 doi:10.20944/preprints201901.0069.v1

Article

RGB Imaging as Tool to Analyze the Growth of *Fusarium graminearum* in Infected Oats (*Avena sativa*) and Rice (*Oryza sativa*)

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Abstract: *Fusarium graminearum* is a cereal pathogen responsible for economic losses worldwide every year. An understanding of its growth is key to control its infection, but current growth models are limited because their size-based approach provides little information about the mold's metabolism. Recently, a RGB (red, green and blue) imaging analysis demonstrated the predictability of *F. graminearum* color change as it grows in yeast extract agar (YEA). This study aimed to verify the same phenomenon in oats (a_w = 0.94, 0.97 and 0.99) and rice (a_w = 0.97, 0.98 and 0.99). Photos were taken using a professional camera and a smartphone (iPhone 6) after incubation and during the subsequent 16 days, and average RGB was quantified using ImageJ software. The photos showed very similar color variations, regardless of the type of grain or a_w. The mold first adopted a *k*-selection strategy by growing as a mycelium and then a *r*-selection strategy, increasing spore production. All RGB channels showed positive Pearson correlations between them ($p < 0.001$) and it was possible to design a model showing two lag phases, the first prior to a mycelial phase and the second prior to a sporular phase at the end of the experiment.

Keywords: *Fusarium graminearum*, RGB, oats, rice, growth

1. Introduction

Fusarium spp. are the most common pathogens for many cereal crops, causing ear rot or head blight diseases and mycotoxin contamination [1,2]. For the case of *F. graminearum*, its host range includes oats, rice, wheat, barley and other plants in which they do not cause disease [2]. There have been recent outbreaks of *Fusarium* head blight (FHB) in several areas including Asia, Canada, United States, Europe and South America, and the disease has been expanding worldwide [3]. In the United States, FHB has caused losses of around US$2.7 billion from 1998 to 2000, and it still reduces considerably the yield and quality of cereals, forcing the reduction of prices [4,5]. In Japan, there were several outbreaks during the 20th century, including a record in 1998 of rice with deoxynivalenol (DON), a toxin associated with the disease, just after a typhoon [6]. The Japanese Government has established a provisional limit for DON in grains and derivatives to 1 µg/g.

To better understand how *F. graminearum* propagates, it is important to analyze its growth pattern. According to Garcia, *et al.* [7], the most commonly used growth models for fungi are "imported" from bacterial studies, namely the linear model and the ones developed by Gompertz and Baranyi. For molds, these models use their spatial expansion (e.g. radius or diameter) as a growth measure assuming that the organism matures as it becomes larger, by the same logic used for plant animals and other types and organisms. However, this approach has several limitations because it provides little information about the organism's metabolism and it may result in

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misinterpretations if the mold grows irregularly or if the mold attains the maximum size in a closed system [8,9]. A viable alternative to this issue is digital imaging combined with statistics, an approach nowadays gaining relevance in studies of FHB because of their potential to monitor the extent and severity of *Fusarium* infection in grains [10,11]. For instance, some authors utilized RGB (red, green and blue) imaging to distinguish wheat with symptoms of FHB from apparently healthy grains [10-12]. Furthermore, these methods require computers and digital color cameras, the latter described by Dammer, *et al.* [11] as "cheap, small and lightweight".

When *F. graminearum* is grown in yeast extract agar in a Petri dish, the so-called stationary phase starts when it reaches the plate's border and stops expanding, keeping the same size until it eventually depletes the nutrients and its spores endure in a state of dormancy [8,13]. However, phases like lag and stationary, represented by size-based models as apparently less active, are exactly the circumstances at which the fungus has to employ the highest effort to ensure its survival, in the former because the organism needs to adapt to a new medium and in the latter because the medium becomes exhaust. Besides the well-known fact that molds produce secondary metabolites during the stationary phase [14], a previous experiment demonstrated that *F. graminearum* keeps changing its surface color (and the color of the agar medium) even during the stationary phase [8]. Change in pigmentation is an indicator of active metabolism [13], and it means that the fungus is indeed substantially active, even when there is no more expansion in size. Thus, a color-based growth model might provide valuable information the pattern of the mold's maturation reflecting its variation in metabolic activity.

The predictability of color change in *F. graminearum* as it grows as already been demonstrated in yeast extract agar [8] but not yet in food matrices. Therefore, the current study aims to verify if oats and rice infected with *F. graminearum* present predictable patterns of color variation if incubated for 16 days at different a_w settings. Awareness about such patterns will allow farmers, researchers and other personnel working in agricultural fields to accurately analyze the mold's growth with better awareness about its state of maturity and possibly associate it with particular metabolic activity according to the stage of its lifecycle.

2. Materials and Methods

2.1. Mold Isolate

This study used an *F. graminearum* isolate from the Catalogue of the Japan Collection of Microorganisms (JCM). It is registered as the teleomorph *Gibberella zeae* (Schwabe) Petch, strain TH-5, isolated by Sugiura [16] from rice stubble in Hirosaki, Aomori Prefecture, Japan. It is a known producer of deoxynivalenol, 15-acetyldeoxinivalenol, and zearalenone [17].

2.2. Grain Samples

All samples of raw oats (*Avena sativa*) and rice (*Oryza sativa*) were obtained locally in Hokkaido. Oats were purchased from a local store, all sealed and dry, with husk in plastic bags, each with 250g. The rice was obtained from local producers, also dry and in plastic bags with 500g each, 90% with husk and the remaining brown. All grains with husk showed the ability to germinate. Thus, they were autoclaved to inactivate the embryos and eliminate possible microbial contamination. After autoclaving, the color did not seem to change considerably. For the current experiment, nine samples of oats and nine of rice, all containing 25g, were placed in Petri dishes. Water activity was empirically set using distilled water, and for each grain the range started with the lowest value at which growth was observed. The cereals were split into three groups of three replicates each. For oats, groups had a_w = 0.94, 0.97 and 0.99. For rice, 0.97, 0.98 and 0.99.

2.3. Incubation and RGB Determination

F. graminearum was inoculated in the Petri dishes and left at room temperature (fluctuating between 18 °C and 26 °C). Including the moment just after the inoculation, the fungi were photographed daily for 17 days in a black bucket, vertically from 30 cm above. The cameras were

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Nikon D3200 with a lens *DX SWM VR* (Nikon Corporation, Tokyo, Japan) and an iPhone 6 (Apple Inc., Cupertino, California, USA). The only source of light was a round LED attached to the bucket's lid. The photos were then processed on the *ImageJ* software (*FIJI* edition, National Institutes of Health, Bethesda, MD, USA), developed by the National Institutes of Health [15] using the method described by Cambaza, *et al.* [8]. ImageJ allowed the determination of average intensities of the RGB components from the photos. At the end, the variables to analyze were incubation time (in days) and RGB parameters, converted from the 8-bit notation (0 – 255) to the arithmetic index (0.0 – 1.0).

