Journal of Plant Pathology
https://doi.org/10.1007/s42161-024-01780-3

ORIGINAL ARTICLE

Survey on the occurrence of *Aspergillus* section *Nigri* species in grapes cultivated in Umbria (central Italy) and influence of several factors on their distribution

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Received: 26 April 2024 / Accepted: 24 September 2024
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Abstract

Secondary bunch rots caused by *Aspergillus* species belonging to the section *Nigri*, commonly called black aspergilli, pose a serious threat to grapes yield and quality. Due to their ability to produce ochratoxin A (OTA), they are considered the primary source of contamination of this mycotoxin in grapes. In this study, a three-year survey was realized to assess the occurrence of black aspergilli in grapes harvested in the Umbria region, a very important grapevine cultivation area in central Italy, by fungal isolation and phylogenetic analysis. In addition, the influence of growing season, vineyard location, farming system and grapes growth stages on black aspergilli distribution was examined. Phylogenetic analysis of the 208 obtained isolates showed the presence of three black aspergilli species: *Aspergillus tubingensis*, *Aspergillus welwitschiae* and *Aspergillus uvarum*. Interestingly, *A. carbonarius*, one of the most important OTA producers, was not detected in the surveyed years. *A. tubingensis*, whose ability to produce OTA is controversial, was always the most isolated species followed by *A. welwistichiae* (OTA producer). The species *A. uvarum* (unable to produce OTA) was detected only in one surveyed year with a low incidence. *A. tubingensis* was always the species with the highest incidence, regardless of location, and farming system. Growth stage appeared to influence the incidence of the three species, that, in the case of *A tubingensis*, was lower during setting in comparison to berries pea-size and berries harvest-ripe. Finally, in the setting and berries pea-size stages, a stereomicroscope analysis, showed that flower debris was the substrate from which black aspergilli mainly developed prior to colonizing berries and bunches.

Keywords Grapevine · Black aspergilli · Fungi · *A. tubingensis* · *A. welwistchiae* · *A. uvarum*

Introduction

Grapevine (*Vitis vinifera*) is a very important crop of the Mediterranean basin, even if it can be considered one of the most widely grown fruit plants in the world (Vivier and Pretorius 2002). In the Mediterranean area, Italy is the most important grapevine producer followed by Spain and France (FAOSTAT 2022). In Italy, wine is the main grapevine-derived product (ISTAT 2022) and grapevine cultivation for wine production is largely diffused alongside the entire peninsula. In Central Italy, grapevine is cultivated on about 100,000 ha (ISTAT 2022) and, in this area, the Umbria region in 2022 had 12,400 ha dedicated to grapevine cultivation with a wine production of about 600,000 hL (ISTAT 2022). In Umbria, grapevine and wine are essential elements of the regional agri-food economy as well as of the landscape and of the local traditions. The importance of grapevine cultivation and wine production in Umbria is also attested by the presence of two "controlled and guaranteed denominations of origin" (DOCG) and 13 "controlled denominations of origin" (DOC) growing areas where cultivars of international interest, such as Sagrantino, Sangiovese, Cabernet, Chardonnay and Grechetto, are used to produce red or white wines much appreciated worldwide.

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Published online: 11 October 2024

Grapevine is susceptible to many plant diseases and some of them, affecting the bunch, are considered a serious threat to wine quality and composition (Steel et al. 2013). One of the most important diseases affecting berries is bunch rot, caused by a complex of filamentous fungi also including those belonging to the genus *Aspergillus* (Leong et al. 2004), considered among the most frequent saprophytic fungal pathogens of grapes (Battilani et al. 2003; Lorenzini et al. 2016; Stefanini et al. 2017; Lorenzini et al. 2019).

Members of the genus *Aspergillus* can biosynthesize mycotoxins (Perrone and Gallo 2017). For this reason, their presence in grapes represents an important threat to grapevine cultivation and wine production, resulting in quality reduction due to mycotoxin accumulation in berries as well as in wine (Covarelli et al. 2012; Tini et al. 2020). Generally, *Aspergillus* spp. development and mycotoxin contamination of grapes begin directly in the vineyard or at stages just before winemaking (Freire et al. 2020).

The most important mycotoxigenic *Aspergillus* species in grapes are those belonging to the section *Nigri*, commonly called black aspergilli. The main black aspergilli detected in grapes grown in the Mediterranean area are represented by species like *Aspergillus niger*, *Aspergillus carbonarius* and *Aspergillus tubingensis* (Somma et al. 2012; García-Cela et al. 2014a, b; Cabañes and Bragulat 2018), however, also *Aspergillus welwitschiae*, *Aspergillus uvarum*, *Aspergillus aculeatus* and *Aspergillus ibericus* have been frequently found (Cabañes and Bragulat 2018; Tini et al. 2020).

Black aspergilli are considered the primary source of the mycotoxin ochratoxin A (OTA) in grapes (Cabañes and Bragulat 2018), produced in the berries during the growing season mainly from veraison to ripening (Somma et al. 2012). OTA is a potent nephrotoxin, classified as a possible human carcinogen (group 2B) (International Agency for Research on Cancer 1993). For this reason, to safeguard consumers' health in the European Union, maximum OTA levels in many grape-derived products have been fixed (Commission Regulation 2023).

Despite black aspergilli are considered the main source of OTA in grapes and grape-derived products, only a few of them have been confirmed to be OTA producers (Cabañes and Bragulat 2018). In particular, *A. carbonarius* is considered the main OTA producer because this species is very consistent in the production of this mycotoxin and non-OTA-producing isolates are uncommon (Cabañes et al. 2013; García-Cela et al. 2014a, b). Conversely, *A. niger* represents a lower OTA risk in grapes, while for *A. tubingensis* the ability to produce OTA remains controversial (Samson et al. 2004; Cabañes and Bragulat 2018; Mikušová et al. 2020) as the genomic analysis of this species revealed the absence of the genes responsible for OTA production (Choque et al. 2018). Also, for other black aspergilli, a different ability in OTA biosynthesis was reported. For example, *A. welwitschiae* is reported to be an OTA producer (Susca et al. 2016), while *A. uvarum* showed no ability to produce this mycotoxin (Perrone et al. 2008).

Several factors such as seasonality, vineyard location, cultivar, farming system and grapes developmental stage can have an influence on the distribution of black aspergilli as well as on their disease severity during infections (Ponsone et al. 2007; Chiotta et al. 2009; Palumbo et al. 2016; Freire et al. 2017; Dachery et al. 2019; Testempasis et al. 2022; Giorni et al. 2023).

