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Reconsidering the Co-Occurrence of *Aspergillus flavus* in Spanish Vineyards and Aflatoxins in Grapes

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Abstract: *Aspergillus flavus* is a xerophilic fungus whose geographical distribution is expected to change due to the current climate change scenario. Grapes are one of the most important crops worldwide, and it is essential to evaluate the risk posed by their contamination with potential mycotoxigenic species. Recently, a few reports have described *A. flavus* as an emerging contaminant in vineyards, which has led to a discussion on the need to legislate aflatoxin contents in grapes. Using a specific PCR assay, the occurrence of *A. flavus* was demonstrated in 43 out of 61 grape samples collected from Spanish vineyards. Considering the high incidence observed, the risk of the grapes becoming contaminated with aflatoxin was subsequently evaluated. *Aspergillus flavus* isolates from grapes can grow in grape-based media under a variety of environmental conditions, but they were unable to produce either aflatoxin B_1 (AFB_1) or aflatoxin B_2 (AFB_2) even though their ability to produce these toxins was confirmed in a permissive medium (CYA). These results confirm that climate change is affecting the distribution of mycotoxigenic fungi, thereby increasing the occurrence of *A. flavus* in vineyards, although the risk of the grapes becoming contaminated with aflatoxin needs to be reconsidered.

Keywords: *Aspergillus flavus*; vineyards; climate change; aflatoxins

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1. Introduction

Grapes are a very prominent crop grown worldwide. According to the International Organization of Vine and Wine (OIV), vineyards cover more than 7 million hectares worldwide, and Spain is the country with the largest vineyard area (955.000 hectares). Italy, France, and Spain are the three main wine producers, representing 51% of global production. In 2022, the global wine export value reached EUR 37.6 billion, which was 9% higher than that in 2021 [1]. In addition, wine is not the only product derived from vineyards; the export value of grapes and grape-based products is also significant. In the last report produced by the OIV, the value of the grape market reached EUR 7.5 billion, and the total global production levels of grapes and raisins reached 30.1 and 1.4 million tons, respectively [2].

Filamentous fungi are a major threat to vineyards. Fungal contamination precipitates a decrease in grape quality and vineyard yield and negatively affects the organoleptic properties of the resulting wine [3]. Mycotoxigenic fungi commonly occur in vineyards, and the mycotoxins they produce have a great impact on human and animal health [4]. The maximum levels of mycotoxins of some products suitable for human consumption are strictly legislated. In the case of wine and other grape products, ochratoxin A (OTA) is the only regulated mycotoxin in the European Union with a maximum of 2 µg/kg [5].

Among all the mycotoxigenic fungi that can contaminate grapes, *Aspergillus carbonarius* and *Aspergillus niger* are traditionally considered the most relevant. *A. niger* is usually the most prevalent, although the risk it poses is limited since only 23% of its strains are OTA

producers [6]. In this context, *A. carbonarius* is considered the main species responsible for the OTA contamination of grapes since it is commonly present in this product and almost all strains are able to produce OTA [7,8]. Less frequently, OTA occurs in grapes via contamination with other mycotoxigenic fungi such as *Aspergillus steynii* or *Aspergillus westerdijkiae* [9].

However, it is well known that a shift in fungal distribution is occurring due to climate change. This scenario is related to an increase in temperatures and in the number of drought episodes in Southern Europe, with a simultaneous increase in precipitation in Northern and Central Europe, among other factors [10]. Mediterranean Europe has been predicted to be a hotspot for extreme changes in temperature, rainfall, and CO_2 concentrations [11]. *Aspergillus flavus* is one of the mycotoxigenic fungi whose geographical distribution is expected to change due to this climate change scenario. *Aspergillus flavus* is a xerophilic fungus with an optimal in vitro growth at 30–35 °C and a water activity (a_w) of 0.99 [12,13], though this range varies depending on the substrate [14]. Aflatoxin production occurs in narrower ranges of temperature and a_w, and the optimal in vitro production is a bit different from the optimal growth conditions (25–30 °C and 0.95–0.99 a_w) [12]. Apart from temperature and a_w, there are other factors that can also affect aflatoxin production like CO_2, pH, light, or the nitrogen/carbon source [15,16].

