

1 Article

2 ***Fusarium graminearum* Colors and Deoxynivalenol  
3 Synthesis at Different Water Activity**4 **Edgar Cambaza** <sup>1,2,\*</sup>, **Shigenobu Koseki** <sup>1</sup>, **Shuso Kawamura** <sup>1</sup>5 <sup>1</sup> Laboratory of Food Process Engineering, Graduate School of Agriculture, Hokkaido University, Sapporo,  
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11 **Abstract:** Deoxynivalenol (DON) is a well-known mycotoxin, responsible for outbreaks of  
12 gastrointestinal disorders in Japan. *Fusarium graminearum*, a parasite of cereal crops, produces this  
13 toxin and this is one of the reasons why it is important to understand its metabolism. It is possible  
14 to predict the mold's color change and the quantity of DON synthesized throughout its lifecycle.  
15 Furthermore,  $a_w$  has been found to affect the amount of DON. This study aimed to analyze the  
16 potential of *F. graminearum* surface color as a predictor of DON concentration at  $a_w$  = 0.94, 0.97 and  
17 0.99. Thus, 36 specimens were incubated at 25 °C, 12 at each  $a_w$ . After 4, 8, 12 and 16 days, 3  
18 specimens from each  $a_w$  were collected for color analysis and DON quantification. For color  
19 analysis, photos were taken and red, green and blue (RGB) channels were measured on *ImageJ*  
20 software. DON was quantified through liquid chromatography (HPLC). Color changes were only  
21 observed at  $a_w$  = 0.99 because at lower  $a_w$  the molds presented high growth of white mycelium. Yet,  
22 DON increased in all cases. It was only possible to relate the colors with DON concentration at  $a_w$  =  
23 0.99, where they presented inverse proportionality.

24 **Keywords:** *Fusarium graminearum*, deoxynivalenol, RGB, water activity.  
2526 **1. Introduction**

27 Mycotoxin studies have been gaining prominence since the second half of the 20<sup>th</sup> century, and  
28 deoxynivalenol (DON) is among the most well known among these toxins. It belongs to the class of  
29 trichothecenes and causes gastrointestinal disorders including regurgitation [1]. DON was identified  
30 as the cause of at least eight outbreaks of intoxication in Japan, including two cases in the Hokkaido  
31 prefecture. The toxin is among the natural contaminants described by the country's Ministry of  
32 Health, Labor and Welfare as a potential threat for public health [2].

33 Water activity ( $a_w$ ) is among the environmental factors with impact on the quantity of DON  
34 produced by *Fusarium graminearum* (teleomorph: *Gibberella zae*) [3-5]. Though there are still some  
35 inconsistencies on how they are related, increased  $a_w$  seems to favor higher DON production [6].  
36 Furthermore,  $a_w$  is frequently used in models to predict mycotoxin concentration *in vitro*, together  
37 with temperature and other variables such as concentration of nutrients or fungicides [7]. Thus, it is  
38 important to know how  $a_w$  affects DON synthesis by *F. graminearum*.

39 The RGB (red, green and blue) components of *F. graminearum* surface color were recently found  
40 to exhibit predictable changes over time [8], and this feature is desirable as an alternative to size  
41 measurement to estimate the mold's maturity because size is highly dependent limitations such as  
42 the borders of a Petri dish and it does not provide much information about the metabolism [8]. Since  
43 both DON concentration [4,9] and surface color [8] are predictable for *F. graminearum* over time, it is  
44 reasonable to admit the possibility that both can be related at certain degree. Furthermore, surface  
45 color and toxin concentration are manifestations of the mold's state of maturity [8,10].

46 This study aims to demonstrate that *F. graminearum* surface color can be used to predict how  
 47 much DON the fungus produces taking  $a_w$  in consideration. These analyses will substantiate the  
 48 idea that color is a viable alternative to size in *in vitro* mold growth studies.