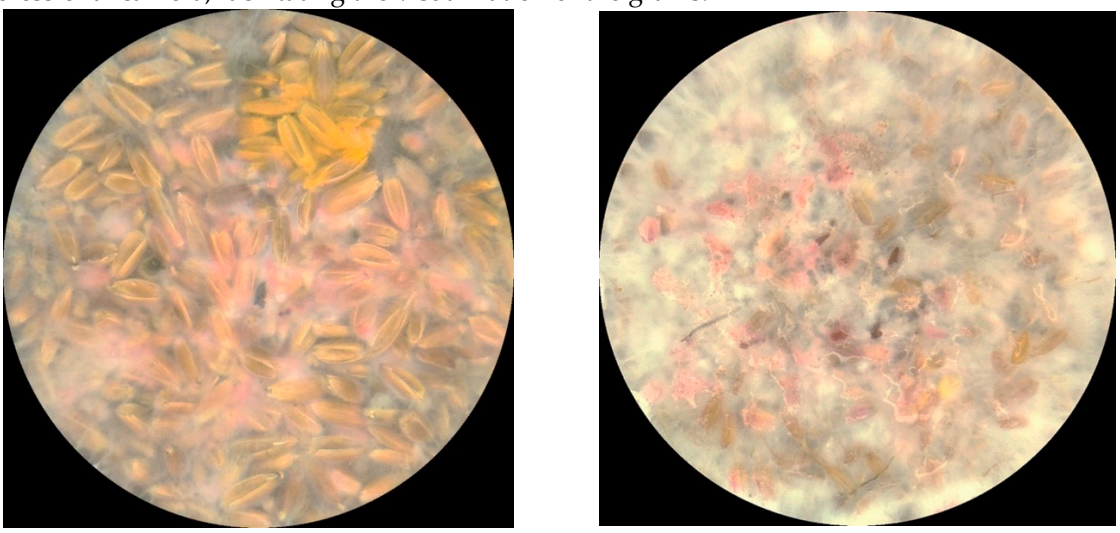
2.4. Statistical Analysis and Model Design

The statistical analysis was performed on JASP 0.9 (The JASP Team, Amsterdam, The Netherlands), Jamovi 0.9 (Jamovi Project, Amsterdam, The Netherlands) and Microsoft Excel (Version 14.5.8, Microsoft, Redmond, Washington, WA, USA). All the hypotheses tested were carried out with $\alpha = 0.05$. The distribution of intensities of red, green, and blue were compared through analysis of covariance (ANCOVA) to find if their differences were significant. After that, some plots illustrated the variation of the RGB components at distinct a_w through 16 days. In the end, Adobe Photoshop CC 2018 (Adobe Inc., San Jose, California, U.S.) was used to design a proposed RGB-based growth model for *F. graminearum* in grains.

3. Results and Discussion

3.1. Qualitative Description

All photos were successfully taken during the 16 consecutive days. For the sake of comprehensiveness on how heavily infected grains appear, this report shows first photos taken using a smartphone presenting the grains on the final phase of infection of the present experiment (Figure 1). The smartphone seemed to present photos much brighter than the ones taken using the professional camera, facilitating the visualization of the grains.



(a) (b)

Figure 1. Contaminated (a) oats at $a_w = 0.97$ and (b) rice at $a_w = 0.98$ with *F. graminearum* after 16 days, taken from a smartphone. The contrast was reduced in 40% to facilitate the visualization.

As the photos show, heavily contaminated grains were involved in whitish mycelium and it was possible to notice some pigmentation (more easily noticeable in the photo of oat grains because the rice presents higher density of the white mycelium). There are three pigment colors: orange-yellow, pink and black. Colors such as pink to salmon-orange are frequently found in infected spikes, and they result from the formation of spore masses in prolonged periods of high humidity [2]. The photos do not show very well but even more extreme cases of contamination presented more clearly several dark dots, perhaps black perithecia [16] or, in some cases, necrotic

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lesions, described by Goswami and Kistler [2] as "brown, dark purple to black". The abundance of white and pinkish colors was expected as they are common visual features in infected kernels [2,10].

The reminder of this study will be based on the photos taken through the professional camera because they significantly differ from the ones taken through the smartphone, as paired t-tests between the images acquired through both devices just at the end of the experiment showed, with p-value consistently below 0.001 (Table 1).

Table 1. Differences between RGB measurements of the photos F. graminearum grown in the cereal grains after 16 days.

Commodity	Color	$p_{t\text{-test}}$	Device	N	Mean	Median	SD	SE
Oats	R	< 0.001	Camera	9	0.16	0.16	0.02	0.01
			Smartphone	9	0.92	0.90	0.08	0.03
	G	< 0.001	Camera	9	0.12	0.12	0.02	0.01
			Smartphone	9	0.80	0.79	0.03	0.01
	B	< 0.001	Camera	9	0.01	0.01	0.01	0.00
			Smartphone	9	0.52	0.54	0.10	0.03
Rice	R	< 0.001	Camera	9	0.20	0.20	0.03	0.01
			Smartphone	9	0.92	0.91	0.03	0.01
	G	< 0.001	Camera	9	0.18	0.19	0.04	0.01
			Smartphone	9	0.87	0.86	0.02	0.01
	B	< 0.001	Camera	9	0.12	0.16	0.08	0.03
			Smartphone	9	0.66	0.71	0.10	0.03

N - sample size; SD - standard deviation; SE - standard error

Even though the number of samples was considerably small per comparison (N = 9), the average and median RGB values between the devices presented very different orders of magnitude, and both standard deviation and standard error are small if compared to the measures of central tendency. There is a considerable body of research demonstrating the potential of smartphones for scientific enquiries [17-19] but for studies like the present they would require validation and it seems to be a good idea for a near future considering how widespread they are among common citizens.

All infected grains exhibited similar patterns of variation, regardless of the type of grain or a_w at which it was subjected (Figure 2 – oats; Figure 3 – rice). It seemed easier to see the color variation in oats in relation to rice because the grains were brighter and presented less contrast through the experiment but both showed similar behavior. All started relatively bright and darkened up to the 15th day. There was a notable reduction in brightness between the 1st and 2nd day, followed by a slight process of darkening with fluctuations up to the 8th day, when brightness declined substantially and the grains exhibited dark brown shades. This shade prevailed with some fluctuations up to the end of the experiment.

Part of the color change observed seems very consistent with the well-known models described by Garcia, *et al.* [7], presenting the lag, log and stationary phases. It has been also observed in a study from which the current derived [8]. The first day comprises the mold's adaptation to the new medium (lag). Just after that, it expands in form of the whitish mycelium frequently described in the literature [10], corresponding to the exponential growth. This explains the increased paleness of the grains, initially with very live brown color.

The mycelium expands as much as it can exponentially, maximizing its surface for nutrient uptake. After reaching its maximum expansion and facing stress, possibly caused by access to nutrients (the grain's resistant husk certainly contributes to such condition), on the 8th day, the mold starts producing spores to ensure its survival. *F. graminearum* has high competitive saprophytic ability (CSA) [2] and the rapid production of spores, and also possibly the toxin synthesis contribute for this aptitude.