The geographical distribution of *A. flavus* is traditionally centered in temperate regions such as the Midwest United States or North African regions [17]. On the other hand, *A. flavus* is one of the main aflatoxin producers, and its presence has been reported in a variety of matrices, including nuts, maize, dried figs, small grains, and rice [18]. Aflatoxin B_1 (AFB_1) is the most relevant mycotoxin due to its great impact on human health. The International Agency for Research on Cancer (IARC) has categorized this toxin as a Group 1 carcinogen, and AFB_1 is currently considered the most potent natural carcinogen [19].

Battilani et al. [20] predicted a shift in mycotoxin occurrence in crops, and they highlighted a possible increase in AFB_1 contamination in maize, assuming a two-degree increase in European temperatures. In vineyards, some authors, like Khoury et al. [21], have already reported a high incidence of *A. flavus* in Lebanese grapes (43.1% of samples were contaminated). Likewise, in a recently published study conducted by our group using a metataxonomic approach, a high occurrence of *A. flavus* in Spanish grapes was also observed [22]. As previously mentioned, OTA is the only regulated mycotoxin in grape and grape-derived products, since aflatoxin-producing species have not been considered as important contaminants in vineyards. However, considering the emerging presence of *A. flavus* in grapes, it is essential to evaluate the potential risk posed by this species to determine whether it is necessary to address the legislation of aflatoxins in this product.

Medina et al. [23] studied, for the first time, the effect of the three-way climate factors (CO_2, temperature, and water stress) on *A. flavus* growth and the expression of genes of the AFB_1 biosynthetic pathway. The authors concluded that whereas *A. flavus* growth might be unaffected by climate change, the AFB_1 biosynthetic pathway was highly stimulated by these conditions. However, to the best of our knowledge, there are no studies focused on how changes in climatic parameters could affect *A. flavus* distribution in vineyards.

In this work, we studied the occurrence of *A. flavus* in 61 samples of grapes collected between 2019 and 2021 in Spanish vineyards. In addition, the production ability of *A. flavus* isolates from grapes was tested in four different grape-based media (based on red grapes, white grapes, red grape juice without pomace, and white grape juice without pomace) and compared with their production in a permissive medium. Furthermore, the impact of different environmental factors such as temperature and water activity on *A. flavus* growth and its ability to produce AFB_1 and aflatoxin B_2 (AFB_2) in a grape-based medium was also studied.

2. Materials and Methods

2.1. Preparation of Plant Material

A total of 61 grape samples were collected randomly at vineyards from different regions of Spain: La Rioja, Valencia, Madrid, Castilla-La Mancha, and Andalucía. They were destemmed, superficially disinfected using 2.5% bleach, and rinsed with water for 2 min. Subsequently, the grapes were freeze-dried for 2 days using a lyophilizer (Cryodos, Telstar, Madrid, Spain) and then ground with a mortar and a pestle to obtain a fine powder. This powder was kept at −80 °C until analysis.

2.2. Occurrence of Aspergillus flavus in Spanish Grapes

Total genomic DNA was extracted from 100 mg of powder using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany). Isolated DNA was amplified for the specific detection of *A. flavus* in accordance with the PCR protocol described by González-Salgado et al. [24] starting with 8 µL of DNA template. Then, reamplification was performed using the same protocol and template 2 µL of the amplification product from the first PCR. All assays were performed in triplicate in an Eppendorf Mastercycler Gradient (Eppendorf, Hamburg, Germany). Each reaction contained 1 µL of each primer (20 µM) (Metabion, Planegg, Germany), 12.5 µL of NZYTaq II 2x Green Master Mix (NZYTech, Lisbon, Portugal), and 8.5 µL of DNA-free water (Panreac Applichem, Barcelona, Spain). The amplified products were visualized via 1.5% agarose gel electrophoresis (Pronadisa, Madrid, Spain) using 1× TAE buffer (Tris-acetate 40 mM and EDTA 1.0 mM) and 3 µL of Green Safe Premium (1 µg/mL) (NZYTech, Lisbon, Portugal). NZYDNA Ladder V (NZYTech, Lisbon, Portugal) was used as a molecular size marker. Electrophoresis was performed at 80 V for 25 min and then visualization was performed under UV light (ETX-20-M, Vilber Lourmat, France). A sample was considered contaminated by *A. flavus* when at least two of the triplicates yielded a specific amplification band.