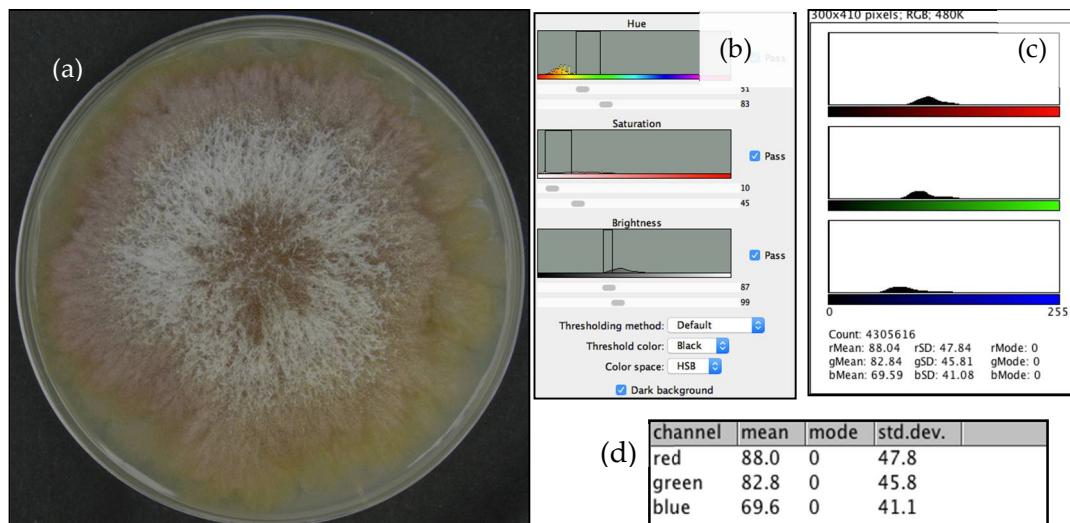
49 **2. Materials and Methods**

50 *2.1. Mold Isolate*

51 This study used an *F. graminearum* isolate from the Catalogue of the Japan Collection of  
 52 Microorganisms (JCM). It is registered as the teleomorph *Giberella zeae* (Schwabe) Petch, isolated by  
 53 Sugiura [11] from rice stubble in Hirosaki, Aomori Prefecture, Japan. It is a known producer of  
 54 deoxynivalenol, 15-acetyldeoxinivalenol and zearalenone [12].

55 *2.2. Experimental Procedure*

56 Thirty-six specimens of *F. graminearum* were grown at 25 °C on yeast extract agar (YEA) at three  
 57 water activity ( $a_w$ ) settings experimentally prepared using glycerol: 0.94, 0.97 and 0.99. From the 4<sup>th</sup>  
 58 incubation day, 3 replicates per temperature were taken for DON quantification. Before the  
 59 extraction, the fungi were photographed in a black bucket, vertically from 30 cm above. The camera  
 60 model was *Nikon D3200* with a lens *DX SWM VR*. The only source of light was a round LED attached  
 61 to the bucket's lid. The photos were then processed on the *ImageJ* software (*FIJI* edition), developed  
 62 by the National Institutes of Health [13] using the method described by Cambaza *et al.* [8] (Figure 1).  
 63 *ImageJ* allowed the determination of average intensities of the RGB components from the photos.  
 64 The analysis considered only the fungal surface, excluding any background including the plate  
 65 borders or agar. At the end, the variables to analyze were incubation time (in days),  $a_w$  and the RGB  
 66 parameters, converted from the 8-bit notation (0 – 255) to the arithmetic index (0.0 – 1.0).



67

68 Figure 1. Process of *F. graminearum* color analysis using *ImageJ*: (a) sample photo of the mold;  
 69 (b) *ImageJ* panel used to remove the background by filtering colors; (c) color measurement panel;  
 70 (d) color measurement table.

