Article

The Use of Colors as an Alternative to Size in Mold Growth Studies

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Abstract: Size-based fungal growth studies have limitations. For example, the growth in size stops in closed systems once it reaches the borders and poorly describes the metabolic status, especially in the stationary phase. This might lead mycotoxin studies to unrealistic results. Color change could be a viable alternative as pigments are results of the mold's metabolic activity. This study aimed to verify the possibility of using gray values and the RGB system to analyze the growth of *Fusarium graminearum*. It consisted color and area measurement using the ImageJ software for specimens grown in yeast extract agar (YEA). The results suggest the usability of color and gray values as reliable tools to analyze the growth of *F. graminearum*.

Keywords: *Fusarium graminearum*, mycelial growth, RGB, gray scale.

1. Introduction

Mycelial size is widely regarded as the "golden standard" for mold growth studies [1], regardless if it focuses radius, diameter, perimeter or area. However, this approach has some drawbacks: (1) molds theoretically do not stop growing if the conditions allow, (2) the size do not tell much about the metabolism, especially in the field, (3) in closed systems, the growth in size is limited by the container and the mold takes its shape, (4) it is vulnerable to biotic or abiotic factors without explaining much about the metabolic variations, (5) it hardly describes noncircular or irregular shapes and (6) usually overlooks the volume.

A constraint occurs when the mold is grown in Petri dishes. Radius and diameter are very effective growth predictors during the lag and exponential phases. This explains how Marin, *et al.* [2] and many other authors have been using size in their studies for many years. However, as the fungus reaches the plate's border, it slows down the expansion and increases it's thickness. It does not necessarily stop growing, probably misleading some interpretations. Indeed, Deacon [3] says most of the secondary metabolism happens during this period. Thus, there is a need for viable alternatives.

Color is probably a good predictor of both physical and chemical changes for any organism. Mendel set the foundations of genetics using colors among the main traits and many other scientists followed his path [4]. It would be convenient to *F. graminearum*'s growth studies because it is a response to its metabolism and maturation processes such as spore production and wall formation, it reflects the state of the fungus in both closed systems and field, under antagonism or any other condition, and nowadays it can be measured using accessible electronic tools.

If the colors are validated as tools to measure mold growth, they might enhance the overall quality of research on mycotoxins, so as other metabolites such as antibiotics or enzymes. In the current context, in the color change is effectively demonstrated as related to growth, it would open the possibility for researchers or farmers to know if *F. graminearum* is producing deoxynivalenol (DON) and zearalenone (ZEA) just by spotting its color.

2. Materials and Methods

2.1. Isolate

This study will use a *F. graminearum* isolate from the JCM Catalogue. It is registered as the teleomorph *Giberella zeae* (Schwabe) Petch isolated by Sugiura [5] from rice stubble in Hirosaki, Aomori Prefecture, Japan. It is a known producer of deoxynivalenol, 15-acetyldeoxinivalenol and zearalenone [6].