2.3. Growth of Aspergillus flavus Isolated from Grapes and Production of AFB_1/AFB_2

The *Aspergillus flavus* isolates used (19.1.1, 19.4.1, and 19.7.1) were isolated from grape samples collected previously in our laboratory [25], and they were identified as *A. flavus* by sequencing a partial region of the β-tubulin gene [26]. They were stored in 15% glycerol (Fisher Chemical, Loughborough, UK) at −80 °C until required. These isolates were selected from the other six due to their ability to produce high levels of AFB_1 in vitro.

All the isolates used were first cultured in Potato Dextrose Agar (PDA) (Pronadisa, Madrid, Spain) plates for 5 days at 28 °C. Afterwards, spore suspensions of each fungus were prepared using sterile saline solution (9 g/L of sodium chloride). Spore concentrations were measured using a Thoma counting chamber (Marienfeld, Lauda-Königshofen, Germany) and adjusted to 10^6 spores/mL.

For the preparation of grape-based media, red and white grapes of Tempranillo and Airén varieties, respectively, were obtained from a local market in Madrid (Spain). Grapes were destemmed, ground using a blender, and conserved at −20 °C for further analysis.

Four different types of grape media were prepared using red grapes, white grapes, red juice without the pomace, and white juice without the pomace. All media contained 20 g/L of bacteriological agar (Pronadisa, Madrid, Spain) and 30 g/L of the corresponding grape extract. pH was measured in all media, and values between 5.43 (white grape) and 5.87 (white grape without pomace) were obtained. CYA medium plates (45.5 g/L of modified Czapek–Dox agar (Pronadisa, Madrid, Spain) and 5 g/L of yeast extract (Pronadisa, Madrid, Spain)) were used as controls for fungal growth and aflatoxin production.

The plates were inoculated in the center with 2 µL of the spore suspension of the corresponding *A. flavus* isolate and incubated for 7 days at 28 °C and 0.99 a_w. Fungal colony diameter was measured daily in two directions until the plates were fully grown (7 and 6 days for the grape-based and CYA media, respectively). Growth rate was calculated in all plates using a linear model by plotting diameter (mm) against time (day). All assays were performed in triplicate.

After incubation, three agar plugs (5 mm) were removed from the inner, middle, and outer parts of the fungal colony for mycotoxin analysis. AFB_1/AFB_2 were extracted using 1 mL of chloroform and shaking for 20 min. Then, chloroform was evaporated in a centrifugal vacuum concentrator (Eppendorf, Hamburg, Germany) and finally resuspended in 1 mL of methanol. Prior to quantification, all samples were filtered through 0.45 µm filters (Branchia, Labbox Labware S.L., Barcelona, Spain) and then stored at −20 °C until analysis (Section 2.5).

2.4. Grape-Based Media at Different Water Activity (a_w) and Temperature Levels

The effects of five a_w levels (0.99, 0.96, 0.93, 0.90, and 0.85) and four temperatures (28 °C, 32 °C, 37 °C, and 42 °C) on the growth and AFB_1/AFB_2 production of *A. flavus* in a grape-based medium were evaluated. In this experiment, grape-based medium was prepared as explained above, using 30 g/L of red juice without pomace and 20 g/L of agar.

Water activity was adjusted using glycerol (as reported by Dallyn et al. [27]). Assays were carried out in triplicate using *A. flavus* 19.1.1, 19.4.1, and 19.7.1.

Fungal inoculation was performed as described in Section 2.3. Plates were incubated at the corresponding temperatures. Mycelial growth was measured daily until the colony mass reached the end of the plate (Table S1), and growth rate was calculated as explained above. Once the mycelial growth reached the end of the plate, agar plugs were removed for AFB_1 and AFB_2 quantification, as explained in Section 2.3.

To analyze the production of AFB_1/AFB_2 after long incubation periods, plates corresponding to 0.99 a_w were incubated for 1 month at the three permissive temperatures tested.