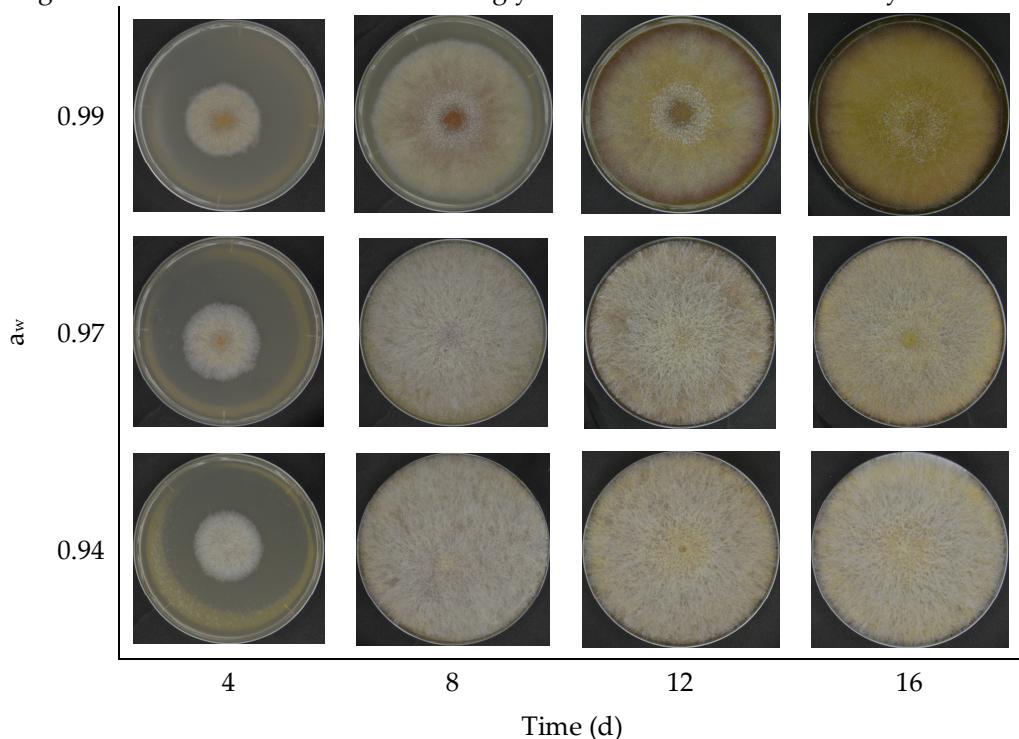
71 *2.3. Statistical analysis*

72 The statistical analysis was performed on JASP 0.9, Jamovi 0.9 and Microsoft Excel. All the  
 73 hypotheses tests were carried out with  $\alpha = 0.05$ . The distribution of intensities of red green and blue  
 74 was compared through analysis of covariance (ANCOVA) to find if their differences were  
 75 significant. Then, the relationships between the colors were analyzed through a scatter plot matrix.  
 76 Subsequently, the focus oriented towards each color. For each, a Kruskal-Wallis test determined if

77 the distribution of color intensity between the samples grown at distinct  $a_w$  presented significant  
 78 differences. The final step analyzed the impact of  $a_w$  on the pigmentation and DON concentration.

79 **3. Results**

80 All the specimens grew throughout the 16 days and measurements were successfully carried  
 81 out. The ones grown at distinct  $a_w$  presented notable visual differences in color and texture (Figure  
 82 2), particularly the molds grown at  $a_w = 0.99$  in relation to the others. However, all specimens were  
 83 mostly similar up to the 4<sup>th</sup> day, developed into a white mycelium with a diameter of approximately  
 84 3 cm with a yellow spot at the center, resembling a fried egg. The central spot was less visible in the  
 85 specimens grown at  $a_w = 0.94$  and it was increasingly noticeable as the water activity increased.



86 Figure 2. Surface color of *F. graminearum* grown at different  $a_w$  for 16 days.

87 The specimens grown at  $a_w$  of 0.94 and 0.97 showed high rate of mycelial growth up to day 8,  
 88 covering the entire plate with its radially dispersed hairy whitish surface, and seemed to remain  
 89 unchanged until the end of the experiment. In some cases, the mycelial growth was immense,  
 90 touching the Petri dish's lid. However, the molds incubated at  $a_w$  of 0.99 did not produce as much  
 91 mycelial growth and exhibited more clearly visible concentric areas with distinct colors, each with  
 92 notable changes from one measurement to the following. Its central spot changed to reddish, brown  
 93 and finally pale, seemingly because of some white mycelial growth on top. Its borders developed a  
 94 wine red tone and the surface became increasingly yellow. These observations suggest that *F.*  
 95 *graminearum* surface color is highly sensitive to  $a_w$ , and  $a_w$  reduction promotes mycelial growth,  
 96 possibly as a stress factor.