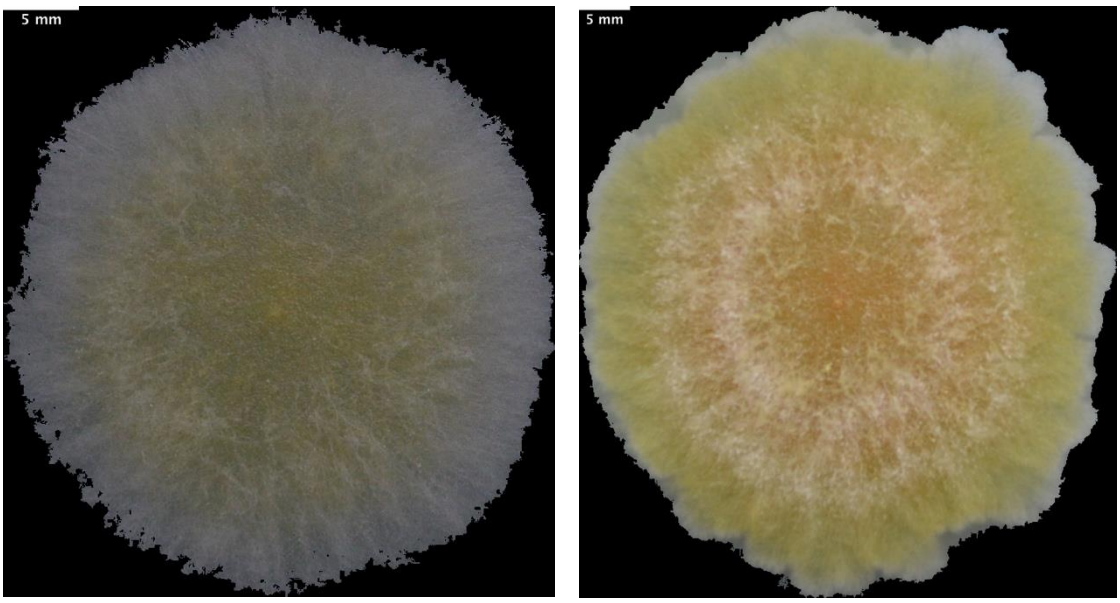
2.2. Procedure

Three replicates were grown inside a chamber at room temperature on yeast extract agar (YEA) in Petri dishes. The plates were inside a black box minimize light interference and maximize the contrast between the objects and the background.

Daily photos from upper view from approximately 25 cm were taken, using a professional camera Nikon D3200. The photos were taken for 20 days and used for color determination using ImageJ software.

First, the images of the fungi were separated from the background using color threshold, in some cases assisted by few instances of cropping. There were three basic processes of measurement: gray quantification, RGB analysis and area determination. The former included mean, mode, minimum, maximum, skewness and kurtosis. The following consisted basically in measuring mean and mode for each color. The latter was measured to validate the mean and modal gray values.

Mode and mean were the chosen parameters to represent the color change as they show central tendency and are probably the best-simplified description of the phenomenon. The mean is good because it results from the input of all values, while mode is focused on the most abundant value. For the case of *F. graminearum*, one has to be careful with mean, as its surface has no homogeneous color distribution. In Petri dish, it forms concentric rings of alternated colors (**Figure 1**).



(a) (b)

Figure 1. *F. graminearum* aspect after 2 (a) and 6 (b) days.

Furthermore, mean might not be the best approach to measure the secondary metabolites such as mycotoxins because it accounts the nearly white immature areas, with probably low levels of toxicity. Thus, mode can be a good alternative as it captures the essential and it will be particularly important if the most pigmented areas are also the ones producing more toxins.

Nevertheless, what counts the most is the consistency of the parameter and its easiness to use in mathematical and statistical processing. For this reason, the levels of dispersion of mean and gray

modal values were compared. Additionally, the raw version of the 6-day photo (**Figure 1**a) was given to a panel of 21 university students with basic instructions on to isolate the image from the background and determine the mean and mode. The most consistent parameter was supposed to show higher proximity between the central tendency measurements and less dispersion.

3. Results and discussion

3.1. Qualitative description

At the peak of the maximum growth rate, *F. graminearum* formed a yellowish mycelium forming a gradient densely pigmented at its center (**Figure 2**). The lag phase took 2 days, followed by a 9-day exponential growth. It finally covered the entire surface of the Petri dish in the 11th day. From this moment, it became compact and a central reddish color expanded towards the borders.

