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## Conidia survival of *Aspergillus* section *Nigri*, *Flavi* and *Circumdati* under UV-A and UV-B radiation with cycling temperature/light regime

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### Abstract

Bio-geographical differences in fungal infection distribution have been observed around the world, confirming that climatic conditions are decisive in colonization. This research is focused on the impact of ultraviolet radiation (UV) on *Aspergillus* species, based on the consideration that an increase in UV-B radiation may have large ecological effects.

Conidia of six mycotoxigenic *Aspergillus* species isolated from vineyards located in the northeast and south of Spain were incubated for 15 days under light/dark cycles and temperatures between 20 and 30 °C per day. Additionally, 6 h of exposure to UV-A or UV-B radiation per day were included in the light exposure. UV irradiance used were  $1.7 \pm 0.2$  mW cm<sup>-2</sup> of UV-A (peak 365 nm) and  $0.10 \pm 0.2$  mW cm<sup>-2</sup> of UV-B (peak 312 nm). The intrinsic decrease in viability of conidia over time was accentuated when they were UV irradiated. UV-B radiation was more harmful.

Conidial sensitivity to UV light was marked in *Aspergillus* section *Circumdati*. Conidia pigmentation could be related to UV sensitivity. Different resistance was observed within species belonging to sections *Flavi* and *Nigri*. An increase in UV radiation could lead to a reduction in the *Aspergillus* spp. inoculum present in the field (vineyards, nuts, cereal crops). In addition, it could unbalance the spore species present in the field, leading to a higher predominance of dark-pigmented conidia.

### INTRODUCTION

*Aspergillus* can contaminate agricultural products at different stages, including pre-harvest, harvest, storage, processing and handling. *Aspergillus* species have been isolated in field surveys from several commodities such as corn, rice, wheat, Brazil nuts, peanuts, pistachios, figs, grapes, onions, pepper, coffee and cocoa.<sup>1-12</sup> *Aspergillus* species are divided into different morphological sections according to the different colours of their pigments. *Aspergillus* sections *Nigri* (black), *Flavi* (green) and *Circumdati* (yellow) are the most studied sections in this genus owing to their potential toxicity. Fungal infection of crops can result in food secondary rots, with the possible accumulation of mycotoxins.<sup>13</sup> Moreover, climatic

conditions directly affect fungal infection and the subsequent mycotoxin contamination in foods and raw materials.

Recently, the Intergovernmental Panel on Climate Change (IPCC) published a Fifth Assessment Report (AR5), in which it was concluded that climate warming is 'unequivocal', and that it is likely that anthropomorphic greenhouse gas emissions contribute to current warming trends.<sup>14</sup> The impact of climate change has been identified as an emerging issue for food and feed safety,<sup>15</sup> and its possible consequences on mycotoxin production in crops have been theorized by several research studies.<sup>15-20</sup> Moreover, the World Meteorological Organization (WMO) has also highlighted that human emissions of chlorofluorocarbons (CFCs) and other chemicals have an important role in atmospheric changes by damaging the stratospheric ozone layer, which filters out harmful ultraviolet radiation (UV).<sup>21</sup> UV radiation affects fungal spore germination, growth and sporulation, the effect depending on time exposure, UV wavelength and fungal species.<sup>22-27</sup>

The United Nations Environment Programme (UNEP) considered that increased UV-B radiation, interacting with other global change factors, may affect many of the important ecosystems processes and attributes, such as plant biomass production, plant consumption by herbivores including insects, disease incidence of plants and animals, changes in species abundance and composition and mineral nutrient cycling, with important implications for food security and food quality.<sup>28, 29</sup>

Although information concerning the effects of UV-B on fungi and bacteria is scarce, studies on microfungal communities in soils of extreme habitats, such as desert (very high solar radiation, drought, and extreme temperatures), suggest the dominance of dark-coloured microfungi with large multicelled conidia.<sup>30, 31</sup> Likewise, the main airborne fungal species identified at a coastal station after exposure to solar radiation were predominantly *Aspergillus niger*, *Alternaria alternata*, *Cladosporium cladosporoides* and *Arthrinium phaeosporum*.<sup>32</sup> Therefore, *Aspergillus* species could prevail over other species under high-UV-radiation environments. Moreover, species of the genus *Aspergillus* (mainly *A. fumigatus*) and teleomorphic ascomycetes show a certain thermotolerance.<sup>30</sup>

The main goal of our investigation was to assess survival under UV radiation of conidia from six species belonging to the *Aspergillus* genus. The effects of isolate origin and pigmentation of conidia on resistance to UV light were also assessed.