97 Table 1 confirms the impact of  $a_w$  on the mold's color, especially the green and blue components  
 98 ( $p_{\text{ANCOVA}} < 0.05$ ). Red color did not seem to be significantly affected by  $a_w$ , even after Tukey's *post hoc*  
 99 comparisons. Green and blue showed exactly the same profile of significance considering the  
 100 different  $a_w$ , though green showed highest levels of discrepancies in all cases. The overall  
 101 differences, measured through ANCOVA were significant. Regarding the *post hoc* comparisons, the  
 102 significant differences occurred between the specimens incubated at  $a_w$  of 0.99 and the others. These  
 103 observations are consistent with the visual analysis in which  $a_w$  reduction drastically affects *F.*  
 104 *graminearum* color pattern.

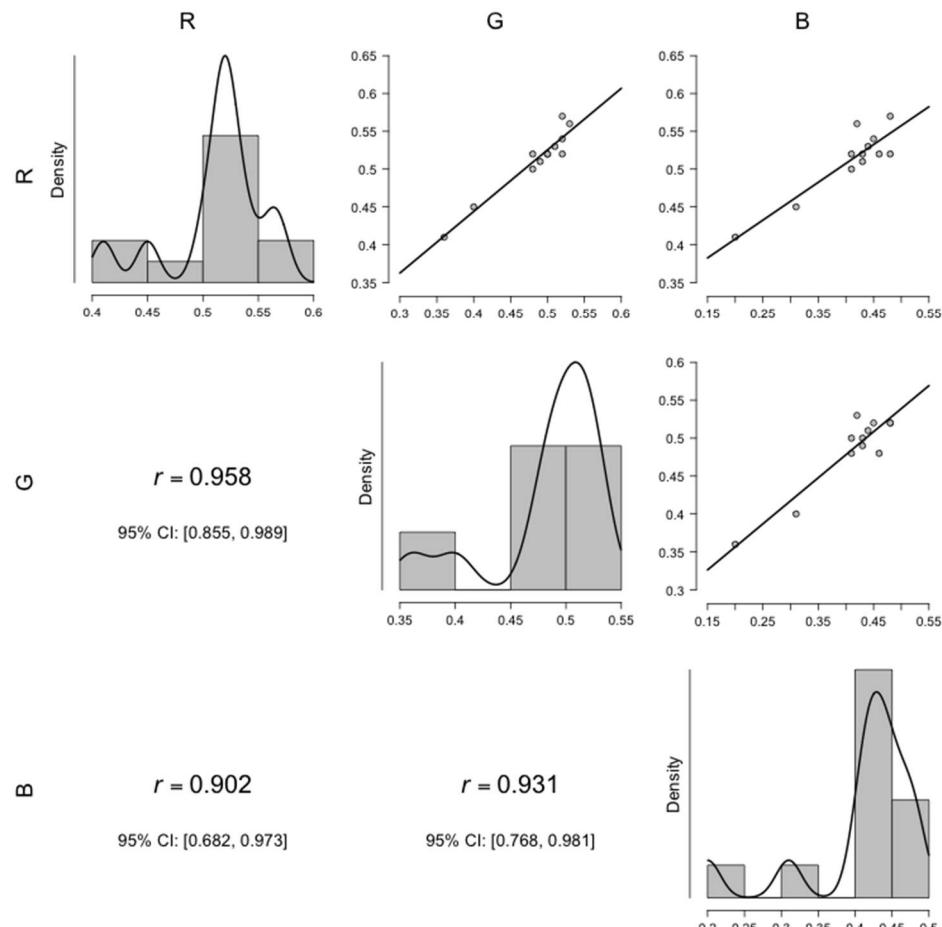
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Table 1. Color intensity differences between the specimens grown under different water activity.

RGB channel	p <sub>ANCOVA</sub>	$a_w$	Post hoc color comparison					
			Mean Difference	SE	df	t	pTukey	
R	0.169	0.94	0.97	0.02	0.03	8	0.63	0.809
		0.97	0.99	0.06	0.03	8	2.06	0.159
		0.97	0.99	0.04	0.03	8	1.44	0.369
G	0.007	0.94	0.97	0.02	0.01	6	2.22	0.145
		0.97	0.99	0.07	0.01	6	8.06	<.001
		0.97	0.99	0.05	0.01	6	5.84	0.003
B	0.02	0.94	0.97	0.03	0.04	8	0.69	0.778
		0.97	0.99	0.13	0.04	8	3.43	0.022
		0.97	0.99	0.1	0.04	8	2.74	0.059

