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Diversity of *Aspergillus* section *Nigri* species from vineyards with different agro-climatic conditions in Catalonia, Spain

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ABSTRACT

A few *Aspergillus* section *Nigri* species are involved in the ochratoxin A (OTA) contamination in grapes worldwide, and its occurrence is determined by the agro-climatic conditions of each region. The aim of this study was to examine the diversity of black aspergilli isolated from grapes, soil, and air from vineyards with different agro-climatic conditions. A total of four vineyards located in Catalonia were studied. Two grape varieties were sampled at harvesting time during two consecutive years, and soil and air samples were collected from the same vineyards along the four seasons. The occurrence of black aspergilli was higher in grapes than in soil and air samples. In soil, black aspergilli counts were relatively stable throughout the year, being higher in acidic soil. Seasonal fluctuations were seen in air samples, with higher counts in autumn. In all samples, the *A. niger* aggregate was the predominant group, followed by *A. carbonarius* and uniserate species. Agro-climatic factors influenced the distribution of black aspergilli species. A high occurrence of *A. carbonarius* was found in grapes from vineyards with high temperature and humidity whereas its occurrence in soil and air was very low. In the northern vineyard, *A. brasiliensis* was predominant in grapes and soil. In southern vineyards, *A. welwitschiae* was predominant in soil while *A. tubingensis* predominated in grapes and air. Within uniseriate species, we described for the first time the isolation of *A. trinidadensis* from grapes. All *A. carbonarius* isolates and three isolates identified as *A. welwitschiae* were able to produce OTA.

1. Introduction

Aspergillus section *Nigri*, commonly known as black aspergilli, comprises several species which may contaminate a wide variety of food products and cause food spoilage (Pitt and Hocking, 2009). Black aspergilli have been detected in grapes all over the world (Cabañes and Bragulat, 2018) and some species can produce mycotoxins such as ochratoxin A (OTA) and fumonisins (Abarca et al., 1994; Cabañes and Bragulat, 2018; Frisvad et al., 2011). In Europe, wine has been determined as the second major source of human exposure to OTA after cereals and followed by beer and coffee (European Commission, 2002). Thus, the European Commission has fixed the maximum limit for OTA in wine and grape juice at 2.0 μg/kg, and dried vine fruit at 8.0 μg/kg (European Commission, 2023).

OTA is produced during the infection of grapes in vineyards, mostly from the veraison to harvest, and toxigenic strains of species belonging to *Aspergillus* section *Nigri* are the main fungi responsible for OTA contamination in grapes and wine (Bau et al., 2005; Gómez et al., 2006; Leong et al., 2006). Although other OTA-producing species of *Aspergillus* section *Circumdati* have been isolated from grapes (Battilani et al., 2003; Bau et al., 2005; Bellí et al., 2006; Cabañes et al., 2002; Chulze et al., 2006; Díaz et al., 2009; Gómez et al., 2006; Leong et al., 2006; Serra et al., 2005; Tjamos et al., 2006), its presence is scarce and their contribution to OTA contamination has not been established. Vineyard soils are important reservoirs for black aspergilli, as they live as saprophytes in the superficial layer. It has been postulated that air movement deposits spores from the soil onto the grape berry surface, thus the risk of contamination with OTA in wines might be related to the presence of toxigenic strains in the soil (Leong et al., 2007). During the last decades, numerous studies have been conducted in several countries aiming to identify the main species of *Aspergillus* section *Nigri* involved in mycotoxin contamination of grapes and its derivatives. Many studies have shown that *A. carbonarius* is the main responsible source of OTA in wine or dried vine fruits from different viticultural regions worldwide (Abarca et al., 2003; Battilani et al., 2003; Bau et al., 2005; Bejaoui et al., 2006; Bellí et al., 2006; Cabañes et al., 2002; Chulze et al., 2006; Díaz

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et al., 2009; Gómez et al., 2006; Kizis et al., 2014; Leong et al., 2006; Palumbo et al., 2011; Serra et al., 2005; Tjamos et al., 2006; Pantelides et al., 2017; Qi et al., 2016). In the *A. niger* aggregate, the phylogenetically close species *A. niger* and *A. welwitschiae* are considered OTA producers, and they are also found in grapes. However, while *A. carbonarius* isolates consistently produce large amounts of ochratoxin A (Cabañes et al., 2013), the reported percentages of OTA-producing strains in the *A. niger* aggregate are much lower (Bau et al., 2005, 2006; Cabañes et al., 2002; Frisvad et al., 2011; Leong et al., 2004; Palumbo et al., 2011; Serra et al., 2005).

Black aspergilli are one of the most difficult groups concerning classification and identification. Classification based on morphological criteria is complicated and can lead to misidentification. In the last revision of the genus *Aspergillus*, where calmodulin gene (CaM) was used as a marker, 27 species were accepted in the *Aspergillus* section *Nigri* (Samson et al., 2014; Houbraken et al., 2020). This section includes biseriate and uniseriate species and they are classified in five series: *Carbonarii*, *Nigri* (formerly known as *A. niger* aggregate), *Heteromorphi*, *Japonici* and *Homomorphi* (Houbraken et al., 2020). Species frequently isolated from grapes belong to the biseriates series *Carbonarii* (*A. carbonarius* and *A. ibericus*) and *Nigri* (*A. niger*, *A. welwitschiae*, *A. brasiliensis*, and *A. tubingensis*), and the uniseriate series *Japonici* (*A. aculeatus*, *A. japonicus*, *A. labruscus* and *A. uvarum*) (Cabañes and Bragulat, 2018). Recently, the re-examination of species boundaries in the series *Nigri* using genomic analysis supported a significant reduction in the number of species (Bian et al., 2022), suggesting that *A. niger* and *A. welwitschiae* represent a single species. However, this proposal has not been widely adopted and a phylogenomic analysis conducted by Steenwyk et al. (2024) supported the presence of two distinct clades.

It is a consensus in the literature that the distribution of *Aspergillus* section *Nigri* species relied on several factors and is determined by the agro-climate condition of each region (Battilani et al., 2006b; Chiotta et al., 2013). Despite the extensive research in this field, most studies are focused on grapes, and information on the black aspergilli population structure and its dynamics in terms of relative abundance over time in vineyard environments, such as soil and air, is scarce. Therefore, this study was initiated to investigate differences in the *Aspergillus* section *Nigri* population structure in four vineyards with different agro-climatic conditions located in Catalonia, Spain. Also, the mycotoxigenic capacity of *Aspergillus* section *Nigri* isolates was evaluated.

2. Material and methods

2.1. Samples

During 2012–2013 season, a total of 4 vineyards located in different areas of Catalonia (northeastern Spain) were sampled. The chosen vineyards belonged to three grape-growing provinces along the mediterranean coast, from north to south as shown in Fig. 1: Girona (vineyard G), Barcelona (vineyards B1 and B2), and Tarragona (vineyard T). Although these areas have predominantly Mediterranean climatic influences, they have particular agro-climatic conditions. Vineyard G is in a coastal region close to the Pyrenees, which causes sharp contrasts with high sunshine, notable rainfall, and strong winds known as tramontana. The soil is also acidic and poor in organic matter. Vineyards B1 and B2 are in the central part of the province of Barcelona. Vineyard B1 has an oceanic climate inland with dry summers, whereas vineyard B2 is near the sea with a Mediterranean climate with humid summers. In both vineyards, the soil is considered calcareous, poor in organic matter, and good draining. Additionally, silt and clay dominate the texture of these soils giving structure and color to the vineyard. Finally, vineyard T is located in the southern part with a hot-summer Mediterranean climate, with hot and dry summers. Their vineyards are cultivated on slopes and the soil is rich in limestone, poor in organic matter and has good drainage. Average annual temperatures and precipitation, altitude and wind for each vineyard are shown in Table 1. For each vineyard two grape varieties were included: Cabernet Sauvignon, and Grenache.

Grapes were sampled at harvesting time (September/October). At each sampling site, 10 bunches were obtained from 10 different vines located approximately along two crossing diagonals of the vineyard. Every bunch was collected in a separate paper bag. Samples were sent to the laboratory as soon as they were collected and were analyzed within 24–48 h maximum. From each bunch, five berries were randomly selected and plated directly onto malt extract agar (MEA) (Pitt and Hocking, 2009) supplemented with 100 ppm of chloramphenicol and 50 ppm of streptomycin and five berries more onto Dichloran Rose Bengal Chloramphenicol (DRBC, Oxoid, Basingstoke, UK) agar (Pitt and Hocking, 2009). In total, 1600 berries were analyzed (800 in 2012 and 800 in 2013, 100 per each variety and location). Plates were incubated at 25 °C for 7 days.