## **MATERIALS AND METHODS**

### **Isolates**

Isolates from berries collected at harvesting time in two different Spanish wine-growing regions were used in this study (Table 1). The vineyards were located in Catalonia and Andalusia, which are located in the northeast and south of Spain, respectively. The climate in the vineyards sampled in the northeast is defined as cold steppe (BSk), while in the south it is temperate with dry or hot summer (Csa) according to the Köppen Climate Classification of the Iberian Peninsula Climate Atlas.<sup>33</sup> A similar cropping system is used in both areas, except for irrigation, which is by the drip method in the case of the northern fields, whereas in the

southern fields there was no irrigation. Isolation and identification of *Aspergillus* spp. have been detailed by García-Cela et al.<sup>34</sup>

Six species belonging to three aspergilli sections (two species each) were chosen for their mycotoxin production ability, and for the presence of such mycotoxins in food commodities such as grapes (ochratoxin A, from *A. carbonarius*), pistachio nuts (aflatoxins) and wheat (ochratoxin A, from several *Aspergillus* species belonging to sections *Nigri* and *Circumdati*). Thus one isolate each of *A. carbonarius* and *A. tubingensis*, *A. flavus* and *A. parasiticus*, and *A. ochraceus* and *A. steynii*, from sections *Nigri*, *Flavi* and *Circumdati*, respectively, were used.

#### Experimental design

For inocula preparation, different culture media were used for each species, simulating frequent commodities from which they are commonly isolated. *Aspergillus* sections *Nigri*, *Flavi* and *Circumdati* were inoculated on to synthetic nutrient medium (SNM),<sup>35</sup> pistachio-based medium (PBM) and wheat-based medium (WBM), respectively. For the PBM and WBM preparation, 30 g pistachio or wheat were ground and boiled in 1 L distilled water for 30 min. Subsequently, the extract was filtered with gauze and 15 g agar were added and the volume made up to 1 L. After that, media were autoclaved for 15 min at 121 °C. Isolates were grown for 7 days at 25 °C in the dark to enable significant sporulation. After this, spores were removed from Petri dishes and suspended in 80 mL sterile water containing 0.05% (w/v) Tween 80 to reach a final concentration of  $10^5$  conidia mL<sup>-1</sup>. Aliquots (5 mL) of conidial suspensions were filtered under vacuum on to individual sterile filter membranes (pore size 0.45 µm, 25 mm diameter, cellulose acetate filter) (Sartorius Biolab Products, Göttingen, Germany). A total of 12 membranes for each isolate were used (six replicates for each treatment, irradiated and control). Membranes were enclosed in pairs in 5 cm diameter plastic Petri dishes and dried at 37 °C in microbiology incubators overnight.<sup>36</sup> Spores deposited on filter membranes were exposed to radiation and temperature cycles for 15 days, as described latter.

The strains were incubated under particular photoperiod and temperature conditions in order to simulate the conditions characteristic for the period of grape ripening in Spain (August). Photoperiod values were obtained from the National Spanish Geographic Institute (IGN), while temperatures were obtained from the Spanish Meteorological Association (AEMET). Daily light and temperature conditions for incubation are described in Fig. 1, following the methodology described in García-Cela et al.<sup>37</sup>

UV-irradiated and non-irradiated isolates were incubated in parallel under the same conditions in two cooled incubators (Memmert ICP-600, UK). Daylight was simulated with four cold-white fluorescent lights (standard illuminant D65, 6500 K) located in the incubators. UV irradiation was generated with a Vilber Lourmat lamp VL-215.LM (Germany). The lamp consisted of two fluorescent tubes of 15 W each and a filter to minimize light interference. UV-A extends from 320 to 400 nm with an energy peak at 365 nm and UV-B runs from 280 to 370 nm with an energy peak at 312 nm (Fig. 2). Petri plates were placed at a distance of 8 and 32 cm from the UV-A and UV-B lamps, resulting in an irradiation of  $1.7 \pm 0.2$  mW cm<sup>-2</sup> and  $0.10 \pm 0.2$  mW cm<sup>-2</sup> for UV-A and UV-B, respectively. The irradiation was measured with a portable UV light meter (UVA-UVB PCE-UV34, PCE Iberica SL, Spain). Values of radiant energy were chosen

according to annual values characteristic for the south of Spain, with a high sum of annual hours of sun.<sup>38</sup> Annual values for UV-A ranged from 0.06 to 2.49 mW cm<sup>-2</sup>, with a mean value of 1.17 mW cm<sup>-2</sup>, while for UV-B they were from <0.001 to 0.23 mW cm<sup>-2</sup>, with a mean value of 0.09 mW cm<sup>-2</sup>. Considering the doses and exposure time, the daily accumulated UV-A and UV-B radiation in the experiments was 0.367 and 0.022 MJ m<sup>-2</sup>, respectively. The mean daily accumulated annual UV-A radiation was 0.464 MJ m<sup>-2</sup> and, focusing on harvest months, values were 0.632 MJ m<sup>-2</sup> in August and 0.539 MJ m<sup>-2</sup> in September. The UV-B mean daily accumulated annual value was 0.035 MJ m<sup>-2</sup>, whereas in August it was 0.050 MJ m<sup>-2</sup>. Direct UV doses used in the study were higher than the global (direct + diffuse) UV mean values recorded in the south of Spain, but daily accumulated irradiation was lower due to the lower number of hours of exposure.