R = red; G = green; B = blue; ANCOVA = analysis of covariance; SE = standard error;

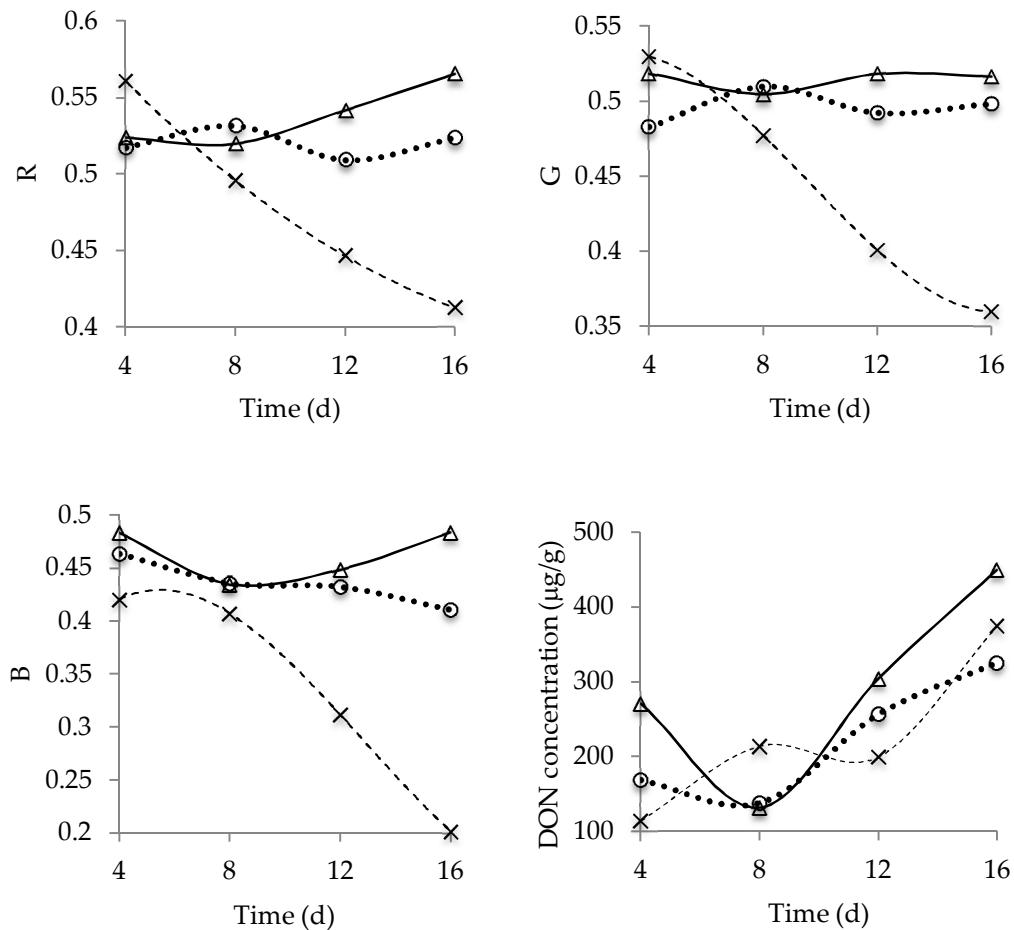
df = degrees of freedom; t – student's t statistics.

106  
107Despite of the differences between the colors at distinct  $a_w$ , all three RGB components seemed highly correlated (Figure 3), with Pearson's correlation  $r$  above 0.9.108  
109  
110Figure 3. Pearson's correlations between the RGB components. The diagonal charts show the intensity of the colors. CI = confidence interval;  $r$  = Pearson's coefficient.111  
112

The data suggest direct relationships between them, and all colors showed considerably high density of their lighter shades. Red and green were the most strongly correlated, followed by blue

113 and green. Thus, even though the red component seemed to be consistently the same through at  
 114 different  $a_w$ , unlike the others, its slight variations presented a similar profile to the ones exhibited by  
 115 the green and blue channels.