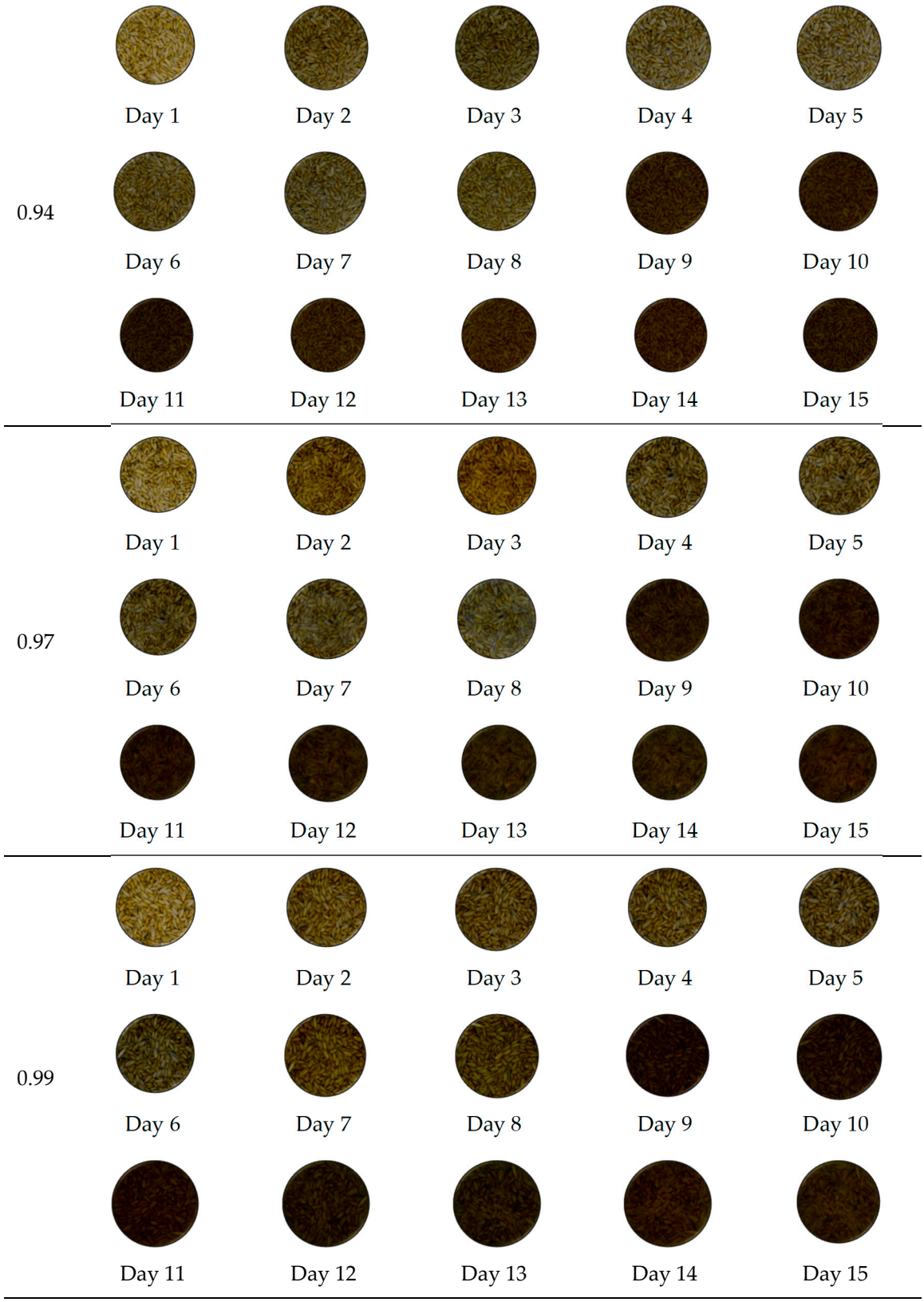

Figure 2. Color variation of oats contaminated with *F. graminearum* at different a_w during 15 days.

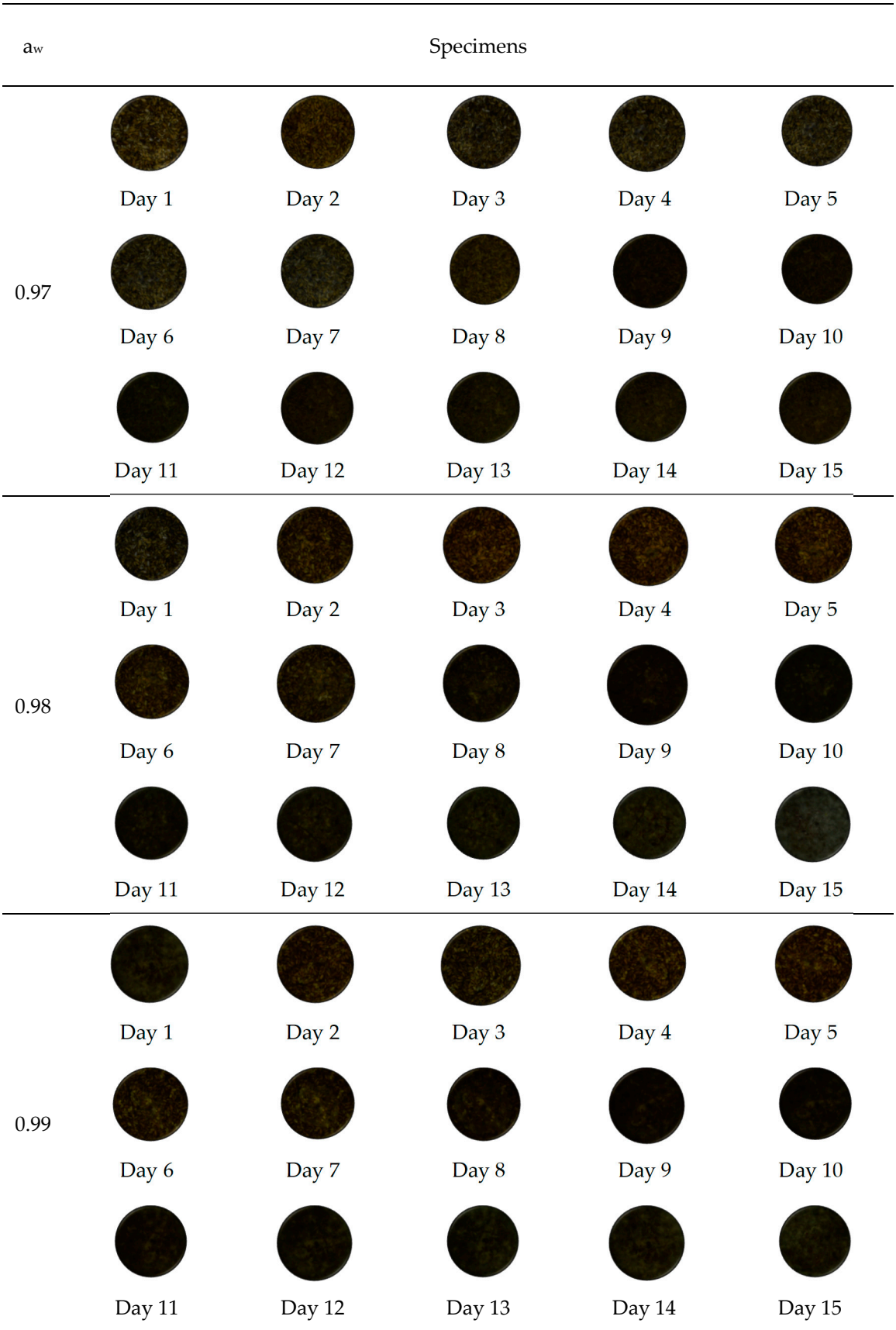

Figure 3. Color variation of rice contaminated with *F. graminearum* at different a_w during 15 days.