During the 2013 season, soil and air samples were taken from the same vineyards and both grape varieties during winter (February), spring (May), summer (July), and autumn (November). At each

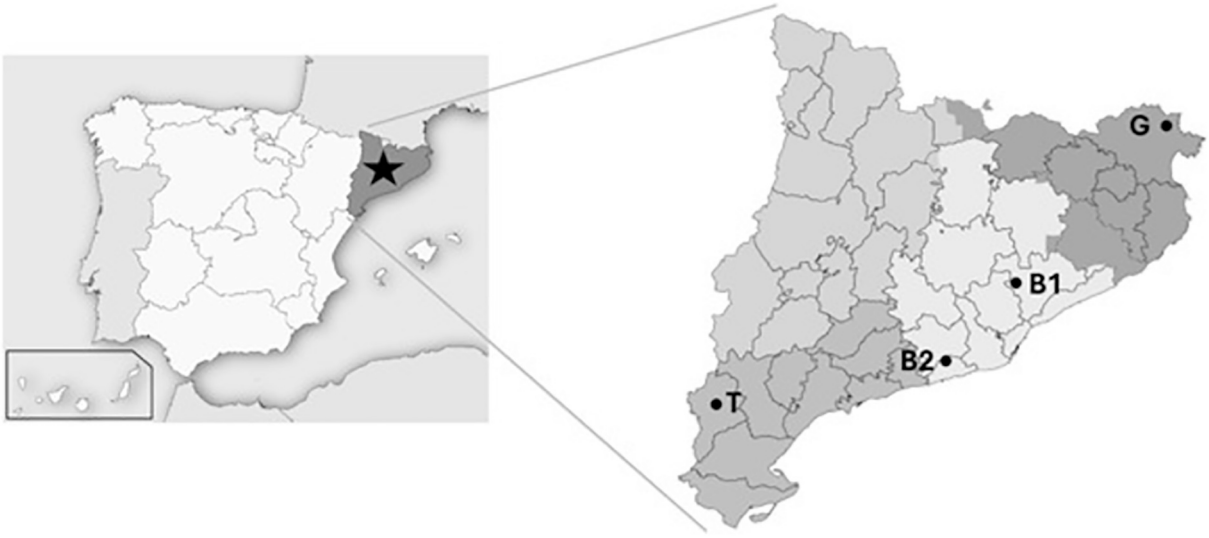

Fig. 1. Map of the sampling sites. The vineyards are located in Catalonia, Northeast of Spain (★). Vineyard G located in Girona, vineyard B1 and vineyard B2 located in Barcelona and vineyard T located in Tarragona.

Table 1
Climatic conditions of the different vineyards included in this study.

			2012					2013				
Vineyard	Altitude (m)	Wind	T max[a]	T mean[b]	T min[c]	RH[d]	Rainfall[e]	T max[a]	T mean[b]	T min[c]	RH[d]	Rainfall[e]
G	124	NW	21.4	15.6	9.7	62	583.2	20.8	15.1	9.5	64	588.5
B1	203	NW	21.5	14.5	8.1	67	513.1	21.0	14.1	8.1	69	678.9
B2	44	S	22.1	16.1	11.6	65	459.5	21.8	15.8	11.3	66	618.6
T	368	W	21.0	15.0	9.9	64	437.4	20.0	14.3	9.4	67	561.0

Data from the nearest meteorological station of each vineyard obtained from the Servei Metereologic de Catalunya, Anuari de dades (https://www.meteo.cat/wpweb/climatologia/dades-i-productes-climatics/anuari-de-dades/) on March 2024.
[a] Average maximum temperature (°C).
[b] Average annual temperature (°C).
[c] Average minimum temperature (°C).
[d] Average relative humidity (%).
[e] Total accumulated precipitation mm.

sampling site, two soil samples were collected of approximately 20 g of the topmost 5 cm of soil, approximately 0.5 m away from each of the trunks of the same vines used for berry sampling. Soil samples were stored at 4 °C until use. A total of 64 samples were collected and processed. To enumerate fungal propagules, each soil sample was mixed by hand, and a 10 g subsample was suspended in 90 ml of sterile 0.05 % Tween 80. The suspension was vortexed for 1 min, and tenfold dilutions were plated on DRBC agar. Dilution plates were incubated at 35 °C for 7 days, after which fungal colonies were enumerated and the results expressed as CFU/g of soil.

Also, a total of 96 air samples were collected at 80 cm above the surface of the soil under the vines using a portable spore trap (SAMPL'AIR™, Biomerieux, Spain) provided with 90 mm diameter Petri dishes containing DRBC agar. For each sampling, the spore trap sampled air for two minutes (aspiration amount 100 l/min). From each vineyard, a total of three air samples were obtained at each sampling date. Plates were incubated at 35 °C for 7 days, after which fungal colonies were enumerated and the results expressed as CFU/m^3 of air.

2.2. Mycological study

On the last day of incubation, all fungi considered belonging to *Aspergillus* section *Nigri* were isolated and transferred to slants and then to Czapek Yeast Extract Autolysate agar (CYA agar) (Pitt and Hocking, 2009) for identification. After incubation at 25 °C for 7 days, black aspergilli isolates were identified using morphological criteria (e.g. conidial head, conidia) as uniseriate species, *A. niger* aggregate and *A. carbonarius* (Abarca et al., 2004). Isolates were preserved at −80 °C.

2.3. OTA production ability

All isolates belonging to *Aspergillus* section *Nigri* were evaluated using a previously described HPLC screening method (Bragulat et al., 2001). Briefly, *A. carbonarius* isolates were grown on Czapek Yeast extract Agar (CYA) and incubated at 30 and 35 °C for 10 days. The rest of black aspergilli isolates were grown on Czapek Yeast extract Agar (CYA) and Yeast extract Sucrose agar (YES) (Samson et al., 2000) and incubated at 25 °C for 10 days.

The isolates were three-point inoculated. Three agar plugs were removed from the central area of the colony and extracted with 0.5 ml of methanol. The extracts were filtered and injected into the HPLC. OTA detection and quantification was carried out by a Waters LCM1 chromatograph with a fluorescence detector Waters 2475 (excitation wavelength: 330 nm/emission wavelength: 460 nm), and with a C18 Spherisorb S5 ODS2, 250 × 4.6 mm column. Twenty microliters of each extract were injected. The mobile phase, with a flow rate of 1 ml/min, consisted of an isocratic program of 57 % acetonitrile, 41 % water and 2 % acetic acid. The limit of quantification of the HPLC technique with the extraction procedure was 0.06 μg/g for OTA.

2.4. Molecular identification

A total of 188 isolates were subjected to CaM gene sequence analysis. Due to the distinctive morphological traits of *A. carbonarius*, only two strains isolated from grapes (one from each harvest) were sequenced. All strains belonging to uniseriate species (n = 43) and all biseriate species able to produce OTA (n = 3) were also identified by CaM gene sequencing. A total of 140 non-OTA producing isolates identified as *A. niger* aggregate were selected for molecular identification. Representative isolates from grapes from both years and grape varieties, and all vineyards (n = 77) were included. Also, representative isolates from soil (n = 43) and air (n = 20) samples from both grapes' variety grown and all seasons and vineyards were included.

DNA was extracted and purified from 48 h old cultures in malt extract broth according to the FastDNA Spin kit protocol with the FastPrep FP-24 instrument (MP Biomedicals, Biolink, Barcelona, Spain). The DNA was kept at −20 °C until used as template for PCR amplification. The CaM gene region was amplified using the primers CL1 (5′ CCG AGT ACA AGG AGG CCT TC 3′) and CL2A (5′ CCG ATA GAG GTC ATA ACG TGG 3′) described in O'Donnell et al. (2000). Both strands of purified gene fragments were sequenced with BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA) and an ABI 3500XL Genetic Analyzer (Applied Biosystems, USA). The partial CaM sequences were subsequently aligned with those from *A.* section *Nigri* available in the GenBank database and sequence alignments were carried out using MUSCLE implemented in MEGA 11 software (Tamura et al., 2021). Maximum likelihood analysis was conducted using MEGA 11 software with 1000 bootstrap replicates. A suitable substitution model was determined. The initial tree for heuristic search was obtained by applying the Neighbour-Joining method to a matrix of pairwise distances estimated using the Maximum Composite Likelihood approach. Clades that were supported by bootstrap values (bs) of ≥70 % were regarded as strongly supported. Sequence of *Aspergillus flavus* CBS 569.65^T was selected as outgroup for the tree construction.