116 Figure 4 shows the variations in RGB components and DON concentration over time  
 117 considering the different  $a_w$  settings. The colors seemed to exhibit very similar patterns of variation  
 118 over time.



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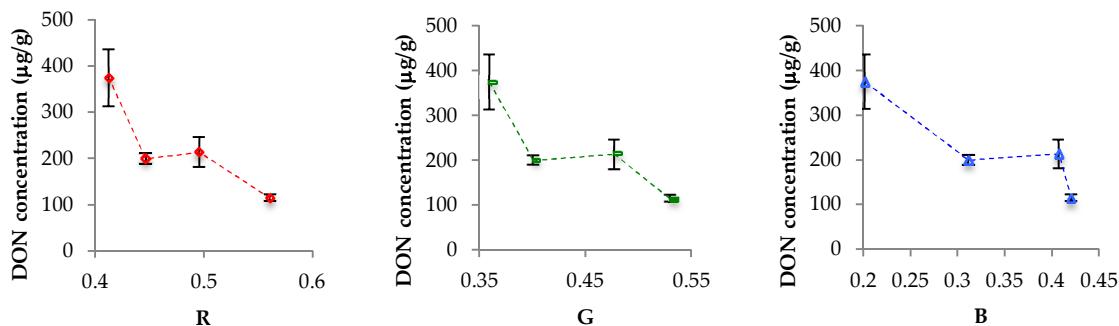
121 Figure 4. Variation of RGB components and DON concentration over time under different  $a_w$ . Note: R  
 122 = red; G = green; B = blue;  $a_w = 0.94$  ( $\Delta$ );  $a_w = 0.97$  (O);  $a_w = 0.99$  ( ).

123 The specimens grown at  $a_w = 0.99$  decreased in color intensity (all RGB channels) while the  
 124 others apparently remained constant and considerably high. This is consistent with the photos,  
 125 where the lowest  $a_w$  incubation setting resulted in predominantly whitish surface during virtually  
 126 the entire experiment. At  $a_w = 0.99$ , the best simple algebraic representations were  $y = 0.0005x^2 -$   
 127  $0.0222x + 0.6416$  for red,  $y = 0.0002x^3 - 0.0044x^2 + 0.023x + 0.4992$  for green and  $y = 0.0002x^3 - 0.0068x^2 +$   
 128  $0.0588x + 0.2831$  for blue, all with  $R^2 = 1$ , assuming  $x$  as time in days and  $y$  as the RGB component  
 129 within the scale 0 to 1. DON concentration seemed to increase in general for all  $a_w$  settings, though  
 130 there are incidental cases of reduction.

131 It is hard to explain why there are reductions because the toxin is expected to accumulate over  
 132 time but it might have been due to some errors. An analysis of covariance (ANCOVA) shows no  
 133 significant differences between DON concentrations ( $p = 0.347$ ) of samples incubated at different  $a_w$ .  
 134 Since the colors change their pattern of variation when the molds are subjected to distinct  $a_w$  but it  
 135 does not happen to DON, the high superficial mycelial growth in the specimens at lower  $a_w$ , the  
 136 fungus seems to keep the ability to produce the toxin even when there is higher mycelial growth. It  
 137 is possible that the layer of whitish hyphae is masking an inferior highly pigmented layer in the

138 specimens grown at lower  $a_w$ . The bottom-line is perhaps the fact that lowering  $a_w$  caused the white  
 139 mycelium to remain abundant throughout the experiment while DON kept accumulating.

140 Only the RGB channels at  $a_w = 0.99$  could be used as independent variables to plot with DON  
 141 concentration (Figure 5) because colors did not change significantly at lower  $a_w$ .



142

143 Figure 5. Relationship between color variation and DON concentration at  $a_w = 0.99$ .

144 All colors decreased in value, oscillating once. The major differences between the colors seemed  
 145 to be the wideness and position of their dominium (range of abscissae). Considering x as RGB  
 146 channel and y as DON concentration, as one observed from the origin of x towards 1, blue presented  
 147 the lowest values but also the widest range, followed by green with intermediate values and range,  
 148 and red. The considerably narrower range of the red component may explain why it did not present  
 149 significant differences across  $a_w$ .