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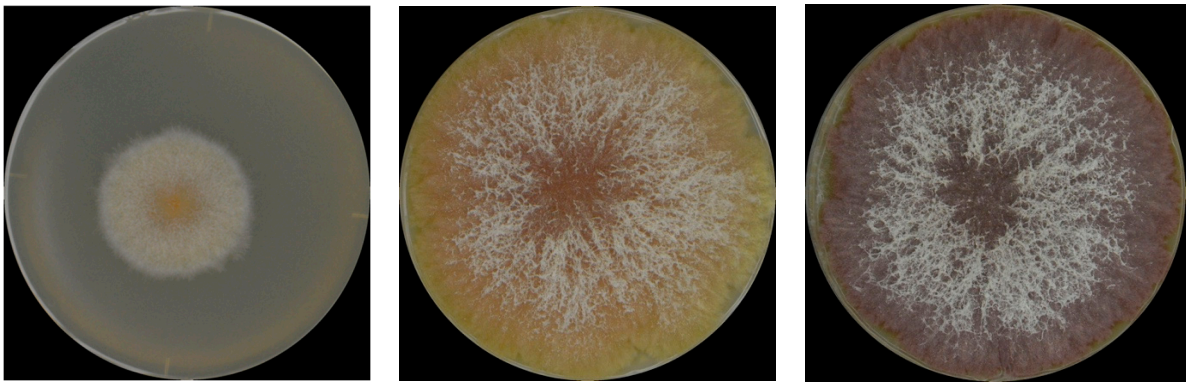
Spores can explain why the color declines in paleness between days 8 and 9. This change corresponds to the beginning of the stationary phase, when part of the mold enters in dormancy. There are remarkable differences between this growth pattern and the one observed in the previous study using yeast extract agar (Figure 4) [8]: (1) the easiest to notice are perhaps the shape and colors of the fungus, more pronounced in specimens grown agar; (2) the grains initially became pale due to the increasingly dense mycelial network, while the specimens grown in agar expanded on the surface forming concentric layers of contrasting colors (3) though the lag phase took also a single day, in agar the exponential phase took longer (seemingly up to the 14th day) and the transition to the stationary phase was smoother.



(a) (b) (c)

Figure 4. *F. graminearum* grown in yeast extract agar after (a) 4 days, (b) 8 days and (c) 12 days. These photos were taken for previous experiments [8,9,20].

The differences in growth strategy can be explained through the concept of *r* and *k* selection discussed in detail by Taylor, *et al.* [21], but it is first important to understand the differences between cereals and agar as media. The major difference is perhaps the fact that agar has nutrients readily accessible to the mold while grains have husk as a polysaccharide barrier [22]. Fungi growing on cereals opted by a *k*-strategy, in which the organism becomes stronger and more capable to face adversity by itself, rather than producing a high quantity of conidia. In the current experiment, *F. graminearum* maintained a mature form (mycelium) in order to create structures such as haustoria, able to penetrate the kernels [14]. Moreover, mycelial growth also provides the fungus the possibility to search for cracks or other openings from which it can invade the grains. This can explain its increased density, resulting in the observed paleness. In agar, the specimens adopted an *r*-strategy, producing high quantity of conidia. It might seem counterintuitive given the circumstances, because the mold has high availability of nutrients and no need to propagate, but in the field abundance of nutrients provides the fungus an opportunity to disperse the spores so that they can try to colonize more habitats around. The high quantity of spores maximizes the possibility that at least some are able to thrive. Assuming the veracity of the *r* and *k* theory, once the fungus is able to penetrate the kernels, it rapidly shifts strategy to *r* and starts producing spores. This phenomenon can explain the sudden reduction in paleness between the 8th and 9th day up to the end of the experiment. In the end, there is a mixture of spores and mycelium all around the grains.

Another difference to examine is the relatively wider surface area in cereals due to their granular nature. Wider space implies more space for the fungus to expand and for that mycelial growth is intuitively better than spore production. It is important to know that spores do not move by themselves, except the zoospores of chytridiomycota, oomycote and slime molds [14]. In the field, wind, insects, water and other agents can spread them [2], but not in a Petri dish. *F. graminearum* never seemed to "dive" into the layer of agar. Instead, it grows on the surface and remains there, certainly absorbing the nutrients until the agar runs dry. It perhaps uses the main mechanism in grains, involving all but never going beyond the surface. These facts, and the high quantity of available nutrients, can explain the presence of a bulky organism growing on the surface of yeast extract agar while the same opts to rather grow in form of disperse hyphae when in grains.

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One final aspect to consider is the homogeneous distribution of water and nutrients in agar. Regarding grains, each has a hull, a layered bran, endosperm and embryo, besides the fact that individual grains differ in size and weight. Moreover, their spatial disposition or superposition is also likely to affect the overall distribution of nutrients. Thus, there is surely a remarkable difference between the nutrient homogeneity of cereals and agar. A major feature of mycelial fungi is their tendency to grow towards areas with the highest concentration of nutrients [14]. This is perhaps one of the reasons why *F. graminearum* presented a radial growth (in ring-like concentric layers) in agar and an apparently irregular pattern in the grains.

In conclusion of this sub-section, since the color of infected grains changed accurately over time in exactly the same fashion, even though the experiment was carried in two different types of cereals and distinct water activity, it is so far reasonable to assume the existence of a pattern of color change and thus, once again the color seems fit as a reliable approach to analyze the growth or state of maturity of *F. graminearum*. Through observation, it was possible to visualize the lag phase (very bright grains), exponential growth (where the grains became pale) and the stationary phase (when the grains became darker and with a less pale tone). Furthermore, the onset of the phases had exactly the same timing.

3.2. RGB Analysis

In general, throughout the experiment oats presented balanced predominance of all three RGB components, all showing positive skewness, while rice exhibited almost neutral skewness for red and green and positive for blue. For both oats and rice, all colors were significantly correlated ($p < 0.001$), exhibiting Pearson coefficients above 0.5 (Figure 5). It means that all colors presented a positive linear relationship. Oats presented the highest correlations (none below 0.9) for all RGB components, though the pattern of correlations between pairs of color components was similar for both cereals: red and green presented the highest values, followed by green and blue. Such high correlations between all RGB components, and even the relative extent of differences between pairs of the RGB components, have been consistently found in previous studies [8,9,20] and are likely due to the fact that very few pigments (aurofusarin, rubrofusarin and carotenoids) contribute for the overall colors of *F. graminearum* and their colors do not differ substantially, all ranging from orange-yellow to wine-red [13,20]. The dark perithecia do not contribute as much as the other pigments for the overall color [13].