2.5. Statistical analysis

Data obtained from grapes was analyzed statistically by means of one-way analysis of variance test, Student's *t*-test, and Kruskal-Wallis nonparametric ANOVA with pairwise comparison test adjusted with Bonferroni correction, and general linear model. Pearson correlation coefficient was used to evaluate the relationship between meteorological variables and frequency uniseriate species, *A. niger* aggregate and *A. carbonarius*. The effect of interaction between variables was analyzed with a General Linear Model (GLM).

Data obtained from soil and air samples was analyzed statistically by Kruskal-Wallis nonparametric ANOVA with pairwise comparison test adjusted with Bonferroni correction. All statistical analyses were performed using SPSS software (version 29.0.1.0).

3. Results

3.1. Grape samples

The frequency of occurrence of black aspergilli varied according to the vineyard and the vintage evaluated, as shown in Table 2. Black aspergilli were found in all grape samples and the highest occurrence of these fungi was observed in 2012 vintage ($p < 0.05$), ranging the percentage of colonized berries from 59.5 % to 87.5 % (mean = 78.3 %), and from 34 % to 82 % (mean = 48.1 %) in 2013 vintage. In both vintages, vineyard B2 had the highest mean occurrence of black aspergilli, being significantly higher in 2013 ($p < 0.05$). Grenache variety had a higher mean occurrence of black aspergilli compared to Cabernet Sauvignon in 2012 ($p < 0.05$), but not significant differences were observed between both grape varieties in 2013.

The percentage of colonized berries with *A. niger* aggregate, *A. carbonarius*, uniseriate species and other *Aspergillus* spp. at each vineyard and grape variety is shown in Fig. 2. In both vintages, *A. niger* aggregate was found to be the most frequently occurring group in grapes from all vineyards and grape varieties except for Cabernet Sauvignon from vineyard B2 where *A. carbonarius* was the most frequently occurring species in both vintages. In fact, and regardless of the vintage, *A. carbonarius* was found in all vineyards and its occurrence varied according to the vineyard. In vineyards B1 and T the percentage of contaminated berries with this species was significantly lower ($p < 0.001$) than in vineyards G and B2. In vineyard G the percentage of contaminated berries ranged from 10 % to 29 % in Cabernet variety and 3 % to 15 % in Grenache variety and vineyard B2 showed the highest occurrence, with 77 % to 80 % of Cabernet berries contaminated with *A. carbonarius*, and 17 % of Grenache variety. The mean percentage of grapes contaminated with this species was higher in Cabernet Sauvignon variety (23–26.8 %) than in Grenache variety (6–8.5 %) in both years although not significant differences were observed. However, the mean percentage of grapes contaminated with *A. niger* aggregate was significantly higher in Grenache variety (39.5–86.5 %) than in Cabernet Sauvignon variety (34.5–48.3 %) ($p < 0.05$). Uniseriate species occurrence was very low although its occurrence was significantly higher in 2013 ($p < 0.05$). The frequency of occurrence of other *Aspergillus* species was very low in all vineyards and both vintages.

A total of 1205 isolates were identified as belonging to *Aspergillus* section *Nigri*. As shown in Table 3, most of them were morphologically identified as species belonging to *A. niger* aggregate, being the predominant group in all vineyards, and accounting 75 % of the total black aspergilli. *Aspergillus carbonarius* isolation frequency was lower than *A. niger* aggregate, representing 22.1 % of the total black aspergilli. Uniseriate species were recovered from three of the four vineyards in a low percentage (2.9 %).

The correlation analysis showed a significant positive correlation between maximum ($r = 0.570$, $p < 0.05$), mean ($r = 0.621$, $p < 0.05$), and minimum ($r = 0.524$, $p < 0.05$) temperature and the occurrence of *A. carbonarius* whereas a significant negative correlation was found between *A. carbonarius* and altitude ($r = -0.624$), $p < 0.05$). The general linear model revealed a positive effect of the interaction between mean temperature and relative humidity on the occurrence of *A. carbonarius* ($r = 0.426$, $p < 0.001$). The occurrence of *A. niger* aggregate was positively correlated with maximum temperature ($r = 0.398$, $p < 0.01$) while a negative correlation was obtained with rainfall ($r = -0.508$, $p < 0.05$). Finally, the occurrence of uniseriate species positively correlated with rainfall ($r = 0.453$, $p < 0.05$).

3.2. Soil

Black aspergilli were detected in 95.3 % (61/64) of soil samples analyzed. Total fungal counts and black aspergilli counts (log CFU/g) in soil samples are shown in Fig. 3. No significant differences were found in either total fungal counts and black aspergilli counts among seasons or between grape varieties grown. Significant differences were found among vineyards.

Soil samples from vineyard G had the highest total fungal counts (mean = 4.3 log CFU/g), which were significantly higher than those from the other vineyards ($p < 0.05$). Significant differences were also found between total fungal counts in vineyard B2 (mean = 4.1 log CFU/g) and vineyard T (mean = 3.4 log CFU/g) ($p < 0.05$). Soil samples collected in vineyard T which showed the lowest total fungal counts.

Regarding black aspergilli, soil samples from vineyard G showed the highest black aspergilli counts (mean = 3.4 log CFU/g), being significantly higher than those from vineyard T (mean = 2.7 log UFC/g).

In those vineyards with lower total fungal counts, such as vineyards B1 and T, black aspergilli accounted for 22.9 % and 23.3 % of the total fungal species (Fig. 4). In vineyards G and B2, with higher total fungal counts, black aspergilli accounted for 10.5 % and 8.8 % of the total fungal species, respectively.

A total of 1533 black aspergilli isolates were recovered from soil samples, accounting for 41.6 % of the total *Aspergillus* isolates. As shown in Table 4, in all vineyards *A. niger* aggregate was the predominant group, representing 99.4 % of the total black aspergilli isolates. Only two isolates of *A. carbonarius* were recovered, an isolate from vineyard G and an isolate from vineyard B2. Uniseriate species were recovered only from vineyard B1 (n = 7).

3.3. Air

Black aspergilli were detected in 45.9 % of the air samples and they were found in all vineyards. The value of total fungal counts and black aspergilli counts per m^3 of air at each season and grape variety are shown in Fig. 5. Neither total fungal counts nor black aspergilli counts were significantly different among vineyards. Significant differences were found among seasons.

Total fungal counts ranged from 4.3×10^1 to 3.7×10^2 CFU/m^3, being significantly lower in winter (mean = 4.3×10^1 CFU/m^3) than in the other seasons ($p < 0.05$). In spring and autumn, the total fungal counts were higher (mean = 2.4×10^2 CFU/m^3 and mean = 3.7×10^2 CFU/m3, respectively). No significant differences were found between grape variety grown.

Black aspergilli counts ranged from 5.4 to 15.6 CFU/m3. Black aspergilli counts were significantly higher in autumn ($p < 0.05$) and in

Table 2
Occurrence of *Aspergillus* section *Nigri* in grapes (percentage of colonized berries) at different vintages, grape variety and vineyard.

Year	Grape variety	Vineyard G	Vineyard B1	Vineyard B2	Vineyard T	Mean grape variety [b]	Mean year [c]
2012	Cabernet Sauvignon	73 %	22 %	96 %	66 %	64.3 %b	
	Grenache	91 %	97 %	79 %	100 %	91.8 %a	
	Mean vineyards[a]	82 %a	59.5 %b	87.5 %a	83 %a		78.3 %a
2013	Cabernet Sauvignon	30 %	21 %	98 %	54 %	50.8 %c	
	Grenache	38 %	62 %	66 %	16 %	45.5 %c	
	Mean vineyards[a]	34 %a	41.5 %a	82 %b	35 %a		48.1 %b

[a] . In rows, means followed by the same letter are not significantly different ($p > 0.05$).
[b] . In column, means followed by the same letter are not significantly different ($p > 0.05$).
[c] . In column, means followed by the same letter are not significantly different ($p > 0.05$).