2.5. Aflatoxin Quantification

All methanolic extracts were diluted in a 1:1 ratio in water (v/v). The chromatography equipment used was a separation Module Alliance 2695 Waters (Waters, Milford, MA, USA), with a C18 analytical column (5 µm Waters Spherisorb, 4.6 × 150 mm ODS2) and a Multi λ Fluorescence Detector, Waters 2475. The temperature of the column was set to 40 °C, and 100 µL of the extract was injected into the HPLC system. The presence of AFB_1/AFB_2 was detected and quantified via fluorescence detection (λexc 365 nm; λem 455 nm). In order to enhance and confirm AFB_1 detection, a post-column derivatization with an LCTech UVE photochemical system (LCTech GmbH, Obertaufkirchen, Germany) was performed. The mobile phase (with a water/acetonitrile/methanol ratio of 70:17:17) was pumped at 1.2 mL/min. The detection limits of the analysis were 0.6 ng/g of AFB_1 and 0.3 ng/g of AFB_2 based on a signal-to-noise ratio of 3:1.

2.6. Statistical Analysis

Statistical analyses were performed using StatGraphics Centurion XVII V.19 software (Statpoint Technologies Inc., Warrenton, VA, USA). Shapiro–Wilk and Barlett tests were used to assess the data's normality and homoscedasticity, respectively. The growth rates under the different conditions tested were analyzed via analysis of variance (ANOVA) to assess the differences between the group means. In the cases in which normality or homoscedasticity were not assessed (growth rate at different temperatures and a_w), the non-parametric Friedman test was performed using the Statistic software Infostat V2020 (National university of Córdoba, Córdoba, Argentina).

3. Results

3.1. Aspergillus flavus Occurrence in Vineyards

Aspergillus flavus was detected in 70.49% of the grape samples analyzed, which corresponds to 43 positive samples out of the 61 analyzed. All the different Spanish regions analyzed presented positive samples.

3.2. Growth and AFB_1/AFB_2 Production of *Aspergillus flavus* Isolated from Grapes

Due to the high incidence of *A. flavus* observed, the risk of AFB_1/AFB_2 contamination in grapes was evaluated. The *A. flavus* isolates mentioned in Section 2.3. were used in this study to evaluate their growth and AFB_1/AFB_2 production in a permissive medium (CYA) and in different types of grape-based media under permissive conditions.

The results regarding the fungal growth of the three isolates of *A. flavus* tested in the grape-based media are presented in Figure 1. The fungal growth rates of isolates 19.4.1 and 19.7.1 in the red-grape-based medium were significantly lower when compared to the results observed in other grape-based media. In general, CYA was the medium that most favored fungal growth, and the highest growth rate was obtained in this medium in the case of *A. flavus* 19.1.1 and 19.4.1. There were no differences between the growth rates observed in the other three grape-based media tested (namely, white grape, red grape without pomace, and white grape without pomace).