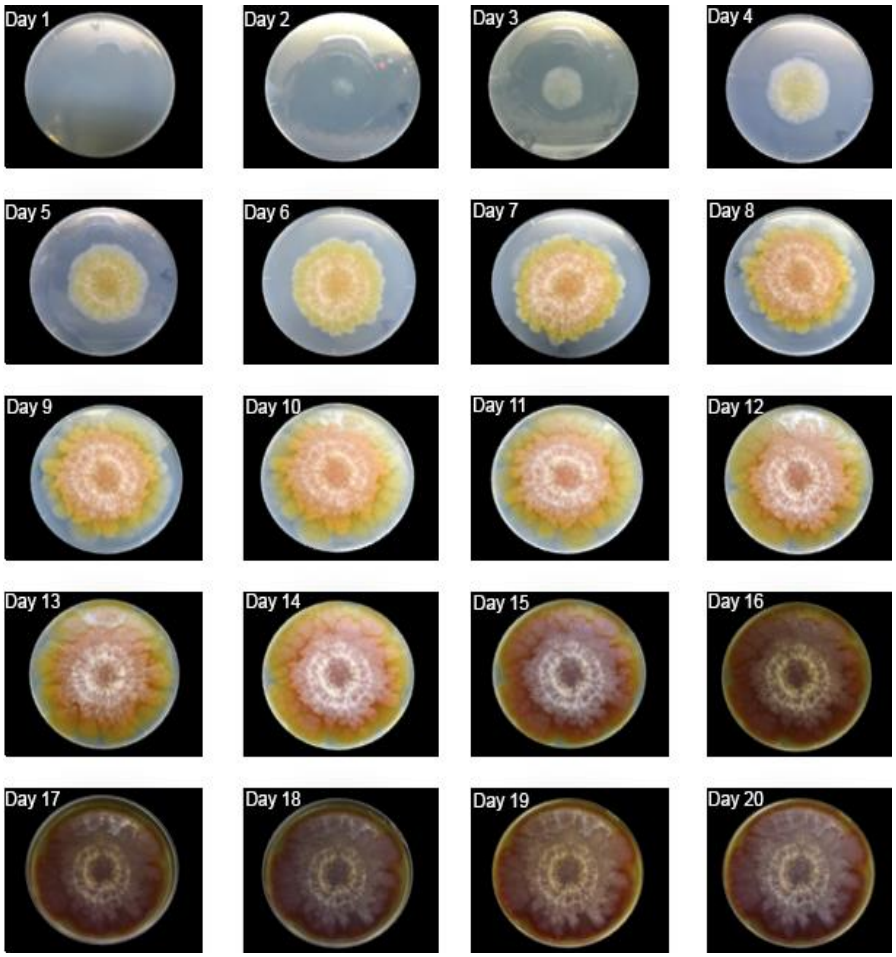


Figure 2. Daily growth of *F. graminearum.*

From day 15, there is a notorious general reduction in the brightness, probably reflecting a low metabolic rate. A white mycelial layer increases it also changes the color, becoming slightly brown. This is probably the senescence of the hyphae. This is probably the moment described by Deacon [3], when the fungus starts releasing the secondary metabolites, including toxins.

3.2. Mean versus mode

The boxplot below (**Figure 3**) illustrates de differences between the mean and modal gray value. The former shows less dispersion, besides some extreme dislocated values. Indeed, the mode showed higher standard error (7.29 against 5.56) and basically all other measures of dispersion.

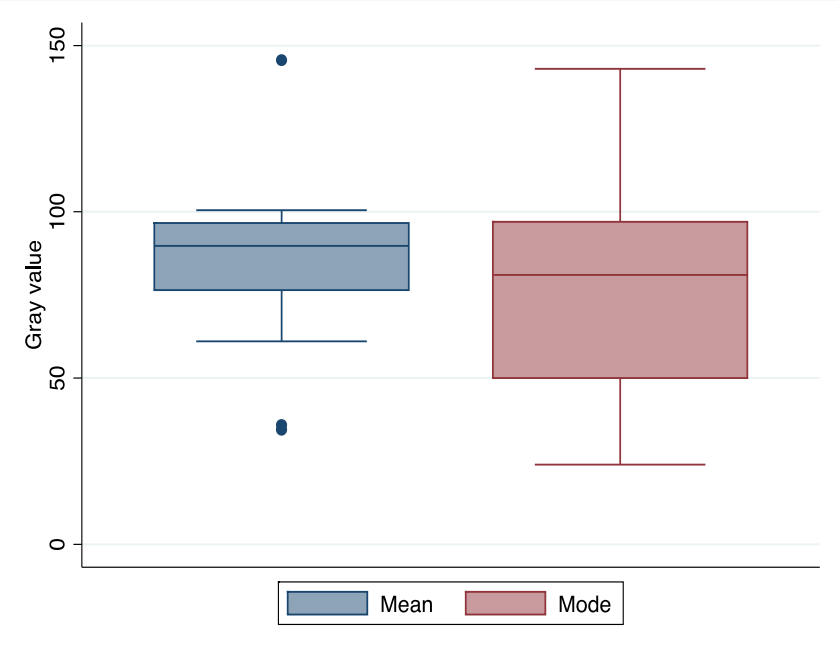


Figure 3. Comparison between the mean and modal gray values from the experimental data.

