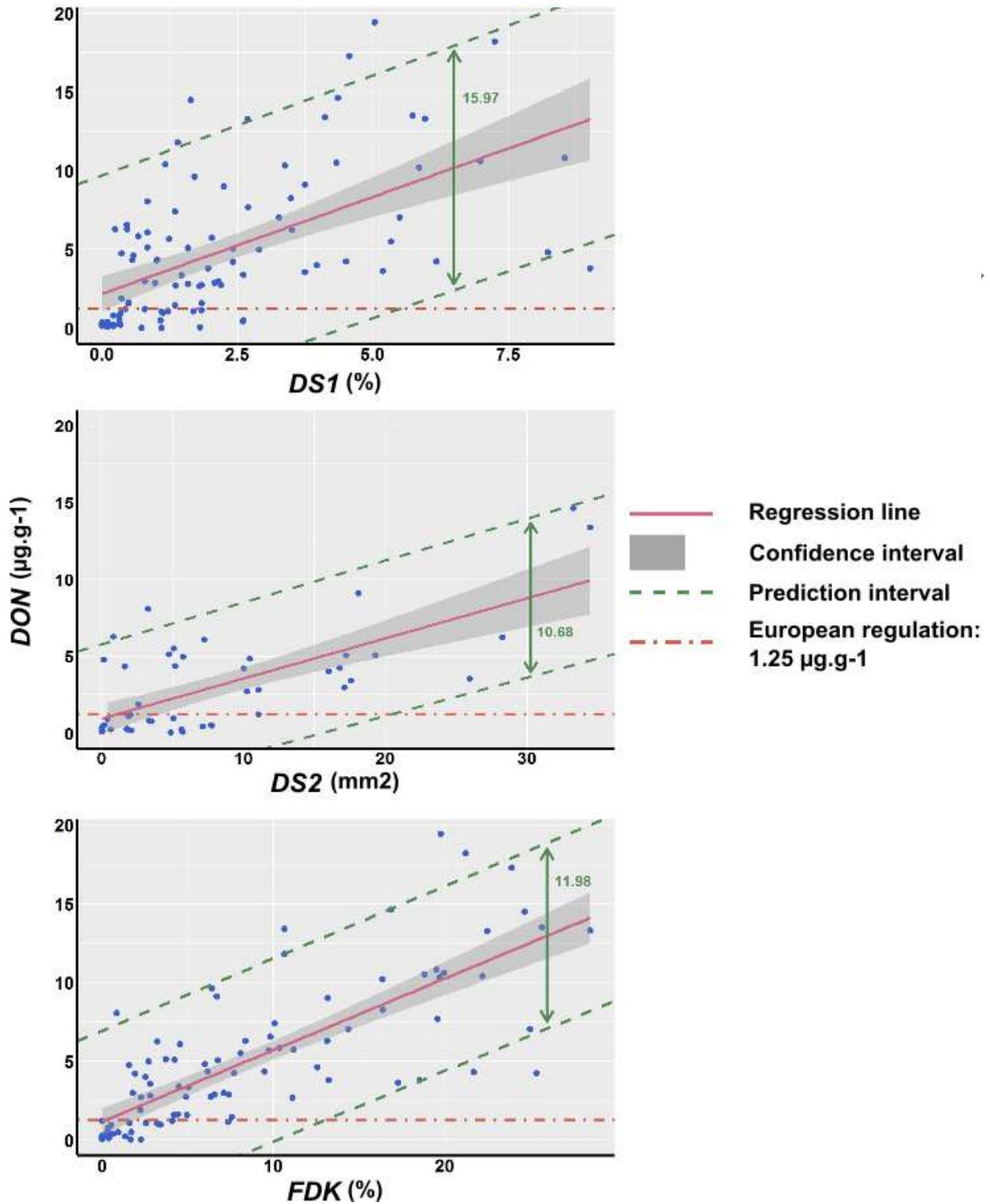
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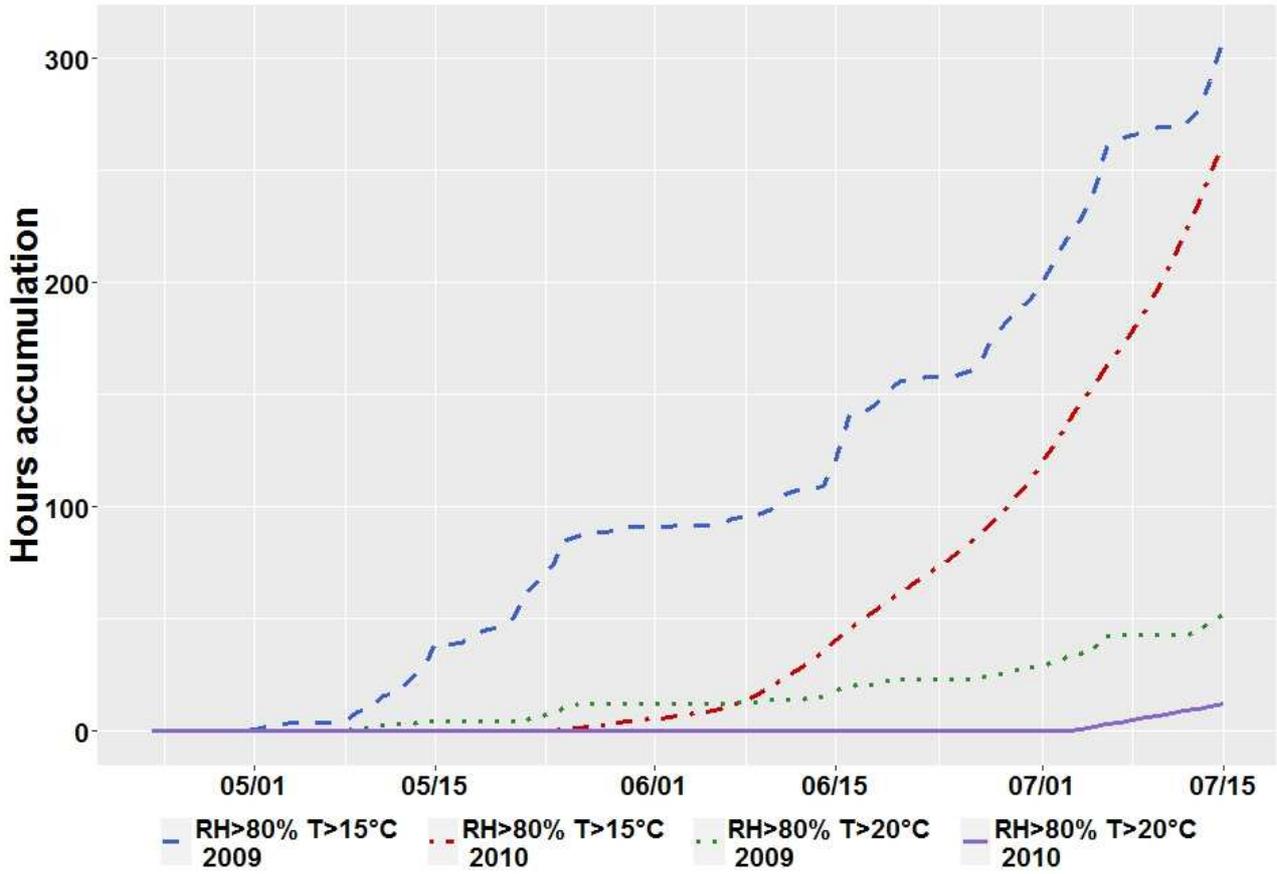
803 Fig. 4

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805 Fig 5.

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