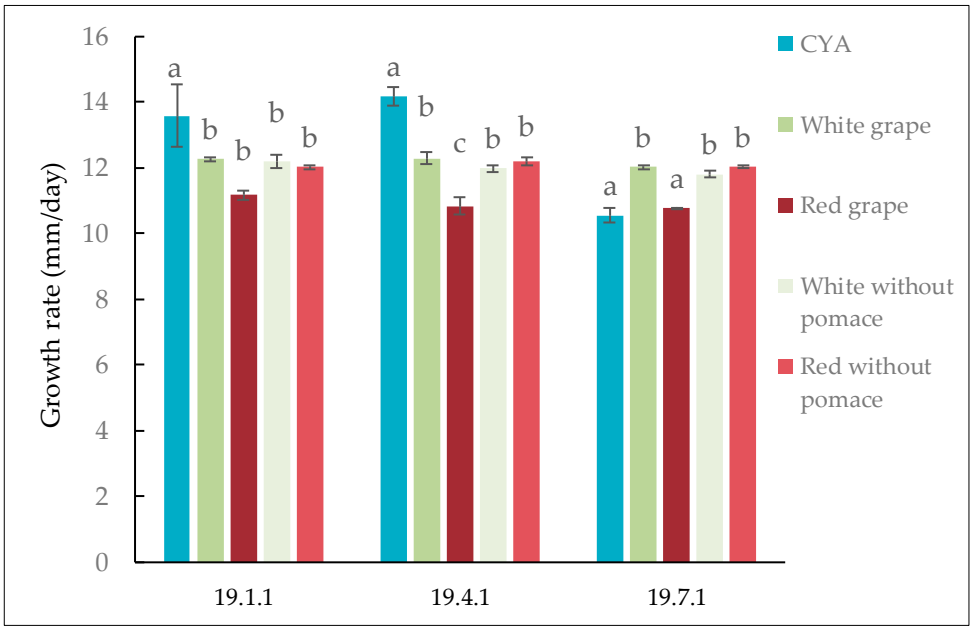

Figure 1. Growth rate of *A. flavus* isolates obtained on CYA and in the four types of grape-based media (Incubation time of 6 days (CYA) or 7 days (grape medium), 28 °C and 0.99 a_w). All values represent the means of the three replicates. Thin bars correspond to the standard error of the data. Different letters represent significantly different groups ($p < 0.05$).

Previous studies conducted in our laboratory [25] revealed the ability of these three isolates to produce AFB_1/AFB_2, and in the present study, both mycotoxins were indeed detected in the CYA cultures at variable levels. The mean AFB_1 concentrations determined were 9.01 ± 3.89 µg/kg, 0.61 ± 0.31 µg/kg, and 615.67 ± 90.71 µg/kg in the case of *A. flavus* 19.1.1, 19.4.1, and 19.7.1, respectively. In the case of AFB_2, the mean concentrations were 0.27 ± 0.12 µg/kg, 0.21 ± 0.08 µg/kg, and 6.28 ± 1.02 µg/kg for 19.1.1, 19.4.1, and 19.7.1, respectively. On the other hand, neither AFB_1 nor AFB_2 was detected in any of the grape-based media tested.

3.3. Effect of Temperature and Water Activity on *Aspergillus flavus* Growth and AFB_1/AFB_2 Production in a Grape-Based Medium

Since no aflatoxin production was detected in any of the grape-based media tested, the following experiments focused on checking if aflatoxin production could be triggered in the more extreme environmental conditions that could occur in the current climate change scenario.

The growth rates of the three *A. flavus* isolates observed at different temperatures and a_w levels are represented in Figure 2. First of all, it is important to highlight that none of the *A. flavus* isolates were able to grow at 42 °C or 0.85 a_w, even after 15 days of incubation. Considering only the effect of temperature, in general, the highest growth rates were observed at 32 °C, whereas the lowest values were observed at 37 °C with the exception of 19.1.1 and 0.93 a_w. However, the results were significantly different in the grape-based plates with 0.90 a_w, in which differences between the three temperatures seemed to be minimized, except at 37 °C, where the growth was significantly lower.