#### 150 4. Discussion

151 In summary,  $a_w$  had a major impact on *F. graminearum* surface color. Up to day 4, all the  
 152 specimens were mostly similar in appearance, with a yellowish center surrounded by a whitish  
 153 mycelium, resembling a fried egg. However, a reduction in  $a_w$  seemed to promote *F. graminearum*  
 154 mycelial growth, masking its conidial pigmentation. As consequence, the specimens grown at  $a_w =$   
 155 0.97 and 0.94 remained whitish throughout the entire experimental period, with RGB channels  
 156 presenting no significant variations, unlike the molds grown at  $a_w = 0.99$ . In any case, the RGB  
 157 components appeared highly correlated, with Pearson's coefficient  $r > 0.9$  when the colors were  
 158 considered two at a time. Yet, only green and blue components exhibited significant variations, even  
 159 though all colors had the same pattern of variation. The significant differences in green and blue  
 160 were only between the samples incubated at  $a_w = 0.99$  and the others, and this supports the previous  
 161 observations from the photos. The highest  $a_w$  was marked by a reduction of RGB components, all fit  
 162 to polynomial functions. The lowest  $a_w$  settings presented notably constant trends. Still, DON  
 163 concentration increased in all  $a_w$  settings, independently of the surface color. Thus, only the highest  
 164  $a_w$  was considered to build graphs relating DON concentration with color variation. They seemed  
 165 inversely proportional if colors represented as the abscissae and DON concentration the ordinate. As  
 166 one moved from the origin of abscissae, the blue, green and red ranges appeared (overlapping), each  
 167 narrower than the previous but all with the same shape.

168 Water activity is among several factors affecting the pigmentation of *F. graminearum* [8,14-16].  
 169 The way it affects can be very complex because the mold's surface color results from the  
 170 combination of several different pigments, some with quite different chemical properties [15,17-20].  
 171 For instance,  $a_w$  partially affects its chromatic attributes of the polyketide aurofusarin, perhaps the  
 172 most influential pigment, notable for its yellow and red coloration [21]. Yet, the color differences  
 173 appeared more associated with the increased growth of white mycelium on top of the mold,  
 174 covering the entire dish, rather than caused by changes in nature or quantity of pigments. The  
 175 simple fact that lower  $a_w$  stimulated higher mycelial growth might look counterintuitive and also  
 176 contradicts previous observations [4,22], but it makes some sense that the shortage of water leads the  
 177 fungus to expand its hyphae in search for new sources [23]. The initial similarity between the

178 specimens grown at distinct  $a_w$  perhaps occurred because the molds were very small and the  
179 shortage of water was not yet impacting the mycelia. As they grew, the ones grown at lower pH  
180 experienced early exhaustion of water and seemed to react by expanding hyphae to all directions  
181 including upwards. Furthermore, during day 4 they were still at exponential growth [8], with minor  
182 differentiation.

183 The fact that all RGB components were highly correlated supports the idea that a small set of  
184 pigments with similar colors is producing them. Otherwise, one should expect each RGB component  
185 to exhibit its own pattern of variation if there were a wide variety of pigments with different colors,  
186 especially if the pigments were chemically diverse. The literature identifies aurofusarin [24], as  
187 already mentioned, and the carotenoid neurosporaxanthin [25,26] as the major pigments influencing  
188 the surface color of *F. graminearum*. They are both yellow, though slightly different. The former is  
189 frequently described as “golden yellow”, though its hue varies to orange and wine red as it changes  
190 to derivatives [15,17,24,27], and neurosporaxanthin was described as “orange-yellowish” [28], just  
191 like most carotenoids. There are also the polyketide rubrofusarin [15] and the carotenoid torulene  
192 [19], both red but not as abundant as the previously mentioned. There are more pigments but they  
193 have minor influence on the overall color [28] or only during differentiation [29,30]. As bottom-line,  
194 the only pigments actually influencing the color have similar or close-related hue ranging from  
195 golden yellow to wine red. It is worth mentioning that the polyketides (aurofusarin and  
196 rubrofusarin) are highly bioactive and possibly essential part of the competitive saprophytic ability  
197 (CSA) of *F. graminearum* [27,31], the carotenoids are not likely and the latter tend to respond mostly  
198 to light rather than nutrients [18], certainly except in extreme cases of shortage of some nutrient  
199 essential for synthesis of such pigments. Thus, the polyketides, especially aurofusarin, appeared to  
200 be key pigments contributing *F. graminearum* surface color variation in the current experiment.