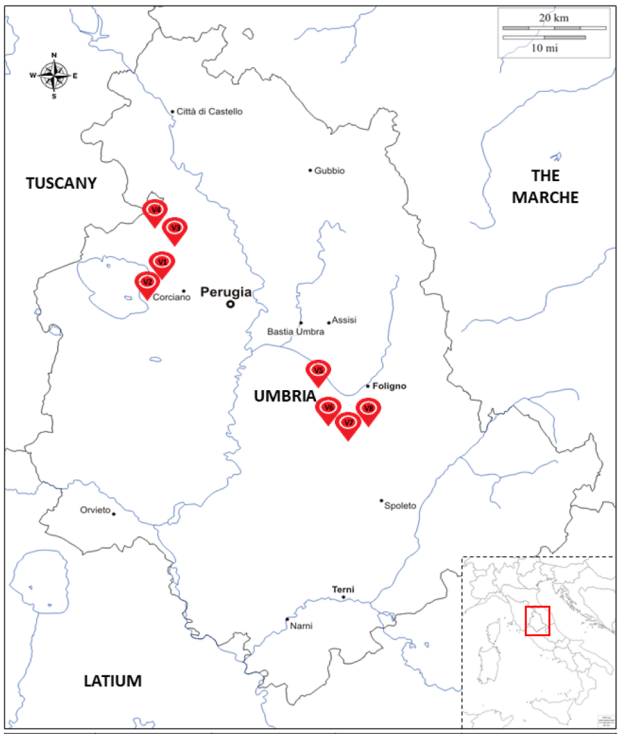
Thus, it is extremely relevant to determine the presence and the population composition of black aspergilli in wine grapes in highly important Mediterranean wine-making areas such as the Umbria region in central Italy. For this reason, in this study, the occurrence of *Aspergillus* section *Nigri* in grapes was determined by isolation and molecular characterization: (a) during three years (2013, 2014 and 2015); (b) in eight different Umbrian vineyards with five different cultivars (Chardonnay, Cabernet, Grechetto, Sangiovese and Sagrantino); (c) cultivated under two different farming systems (organic or integrated); (d) through four different grapes growth stages (setting, berries pea-size, veraison and berries harvest-ripe).

It should be mentioned that the present survey, conducted during the three years 2013–2015, may not reflect the current composition of the black aspergilli community associated with grapes due to climate change (Singh et al. 2023) but also to seasonal weather conditions which can vary from year to year.

Materials and methods

Vineyards and sampling

Sampling was carried out in 2013, 2014 and 2015 in eight different vineyards (V1-V8) all located in the Umbria region (central Italy) and cultivated under organic or integrated systems (Fig. 1). Five grape cultivars were included in the sampling: Chardonnay (V1 and V3), Cabernet (V2, V4, V5 and V8), Sangiovese (V5), Grechetto (V5) and Sagrantino (V5, V6 and V7). In 2013, samples were collected from vineyards V1, V2, V3, V4 and V5 from June to October at four growth stages (Coombe 1995): setting (BBCH 71), berries pea-size (BBCH 75), veraison (BBCH 81) and berries harvest-ripe (BBCH 89). The same sampling schedule was followed also in 2014 (V3, V4 and V5) and 2015 (V3, V4, V5, V6, V7, V8). At each sampling time, ten bunches were collected from ten grapevines located along two crossing diagonals of each vineyard. Each bunch, collected in a



Vineyard	Location	Year of sampling	Farming system	Cultivar
V1	Magione	2013	Integrated	Chardonnay
V2	Magione	2013	Integrated	Cabernet
V3	Castel Rigone	2013; 2014; 2015	Integrated	Chardonnay
V4	Castel Rigone	2013; 2014; 2015	Integrated	Cabernet
V5	Cannara	2013; 2014; 2015	Organic	Grechetto
				Cabernet
				Sangiovese
				Sagrantino
V6	Bevagna	2015	Integrated	Sagrantino
V7	Bevagna	2015	Integrated	Sagrantino
V8	Bevagna	2015	Integrated	Cabernet

Fig. 1 Map of the Umbria region (central Italy) showing the different vineyards from which grapes were sampled. In the right bottom corner (outlined box) a map of Italy indicates the location of the Umbria region (red square) in the national geographical context. Under the maps, details of the samples are reported

separate plastic bag, was placed at 4 °C and analysed within 24–48 h.

Obtainment of black aspergilli isolates from grapes

Sixteen randomly chosen berries were selected from each bunch and surface-sterilized for 2 min with a water-ethanol (95%, Sigma Aldrich, Saint Louis, MO, USA)-sodium hypochlorite (7%, Carlo Erba reagents, Milan, Italy) solution (82:10:8 vol%) and then rinsed twice in sterile water for 1 min. Eight berries were chopped into small pieces with a laboratory scalpel and placed in 8 Petri dishes (5 pieces per dish from 1 berry) (90 mm diameter, Nuova Aptaca, Asti, Italy) containing malt extract agar (MEA) (Pitt and Hocking 1997) amended with streptomycin sulphate (0.16 g/L, Sigma Aldrich). Other chopped pieces, coming from the remaining eight berries, were placed in 8 Petri dishes (5 pieces per dish from 1 berry) (90 mm diameter, Nuova Aptaca) with potato dextrose agar (PDA, Biolife Italiana, Milan, Italy) amended with streptomycin sulphate (0.16 g/L, Sigma Aldrich). All Petri dishes (MEA and PDA) were incubated at 22 ± 2 °C in the dark for 7 days. Successively, after stereomicroscope (SZX9, Olympus, Tokyo, Japan) and optical microscope (Axiophot, Zeiss, Oberkochen, Germany) observations, all the colonies morphologically identified as belonging to the genus *Aspergillus* section *Nigri*, were transferred into new Petri dishes (60 mm diameter, Nuova Aptaca) containing PDA (Biolife Italiana) and placed at 22 ± 2 °C in the dark.

The sampled bunches were also placed in humid chambers (HC). In detail, bunches were sectioned into several pieces (about 5 cm) and then placed onto three sterilized layers of filter paper (150 mm diameter, grade 1, Whatman, GE Healthcare, Amersham Place, UK) previously supplemented with 15 mL of sterile deionized water into 4 Petri dishes (150 mm diameter, Nuova Aptaca), and maintained at room temperature for 2 days. Each bunch was observed under a stereomicroscope (SZX9, Olympus) and all *Aspergillus* section *Nigri* colonies developed from the bunches/berries were transferred into new Petri dishes (60 mm diameter, Nuova Aptaca) containing PDA (Biolife Italiana) and placed at 22 ± 2 °C in the dark. During stereomicroscope observations of the fungal structures developed from bunches placed in HC, pictures were taken using a BEL Imaging System (BEL Engineering, Monza, Italy).

All *Aspergillus* section *Nigri* isolates coming from the three different isolation methods (PDA, MEA and HC) were stored at −80 °C after obtaining monosporic cultures and then stored in the fungal collection of the Department of Agricultural, Food and Environmental Sciences of the University of Perugia (Italy).