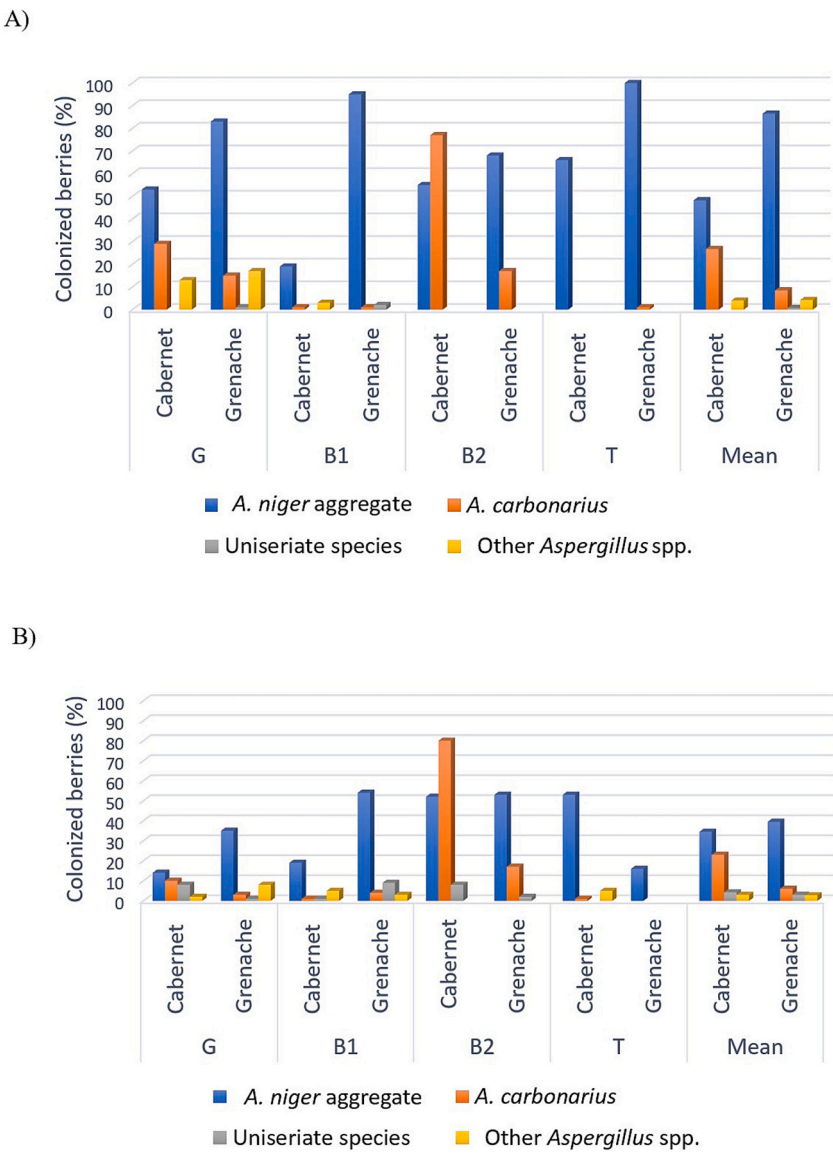

Fig. 2. Occurrence of *A. niger* aggregate, *A. carbonarius*, uniseriate species and other *Aspergillus* spp. at each vineyard and grape variety in 2012 (A) and 2013 (B).

Table 3
Number of isolates of *Aspergillus* section *Nigri* recovered from grapes at different vineyards and grape variety.

Species	G		B1		B2		T		
	n (C/GR)[a]	%[b]	n (C/GR)	%	n (C/GR)	%	n (C/GR)	%	Total
A. niger aggregate	194 (72/122)	73.2	203(40/163)	90.6	251 (122/129)	54.8	256 (126/130)	99.2	904 (75.0%)
A. carbonarius	59 (40/19)	22.3	8 (2/6)	3.6	197 (161/36)	43.3	2 (1/1)	0.8	266 (22.1%)
Uniseriate species	12 (10/2)	4.5	13(1/12)	5.8	10 (9/1)	2.2	0 (0/0)	0	35 (2.9%)
Total	265 (122/143)		224 (43/181)		458 (292/166)		258 (127/131)		1205

G, vineyard G; B1, vineyard B1; B2, vineyard B2; T, vineyard T.
[a] C, Cabernet Sauvignon variety; GR, Grenache variety.
[b] Percentage in relation to total isolates of black aspergilli.

vineyards where the Grenache variety was grown ($p < 0.05$). Depending on the season, these fungi accounted for 1.0 % of the total fungal species in spring to 11.1 % in winter (Fig. 6).

A total of 161 black aspergilli isolates were recovered from air samples, accounting for the 36.7 % of the total *Aspergillus* species (Table 5). In vineyard B2 black aspergilli accounted for the 68 % of the total *Aspergillus* species, whereas in vineyard G this percentage was lower (26.7 %). As shown in Table 6, most of the strains were isolated from air samples from the Grenache variety. In all vineyards *A. niger* aggregate was prevalent (158/161) in air samples, being the only group found in vineyards G and T. Only two isolates of *A. carbonarius* were recovered, one in vineyard B1 and the other in vineyard B2. Only one isolate of uniseriate species was recovered, and it was from vineyard B1.

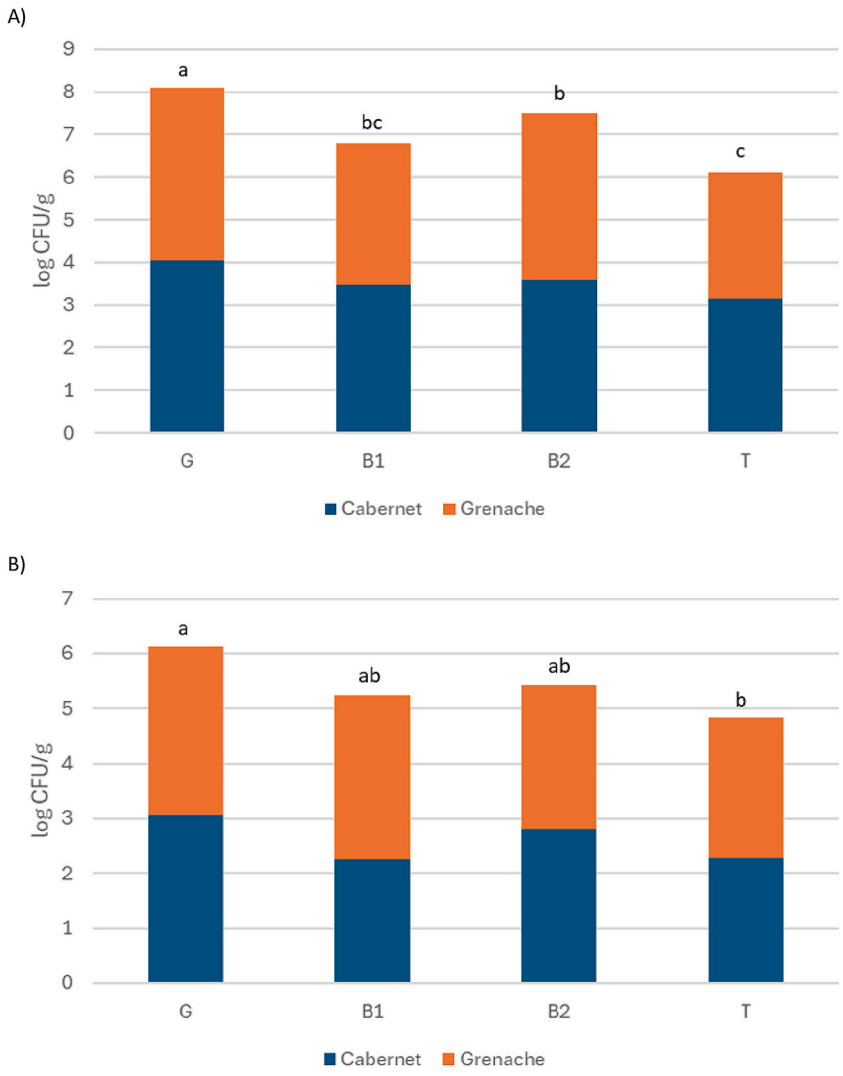

Fig. 3. Total fungal counts (A) and black aspergilli counts (B) in soil samples from vineyard G (G), vineyard B1 (B1), vineyard B2 (B2) and vineyard T (T). Values with the same letter are not statistically different ($p > 0.05$).

3.4. OTA production

A total of 2899 isolates were tested for OTA production. All isolates identified as *A. carbonarius* (n = 270) were able to produce OTA. As shown in Table 6, the concentration of OTA detected on CYA at 35 °C varied from 0.14 to 70.5 μg/g. None of the uniseriate species (n = 43) were able to produce OTA. Regarding *A. niger* aggregate isolates (n = 2586), only three of them were able to produce OTA in low concentrations (range 0.36–2.90 μg/g). One isolate was recovered from grapes (vineyard T) and two isolates were recovered from soil samples (vineyard B1 and B2).