The distance between the average values and medians is 5 units for both, but the mean had higher kurtosis (2.36 against -019), and the mode's interquartile range (47) is much higher than mean's (20). These observations suggest the mean gray value as the best parameter to analyze *F. graminearum*'s brightness. As p = 0.014, it would be unwise to interchange mean and mode. Similar results were observed with RGB mean and modal values from the panel of 21 students (**Table 1**), though it might be acceptable to interchange both parameters (p = 0.3334).

Table 1. Comparison between the mean and modal colors (RGB) from a 6-day photo of *F. graminearum* measured by 21 different people.

Statistics	Mean	Mode
Average	147.89	146.71
Standard error	3.10	3.62
Median	157	160
Mode	157	173
Standard-deviation	24.61	28.74
Variance	605.77	825.98
Kurtosis	-1.52	-1.52
Skewness	-0.53	-0.61
Range	63	66
Minimum	111	107
Maximum	174	173
Sum	9317	9243
Count	63	63

Mode still shows higher standard error and dispersion in general, although the kurtosis is similar for both. The distance between the central tendency measures is smaller for mean and it even has equal median and mode (157).

The observations above suggest mean as a more consistent central tendency measure than mode, thus a more reliable variable to analyze the growth *F. graminearum* based in colors when grown in yeast extract agar.

3.3. Mycelial area and color measurement

The mycelial area increase will be briefly described (**Figure 4**), as it is important to validate the color parameters as growth descriptors. The fungus grew according to the typical "s" pattern. The median lag phase lasted 2 days. Before that, the specimens were not observable without the aid of a microscope. Thus, the major measurements started in the second day and went up to the 20th day. The exponential growth went up to the 10th day and it was possible to observe a considerable change to a darker tone in the overall color.

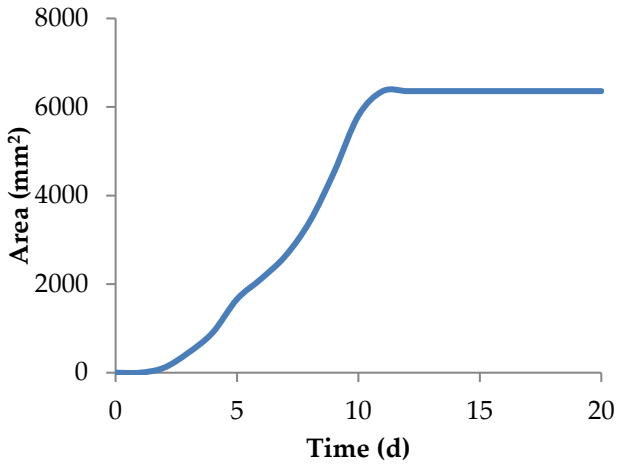


Figure 4. Mycelial area of *F. graminearum* for 20 days.

3.4. Gray value analysis

The colors observed covered the entire range of grey scale (0 to 255) (**Figure 5**). The darkest tone was consistently black (0) but the maximum gray value in general increased from 117 to 255 and remained there from the 18th day. Yet, there are notable variations, such as the peaks in days 6, 10 and others, or the valley from the 16th to 17th day.