Table 2 shows how much the distinct a_w settings differed in terms of colors. All RGB components presented highly significant differences ($p < 0.001$ for most, 0.003 for the B channel in rice), though the visual observation did not provide such impression. This is one more reason why it might not be prudent to rely solely in visual evaluation of kernels. According to Jirsa and Polišenská [10], sometimes differences can hardly be noticeable with the naked eye and digital imaging combined with statistical methods can perform this task more accurately.

The major differences occurred between specimens incubated at $a_w = 0.99$ and the others, except for the case of blue component measurements in rice (it can also be noticed through the difference in skewness in Figure 5b, where the specimens grown at $a_w = 0.98$ were the ones with significant differences in relation to the others. The most frequent pattern of differences will be examined in detail first and then the case of the blue component in rice. It is also important to bear in mind that a_w ranged from 0.94 to 0.99 in oats, with 0.97 as the intermediate value recorded, while in rice the interval was narrower (0.97 to 0.99, with 0.98 as intermediate).

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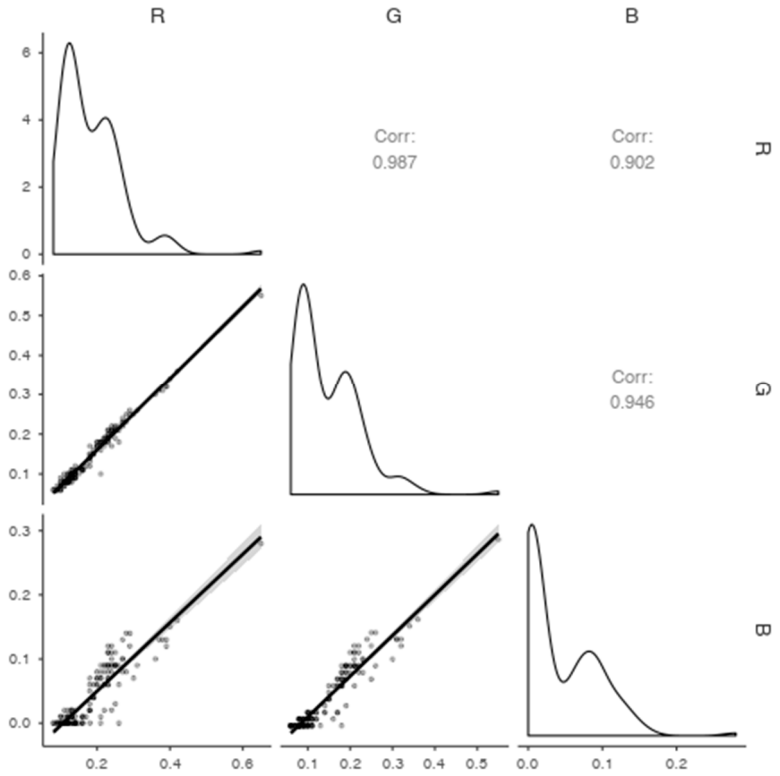
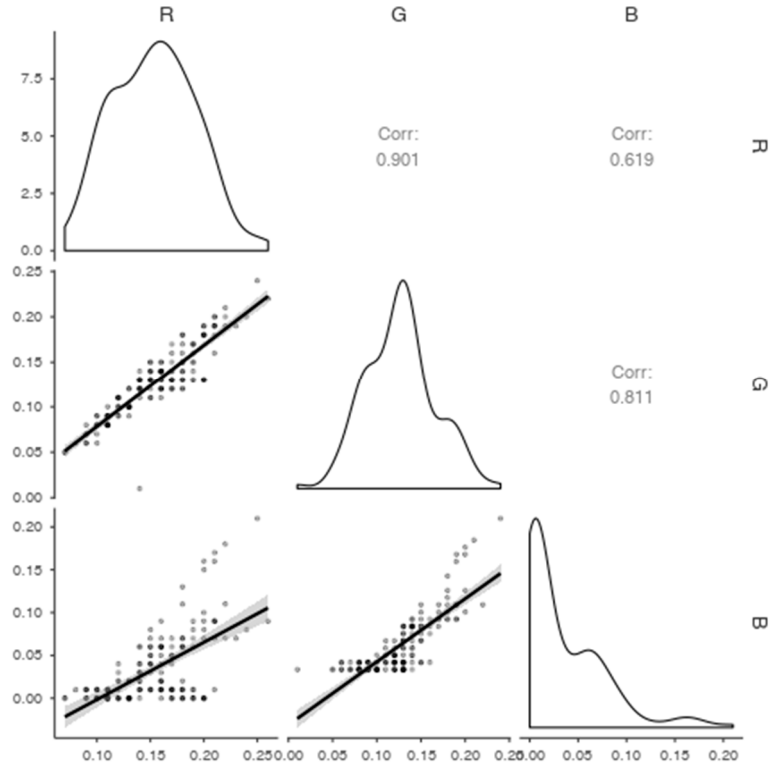


(a)



(b)

Figure 5. Correlations between the RGB components of *F. graminearum* grown in (a) oats and (b) rice.

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Table 2. RGB differences between the infected grains at different a_w.

Commodity	Color	p_{ANCOVA}	a_w		Post Hoc Comparisons				
					MD	SE	df	t	p_{Tukey}
Oats	R	< 0.001	0.94	0.97	0.01	0.01	102	2.64	0.026
				0.99	0.03	0.01	102	6.37	< .001
			0.97	0.99	0.02	0.01	102	3.72	< .001
	G	< 0.001	0.94	0.97	0.01	< 0.01	102	1.95	0.130
				0.99	0.03	< 0.01	102	6.55	< .001
			0.97	0.99	0.02	< 0.01	102	4.6	< .001
	B	< 0.001	0.94	0.97	0.00	< 0.01	102	0.88	0.649
				0.99	0.02	< 0.01	102	5.28	< .001
			0.97	0.99	0.02	< 0.01	102	4.39	< .001
Rice	R	< 0.001	0.97	0.98	0.00	< 0.01	102	1.65	0.230
				0.99	0.02	< 0.01	102	5.77	< .001
			0.98	0.99	0.01	< 0.01	102	4.12	< .001
	G	< 0.001	0.97	0.98	1.96e-4	< 0.01	102	0.06	0.998
				0.99	0.02	< 0.01	102	4.68	< .001
			0.98	0.99	0.02	< 0.01	102	4.62	< .001
	B	0.003	0.97	0.98	-0.01	< 0.01	102	-2.41	0.046
				0.99	0.00	< 0.01	102	0.95	0.609
			0.98	0.99	0.01	< 0.01	102	3.36	0.003

MD - mean difference; SE - standard error; df - degrees of freedom

An article recently published about RGB variation of *F. graminearum* grown in agar [20] provides a valuable hint on what might have happened, as the authors found exactly the same pattern of differences (Figure 6). Indeed, the experiment on agar stressed the color differences in such extent that RGB imaging analysis did not initially seem adequate to predict the mold's growth and toxin production at a_w below 0.99.