3.5. Molecular identification

A total of 188 selected isolates were subjected to CaM gene sequence analysis (Supplementary Tables S1, S2 and S3). The maximum likelihood tree representing phylogenetic relationships among their sequences is shown in Fig. 7. The identification of two *A. carbonarius* isolates from grapes was confirmed by calmodulin sequence (isolates A-4216 and A-6889). The three isolates belonging to the *A. niger* aggregate which were able to produce OTA were identified as *A. welwitschiae* (isolates A-4872, A-5119, and A-6616).

Among the uniseriate species, *A. uvarum* was predominant (n = 35), with two different sequence types found (isolates A-5229 and A-5514). This species was recovered from grapes in vineyards G, B1 and B2 and from soil and air samples in vineyard B1. Eight isolates from grapes in vineyard B2 were identified as *A. trinidadensis*. All of them showed the same calmodulin sequence (isolate A-6798).

The non-ochratoxigenic selected isolates of the *A. niger* aggregate group (n = 140) were identified as *A. tubingensis* (n = 75, 53.6 %), *A. welwitschiae* (n = 35, 25 %), *A. brasiliensis* (n = 29, 20.7 %), and *A. niger* s. str. (n = 1, 0.7 %) (A-5282). In *A. brasiliensis*, two different sequence types were found (isolates A-4355 and A-4384), whereas nine genotypes were found both in *A. tubingensis* (A-4800, A-4983, A-5133, A-5785, A-6578, A-6640, A-67788, A-6955, and A-7135) and in *A. welwitschiae* (A-4308, A-4468, A-4497,A-4537, A-5079, A-5240, A-5256, A-5583, A-7117). Representative genotypes for each species have been deposited at the GenBank database (accession numbers PQ510013-PQ510042).

As shown in Fig. 8, most of the selected isolates recovered from grapes were identified as *A. tubingensis* (n = 49), being the predominant species in all vineyards except in vineyard G, where most of the isolates were identified as *A. brasiliensis* (n = 13). *Aspergillus welwitschiae* was recovered from grapes of all vineyards (n = 14) and only one isolate, recovered from vineyard B1, was identified as *A. niger*.

In soil samples, selected isolates were identified as *A. welwitschiae* (n = 18) and *A. tubingensis* (n = 13) in vineyards B1, B2 and T whereas in vineyard G, all the isolates were identified as *A. brasiliensis* (n = 12). In

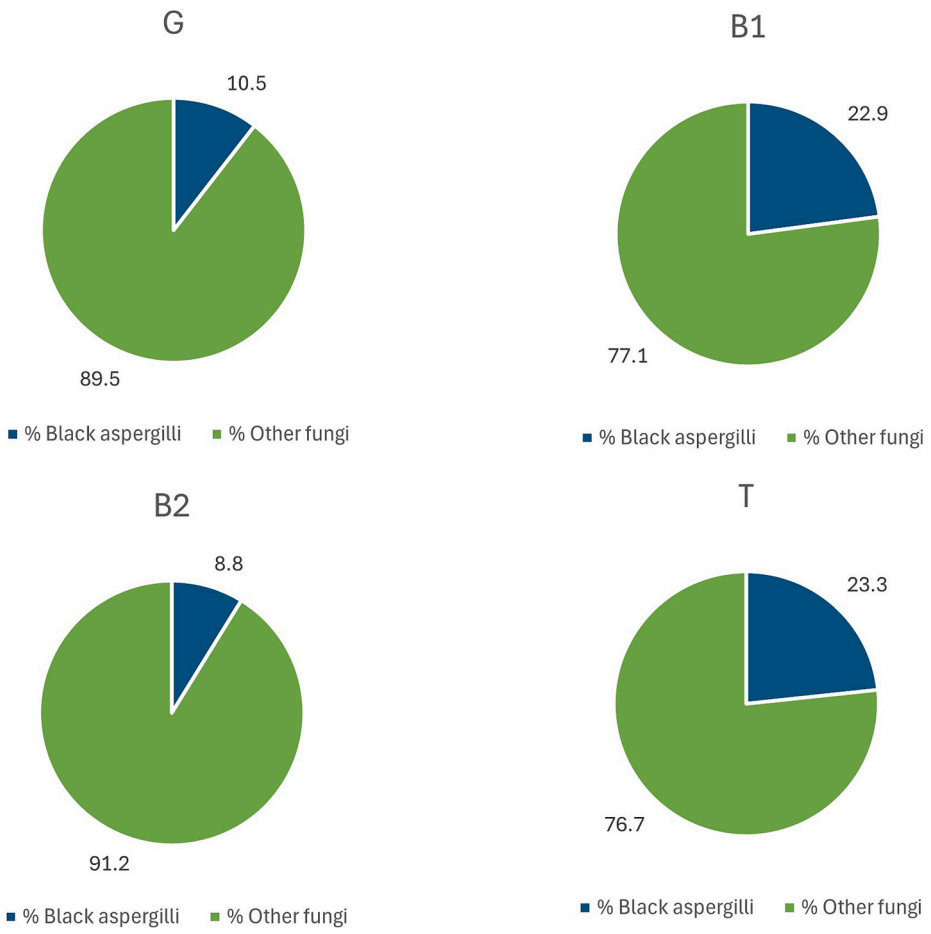

Fig. 4. Occurrence of black aspergilli in soil samples from different vineyards.

Table 4
Number of isolates of *Aspergillus* section *Nigri* recovered from soil samples in different vineyards and grape varieties.

Species	G		B1		B2		T		
	n (C/GR)[a]	%[b]	n (C/GR)	%	n (C/GR)	%	n (C/GR)	%	Total
Aspergillus section *Nigri*	764 (350/414)	33.2	333 (52/281)	72.9	270 (162/108)	38.1	166 (51/115)	46.9	1533 (41.6 %)
A. carbonarius	1 (0/1)		0		1 (1/0)		0		2
A. niger aggregate	763 (350/413)		326 (52/274)		269 (161/108)		166 (51/115)		1524
Uniseriate species	0		7 (0/7)		0		0		7
Total *Aspergillus* spp.	2168 (1040/1128)		457 (91/366)		709 (349/360)		354 (191/163)		3688

G, vineyard G; B1, vineyard B1; B2, vineyard B2; T, vineyard T.
[a] . C, Cabernet Sauvignon variety; GR, Grenache variety.
[b] . Percentage in relation to total isolates of *Aspergillus* spp.

air samples, most of the isolates were identified as *A. tubingensis* (n = 13), followed by *A. brasiliensis* (n = 4) and *A. welwitschiae* (n = 3).

4. Discussion

In the present study, we investigated the *Aspergillus* section *Nigri* occurrence in grape, soil and air samples from four vineyards of Catalonia, each with different agro-climatic conditions. The grape samples analyzed in our study were obtained during two consecutive harvests and a high occurrence of *Aspergillus* section *Nigri* was observed. *Aspergillus* section *Nigri* was present in all the grape samples and represented >90 % of total *Aspergillus* found in grapes. The average of colonized berries ranged from 78.3 % in 2012 to 48.1 % in 2013. Similar occurrences have been reported in other European countries (Bau et al., 2005; Bejaoui et al., 2006; Kizis et al., 2014; Pantelides et al., 2017; Serra et al., 2005), South America (Chiotta et al., 2013; Chulze et al., 2006; Díaz et al., 2009; Ferranti et al., 2018), Australia (Leong et al., 2006) and North America (Palumbo et al., 2016b; Qi et al., 2016). This predominance in grapes could be explained because of their black spores, which provide protection from sunlight and consequently a competitive advantage against other species (Pitt and Hocking, 2009).