201 A previous experiment had already shown that all RGB components exhibit similar pattern of  
202 variation, consistent with 3<sup>rd</sup> degree functions [8]. It is not clear why the red component did not  
203 show significant variation ( $\alpha = 0.05$ ) while the other colors did, but it might be related to the nature  
204 of the most abundant pigments [10]. Perhaps red pigments such as rubrofusarin and torulene,  
205 especially the latter, do not change their colors throughout the mold’s lifecycle, contributing to this  
206 “resistance” to change. However, both pigments probably suffer a considerable reduction because  
207 the former is an intermediate of aurofusarin synthesis [32] and the latter is a precursor of  
208 neurosporaxanthin [33]. Yet, both pigments have been found in *F. graminearum* matrix, even when  
209 the others are present [15,33], from which one can imply the existence of chemical equilibrium  
210 between them. In this case, it is still possible that rubrofusarin and torulene contribute to the  
211 endurance of the red component.

212 RGB values were expected to decrease throughout the experiment, especially for blue, followed  
213 by green and finally red. According to a previous experiment [8], this RGB reduction corresponds to  
214 the darkening process as the fungus grows towards the stationary growth phase. It surely does not  
215 apply in the cases in which the fungi were covered with white mycelia because it did not allow the  
216 pigments to be visible. In the cases where the color changed, the variation of RGB components was  
217 possible of representation through polynomial curves, and this was also observed in the  
218 aforementioned experiment.

219 All samples showed overall increased DON concentration over time, not mattering if there was  
220 high mycelial growth or not on the surface. There is some counterintuitive reduction for the samples  
221 grown at  $a_w = 0.94$  and 0.97 between days 4 and 8, but it was likely due to fluctuations in the results.  
222 Indeed, the ANCOVA test ( $p = 0.347$ ) suggested that the differences between the DON  
223 concentrations at different  $a_w$  were not significant. This result contrasts with some found in the  
224 literature showing significant differences between DON concentrations at distinct  $a_w$  [3-5]. Though it  
225 is difficult to know why these results were counterintuitive, it might have been due to chemical,  
226 genetic (distinct strains) or nutritional differences [6]. All other experiments were performed with  
227 irradiated wheat, while the current was carried out with YEA. The latter is highly nutritive [34] and  
228 this perhaps attenuated the stress caused by  $a_w$  differences. Furthermore, Sorensen and Sondergaard  
229 [35] demonstrated that even different yeast extracts influence DON concentration. Anyway, the

230 studies on wheat showed similar trends disregarding  $a_w$ , and it is intuitive that DON tends to  
231 accumulate over time because mycotoxins are very stable and the fungi do not metabolize them  
232 [23,36].

233 DON concentration seems to have similar relationship with all RGB components at  $a_w = 0.94$   
234 and it will facilitate use colors as an alternative to size in DON analysis at this  $a_w$ . This subsidizes the  
235 previous study demonstrating that *F. graminearum* color variation is predictable throughout its life  
236 cycle [8]. There is also evidence that biosynthesis of the pigment aurofusarin is related to DON  
237 production as histone H3 lysine 4 methylation (H3K4me) is crucial in the transcription of genes for  
238 synthesis of both compounds [10]. Yet, the relationship between the pigment and DON still requires  
239 further biochemical and genetic investigation. In any case, as far as it showed, *F. graminearum* surface  
240 color can be used in microbiological studies to predict DON concentration at  $a_w = 0.99$  but it does not  
241 seem practical for lower  $a_w$ .

## 242 5. Conclusion

243 The current experiment suggested that all RGB channels obtained from photos of *F.*  
244 *graminearum* are correlated and can be used to predict DON concentration produced by the fungus at  
245  $a_w = 0.99$ . However, the colors were not effective predictors at  $a_w = 0.97$  and 0.94 because these  
246 conditions appeared to stimulate the production of white mycelia, barely changing in color. Thus,  
247 the results indicate that *F. graminearum* surface color can only be used as predictor of DON  
248 concentration at  $a_w$  as high as 0.99.

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251 **Conflicts of Interest:** The author declares no conflict of interest.

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