Molecular analysis of isolated black aspergilli

At the end of the three sampling years, all *Aspergillus* section *Nigri* isolates obtained from the cultures grown on MEA, PDA and HC were moved from −80 °C to new Petri dishes (60 mm diameter, Nuova Aptaca), containing PDA (Biolife Italiana). After 7 days at 22 ± 2 °C in the dark, a 5 mm diameter mycelial plug, taken from the edge of the colony of each isolate, was transferred in 50 mL flasks containing 30 mL of Czapek Yeast Broth (CYB, Sigma Aldrich) as reported by Atlas (2010). All flasks were incubated at room temperature in an orbital shaker (Labline Instruments; Melrose Park, IL, USA) for 7 days at 200 rpm. All fungal cultures were then filtered (Miracloth, Millipore Corporation, Billerica, MA, USA) to recover the mycelium which was subsequently freeze-dried with a Heto Powder Dry LL3000 lyophilizer (Thermo Fisher Scientific, Waltham, MA, USA) and finely ground with stainless-steel

beads (Qiagen, Hilden, Germany) and a Mixer Mill MM200 grinding machine (Retsch, Haan, Germany) with a frequency of 25 Hz for 6 min.

Molecular identification of obtained isolates was realized by extracting genomic DNA from the fungal colonies following the method described by Parry and Nicholson (1996) with modifications introduced by Covarelli et al. (2015a, b) and by Beccari et al. (2018). Genomic DNA was visualized on a 2% agarose, trizma base-glacial acid acetic-ethylenediamine-tetraacetic acid disodium salt dihydrate (TAE; all from Sigma Aldrich) gel (1x) containing 0.1 µL/mL of SafeView FireRed (Applied Biological Material, Richmond, BC, Canada). DNA fragments were separated in a 10 cm-long agarose TAE gel, with an electrophoresis apparatus (Eppendorf, Hamburg, Germany) applying a tension of 110 V for 30 min. Electrophoretic runs were visualized using an ultraviolet transilluminator (Euroclone, Milan, Italy). DNA concentration was estimated by comparison with a 1 kb gene ruler (Thermo Fisher Scientific) and by a spectrophotometer Lambda Ez201 (Perkin Elmer Italia, Milan, Italy) reading the DNA absorbance at 260 nm. DNA was diluted in DNase-free sterile water for molecular biology use (5prime, Hilden, Germany) to obtain a concentration of ~30 ng/µL and stored at −20 °C until use.

A phylogenetic analysis was performed using partial ribosomal DNA (rDNA) *Internal Transcribed Spacer* (*ITS*), partial *β-tubulin* (*βt*) and partial *calmodulin* (*CMD*) gene sequences (White et al. 1990; Glass and Donaldson 1995; Hong et al. 2005). A PCR protocol was adopted using a total reaction volume of 50 µL. Each reaction contained 2 µL of DNA, 5 µL of 10x Reaction Buffer (Microtech, Naples, Italy), 5 µL of 10 µM dNTP Mix (Microtech), 3 µL of 25 mM $MgCl_2$ (Microtech), 3.75 µL of Cresol Red (Sigma Aldrich), 2.5 µL of 10 µM of forward and reverse primers (Table 1), 0.25 of 5 U/µL of Taq DNA polymerase (Microtech) and 26 µL DNase-free sterile water (5prime).

The PCR cycle for *ITS* amplification consisted of an initial denaturation step (95 °C for 4 min), followed by 35 cycles of denaturation (94 °C for 45 s), annealing (57 °C for 50 s) and extension (72 °C for 30 s), and a final extension (72 °C for 8 min). The PCR cycle for *βt* amplification consisted of an initial denaturation step (95 °C for 4 min), followed by 35 cycles of denaturation (94 °C for 1 min), annealing (67 °C for 1 min) and extension (72 °C for 1 min), and a final extension (72 °C for 8 min). Finally, the PCR cycle for *CMD* amplification consisted of an initial denaturation step (95 °C for 4 min), followed by 35 cycles of denaturation (95 °C for 1 min), annealing (55.5 °C for 1 min) and extension (72 °C for 2 min), and a final extension (72 °C for 8 min).

PCR assays were performed on a T-100 thermal cycler (Bio-Rad, Hercules, CA, USA). PCR fragments were visualized on TAE 1X agarose gel (2%) containing 0.1 µL/mL of SafeView FireRed (Applied Biological Material). DNA fragments were separated at 110 V for ~40 min. Electrophoretic runs were observed with an ultraviolet transilluminator (Euroclone). The size of the amplified fragments was obtained by comparison with HyperLadder 100–1000 bp (Bioline Meridian Bioscience, Cincinnati, OH, USA). PCR fragments were purified and sequenced by an external sequencing service (Genewiz Genomics Europe, Takeley, UK).

Phylogenetic analysis

The taxonomic identity of the *Aspergillus* section *Nigri* isolates obtained in this study was investigated by phylogenetic analysis of *ITS*, *βt*, and *CMD* combined gene regions. The newly generated sequences were submitted to the Basic Local Alignment Search Tool nucleotide (BLASTn) search and aligned with the nucleotide sequences of reference isolates of *Aspergillus* section *Nigri* retrieved from the National Center for Biotechnology Information (NCBI) GenBank database (National Center for Biotechnology Information 2024), based on Houbraken et al. (2020). The sequences were aligned with Muscle on MEGA XI v.11.0.8 (Tamura et al. 2021) and were trimmed and concatenated to generate the multi-sequence alignment. IQ-TREE version 2.3.1 (Minh et al. 2020) was used to calculate the best-fit evolution model according to BIC with the implemented ModelFinder (Kalyaanamoorthy et al. 2017), and to infer the Maximum likelihood phylogenetic tree, based on 10,000 ultrafast bootstrap support replicates (Hoang et al. 2018), on the partitioned dataset (Chernomor et al. 2016).

Table 1 Information about the primers used in this study

Target	Primer name	Sequence (5′-3′)	Size	References
Internal Transcribed Spacer (ITS)	ITS1	TCCGTAGGTGAACCTGCGG	290 bp	White et al. (1990)
	ITS4	TCCTCCGCTTATTGATATGC		
β-tubulin (βt)	Bt2a	GGTAACCAAATCGGTGCTGCTTTC	540 bp	Glass and Donaldson (1995)
	Bt2b	ACCCCTCAGTGTAGTGACCCTTGGC		
Calmodulin (CMD)	CMD5	CCGAGTACAAGGAGGCCTTC	580 bp	Hong et al. (2005)
	CMD6	CCGATAGAGGTCATAACGTGG		

Statistical analysis

It is necessary to point out that this study is to be regarded as an observational survey and not as a designed experiment. Consequently, there is a certain degree of unbalance and the experimental factors are never fully crossed, which has been considered by the authors in the interpretation of the results.

The data about the presence/absence of fungal colonies (four exclusive outcomes: no colony, *A. tubingensis*, *A. welwitschiae* or *A. uvarum*) were analysed with a multinomial GLM with logit-link. As the first step, a set of univariable models was fitted, by considering the effects of the year, location, farming system and growth stage separately (Agresti 2002). The significance of pairwise differences (between different years, locations, farming systems and growth stages) was tested with a generalized linear contrast testing procedure, with single-step multiplicity adjustment (Bretz et al. 2011). All analyses were performed using the R statistical environment (vers. 4.2.3; R Core Team 2023) together with the package 'emmeans' (Lenth 2022).