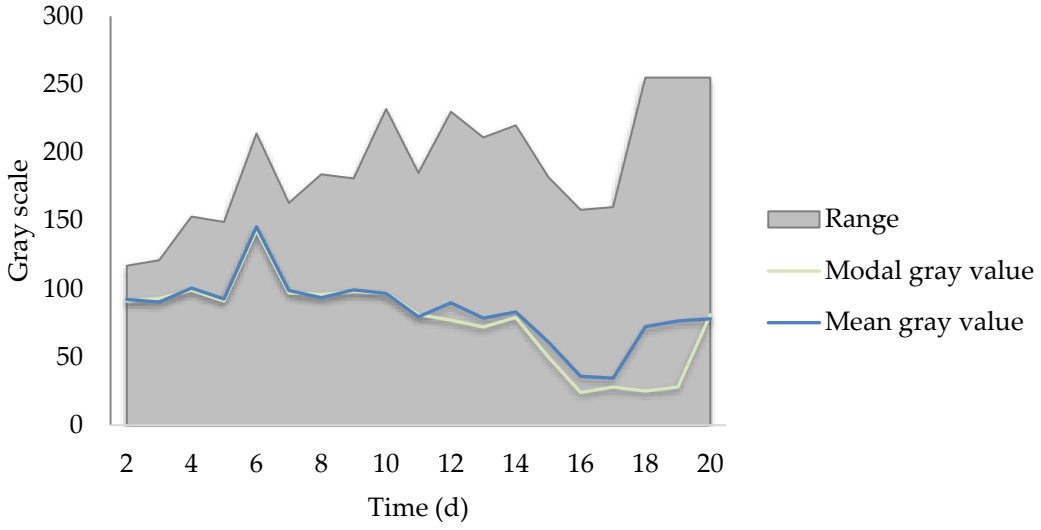


Figure 5. Gray value variation in the samples. The equation represents the trend line for the mean gray value.

A peculiar phenomenon to consider is a daily slope shift. The brightness increases and then decreases, with only two exceptions in the final days. This might be related with pinocytosis followed by metabolism in a repetitive fashion until it reaches the stationary phase. The long valley between the 14th and 18th days is probably because of high shortage of nutrients at the surface. The fungus is

thus forced explore deeper the agar, pulling more nutrients to the surface and it would explain the high rate of color change and a final shortage.

There are also instances where the fungus apparently shrinks (**Figure 4**). This is certainly due to few errors in the measurement but there is also an alternative or complementary biological explanation. As the mycelium starts to die, the fungus isolates the area using Woronin bodies and then the internal enzymes start digesting it [3]. Than the newer areas of the fungus reabsorb the contents. Yet, it probably happens only at a very low rate, as most of the fungus remains intact.

Unlike the maximum gray value, the central tendency measures tends to globally decrease, though the mean and mode are, as shown before, significantly different. By the shape, they have similar trends up to day 11 (beginning of the stationary phase), when the darker tones increase in dominance. The mean is considerably consistent with a cubic function ($R^2 = 0,65$), thus it can be used to analyze the growth *F. graminearum* and probably mycotoxin production, especially with some increase in degree.

The skewness and kurtosis of the gray scale were also analyzed (**Figure 6**). The skewness increased logarithmically from negative values and becomes positive after the 9th day. It means up to this day there was a dominance of lighter colors and then there was a shift to darker. Such shift might be a good milestone of the actual metabolic start of the stationary phase.