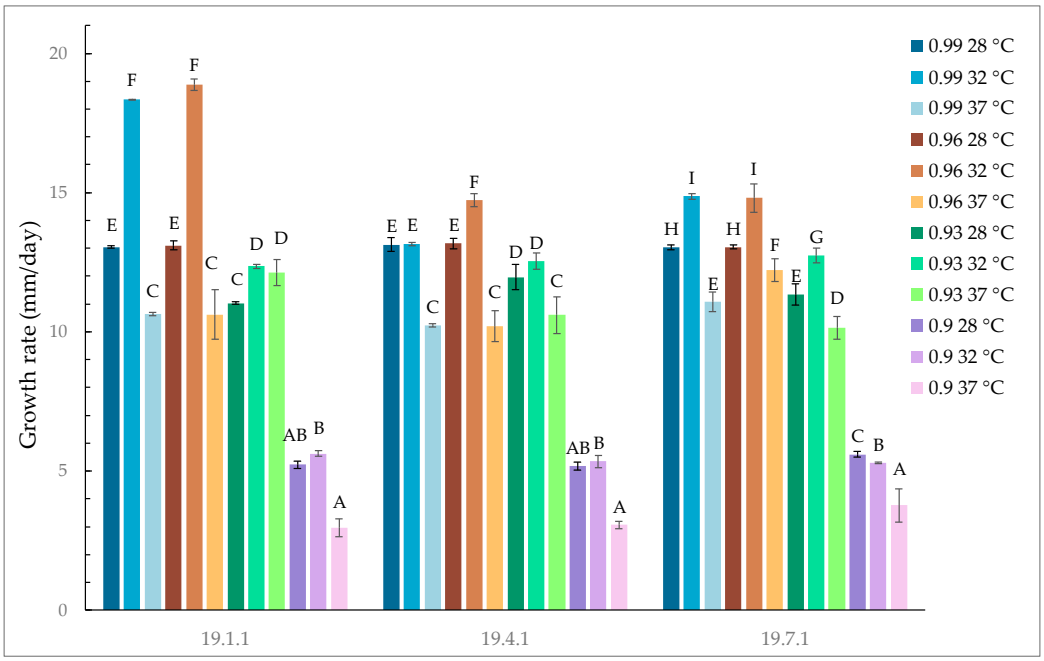

Figure 2. Growth rate of *A. flavus* isolates in a grape-based medium (red grape juice without pomace) at different temperatures (28 °C, 32 °C, and 37 °C) and a_w levels (0.99, 0.96, 0.93, and 0.90). In all cases, the values are the means of three replicates. Thin bars indicate the standard error of the corresponding data. Groups with different letters are significantly different ($p < 0.05$).

Regarding the different a_w levels tested, only slight differences in growth rates were observed between 0.99 and 0.96 at the same incubation temperatures (28 °C and 32 °C). Conversely, the growth rates obtained at 0.93 were significantly lower than those observed at 0.99 and 0.96 at the temperature conditions mentioned before. This tendency was not observed when the plates were incubated at 37 °C; in this case, the differences between the growth rates at the a_w levels of 0.99, 0.96, or 0.93 were reduced in the three isolates tested. The lowest growth rate of *A. flavus* was observed at 0.90 a_w, with a growth rate reduction in comparison to the results at 0.99 a_w of 59%, 64%, and 69% when incubated at 28 °C, 32 °C, and 37 °C, respectively.

Regarding AFB_1/AFB_2 production, none of these mycotoxins were detected under any of the conditions tested.

4. Discussion

Vineyards represent an important crop in Spain [1]; thus, it is essential to ensure the safety of the products derived from grapes grown there. Concerning the mycotoxin contamination of grape-derived products, ochratoxin A (OTA) is the most relevant since its maximum levels in wine and other grape products are legislated [5]. The OTA-producing species *Aspergillus carbonarius* and *A. niger* have so far been the focus of studies on the risk posed by mycotoxins in vineyards [28,29]. Traditionally, *A. flavus* has never been considered a threat to this crop. However, many reports have warned of an increase in *A. flavus* contamination in other relevant matrices such as maize [20,30]. Such an increase

is associated with climate change and would probably entail a risk of aflatoxin presence in foodstuffs [31–33]. In this context, a previous study conducted in our laboratory has already pointed out an increase in the occurrence of *A. flavus* in grapes [22]. A similar situation was also recently observed in Brazilian vineyards, where an increase in *A. flavus* presence was reported [34]. Accordingly, the first objective of this work was to perform a more in-depth study to evaluate *A. flavus* contamination in Spanish vineyards. The results of our study confirm the high level of contamination of Spanish grapes with this fungus, which was detected in more than 70% of the samples analyzed. A study performed less than a decade ago reported that *Aspergillus* section *Flavi* was a minority since its isolates only represented 3% of the total *Aspergillus* spp. found in grape samples [8]. Thus, according to the results obtained in the present work, a clear shift in the prevalence of *A. flavus* is occurring in Spanish vineyards. However, it is well known that the presence of a mycotoxigenic fungi does not always correlate with mycotoxin contamination [35]. Therefore, the characterization of the ability of *A. flavus* to produce AFB_1 in grapes was essential in order to evaluate the real risk of contamination by this toxin.