Results

Identification of black aspergilli

The individual BLASTn search results of *ITS*, *βt*, and *CMD* sequences indicated that all the isolates obtained in this study effectively categorized in the *Aspergilllus* section *Nigri* clade. *Aspergillus flavus* was used as the outgroup taxon. The combined alignment has 240 sequences with 1453 total sites, 689 distinct patterns, 447 informative, 175 singleton sites, and 831 constant sites. The partitioned models were TNe+G4 for *ITS*, and TNe+I+G4 for *βt* and *CMD* sequences, respectively. The phylogenetic analysis (Fig. 2 and Figure S1) confirmed the identification of the isolates obtained in this study as *A. tubingensis*, *A. welwitschiae* and *A. uvarum* (Table S1).

Distribution of black aspergilli species in the three surveyed years

The incidence of each of the three isolated species (*A. tubingensis*, *A. welwitschiae* and *A. uvarum*) did not significantly change across the three years ($p = 0.606$). However, significant differences were observed between the incidences of the three black aspergilli species within each year (Fig. 3). In detail, focusing on 2013 (Fig. 3), the incidence of *A. tubingensis* was significantly higher than that of *A. welwitschiae* (85% vs. 15%; $p = 0.005$). In 2014 (Fig. 3), results were totally similar (88% vs. 12%; $p = 0.0008$). Finally, in 2015 (Fig. 3) *A. uvarum* was isolated for the first time and its incidence was not significantly different from that of *A. welwitschiae* (2% vs. 11%, $p = 0.061$), while both these species were significantly lower than *A. tubingensis* (87%; $p < 0.001$ for both tests).

Role of vineyard location on black aspergilli development

Due to the unbalanced dataset, the effect of location should not be interpreted only concerning climate or soil, but it should be taken as the overall contribution of all differences in vineyard management and cultivars, as grown in each location.

The role of vineyard location (Fig. 4) is highly significant in the *Aspergillus* section *Nigri* species development on grapes ($p = 0.0065$). In detail, among the three black aspergilli species detected in this survey, the occurrence of *A. tubingensis* showed a significant difference across locations. The incidence detected in V2, V5 and V7 was significantly higher than in V6 ($p < 0.043$). No significant differences were detected for the other locations (V1, V3, V4 and V8) in comparison to V6 or the V5-V2-V7 group ($p > 0.343$).

Considering the incidences of the three *Aspergillus* section *Nigri* species detected in this survey within each single location, no significant differences were observed in the isolation from grapes of vineyard V1 (Magione) between *A. tubingensis* (67%) and *A. welwitschiae* (33%) ($p = 0.317$). *A. uvarum* was not isolated from this location (Fig. 4).

Conversely, in the other vineyard located in the Magione area (V2), a significant difference between the isolation incidences of *A. tubingensis* (93%) and *A. welwitschiae* (7%) ($p < 0.0001$) was observed, while *A. uvarum* was not detected.

From the grapes collected in the vineyards V3 and V4, both located at Castel Rigone, *A. tubingensis* (V3 = 75%; V4 = 80%) was isolated with an incidence not significantly different from *A. welwitschiae* (V3 = 25% and V4 = 20%), with $p = 0.500$ and $p = 0.600$, respectively. *A. uvarum* was not isolated from the grapes sampled in both the vineyards (Fig. 4).

Focusing on the vineyard at Cannara (V5), *A. uvarum* (1%) was isolated in a similar amount with respect to *A. welwitschiae* (6%; $p = 0.072$), while *A. tubingensis* (93%) was the most isolated species also from this location ($p < 0.0001$) (Fig. 4).

A. uvarum was abundantly isolated only from grapes sampled in vineyard V6 (Bevagna). Focusing on this location (Fig. 4), no significant differences were observed in the isolation incidences of the three *Aspergillus* species detected: *A. tubingensis* (33%), *A. uvarum* (22%) and *A. welwitschiae* (45%) ($p > 0.679$).