It is a consensus in the literature that black aspergilli occurrence in grapes is determined by various factors and differs depending on agro-climatic regions or conditions (Chiotta et al., 2013; Perrone et al., 2007). It has been shown that the occurrence of black aspergilli at harvesting time was related to the latitude and longitude of vineyards, and a positive gradient was detected moving from west to east and from north to south in Europe. Moreover, higher occurrence rates have been reported in the hottest and driest years (Battilani et al., 2006b). In our study, the occurrence of black aspergilli was higher in grapes harvested in 2012, a year with higher temperatures and lower precipitation than year 2013 (Servei metereologic de Catalunya). Several previous studies

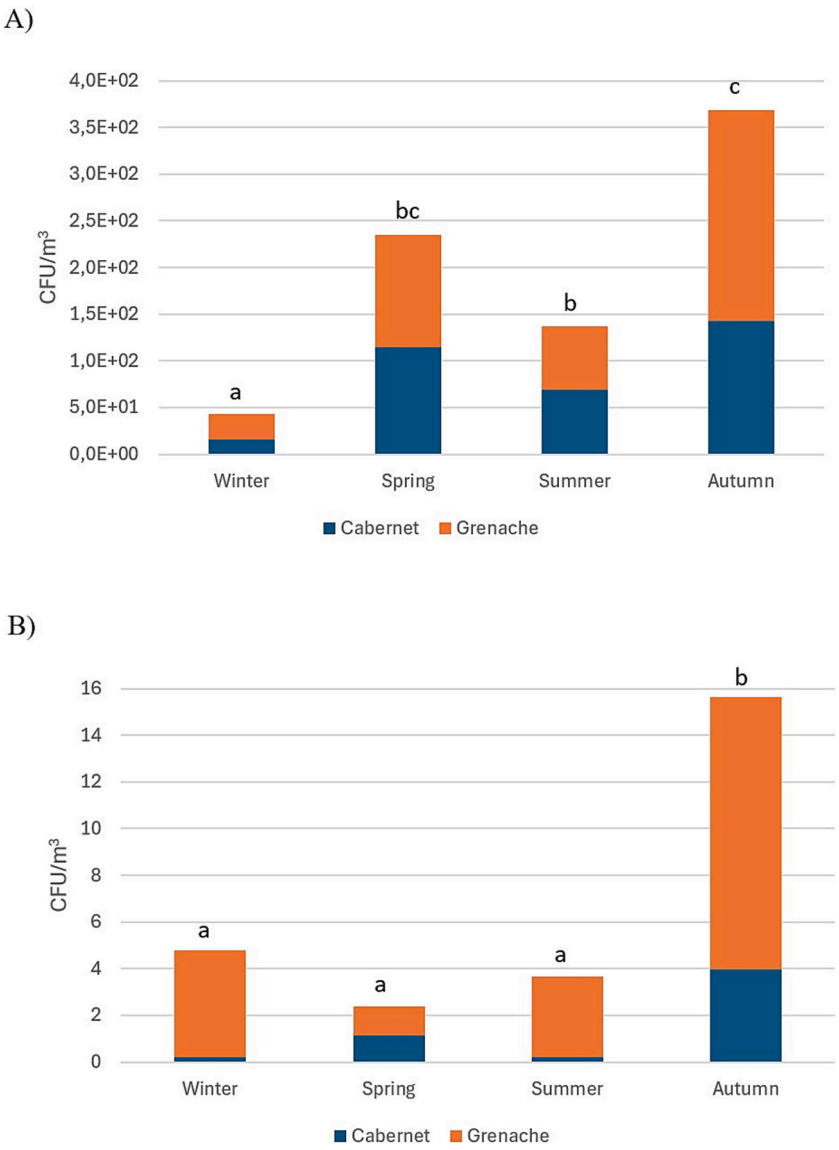

Fig. 5. Total fungal counts (A) and black aspergilli counts (B) in air samples in different seasons and grape variety. Values with the same letter are not statistically different ($p > 0.05$).

correlating meteorological data with the biodiversity of *Aspergillus* section *Nigri* in grapes reported that, in general, *A. niger* aggregate was more frequently isolated in hot and dry areas, while *A. carbonarius* showed higher occurrences in hot and wetter areas (Battilani et al., 2006a; Bellí et al., 2006; Chiotta et al., 2009, 2013). In our study, the agroclimatic conditions significantly influenced the percentage of colonization by a specific species within the *Aspergillus* section *Nigri*. Vineyards located near sea level with hot and humid summers, such as vineyard G and B2 showed a high occurrence of *A. carbonarius*. Even though, *A. carbonarius* occurrence was lower in vineyard G than in vineyard B2, probably because it is exposed to the tramontana wind, a strong, cold and dry wind originating from the Pyrenees that prevents disease and frost. Vineyards B1 and T which were located at higher altitude with lower average annual temperature and hot and dry summers, showed the lowest occurrence of *A. carbonarius* in grapes and the highest isolation frequency of *A. niger* aggregate species. The occurrence of uniseriate species positively correlated with rainfall, being higher in 2013 vintage and in vineyard B1.

The grape variety has been shown to be important in relation to the susceptibility of fungal infection. More compact varieties, such as Cabernet Sauvignon and Grenache, are more susceptible to infection by black aspergilli since they have greater contact surface among berries, which favors fungal growth by being able to retain moisture as well as ease fungal propagation within the bunch. In our study, we observed a higher black aspergilli occurrence on the Grenache variety than in the Cabernet Sauvignon variety in 2012, probably because Grenache has a more compact bunch and is very sensitive to *Botrytis* infection (Chomé et al., 2006). However, in this variety, the colonization with *A. carbonarius* was very low compared to Cabernet Sauvignon. In vitro studies have shown that Cabernet Sauvignon was one of the grape varieties with higher rates of *A. carbonarius* occurrence and OTA content (Battilani et al., 2004).

Although many studies have focused on black aspergilli distribution in grapes, only a few have addressed its occurrence in soil and air in vineyards. In our study, *Aspergillus* section *Nigri* species were present nearly in all the soil samples analyzed and represented a low percentage of the total fungi recovered (<23.3 %) and 41.6 % of the total *Aspergillus* species isolated. These results agree with previous studies that have shown that black aspergilli live as saprophytes in the superficial layer of the vineyard soil, constituting the major inoculum reservoir (Barberis et al., 2014; Leong et al., 2006, 2007; Oliveri et al., 2017; Palumbo et al., 2016a; Qi et al., 2016). Some studies have shown that black aspergilli

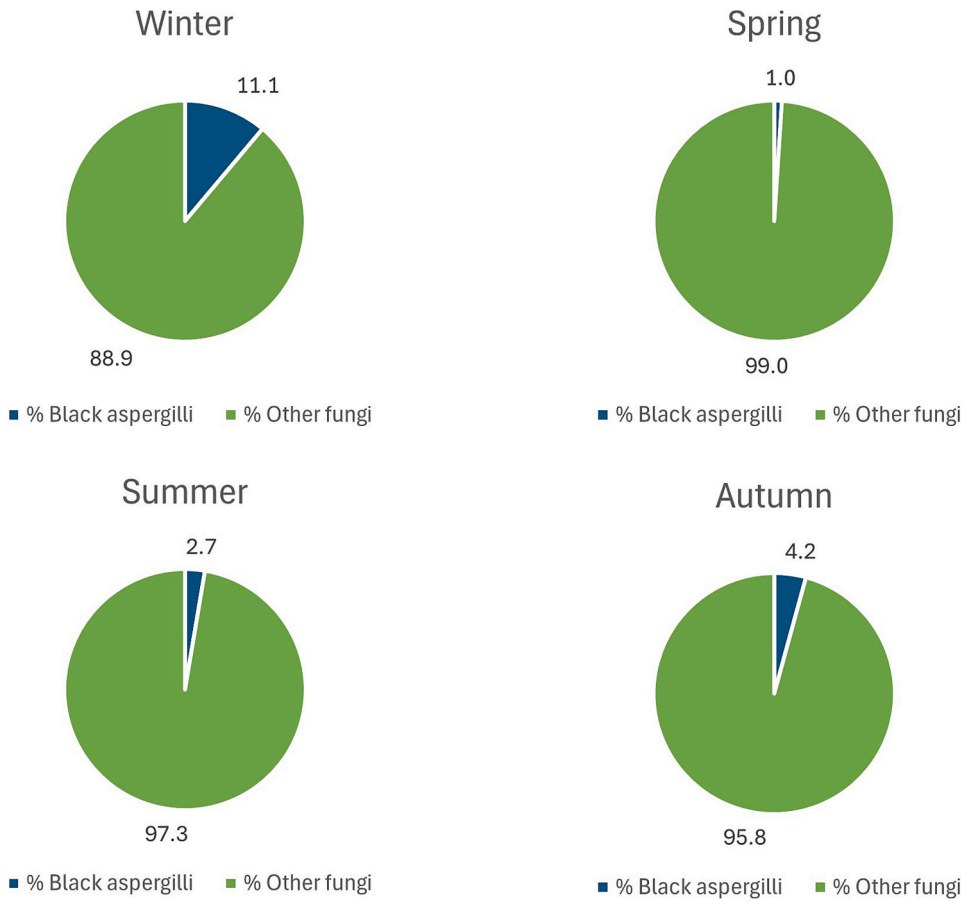

Fig. 6. Occurrence of black aspergilli in air samples in different seasons.