Considering all the above, we characterized the ability of *A. flavus* strains isolated from grapes to grow and produce AFB_1/AFB_2 in grape-based media in comparison to a permissive medium (CYA). Both red grape and white grape extracts were tested, since some authors have reported that the chemical compositions of these two main types of grapes are different and could influence the growth of *A. flavus* [36]. Nevertheless, the effect of pomace was also addressed; thus, it was determined that many bioactive compounds are present in this part of the grape [37]. In general, *A. flavus* growth in grape-based media was lower in comparison to that in CYA, and the presence of red grape induced the smallest growth rate. Moreover, AFB_1/AFB_2 production was only detected in the CYA medium and not in any of the grape-based media. Therefore, these results indicate that the grape matrix somehow affects both growth and AFB_1/AFB_2 production.

Grapes are mainly composed of sugars, such as glucose and fructose, followed by organic acids and phenolic compounds; the latter are mainly present in seeds and grape skin, which constitute grape pomace [38]. Several authors have already described the potential of different phenolic compounds to reduce both the growth and toxin production of some fungi [39]. Among all the polyphenols present in grapes, resveratrol is one of the most predominant, and, in the last decade, several studies have focused on its antimicrobial role [40]. The amount of resveratrol present in grapes varies depending on the grape cultivar, and it mainly accumulates in the berry skin. Moreover, resveratrol concentrations are higher in red grapes than in white ones [41]. These differences in resveratrol concentration could explain the significant reduction in *A. flavus* growth in the red grape agar plates. On the other hand, Wang et al. [42] reported that this compound significantly suppressed the transcription of the genes *aflA* and *aflB* located in the aflatoxin biosynthetic cluster of *A. flavus*. This suppression is related to an insufficient number of precursors and, therefore, to the disruption of the aflatoxin pathway. Consequently, this could explain the inability of *A. flavus* isolates to produce detectable levels of AFB_1/AFB_2 in grape-based media. Other genes involved in mycelial and conidial development are also downregulated when *A. flavus* grows in the presence of resveratrol [42]. Apart from resveratrol, another polyphenol highly present in grapes is quercetin, which is mainly present in grape skin [43], though its concentration can vary from one variety to another [44]. Some authors have demonstrated that this compound is able to inhibit both the growth and AFB_1 production of *A. flavus* through the down-regulation of regulatory genes such as *aflS* [45]. Moreover, recent studies have described the ability of other members of the polyphenol group, such as those present in the peels of citrus fruits like oranges, mandarins, or blood oranges (hesperidin, narirutin, resveratrol, or quercetin), to inhibit both the growth and AFB_1 production of *A. flavus* [46,47]. Taking all this into account, the polyphenolic compounds present in grapes could be determinant factors of the inhibition of aflatoxin production by *A. flavus* observed in this work.

Since no AFB_1/AFB_2 production was observed in the grape-based media tested, we decided to check whether this result would also occur under various conditions of temperature and water stress, including those expected in the context of climate change. As mentioned, traditionally, the research regarding toxigenic mycobiota associated with grapes has focused on the black *Aspergilli* group. In this context, some studies have evaluated how these species could be affected by climate change conditions [48,49]. Medina et al. [23] evaluated the effect of climate change conditions on *A. flavus* growth and aflatoxin B_1 production although they used a synthetic medium (yeast extract sucrose agar, YES). To the best of our knowledge, this is the first study that has analyzed the effect of climatic conditions (temperature and water stress) on *A. flavus* growth and AFB_1/AFB_2 in a grape-based substrate.