Each 5 days (5th, 10th and 15th day) conidia were dislodged from two control and two irradiated membranes into 100 mL sterile peptone (0.1% w/v) solution using a stomacher. Subsequently, serial dilutions from 10<sup>-1</sup> to 10<sup>-4</sup> were made and 100 µL of each dilution were plated on to Dichloran Rose Bengal Chloramphenicol agar (DRBC). DRBC plates were incubated at 25 °C in darkness for 2 days. Afterwards the colonies were counted. The number of viable conidia (N) was calculated using the sum of colonies counted (C) in two consecutive dilutions, considering the number of plates counted at the first (n1) and second dilution (n2), taking into account the volume (V) dispensed to each plate and the dilution from which first count was obtained (d) (Eqn 1). Only Petri dishes with 15–150 colony-forming units (CFUs) were considered and the limit of detection of the whole procedure was 104 CFU.

#### Data analysis

As an equal number of conidia were irradiated for all isolates across the experiment (5 × 10<sup>6</sup> conidia in each membrane), analysis of variance (ANOVA) was applied directly to log CFU data in order to determine the effect of species, sections, irradiation treatment, time and their interactions on conidial survival. In addition, ANOVA considered only data from species isolated from both regions (*A. ochraceus*, *A. flavus*, *A. carbonarius* and *A. tubingensis*) in order to evaluate the influence of geographical origin on conidia survival. Subsequently, significant effects were analysed by Tukey's honestly significant different test (HSD). Significance was defined as  $P < 0.05$ .

## RESULTS

Both types of radiation (UV-A and UV-B) caused significant effects on conidial survival compared to non-irradiated conidia, with a significant effect of the interaction between irradiation treatment and *Aspergillus* section studied and time of exposure ( $P < 0.001$ ) (Table 2).

Some of the species studied in this work were isolated from two different regions (Table 1). Common irradiance values measured in the northeast are lower than values measured in the south. Nevertheless, no significant differences ( $P > 0.05$ ) in the response to irradiation (UV-A or UV-B) were found as a result of the different origin of isolation.

In general, a decrease in viability of conidia was observed over time for all isolates tested (Fig. 3). Species from the section Circumdati showed the greatest loss of conidial viability, both in UV-irradiated and control treatments; moreover, earlier (5 days) significant differences in viability due to UV radiation were observed in this section. *Aspergillus* sections Nigri and Flavi showed reduced viability after 10–15 days, although the relative decrease in viability after 15 days was less than 2 log cycles for all sections.

Mean viability ( $n = 6$ ) of UV-exposed *Aspergillus* conidia over time (expressed as log CFU counts in DRBC plates). Error bars indicate standard deviation. Letters indicate homogeneous groups within time/irradiation treatments (Tukey HSD,  $P < 0.05$ ).

Furthermore, data were analysed separately for each *Aspergillus* section in order to establish significant differences among species (Table 3). Within section Flavi, *A. parasiticus* was significantly more affected than *A. flavus*; similarly within section Nigri *A. tubingensis* was more affected than *A. carbonarius* but only when they were irradiated under UV-B, and no significant differences were observed within section Circumdati (Fig. 4).

The effect of UV-A and UV-B was not compared as both radiations were applied at different levels of intensity; however, as comparable levels of survival were observed and UV-B was applied at a lower irradiance, it is clear that UV-B produces more deleterious effects owing to its shorter wavelength.

## DISCUSSION

Spain is one of the European countries which receives higher amount of radiation but also the country that shows the greatest contrast and radiative gradients and complexity in the distribution of radiative energy.<sup>39</sup> Values used in this study were based on mean values of UV-A and UV-B global (direct and diffused) radiation measured in Seville, since this city has one of the highest intensities of radiation and number of hours of exposure values in Spain. In this work, the effect of UV-A (365 nm) and UV-B (312 nm) radiation on mycotoxigenic fungal conidia survival, which are frequently isolated from crops, has been studied.