Table 5
Number of isolates of *Aspergillus* section *Nigri* recovered from air samples in different vineyards and grape varieties.

Species	G		B1		B2		T		
	n (C/GR)[a]	%[b]	n (C/GR)	%	n C/GR)	%	n (C/GR)	%	Total
Aspergillus section *Nigri*	64 (7/57)	26.7	24 (3/21)	42.8	30 (7/23)	68.2	43 (12/31)	43.4	161 (36.7 %)
A. carbonarius	0		1 (1/0)		1 (0/1)		0		2
A. niger aggregate	64 (7/57)		22 (1/21)		29 (7/22)		43 (12/31)		158
Uniseriate species	0		1 (1/0)		0		0		1
Total *Aspergillus* spp.	240 (28/212)		56 (12/44)		44 (17/27)		99 (36/63)		439

G, vineyard G; B1, vineyard B1; B2, vineyard B2; T, vineyard T.
[a] . C, Cabernet Sauvignon variety; GR, Grenache variety.
[b] . Percentage in relation to total isolates of *Aspergillus* spp.

Table 6
Distribution of *Aspergilllus* section *Nigri* isolates in the different substrates and range of OTA production (μg/g).

Species	Grapes			Soil			Air		
	n	OTA+	Range	n	OTA+	Range	n	OTA+	Range
A. carbonarius	266	266	0.14–70.5	2	2	3.17–5.29	2	2	10.79–12.68
A. niger aggregate	904	1	1.24	1524	2	0.36–2.90	158	0	–
Uniseriate	35	0	–	7	0	–	1	0	–
Total	1205	267		1533	4		161	2	

concentration in vineyard soil are higher in the top layer and in the soil directly beneath the vines compared to the inert-row area. Also, it has been reported that black aspergilli counts in soil can be increased at early veraison (late July/August) compared with the pea berry stage (June/ early July) (Oliveri et al., 2017). In our study, no differences were observed in black aspergilli counts among seasons, suggesting that black aspergilli population is relatively stable throughout the year in vineyard soil. Differences in black aspergilli counts were observed among vineyards. Vineyard G, which has an acidic soil, showed total fungal counts and black aspergilli counts significantly higher than the rest of the vineyards. Although there is no information about the impact of soil pH on black aspergilli, it has been described that an acid pH

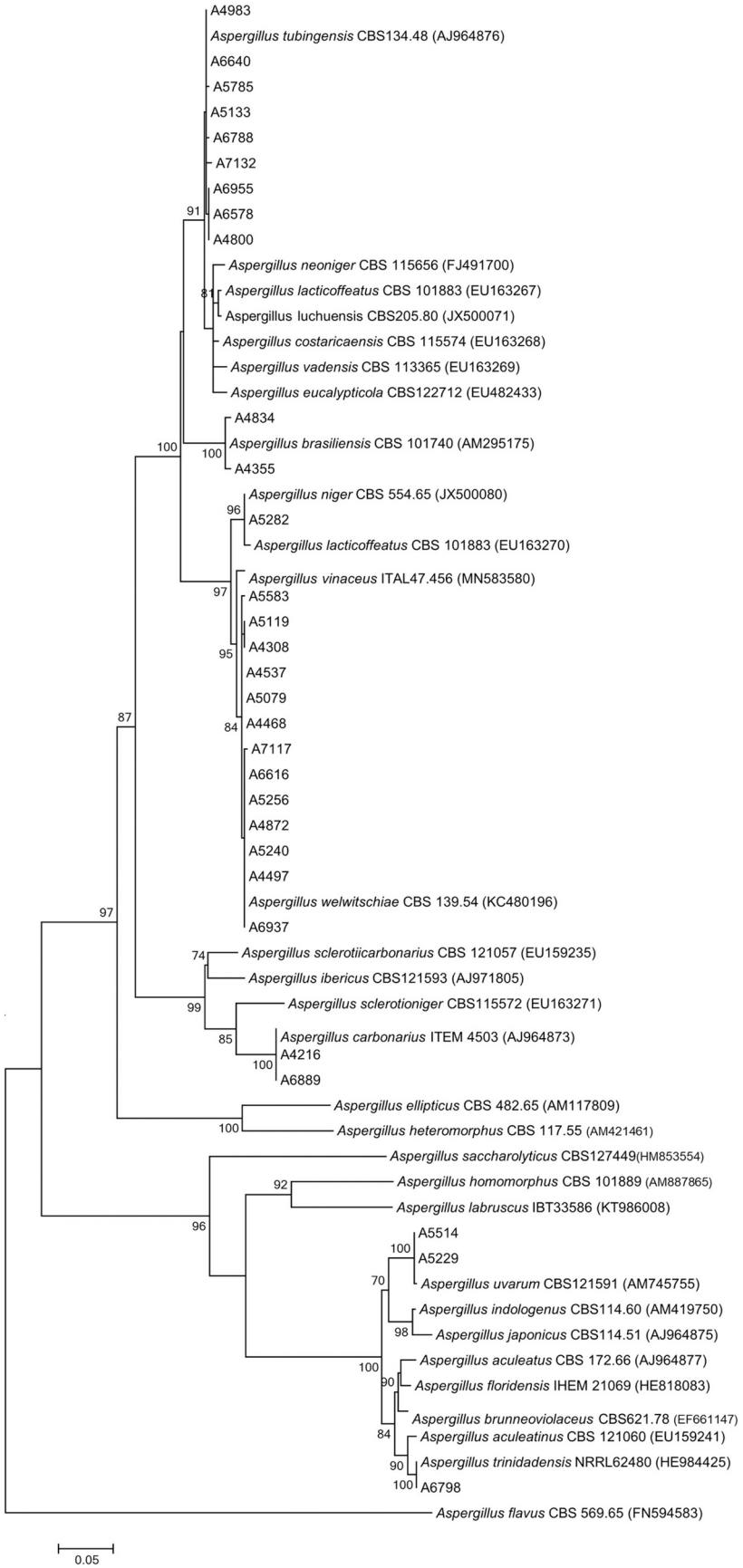

Fig. 7. Maximum likelihood phylogeny of *Aspergillus* section *Nigri* based on calmodulin gene. Bootstrap values>70 % in 1000 replications are shown at nodes. Sequence of *Aspergillus flavus* CBS 569.65^{T} was selected as outgroup for the tree construction.