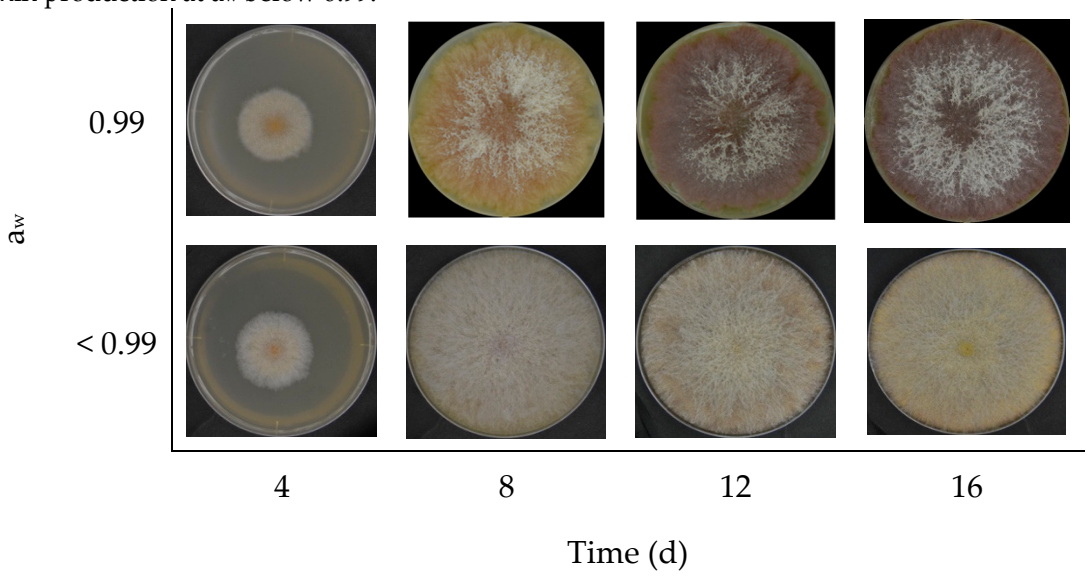

Figure 6. *F. graminearum* growth strategies at a_w = 0.99 and below, as observed in a previous experiment [20]. Note: the mold did not show any growth at a_w < 0.94.

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When grown at a_w = 0.99, *F. graminearum* behaved exactly as it has been already described in the current report, but at lower a_w the mold produces high volume of white mycelium, completely covering the surface of the medium, thus providing a barrier for the visualization of the pigments.

A major distinctive feature of the kingdom Fungi is the organisms' extracellular digestion and nutrient uptake through pinocytosis, and such process makes fungi highly dependent on water [14]. Considerably low a_w might have led the mold to prioritize the *k*-selection survival strategy by growing an increased quantity of white hyphae, thus enhancing its CSA. When in grains, the mycelia did not produce as drastic color variation perhaps because of the wider surface the cereals provided if compared to the same weight of agar.

The case of blue color in rice is unique, considering that it has not happened in oats, agar, and also not with the other channels, even though they are correlated. Another noticeable fact is the p-value (0.003) of the differences in blue component in the specimens incubated in rice, based in an analysis of covariance (ANCOVA), higher than similar calculations for other colors and in oats, implying the existence of some abnormality large enough to differ from the others. Furthermore, this group of measurements of the blue component showed the lowest correlations with red and green, even when compared with results from previous experiments [8,9,20].

A plausible explanation can be the abundance of dark perithecia or necrotic lesions in the specimens grown in rice at a_w = 0.99, particularly at the end of the experiment. These pigments are frequently described as "dark blue, violet, or purple" [13], likely affecting the blue channel more than the others. The higher mycelial density in rice samples in relation to oats is perhaps related to the presence of dehusked rice among the grains, allowing *F. graminearum* to more readily access amylose, proteins and other easily digestible nutrients. Goswami and Kistler [2] said that high moisture and favorable conditions promote the development and maturation of conidia and perithecia.

For the following analysis of RGB variation in the grains it is important to highlight the fact that the specimens described so far as photographed on the first day correspond to the initial time just after inoculation, i.e., time = 0 d (days). Thus, day 2 corresponds to time = 1 d (after 1 day of incubation) and so on. It implies, for instance, that observations between days 8 and 9 correspond to observations in the interval $t \in [7\ d; 8\ d]$. It also means that the only photos of the last day are the ones in the Figure 1 (the ones taken from a smartphone). The photos from day 17 were accounted in the daily analysis, though they are not in Figure 2 and Figure 3, and it did not seem to be a problem because the photos presented were enough to demonstrate the predictability of *F. graminearum* color change in infected grains through visual observation. Furthermore, any particularity of the photos lacking will be explained as accurately as necessary.

Also, the same names of the phases as described in size based growth models through the literature will be employed to the sake of comprehension and comparison with the current state of knowledge. The names are lag, log (or exponential) and stationary phases [23-25]. The phase of senescence or death is not mentioned because the fungus did not die until the end of the experiment and even in nature *F. graminearum* can easily thrive under extreme stress as saprophyte [2]. However, new names will be proposed because they will more accurately reflect the observation. It is important to have in mind that growth should not be seen simply as spatial expansion of an organism, but rather a combination of physiological changes. In any case, it is more prudent to observe first what happens in oats and rice and then propose the names.

All RGB values in oats showed an overall tendency to decrease, regardless of a_w (Figure 7). Variations seemed more consistent among the values of the red component, followed by green and finally blue, where the discrepancies between the specimens at different aw seemed more evident. Yet, the overall shapes of all graphs are remarkably similar, and it confirms the existence of a pattern of *F. graminearum* color variation as previously demonstrated by cultivating the fungus in yeast extract agar [8]. The current observation adds the fact that this phenomenon also occurs in oats and across a_w (within the range where growth is possible).