In this study conidia were dislodged on to membranes, and no source of water or nutrients was available. Moreover, light is a very important signal for fungi: it influences many different physiological responses such as pigmentation, sexual development, asexual conidiation, the circadian clock and secondary metabolism.<sup>40</sup> For this reason, the experimental design consisted of alternating periods of dark and white light with UV radiation exposure included in the light periods, whereas this approach was not used in previous experiments where only UV light was tested.<sup>23, 24</sup>

Some authors have suggested that conidia pigmentation could represent an important protection against UV radiation.<sup>24, 30, 32, 41-44</sup> Three differently coloured conidia of *Aspergillus* were tested in our experiment: yellow, green and black, belonging to sections Circumdati, Flavi and Nigri, respectively. Undoubtedly, conidia belonging to section Circumdati, with the lightest pigmentation, showed greater loss of viability, in both irradiated and non-irradiated treatments. Nevertheless, at the 5th day, when no significant differences compared to the first day were observed in the controls, only significant differences due to irradiation

were noticeable in section Circumdati under UV-A and section Circumdati and Flavi under UV-B. The photo-protective potential under UV-C radiation of fungal pigments of three *A. niger* isolates possessing the same genetic background, but differing in their degree of pigmentation, have been studied recently by Esbelin et al.<sup>45</sup> The authors reported that spores of *A. niger* with fawn and white pigmentation were more sensitive to continuous UV-C radiation than the wild-type *A. niger* isolate, with dark pigmentation. Dominance of dark-coloured microfungi is characteristic for almost all mycologically studied desert soils.<sup>46-48</sup> Therefore, dark-coloured spores could confer more protection against UV-B radiation. In fact, this provides a logical explanation for the high number of black aspergilli on grapes subjected to prolonged sun exposure in countries with high UV irradiance, such as Spain, Italy and Greece.<sup>34, 49, 50</sup>

However, not only differences were found between sections, but also interspecific differences in conidial survival were found within *Aspergillus* sections Flavi and Nigri, showing that other characteristics could give protection to the conidia. Physical characteristics like the projected surface area-to-volume ratio (SAV) of spores or wall thickness have also been emphasized as responsible for conidial survival to radiation.<sup>24, 43, 44</sup> In fact, the projected SAV of spores is an important factor on UV sensitivity, as the lower sensitivity of spores was related to lower SAV values.<sup>24</sup> The spores of *Aspergillus* species tested are spherical, and therefore lower SAV corresponds to bigger radius. Simões et al.<sup>51</sup> described the structural diversity of spores of black aspergilli. Interestingly, *A. carbonarius* showed higher diameters (7.658  $\mu\text{m}$ ) among the section Nigri, compared to other species like *A. tubingensis* (3.972  $\mu\text{m}$ ) or *A. niger* (3.340  $\mu\text{m}$ ). This could be the cause of significant differences between these species when irradiated with UV-B. Similarly, *A. carbonarius* was significantly more resistant than *A. niger* when it was irradiated for a short time under UV-C.<sup>41, 44</sup> The authors also suggested that the resistance of *A. carbonarius* was due to the thicker conidial wall.

Focusing on UV-A, we found a relevant mortality of conidia due to exposure to 1.7  $\text{mW cm}^{-2}$  (1836  $\text{kJ m}^{-2}$ ) around the 5th day on section Circumdati and the 10th day on *Aspergillus* sections Flavi and Nigri (3672  $\text{kJ m}^{-2}$ ). Twice the exposure time (12 h d<sup>-1</sup>) but lower irradiance 0.60–1.250  $\text{mW cm}^{-2}$  (43.2–540  $\text{kJ m}^{-2} \text{d}^{-1}$ ) during one day did not cause any significant difference to germination percentage of sporangia of *Bremia lactucae* compared to dark incubation.<sup>52</sup> Shorter exposure times (3 h d<sup>-1</sup>) but under higher irradiance (3.056–5.556  $\text{mW cm}^{-2}$ ) for 16 days (330–600  $\text{kJ m}^{-2} \text{d}^{-1}$ ) caused different effects on germination depending on the species tested:<sup>24</sup> it was enhanced in *Penicillium purpurogenum*, but reduced in *Cladosporium cladosporoides*.

Under UV-B radiation (0.1  $\text{mW cm}^{-2}$  for 6 h d<sup>-1</sup>, 21.6  $\text{kJ m}^{-2} \text{d}^{-1}$ ), a significant reduction was also observed on conidia viability around the 5th day on *Aspergillus* sections Circumdati and Flavi and the 10th day on Nigri. As for UV-A radiation, irradiation for 16 days at <0.001–0.019  $\text{mW cm}^{-2}$  for 3 h d<sup>-1</sup> (0–2.1  $\text{kJ m}^{-2} \text{d}^{-1}$ ) led to divergent effects depending on the species.<sup>24</sup> Exposure of *B. lactucae* to doses of 0.150 and 0.700  $\text{mW cm}^{-2}$  in a range from 2 to 12 h (10.8–302.4  $\text{kJ m}^{-2} \text{d}^{-1}$ )<sup>52</sup> resulted in highly reduced germination (71–100%) compared to white light and darkness.