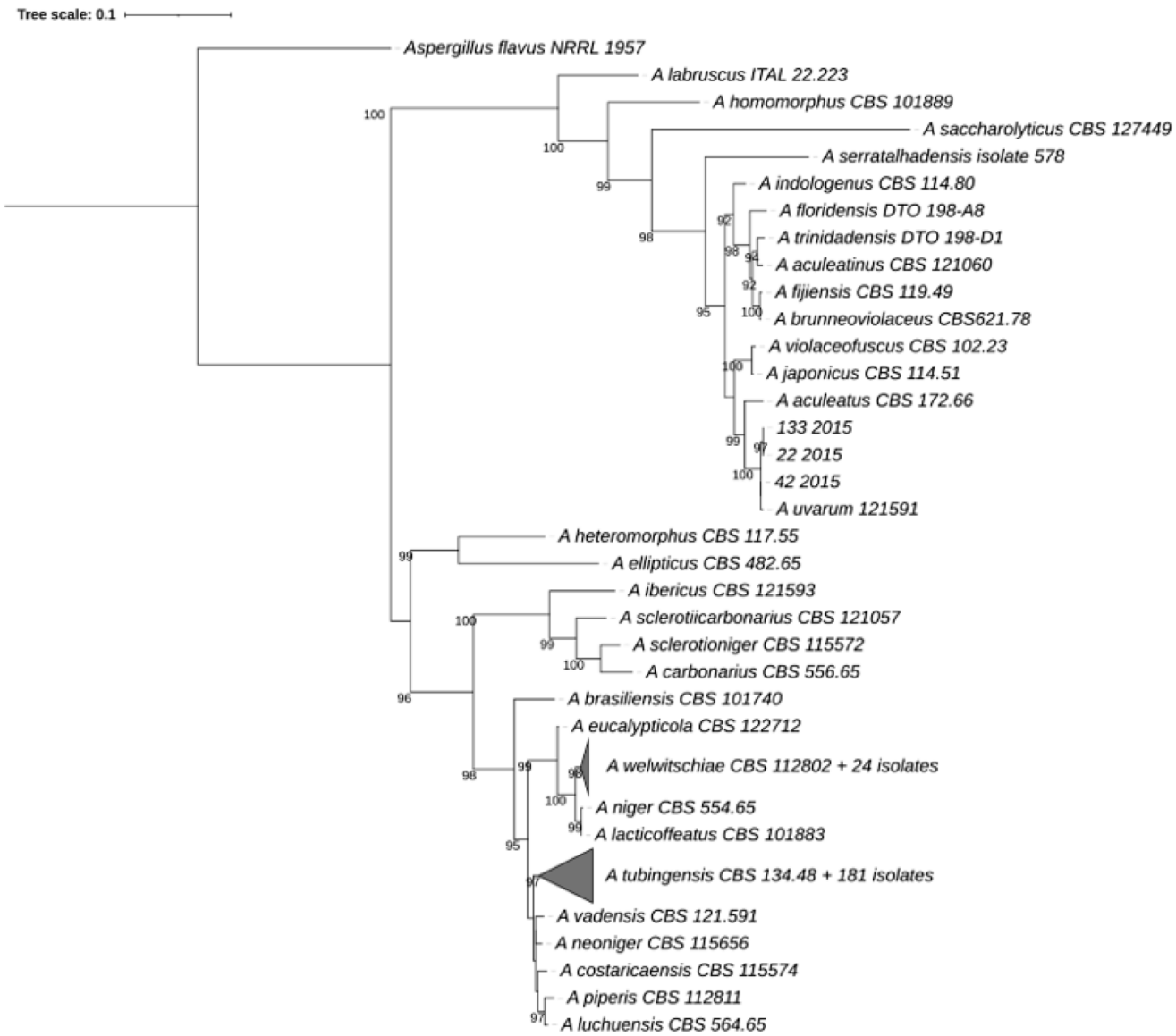


Fig. 2 Maximum Likelihood consensus tree (compressed version) of *ITS*, *βt*, and *CMD* sequences constructed from 10,000 bootstrap trees inferred by IQtree. Numbers are ultrafast bootstrap support (%)

A. tubingensis (94%) was the most isolated species from vineyard V7 (Bevagna), with a significant difference compared to *A. welwitschiae* (6%; $p = 0.0001$), whereas *A. uvarum* was not isolated at all (Fig. 4).

A similar trend was observed in the other vineyards located in the plain in the Bevagna area (V8), with *A. tubingensis* (85%) more frequently isolated with respect to *A. welwitschiae* (15%; $p = 0.0086$). *A. uvarum* was not isolated in this location (Fig. 4).

Distribution of black aspergilli in vineyards grown under different farming systems

The different farming systems are not fully crossed with cultivars and locations and, therefore, direct statistical comparisons between farming systems are not warranted.

Focusing on the organic system, performed only in one location, *A. tubingensis* (93%) was more frequently isolated than *A. welwitschiae* (6%; $p = 0.0001$) and *A. uvarum* (1%; $p = 0.0001$), with no significant difference between these latter two species (Fig. 5; $p = 0.153$). Also, from the grapes sampled in the integrated vineyards (all locations, but V5; all cultivars, but Grechetto and Sangiovese) the most isolated species was *A. tubingensis* (78%), followed

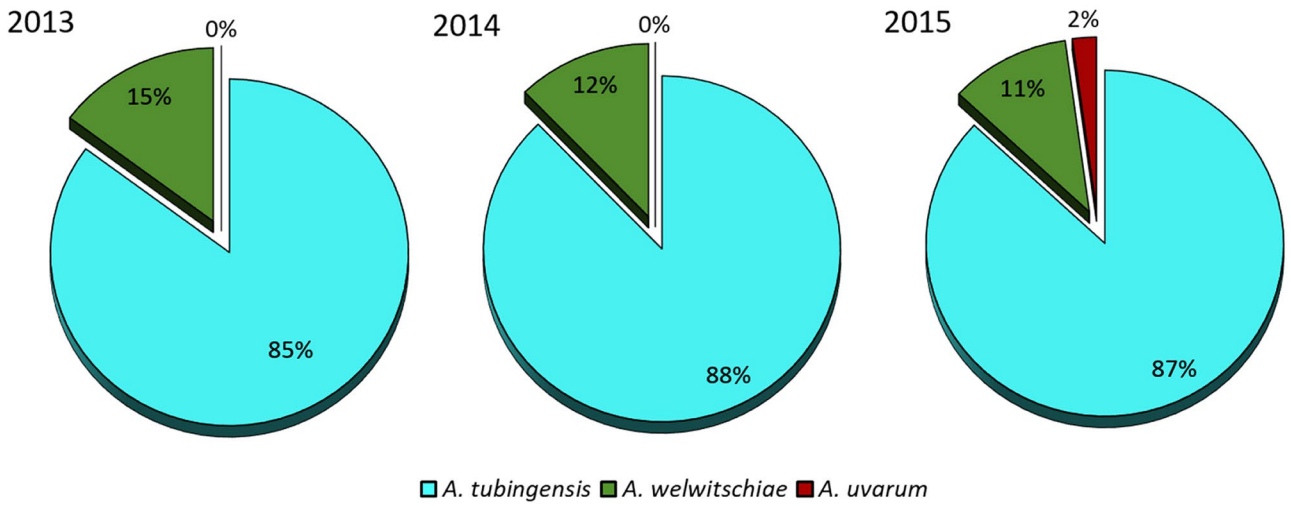


Fig. 3 Incidence (%) of *A. tubingensis*, *A. welwitschiae*, and *A. uvarum* isolated from grapes sampled in 2013, 2014 and 2015 in the surveyed Umbrian vineyards

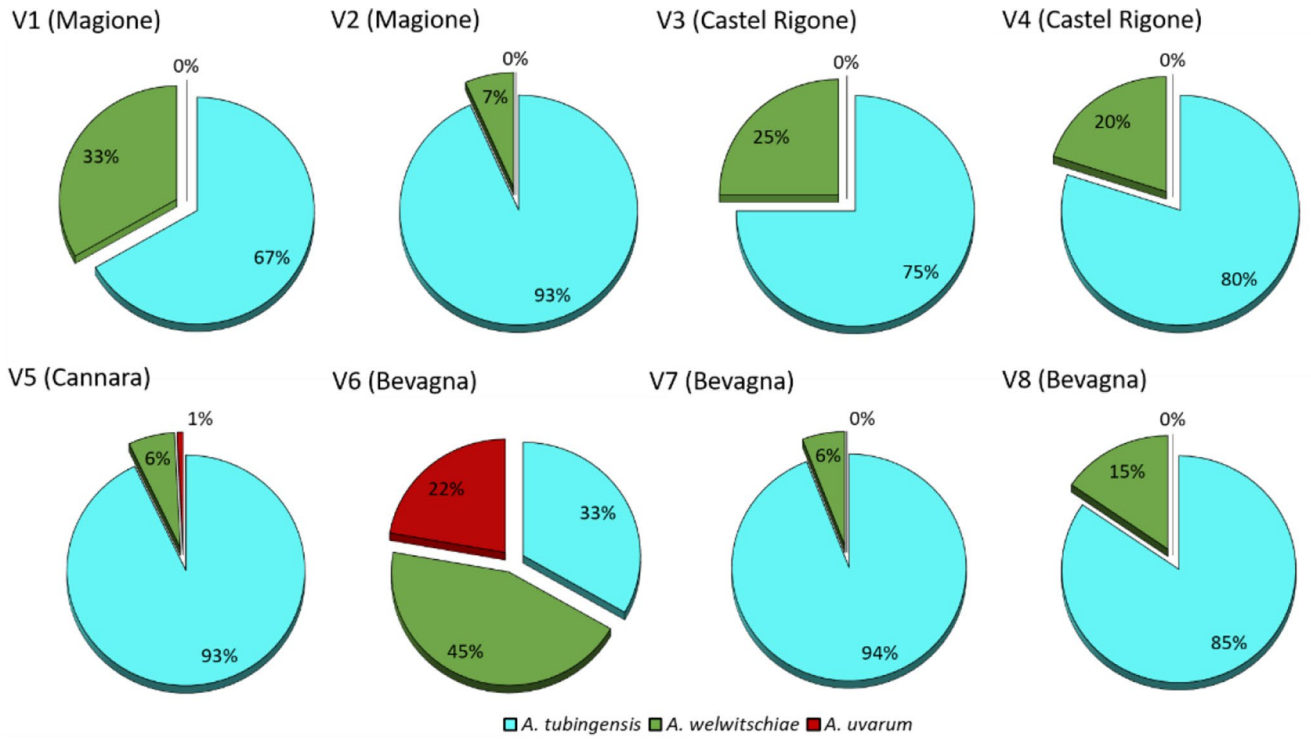


Fig. 4 Incidence (%) of *A. tubingensis*, *A. welwitschiae*, and *A. uvarum* isolated from grapes harvested in eight different Umbrian vineyards (V1–V8) during the three surveyed years