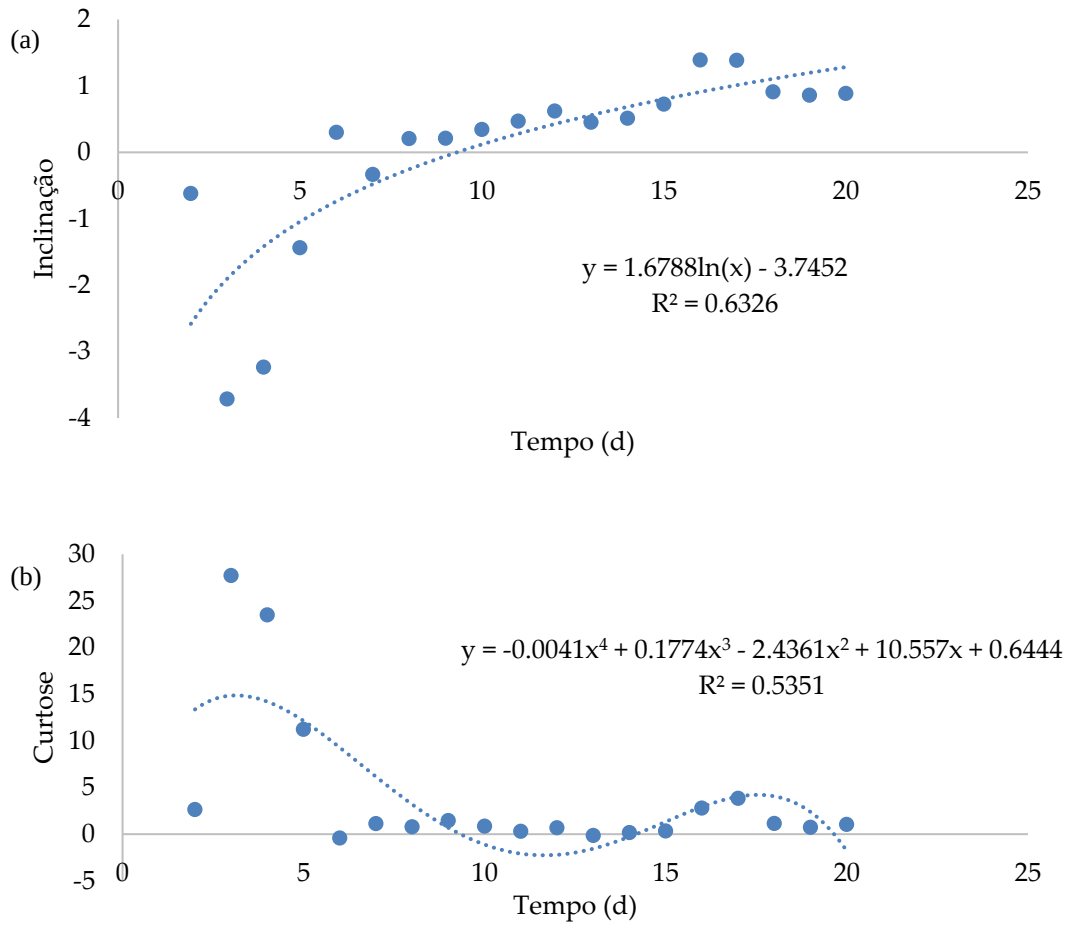


Figure 6. Skewness (a) and kurtosis (b) of *F. graminearum*'s gray scale.

Kurtosis is a good measurement of how narrow is the distribution of tones. Its simplest acceptable fit was a 4th-degree equation. As it shows, there was abundance of relatively few colors during the first days, certainly up to the 6th where there is a peak in gray values. Then, the number of colors increases drastically and remains as such until the end.

Together, skewness and kurtosis suggests a variation from few bright colors to a wide-range of darker colors. Such variety of colors in the end is probably related with the synthesis of a large number of chemicals.

It is also important to know the extent gray value usability if there is a need to discuss results from previous research, all done considering size as the major growth property. One shall assume are as much more realistic than radius and diameter, especially if the mycelium is not regularly rounded.

The **Table 2** correlates the mean and modal gray values and the area. As it shows, both variables have significant correlation with the size at 0.01, especially the mode. It suggests the mode as the best parameter to relate with studies based on fungal area, even though the mean is more appropriate to initiate and develop studies based on colors.

Table 2. Correlation between the gray central tendency measures and mycelial area of *F. graminearum*.

	Parameter	Area (mm²)
Mean gray value	Pearson Correlation	-,533*
	Sig. (2-tailed)	,019
	N	19
Modal gray value	Pearson Correlation	-,577**
	Sig. (2-tailed)	,010
	N	19

* Correlation is significant at the 0.05 level (2-tailed).

** Correlation is significant at the 0.01 level (2-tailed).

3.5. RGB analysis

All RGB channels (**Figure 7**) showed trends similar to the gray value. Likewise, they present the peak in the 6th day and acceptably fit to cubic functions, with an overall decline in value. The cubic regressions resulted in R^2 above 0.8 and it indicates the possibility to combine them in a single equation with approximately 59.5% of probability of effectively represent the simultaneous behavior of the RGB channels. Indeed, the colors seem highly correlated, meaning that some factor is causing them to change in the same fashion.

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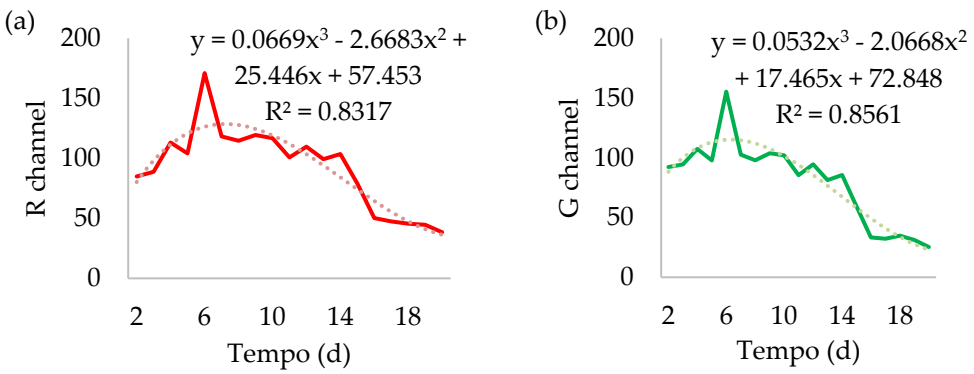