Field studies with sunlight exposure have shown greater conidia mortality than laboratory works<sup>27</sup> for *Alternaria solani* (20%), *Uromyces phaseoli* (40%), *Peronospora tabacina* (93%)

and *Venturia inequalis* (95%).<sup>22, 27, 36, 53</sup> Exposure to direct sunlight showed a higher reduction in the viability of *A. carbonarius* conidia supported on filter membranes after ~1 week than in our case, although a small part of this decrease (~15%) was attributable to the wind, which could have blown some spores from the filter membranes.<sup>36</sup> Similarly, no disease was observed in carnation inoculated with *Fusarium wilt* grown under direct solar radiation, whereas severe disease was observed in plants under 85% shade cover.<sup>53</sup> The effect of solar irradiance in the field cannot be assessed independently of other physical variables, especially temperature<sup>22</sup> or protection by the infected host tissue.<sup>27</sup> For instance, spores on bunches could be somewhat shielded from sunlight, depending on the bunch and canopy architecture.<sup>36</sup>

Duguay and Klironomos<sup>41</sup> suggested that despite spores being able to survive under certain condition, this does not imply that the resulting hyphae have the same advantages. García-Cela<sup>37</sup> studied the effect of UV radiation on conidia deposited on culture medium, which can provide both nutrients and protection. Again UV-A and UV-B reduced germination and mycelial growth of the species tested; however, *A. carbonarius* was the most tolerant to UV radiation. Also, UV radiation reduced the mycotoxin production by *A. carbonarius* and *A. parasiticus* and interestingly affected the distribution of AFs, the ratios of the different AFs (AFB1, AFB2, AFG1 and AFG2) being different when *A. parasiticus* colonies grew under UV-B.

Additionally, the species studied in this work were isolated from two different regions; however, no different sensitivity to UV was observed due to the origin of isolation. By contrast, previous works with *Aspergillus* species showed slightly different ecological profiles due to the origin of isolation.<sup>6, 54</sup>

## CONCLUSION

The overall spore inoculum present in the field may decrease as a consequence of UV increase, as conidia mortality was observed in all species tested. Moreover, an increase in UV radiation may unbalance the surviving spore species present in vineyards, possibly favouring in the future an even higher predominance of black aspergilli.

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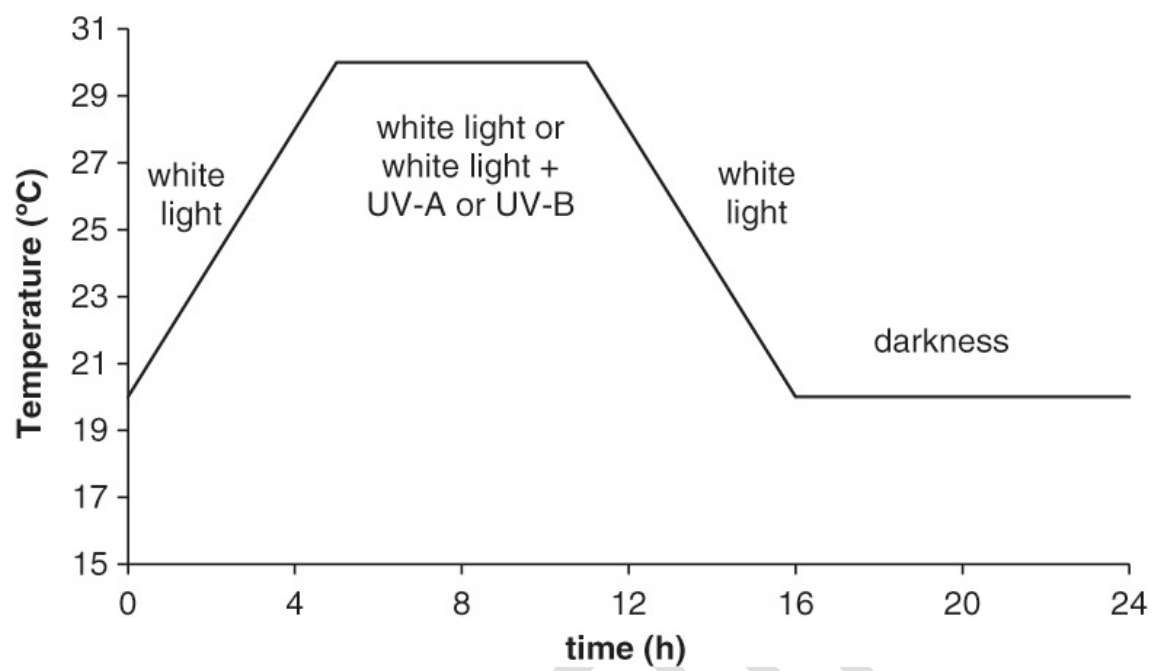


Figure 1

Daily light and temperature conditions for incubation of conidia on filter membranes.