by *A. welwitschiae* (20%) and *A. uvarum* (2%) ($p < 0.0062$) (Fig. 5). Similar to what was detected in the organic vineyards, no significant differences were observed between *A. welwitschiae* and *A. uvarum* ($p = 0.0508$).

Role of grape growth stages on black aspergilli development

In regard to the effect of growth stages, the dataset appears to be more orthogonal than the previously considered effects of location, cultivar and farming system, so some comparisons between growth stages seem to be possible.

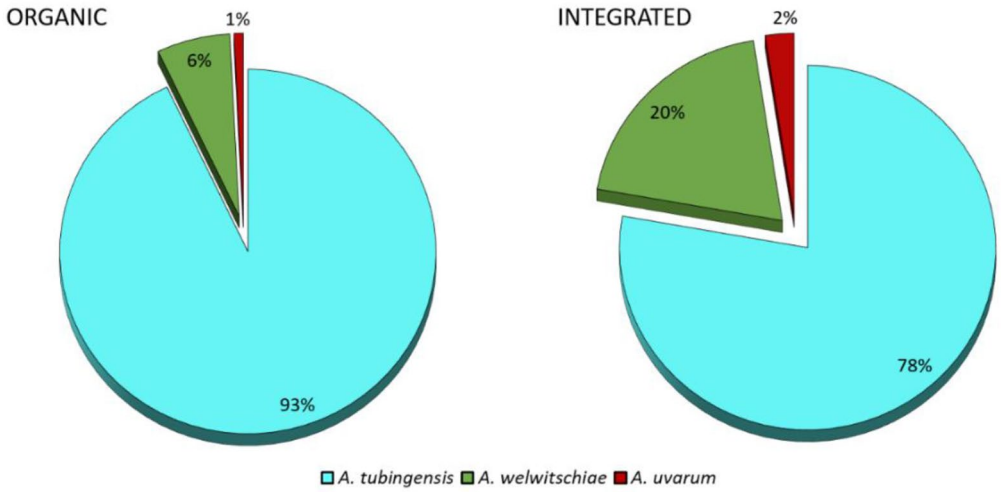


Fig. 5 Incidence (%) of *A. tubingensis*, *A. welwitschiae*, and *A. uvarum* isolated from grapes sampled in vineyards grown under organic and integrated farming systems. Direct comparisons between farming systems were not possible, due to the design of this survey

Growth stages played a significant ($p = 0.032$) role in the development of two *Aspergillus* section *Nigri* species (Fig. 6). In detail, *A. tubingensis* showed the lowest presence at setting in comparison to berries pea-size and berries harvest-ripe ($p < 0.032$), while no significant differences were detected at veraison in comparison to the other growth stages ($p > 0.059$). Considering *A. welwitschiae*, the occurrence detected at the setting stage was significantly higher than that recorded at berries pea-size and at berries harvest-ripe ($p < 0.043$). Also for this species, the incidence detected at veraison was not significantly different compared to what was observed at setting, berries pea-size and berries harvest-ripe ($p > 0.072$). No significant differences among the different growth stages were observed for *A. uvarum* ($P > 0.338$).

Focusing on every single growth stage, from grapes sampled during the setting stage (Fig. 6), *A. tubingensis* (68%) was isolated with a significant ($p = 0.0004$) higher incidence than *A. uvarum* (3%) but not ($p = 0.100$) than *A. welwitschiae* (29%). Despite the higher incidence, this last species was not significantly different ($p = 0.051$) from *A. uvarum*. The isolation carried out by the HC technique provided an opportunity to observe that the most colonized tissue by black aspergilli at setting was represented by flower debris.

A. tubingensis was the species mainly isolated (93%) from the samples collected at the berries pea-size (Fig. 6), in comparison to *A. uvarum* (4%; $p < 0.0001$) and *A. welwitschiae* (3%; $p < 0.0001$). Conversely, these two last species were equally isolated ($p = 1.000$). Similar to what observed at the previous sampling time, the most colonized tissue by black aspergilli at the berries pea-size was represented by flower debris.

A. tubingensis (88%) was the most frequently isolated species also from the samples collected at the veraison stage (Fig. 6) compared to *A. uvarum* (1%; $p < 0.0001$) and *A. welwitschiae* (11%; $p < 0.0001$), while no significant differences ($p = 0.095$) between these last two species were found. At this stage, the most colonized tissue by black aspergilli was represented by the peduncles, from which colonization extended to the berries.

Finally, *A. uvarum* was not isolated from grapes at the berries harvest-ripe (Fig. 6) whereas *A. welwitschiae* (8%) was less frequently isolated ($p < 0.0001$) than *A. tubingensis* (92%). At this stage, the most colonized tissue by *Aspergillus* spp. were the wounds and the cracking present on the berries as well as the whole berries probably colonized from the peduncles. At berries harvest-ripe, the colonization by black aspergilli was also detected on mummified berries.

Discussion

The present paper describes the results of a survey study conducted in the Umbria region (central Italy) to identify the *Aspergillus* species belonging to the section *Nigri* associated with wine grapes. In addition, the effect of sampling year (2013, 2014 and 2015); sampling location, farming system (organic or integrated) and growth stage (setting, berries pea-size, veraison and berries harvest-ripe) was also considered. Due to the nature of the survey, the different effects are not fully crossed and there is a certain degree of unbalance, which requires some prudence when trying to disentangle those effects.