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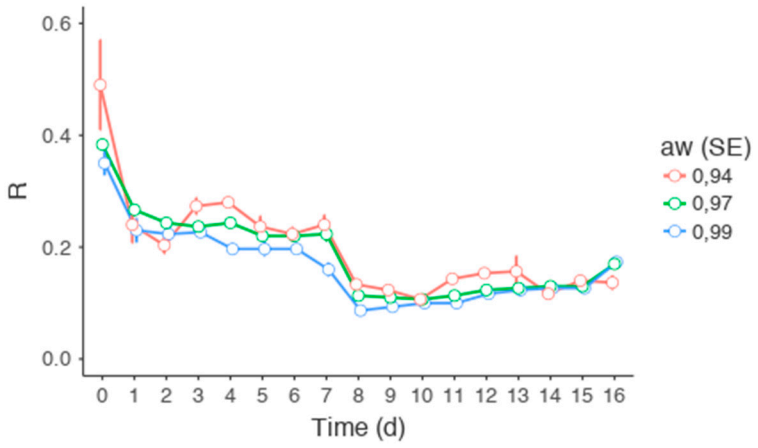

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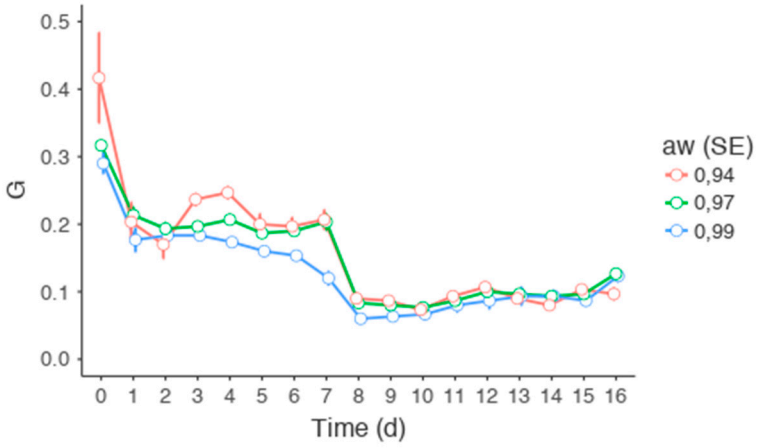
317 (a)



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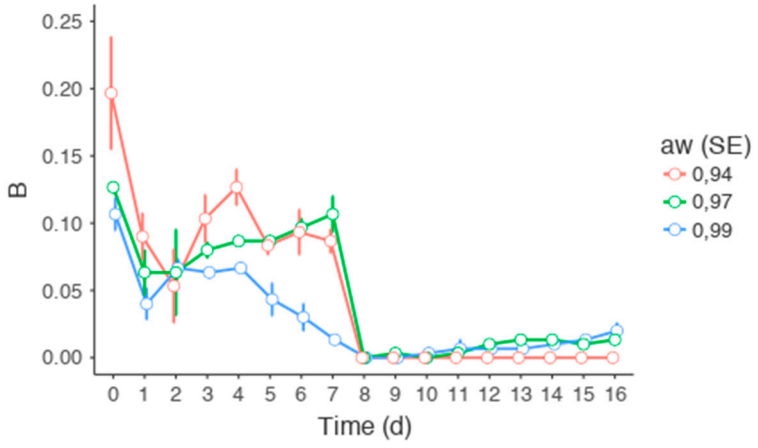

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321 (c)

322 Figure 7. Variations of RGB components in oats infected with *F. graminearum* during 16 days.

323 The RGB-based growth curves for *F. graminearum* in oats seemed to decline in color intensity,
324 and three growth phases can also be distinguished, exactly during the same periods, regardless of
325 the color component and a_w. They accurately reflect the information grasped through visual

analyses. The lag phase lasted for one day, where all components declined remarkably in intensity. The exponential phase comprised the following six days, at which the quantity of pigments was mostly constant, yet showing some fluctuations. This was the phase of dominance of the mycelial paleness. The transition for the following phase lasted one day at which the color intensity further declined. During the stationary phase, it was possible to observe a slight trend for all color components to increase and this was due to spore production and the consequent accumulation of pigments [2,10].

The transitions between the phases seemed smoother as a_w increased, perhaps for the reason already mentioned, based on the experiment in yeast extract agar [20]: water-borne stress led the fungus to rapidly expand the mycelia through the medium, thus affecting more drastically the color. An interesting remark is that even though the colors were initially different because of the distinct levels of humidity, in the end they all seemed to converge, especially from the 8th day until the end of the experiment. This fact suggests that *F. graminearum* shapes its microenvironment or substrate according to its needs up to a point at which it presents the same characteristics, independently of the initial state. Several molds are known to affect the colors of grains [26-28] and in the case of the current experiment the grains acquired very similar colors after 8 to 10 days. This process has been also observed in agar and Dufosse, *et al.* [29] mentioned that *Fusarium* usually releases pigments in the medium.

In rice (Figure 8), the trends presented some similarities and differences in relation to oats, but the basic shape prevails: the color declines in the 1st day, except the red component at a_w = 0.98 and 0.99 (they virtually did not change much), and the green component under the same treatments declined just a little; subsequently it fluctuates around a certain range of values for around 6 days, then it quickly declines for approximately one day and finally rises. Unlike in oats, there was an exponential growth of all components in the last day of the experiment, except for the blue component at aw = 0.97. This rapid RGB increase in intensity would likely have happened oats too if the experiment was carried for longer, probably for only one more day. It corresponds to a phase at which the grains are completely covered with white mycelium as Figure 1b shows. It is reasonable to believe that Figure 1a represents just one step behind, and the rice samples showed more rapid increase in color intensity from day 8 until the end of the experiment. The variations are also more extreme as a_w decreases, around the second half (perhaps from the 7th to 9th day) the trends tend to become similar for the different color components. However, blue was different (as already seen and explained) and now it is possible to see that the samples incubated at a_w = 0.97 exhibited a pattern of variation of the blue component more similar to what happened in oats in relation to the other colors in rice: after the second decline, the intensity grew very smoothly, almost constant. The new observation suggests an idea opposed to what was previously postulated as reason for the difference about the blue component in specimens grown in rice: there was considerably less quantity of perithecia and necrotic spots at this condition but there was high production of conidia (noticeable through the increase of the red component and perhaps green). Indeed, *F. graminearum* requires high humidity to start producing perithecia and this is one of the reasons why maturation of such structures occurs during spring [2].

Differently from the infected oats, rice showed smoother levels of decline in colors, possibly because of the already mentioned existence of some dehulled grains and consequent less stress for the mold to acquire the nutrients. The presence of dehulled grain should also be expected to affect the dynamics of water distribution throughout the medium [30,31], because the cellulose barrier certainly affected the speed at which the grains absorbed the water. Thus, the fungus did not have to employ as much effort as in oats to assure its survival and it was certainly much easier to modify the microenvironment to suit its needs, as part of the medium was already ideal for growth. For instance, in yeast extract agar, *F. graminearum* started producing spores from the 3rd day of incubation [8] instead of the 7th or 8th observed in the current experiment. Thus, one can conclude from these observations that nutrient availability favors smoother color transitions between different growth phases.