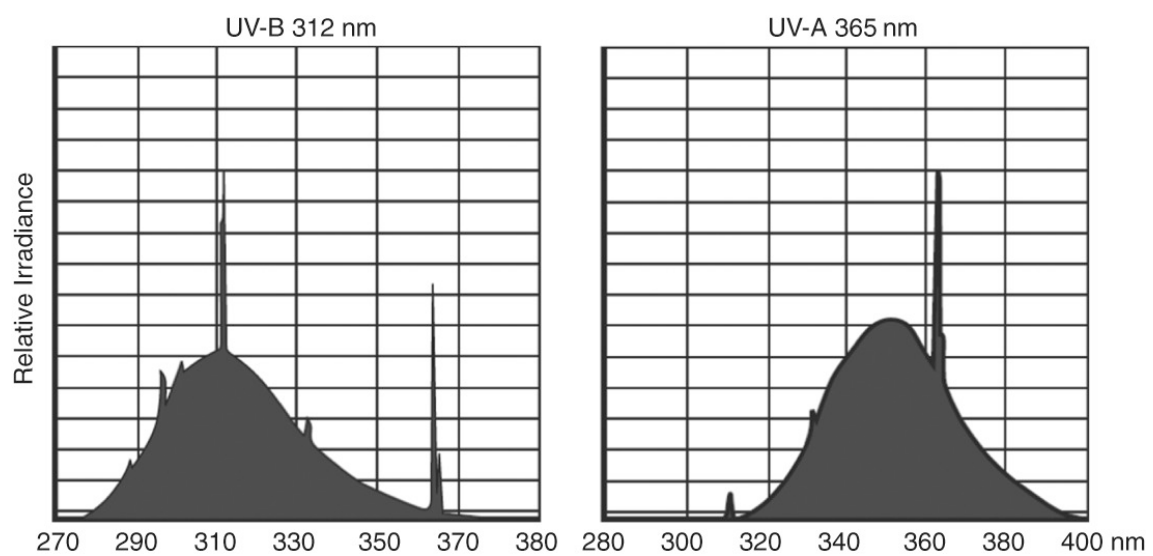


Figure 2

Spectral curves of UV tubes.