The results obtained regarding the composition of the black aspergilli community associated with grapes cultivated in the investigated area are relative to the three years 2013, 2014, and 2015. For this reason, they may not

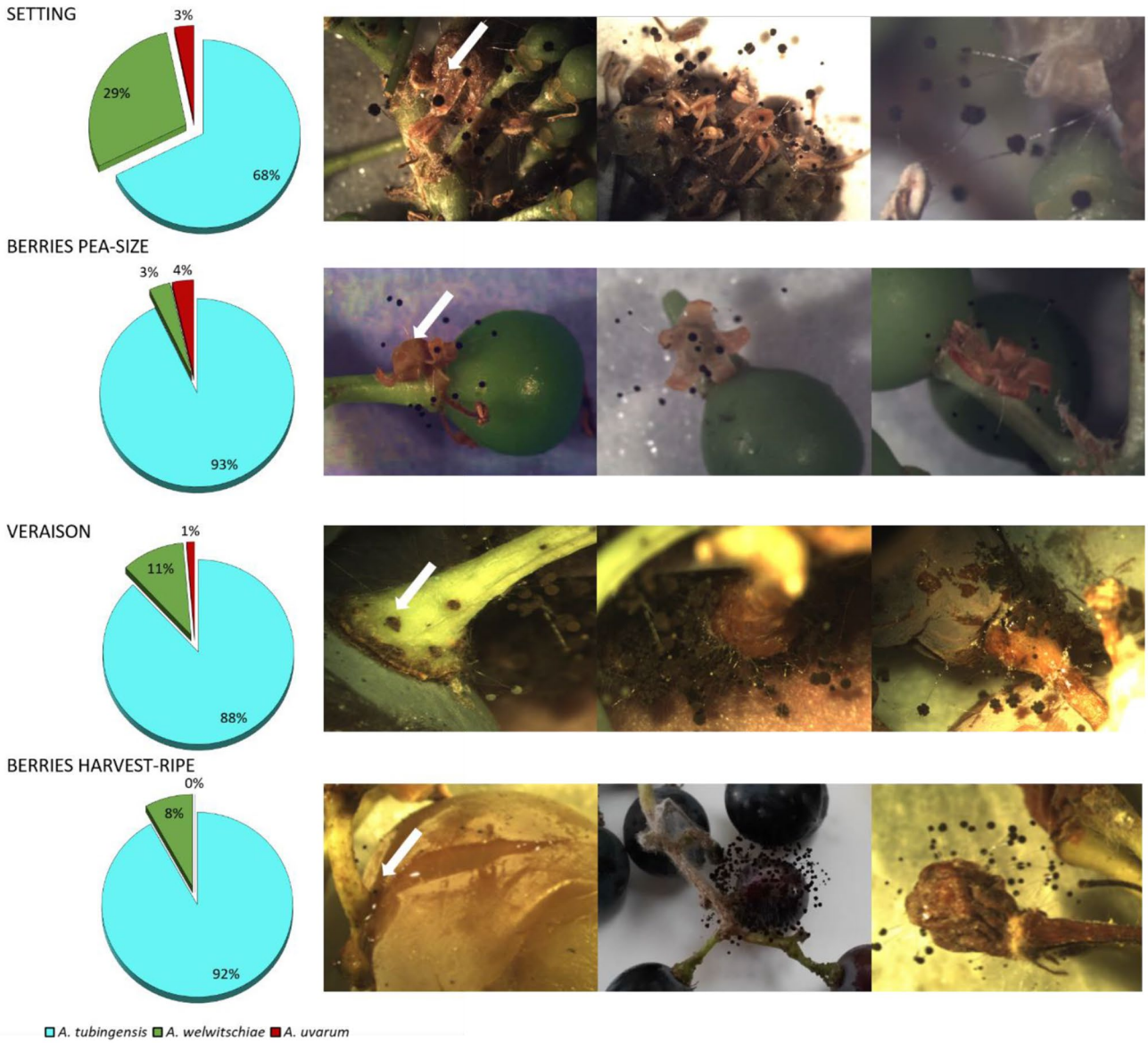


Fig. 6 Incidence (%) of *A. tubingensis*, *A. welwitschiae*, and *A. uvarum* isolated from grapes sampled at different growth stages: setting, berries pea-size, veraison and berries harvest-ripe. For each growth stage, the images show the mainly colonized tissue by *Aspergillus* section *Nigri* spp. In detail, flower debris was the most colonized tissue at setting and berries pea-size stages; peduncles were the most colonized tissues at the veraison stage; cracked berries were mainly colonized at berries harvest-ripe, even if, at this sampling time, berry colonization occurred also as a consequence of infections started from the peduncles, causing also their mummification

correspond to the community currently present in the same cultivation area.

A. tubingensis, together with *A. niger* and *A. carbonarius*, is usually considered one of the main black aspergilli detected on grapes grown in the Mediterranean area (Somma et al. 2012; García-Cela et al. 2014a, b; Cabañes and Bragulat 2018). The other two species detected in this survey (*A. welwitschiae* and *A. uvarum*) are among those found with a certain incidence in the same territory (Cabañes and Bragulat 2018; Tini et al. 2020). Other researchers previously determined the simultaneous presence of these three black aspergilli on grapes in specific surveyed areas. For example, similar results to those obtained in the present study were highlighted by Habib et al. (2021) on table grapes in Lebanon. The authors detected the presence of *A. tubingensis*, *A. welwitschiae* and *A. uvarum* and the absence of other black aspergilli (Habib et al. 2021). These three species were also reported in grapes from non-Mediterranean countries such as Canada (Qi et al. 2016) and China (Huang et al. 2020). However, in these last cases, the authors detected, together with these three species, also other black aspergilli (Qi et al. 2016; Huang et al. 2020). Conversely, a survey conducted

in northern Italy showed, among the other black aspergilli (*A. niger*, *A carbonarius*, *A. aculeatus*, *A. japonicus*), the presence of *A. tubingensis* but not of *A. welwitschiae* and *A. uvarum* (Spadaro et al. 2012). Similarly, in Spain, the species *A. tubingensis* (together with *A. carbonarius* and *A. niger*) was found, while *A. welwitschiae* and *A. uvarum* were not detected (García-Cela et al. 2014a). However, cases where the species *A. welwitschiae* and *A. uvarum* were detected in the grapes in the absence of *A. tubingensis* were reported in Uruguay (Garmendia and Vero 2016). Finally, the presence of *A. tubingensis* together with *A. uvarum* was detected in grapes in Greece (Testempasis et al. 2022), while, *A. tubingensis* was detected with *A. welwitschiae* in grapes in Cyprus (Pantelides et al. 2017) and California (USA) (Palumbo et al. 2016).

In summary, the exclusive detection of *A. tubingensis*, *A. welwistichiae* and *A. uvarum* in a surveyed grapes cultivation area, as reported in this study, is not very common (Fig. 7). Conversely, the presence of these three species (together or separated), in combination with other black aspergilli, is quite common (Fig. 7).

Interestingly, in the present study, *A. carbonarius*, considered for many years one of the most important black aspergilli able to produce OTA in grapes in the Mediterranean area (Gil-Serna et al. 2018; Welke 2019; Mondani et al. 2020), was not detected in any of the analyzed samples. The absence of this species in grapes was reported also during other surveys conducted in Spain (Gómez-Albarrán et al. 2021) and Lebanon (Habib et al. 2021). The decrease of *A. carbonarius* in Mediterranean areas could be explained by the climate change scenario that might have negatively affected the development of this species in grapes (Cervini et al. 2020). The absence of *A. carbonarius* in the area surveyed in the present study could suggest a potential reduction of OTA contamination risk, even if another OTA producing species, *A. welwistichiae*, has been detected during this investigation. However, the percentage of OTA producing isolates within *A. welwistichiae* populations analyzed in other studies was low (Susca et al. 2016) or even absent (Qi et al. 2016). These findings, combined with the fact that *A. tubingensis* and *A. uvarum* are considered, respectively, controversially able (Choque et al. 2018) or not able (Perrone et al. 2008) to produce OTA, would suggest the presence of a low OTA contamination risk in the surveyed area during the investigated years.