The optimum growth rate of *A. flavus* isolates in a grape-based medium was obtained at a temperate temperature (32 °C) and high a_w levels (0.99 and 0.96). This is in agreement with previous published studies using media specifically formulated for the study of fungal secondary metabolism, such as YES or Czapek, in which the maximum growth was reached at 0.99 and 0.95 a_w and 30–35 °C [13,50]. In addition, no noticeable differences were observed in the fungal growth rate at 0.99 compared to 0.96 a_w when the incubation temperature was 32 °C, and only a slight decrease was observed when a_w was set to 0.93. Similar results were observed in a study by Medina et al. [23], in which *A. flavus* growth in a YES medium was unaffected by changes in a_w (0.97 and 0.95) at 34 °C and only a small decrease in growth rate was observed when a_w was reduced to 0.92. All these results suggest that the ability of *A. flavus* to grow in a grape-based medium is affected in a manner similar to that observed in other in vitro media; therefore, CYA or YES can be very useful for performing ecophysiological studies focused on the growth of this important mycotoxigenic fungi. However, the situation is completely different in the case of aflatoxin production. Several authors have described the ability of *A. flavus* isolates to produce AFB_1 in a variety of environmental conditions, with optimal ranges of 25–30 °C at 0.99 a_w and 30–35 °C at 0.95 a_w [12,13]. Other works mentioned that the current climate change scenario might entail a significant increase in the levels of AFB_1 produced by *A. flavus* [23]. Surprisingly, in the current work, neither AFB_1 nor AFB_2 was detected under any of the conditions tested in the grape-based medium in the case of all the *A. flavus* isolates from grapes, whose ability to produce these toxins was confirmed in a CYA medium. Therefore, it can again be stated that the lack of production observed in a grape-based medium may be related to the composition of the substrate, as previously mentioned. Similar results were obtained by Adjovi et al. [51], who found that cassava is highly contaminated with strains of *A. flavus* potentially capable of producing toxins, but they do not seem to be able to produce AFB_1. The authors also relate this inhibition of aflatoxin production to some of the components of cassava.

However, even though AFB_1 synthesis was not observed in *A. flavus* cultures in a grape-based medium in the current work, it is not possible to reject the risk for other grape-based products. For example, dried vine fruits are traditionally obtained through direct sun exposure, mainly in developing countries [52]. Similarly, these sun-dried grapes are used in the production of certain types of widely consumed wine. In the dehydration process, the grapes are exposed to the sun for 10 to 31 days, and this step can allow fungal development due to the favorable temperature and humidity conditions. Subsequently, water stress can lead to the production of aflatoxins. This increase in mycotoxin concentration has already been described for OTA levels in dried grapes [53,54]. Therefore, the risk posed by *A. flavus* to these kinds of grape-derived products must be assessed in the future.

On the other hand, the high occurrence of potential AFB_1-producing *A. flavus* in grapes cannot be disregarded. The boundaries of different fields may be in close proximity, which could lead to the contamination of nearby crops with *Aspergillus flavus*, thus precipitating the production of aflatoxins. Moreover, new agricultural practices in vineyards usually include the use of crop covers to minimize soil erosion and improve nutrient retention [55]. In some cases, these crop covers are the remains of cereals such as oats, wheat, or barley,

which are matrices susceptible to *A. flavus* contamination and may be responsible for increasing the abundance of fungal inocula [56].

This kind of study is essential for identifying new matrices that might pose a risk to food safety via contamination by mycotoxins. For example, this is the case for OTA presence in figs, the maximum levels of which have been recently regulated by the European Union [5]. This decision was based on several works that reported a worrying increase in OTA occurrence in this matrix [57,58]. Currently, there are no regulations setting the maximum levels of aflatoxins in grapes or grape-derived products. The results obtained in the present work indicate that despite the high occurrence of *A. flavus* in vineyards, the isolates do not seem to produce AFB_1 in grapes in a variety of conditions. It is clear that more research is needed, but the currently available data suggest that it would not be necessary to regulate the maximum levels of aflatoxins in grapes.

Overall, the results obtained in this work confirm that climate change is increasing the occurrence of *A. flavus* in non-common matrices like grapes, a crop that, in the past, was not commonly contaminated by this fungus. The ability of this fungus to produce AFB_1 is drastically affected in grape-based media, regardless of factors such as temperature, water stress, or incubation time. However, the risk posed by this fungus as a preliminary inoculum for other crops or regarding the AFB_1 contamination of other grape-based products like raisins should be considered.

5. Conclusions

The results presented here provide a preliminary insight into the emerging risk of *A. flavus* as a contaminant of grapes. According to our work, the frequent occurrence of *A. flavus* in Spanish vineyards cannot be disregarded. However, the results indicate that *A. flavus* isolates from grapes are unable to produce aflatoxins in this matrix, probably due to some compounds present in the substrate. Environmental conditions related to the climate change scenario do not seem to affect the production of these mycotoxins by *A. flavus*. Thus, according to these preliminary results, the regulation of AFB_1 or AFB_2 in grapes or grape-derived products might not be necessary.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agriculture13101998/s1, Table S1: Incubation days of the grape plates under the different conditions tested.

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