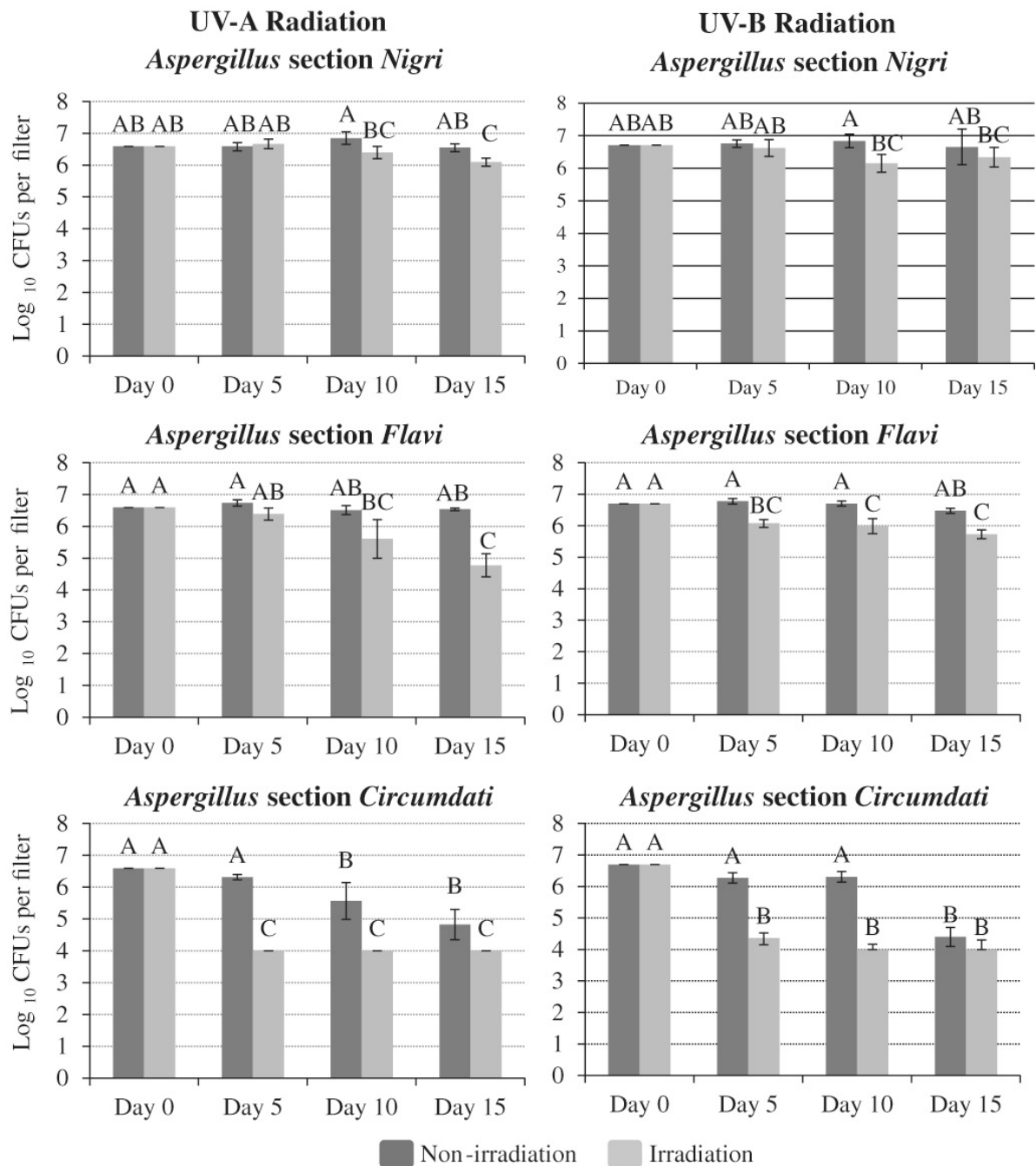


Figure 3

Mean viability ( $n = 6$ ) of UV-exposed *Aspergillus* conidia over time (expressed as log CFU counts in DRBC plates). Error bars indicate standard deviation. Letters indicate homogeneous groups within time/irradiation treatments (Tukey HSD,  $P < 0.05$ )

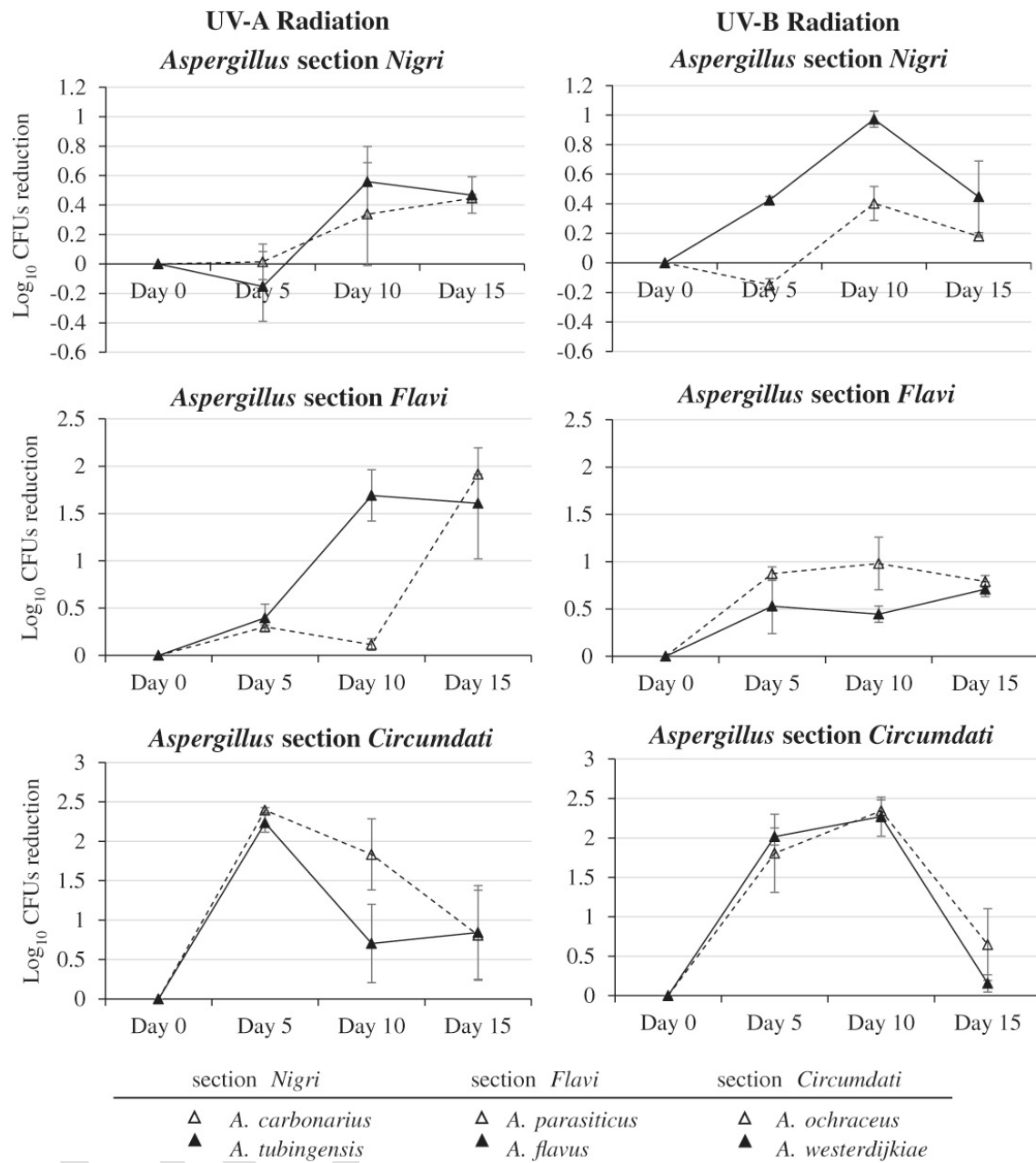


Figure 4

Logarithmic reduction of conidial survival for each species due to the UV irradiation over time