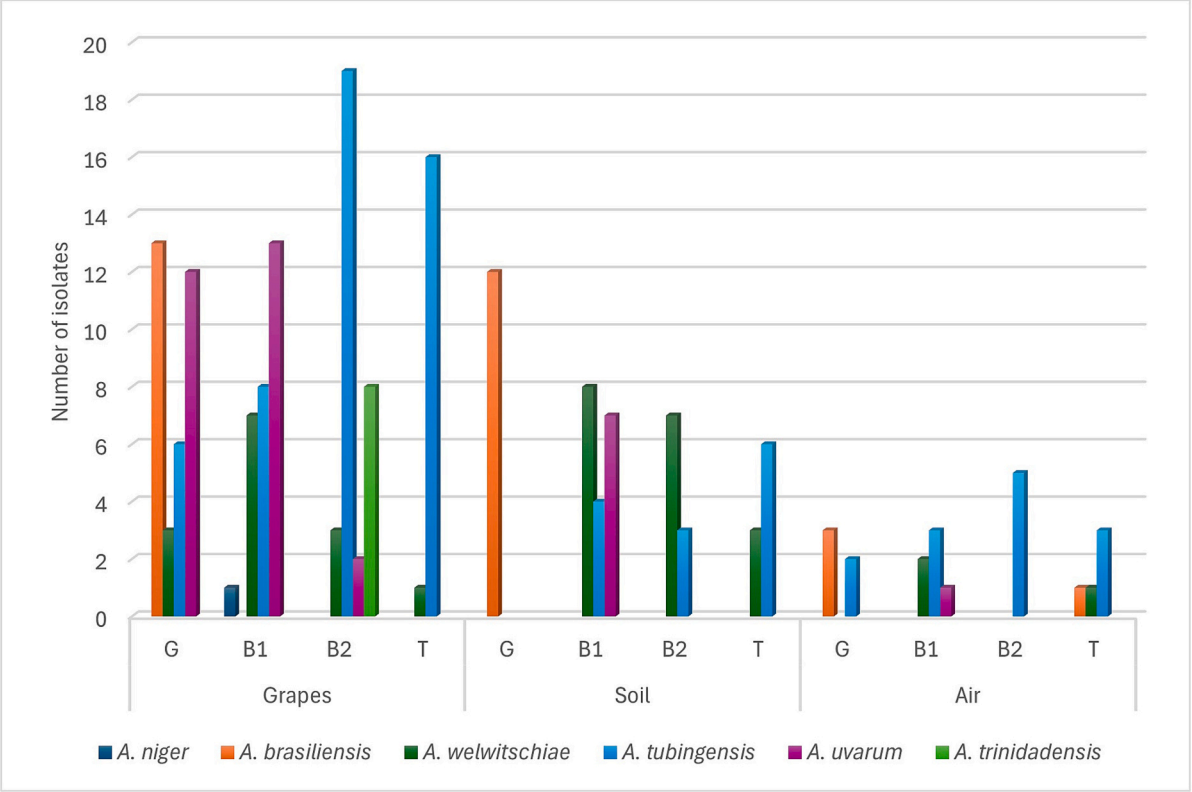

Fig. 8. Distribution of non-ochratoxigenic *Aspergillus* section *Nigri* species obtained from grape, soil, and air samples in the different vineyards. G, vineyard G; B1, vineyard B1; B2, vineyard B2; T, vineyard T.

favored fungal growth on soil (Rousk et al., 2009). Besides, black aspergilli are able to grow in over an extremely wide pH range (1.4–9.8) and produce organic acids (Schuster et al., 2002; Yang et al., 2017).

In our study, black aspergilli were present in the air samples of all vineyards, although its occurrence was lower than in soil. This group of fungi represented a low percentage of the total fungi recovered (<11.1 %) and 36.7 % of the total *Aspergillus* species isolated. Contrary to what was observed in soil samples, black aspergilli counts in air samples showed no differences among vineyards, but differences were observed among seasons. Seasonal variations in the occurrence of black aspergilli have also been described by Oliveri et al. (2017), who reported that early veraison had higher levels of black aspergilli contamination than the pea berry stage. In our study, black aspergilli counts were higher in autumn, after harvesting. This may be due to the residual vegetation of the harvest and later pruning in the vineyard.

In the present study, *A. niger* aggregate species were the most frequently isolated from grapes (75 %), followed by *A. carbonarius* (22.1 %) and uniseriate species (2.9 %). In soil and air samples, *A. niger* aggregate was also the predominant group (>98 %), whereas the occurrence of *A. carbonarius* was very low (<1.3 %), being isolated only from the vineyards which showed the higher occurrence of this species in grapes. Uniseriate species were isolated from soil and air only from one vineyard, which showed the higher occurrence of uniseriate species in grapes. Both, *A. carbonarius* and *A. niger* aggregate, exist as saprophytes in the superficial layer of vineyard soil, from where they are thought to be blown onto bunches. The level of *A. carbonarius* in soil may be reduced by temperatures above or below the optimum temperature for survival, by high soil moisture content, and by modifications to tillage and mulching practices (Leong et al., 2006). The dominance of *A. niger* aggregate over *A. carbonarius* in vineyards soil mirrors the frequent isolation of these species from grapes over the world and it has been attributed to the high optimum and maximum growth temperatures of *A. niger* aggregate compared with *A. carbonarius* and uniseriate species, and its faster growth in most conditions (Battilani et al., 2003; Bellí et al., 2006; Leong et al., 2004).

All isolates from grapes, soil and air were tested for OTA production. All *A. carbonarius* isolates (n = 270) were able to produce OTA. Within the *A. niger* aggregate group, only three isolates out of 2586 produced OTA but in low amount. These isolates were identified by molecular methods as *A. welwitschiae*. Our results agree with other previous surveys showing that *A. carbonarius* is the main OTA producer among all species of *Aspergillus* section *Nigri* (Cabañes and Bragulat, 2018) whereas only a low percentage of *A. niger* and *A. welwitschiae* strains showed this ability (Bau et al., 2005, 2006; Susca et al., 2016). None of the uniseriate species were able to produce OTA.

In our study, most of the *A. niger* aggregate selected isolates were identified as *A. tubingensis*, followed by *A. welwitschiae, A. brasiliensis* and *A. niger*. The species found in grapes correlate well with the species found in soil and air on each vineyard. The occurrence of each species was different depending on the vineyard.

In grape samples, in all vineyards except vineyard G, *A. tubingensis* was the predominant species, followed by *A. welwitschiae*. While *A. tubingensis* has been reported as the most frequently occurring species in grapes from Cyprus, Argentina, Spain, and California (Bau et al., 2005, 2006; Chiotta et al., 2013; Gil-Serna et al., 2019; Palumbo et al., 2019; Pantelides et al., 2017), *A. niger* and *A. welwitschiae* are the predominant species on other countries such as Uruguay (Garmendia and Vero, 2016), Brazil (Ferranti et al., 2018; Massi et al., 2016) and Canada (Qi et al., 2016). In our study only one isolate was identified as *A. niger* s. str., showing the low occurrence of this species in grapes.

In vineyard G, *A. brasiliensis* was the predominant species in grapes and the only species isolated from vineyard soil. This vineyard, located in the northeast corner of Spain, has a precipitation rate of roughly 600 mm per year and the average annual temperature is between 14 and 16 °C. This species has been isolated from vineyard soil in Canada, as well as from grape berries from Portugal and Canada (Qi et al., 2016; Varga

et al., 2007). To our knowledge this is the first report of the high occurrence of this species in Spain.

In our study, *A. welwitschiae* was the predominant species in soil samples, followed by *A. tubingensis*. *Aspergillus welwitschiae* has a worldwide distribution in all continents and it is a common inhabitant of soil (Duarte et al., 2018). Its spores are present in the air (Whitaker et al., 2008) and it can also be found in grapes and raisins (Susca et al., 2016; Qi et al., 2016). It has been isolated also from vineyard soil (Qi et al., 2016) although *A. tubingensis* and *A. niger* were found prevalent in soil samples (Barberis et al., 2014; Leong et al., 2007; Palumbo et al., 2019).

Regarding uniseriate species, *A. uvarum* was the predominant species isolated from grape samples, being the only species isolated from soil and air. This species was frequently found on grapes collected from temperate climate regions (Chiotta et al., 2013; Ferranti et al., 2018; Perrone et al., 2008; Qi et al., 2016; Testempasis et al., 2022). Contrary to some studies which isolated *A. japonicus* as the main uniseriate species in grapes and soil (Barberis et al., 2014; Ferranti et al., 2018; Perrone et al., 2007), this is the first study to describe *A. uvarum* as the prevalent uniseriate species in grapes from Spanish vineyards. In vineyard B2, *A. trinidadensis* was also isolated from grapes. This species has been previously isolated from indoor air samples in Trinidad Tobago and USA (Jurjević et al., 2012). To our knowledge, this is the first report of *A. trinidadensis* isolated from grapes and the first reported isolation in Europe.

One limitation of this study was the fact that samples were collected in 2012–2013, and the environmental and ecological context may have changed since then. Factors such as climate change and shifts in temperature and precipitation patterns, which can vary from year to year, can impact species distribution, population dynamics and ecological relationships.

In summary, results of the current study showed that the occurrence of *Aspergillus* section *Nigri* was lower in vineyard soil and air than in grapes. The occurrence of *A. carbonarius* in grapes was linked to agro-climatic factors, being higher in hot and humid areas, whereas its occurrence in soil and air was very low. The population of black aspergilli remained relatively consistent in soil year-round, whereas fluctuating seasonal variations were seen in the air. In the *A. niger* aggregate, *A. brasiliensis* was the predominant species in the north vineyard whereas in southern vineyards, *A. welwitschiae* was the predominant species in soil and *A. tubingensis* in grapes and air. Only few isolates of *A. niger* aggregate produced OTA at low levels and were identified as *A. welwitschiae*.

CRediT authorship contribution statement

Júlia Marquès: Writing – original draft, Methodology, Formal analysis. **Gemma Castellá:** Writing – review & editing, Methodology, Funding acquisition, Formal analysis, Conceptualization. **M. Rosa Bragulat:** Writing – review & editing, Methodology, Formal analysis, Conceptualization. **F. Javier Cabañes:** Writing – review & editing, Supervision, Resources, Methodology, Funding acquisition, Formal analysis, Conceptualization.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijfoodmicro.2024.111049.

Data availability

The camodulin sequence for selected isolates analyzed in this study are available in the GenBank database.

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