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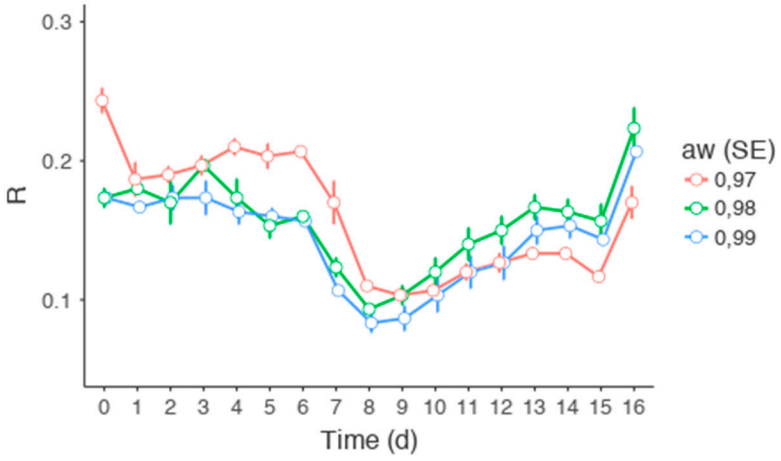

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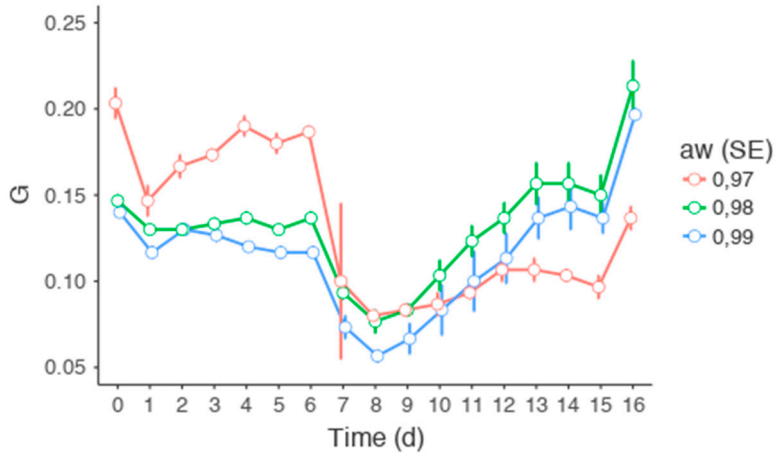

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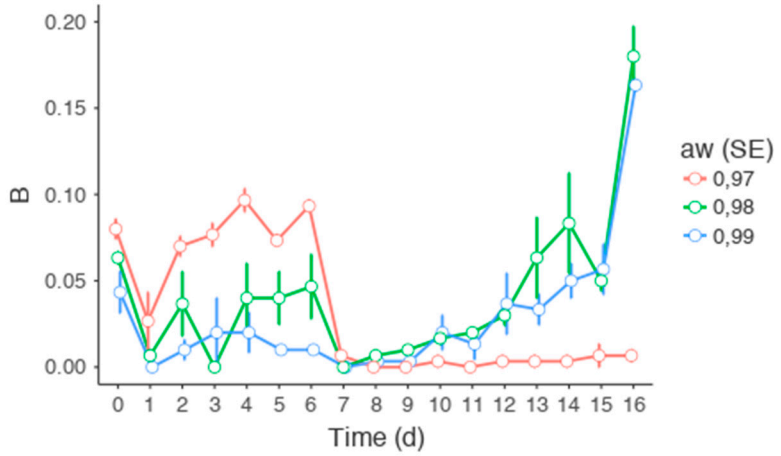

(c)

Figure 8. Variations of RGB components in rice infected with *F. graminearum* during 16 days.

By observing RGB changes in both oats and rice, some features became evident: as a_w decreases, the RGB components tend to fluctuate more and the graph presents a wider codomain (range of ordinates); the blue component presents more fluctuations, possible due to the fact that its values are

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frequently low in relation to the others and thus more susceptible to noise or slight variations; the specimens at the lowest aw always start with higher color intensity in relation to the others but then it equalizes to the others or become lower; the two highest a_w conditions presented almost the same behavior throughout the entire experiment.

Considering what is known about microbial growth models and the observations from the current study plus the ones in yeast extract agar [8,9,20], the authors propose the model showed in Figure 9 to represent the growth of *F. graminearum* regardless of a_w, based on the variation of the RGB components and stressing the alternation of the *k* and *r* selection strategies as response to changes in the micro-environmental settings.

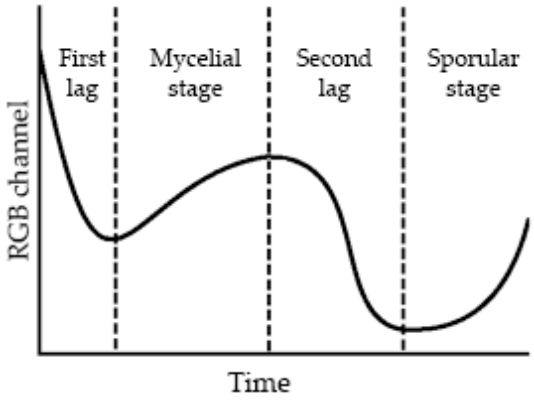

Figure 9. *F. graminearum* growth curve based on RGB measurements of contaminated cereals.

Unlike the models widely used to describe bacterial growth [23-25,32,33] and "borrowed" to explain the pattern of fungal growth [7], the current presents two lag phases, one before a mycelial phase and other before a sporular phase. Also, contrasting the stationary-like lag phases observed in size-based growth models, these lag phases present a decrease in coloration: the first time because the mycelium forms a transparent layer masking the "shiny" grains, and the second time because the mature hyphae initiate the production of spores, also reducing the color intensity established by the mycelia. Just after the first lag (logarithmic negative), the paleness of hyphae restores the intensity of all colors as the mycelial network becomes denser (mycelial phase, logistic). After the second lag (logistic with negative coefficient of the abscissa), color intensity increases as yellow and pink pigments spread through the media, and the mycelia attain their maximum thickness (sporular phase, exponential). The model presents two valleys, each after a respective lag. They could be named valleys of re-adaptation (first and second), as these are circumstances in which a particular selection strategy (*r* or *k*) starts to dominate the microenvironment.

4. Conclusions

The current report is the follow-up and of the study on RGB imaging as a tool to analyze *F. graminearum* growth as an alternative to size-based approaches. The first was performed using yeast extract agar and demonstrated the predictability of the mold's variation of the RGB channels through digital photos taken in successive days using a professional camera and a smartphone. The current study validated the idea for cases in which the fungus grows in oats and rice. Furthermore, it was performed at distinct a_w settings. Thus, the visual analysis of digital photos, so as statistical calculations and plotting confirmed the existence of a growth pattern through time when *F. graminearum* infects oats and rice, and such pattern occurs across the a_w settings at which the fungus can grow in a fairly similar fashion for both grains. The mold starts the colonization of the grains using a *k*-selection strategy, at which the fungus quickly develops a mycelium to endure in the new medium, and later a *r*-selection strategy, comprising high sporulation that in field would ensure its propagation to new habitats or simply a dormant survival for long periods. From the observed pattern, a 4-phased model was proposed, containing a mycelial and a sporular phases preceded by lags or phases of adaptation.

Author Contributions: Conceptualization, Methodology, Formal Analysis and Writing-Original Draft Preparation, Edgar Cambaza; Supervision, Shigenobu Koseki & Shuso Kawamura.

Funding: The Japanese Ministry of Education, Culture, Sports, Science and Technology (MEXT) funded this research.

Conflicts of Interest: The authors declare no conflict of interest.

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