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Peer-reviewed version available at Foods 2018, 7, 100; doi:10.3390/foods7070100

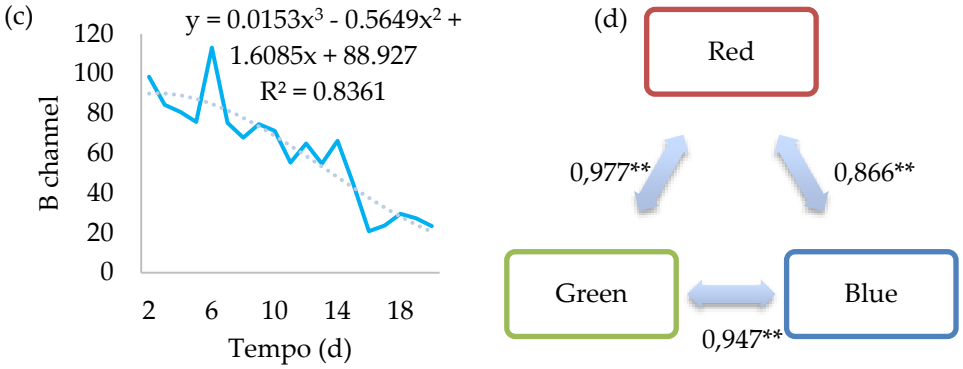

Figure 7. The mean values of the red (a), green (b), blue (c) channels, and the correlations between their means (c). ** Correlation is significant at the 0.01 level (2-tailed).

As it seems, each can individually be used to analyze the growth of *F. graminearum*, but the green component has shown the best performance. However, they cannot replace each other, as a Friedman's two-way analysis showed (p = 0,00). In more advanced analyses, each color might be more appropriated to study a particular chemical or metabolic phenomenon. Until then, it is better to include all and separately.

3.6. How related are the gray and RGB measurements?

As the **Table 3** shows, all colors are correlated to gray parameters and once again green shows stronger relation.

Table 3. Correlations between RGB colors and the central tendency gray measures.

Correlations		Mean gray value	Modal gray value
Red	Pearson Correlation	,825**	,865**
	Sig. (2-tailed)	,000	,000
	N	19	19
Green	Pearson Correlation	,858**	,902**
	Sig. (2-tailed)	,000	,000
	N	19	19
Blue	Pearson Correlation	,846**	,881**
	Sig. (2-tailed)	,000	,000
	N	19	19

** Correlation is significant at the 0.01 level (2-tailed).

The choice of gray scale to analyze color is convenient as it only consists of one parameter. However, it neglects hue and saturation, certainly related to some phenomena contributing for pigmentation. Furthermore, gray scale might be convenient to easily extrapolate observations between species as even fungi from the same genera might substantially differ in color, especially hue. On the other hand, the colors, especially green, seem more consistent.

Unlike most results above show (except for the correlation with area), the modal gray value shows more affinity with the mean RGB values. This observation might require more attention in the future, as some property of the colors was somehow better evidenced through its abundance, even in absence of the hue and saturation. If the modal gray value showed higher correlation with both mean RGB measures and area, it is probably more accurate than mean, though less precise.

5. Conclusions

It is possible to use color to analyze and predict the growth of *F. graminearum* during the first 20 days in yeast extract agar. The color parameters have showed algebraically predictable behaviors either when observed throughout *F. graminearum*'s growth or if related to radius, a well-known growth variable. Even a simple observation shows gradual change, easy to correlate to several biological phenomena.

However, the colors do not necessarily behave like the size, although it is possible to correlate the color change and the fungal area. Thus, it is necessary to develop new models rather than retrieving the already reasonably designed for radius or diameter.

The color analysis is very promising as it keeps changing even in the stationary phase. Indeed, the fungus increases in number of colors in this stage, though it also becomes very dark. This possibility to verify the color change will certainly provide a better understanding of the fungal metabolism than simple size measurements.

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