Table 1. Data on isolates used in the study

Reference <sup>a</sup>	Section	Species	Origin in Spain	Year
AC14-UdLTA	<i>Circumdati</i>	<i>A. westerdijkiae</i>	South	2012
AC18-UdLTA	<i>Circumdati</i>	<i>A. westerdijkiae</i>	South	2012
AC7-UdLTA	<i>Circumdati</i>	<i>A. ochraceus</i>	Northeast	2012
AC16-UdLTA	<i>Circumdati</i>	<i>A. ochraceus</i>	South	2012
AF51-UdLTA	<i>Flavi</i>	<i>A. flavus</i>	South	2012
AF34-UdLTA	<i>Flavi</i>	<i>A. flavus</i>	Northeast	2012
AF41-UdLTA	<i>Flavi</i>	<i>A. parasiticus</i>	Northeast	2012
AF16-UdLTA	<i>Flavi</i>	<i>A. parasiticus</i>	Northeast	2012
311-UdLTA	<i>Nigri</i>	<i>A. carbonarius</i>	South	2011
287-UdLTA	<i>Nigri</i>	<i>A. carbonarius</i>	Northeast	2011
276-UdLTA	<i>Nigri</i>	<i>A. tubingensis</i>	South	2011
68-UdLTA	<i>Nigri</i>	<i>A. tubingensis</i>	Northeast	2011

<sup>a</sup> References are the codes of cultures held in the Food Technology Department Culture Collection of the University of Lleida.

Table 2. ANOVA data for the effect of *Aspergillus* section, time of exposure, UV treatment and interactions among them on survival of conidia

Source	d.f.	Sum of squares		F-ratio	
		UV-A	UV-B	UV-A	UV-B
Time	3	40.45	29.94	46.86**	76.01**
Section	2	62.80	57.81	109.12**	220.16**
Treatment	1	22.73	20.93	78.99**	159.39**
Time × Section	6	22.94	24.59	13.28**	31.22**
Time × Treatment	3	7.75	10.36	8.97**	26.3**
Section × Treatment	2	6.48	6.39	11.25**	24.34**

\*\*  $P$ -value = 0.001;

\*  $P$ -value = 0.05; n.s. not significant.

Table 3. ANOVA data for the effect of species, time, irradiation and their interactions on conidia survival within each *Aspergillus* section

	Source	d.f.	Sum of squares		F-ratio	
			UV-A	UV-B	UV-A	UV-B
Aspergillus section Nigri	Species	1	0.01	0.09	0.12 n.s.	1.42 n.s.
	Time	3	1.34	0.66	5.52*	3.29*
	Irradiation	1	0.70	1.30	8.63*	19.47**
	Species × Time	3	0.01	0.09	0.05 n.s.	0.46 n.s.
	Species × Irradiation	1	0.00	0.50	0.02 n.s.	7.45*
	Time × Irradiation	3	0.96	1.06	3.94*	5.3*
	Species × Time × Irradiation	3	0.01	0.09	0.05 n.s.	0.46 n.s.
Aspergillus section Flavi	Species	1	1.67	0.60	5.99*	11.39*
	Time	3	11.01	3.02	13.17**	18.97**
	Irradiation	1	9.09	4.69	32.59**	88.32**
	Species × Time	3	3.17	0.41	3.79*	2.6 n.s.
	Species × Irradiation	1	0.46	0.23	1.67 n.s.	4.35*
	Time × Irradiation	3	7.09	1.57	8.47**	9.84**
	Species × Time × Irradiation	3	0.01	0.09	0.05 n.s.	0.46 n.s.
Aspergillus section Circumdati	Species	1	0.39	0.04	1.51 n.s.	0.32 n.s.
	Time	3	51.03	51.28	65.47**	124.21**
	Irradiation	1	19.42	21.33	74.76**	155.02**
	Species × Time	3	0.91	0.22	1.17 n.s.	0.54 n.s.
	Species × Irradiation	1	0.39	0.03	1.51 n.s.	0.23 n.s.
	Time × Irradiation	3	11.15	15.22	14.31**	36.86**
	Species × Time × Irradiation	3	0.01	0.09	0.05 n.s.	0.46 n.s.

\*\* *P*-value 0.001;

\* *P*-value 0.05; n.s., not significant.