The three black aspergilli associated with wine grapes detected during the present surveys showed different isolation incidences. In fact, despite the sampling year did not show any effect on the incidence of the different species, *A. tubingensis* was always the most isolated species in each of the three investigated years followed by *A. welwistichiae*. *A. uvarum* was detected only in one surveyed

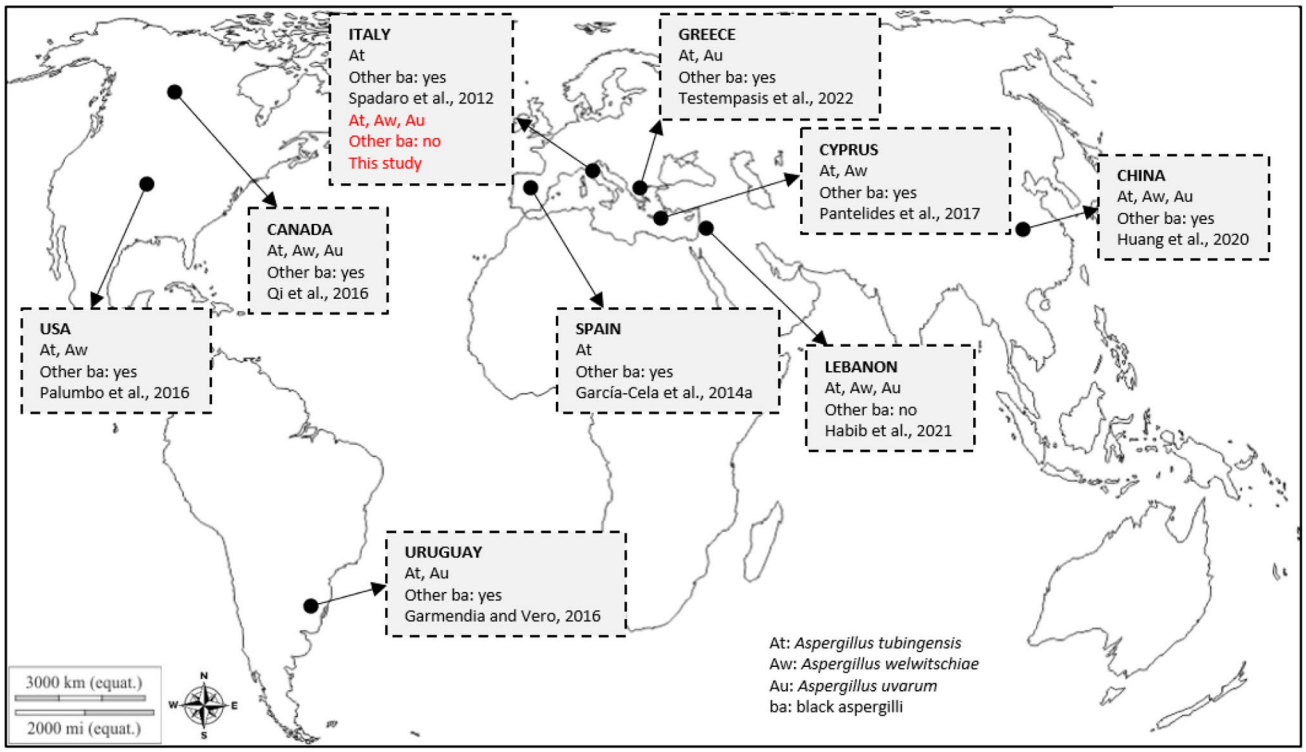


Fig. 7 Map showing the detection of *A. tubingensis*, *A. welwitschiae*, *A. uvarum* and other black aspergilli from grapes as obtained from this survey (in red) and from other surveys in several world countries

year. The predominance of *A. tubingensis* in grapes within a black aspergilli population was also reported in surveys conducted in other countries such as Lebanon (Habib et al. 2021), Canada (one of the three investigated years; Qi et al. 2016), China (Huang et al. 2020), Spain (García-Cela et al. 2014a), Greece (one of the two investigated years; Testempasis et al. 2022) and Cyprus (Pantelides et al. 2017). The results obtained in the present study further contribute to confirming that *A. tubingensis* can be considered, worldwide, one of the most present species within a black aspergilli population in grapes.

The predominance of *A. tubingensis* was significant also in most of the surveyed vineyards (five out of eight). This species was present in each single location analyzed together with *A. welwitschiae*, while *A. uvarum* was reported only in two out of eight locations. Interestingly, one of these two locations (V6) in which the three species were simultaneously present, was the only showing no incidence differences among the three species. These results highlight that in the surveyed area the level of complexity of the black aspergilli community associated with the grapes of a single vineyard was low. A similar result was obtained also by Testempasis et al. (2022) who, investigating the composition of the black aspergilli community in some grapes cultivation areas of Greece, detected no more than three species simultaneously in each surveyed area. The present study showed also that the effect of vineyard location was limited and, when present, restricted to *A. tubingensis*. Similarly, significant differences in the distribution within each single location were detected only for this predominant species with respect to the less represented ones. Conversely, in the surveyed area, the other detected species (*A. welwistchiae* and *A. uvarum*) showed a uniformity of their distribution both across the different locations and within a single location.

Previous studies showed that farming systems may have (Testempasis et al. 2022) or not (Palumbo et al. 2019) a significant influence on black aspergilli populations in grapes. For example, Testempasis et al. (2022), surveying Greek vineyards, detected a higher presence of *A. tubingensis* in organic systems than in conventional ones. In the same survey, *A. uvarum* showed a higher presence in conventional vineyards in comparison to organic ones (Testempasis et al. 2022). Palumbo et al. (2019), comparing the incidence of *A. carbonarius*, *A niger*, *A welwitschiae* and *A. tubingensis* on grapes cultivated in California (USA) under conventional or organic systems, concluded that no difference was detected. In this survey, the comparison between farming systems is not meaningful because they were present in different locations. However, the present study highlighted that all three species were isolated from both farming systems and that *A. tubingensis* was predominant both in organic and in integrated vineyards.

The different berry growth stages play a role in the dynamics of black aspergilli populations. For example, Ponsone et al. (2007) and Palumbo et al. (2016) reported that through the different growth stages, from setting to harvest, grape contamination by black aspergilli increased. In the present survey, the role of growth stage played a significant effect on *A. tubingensis* and *A. welwistchiae* incidence. Specifically, *A. tubingensis* (detected at every sampling time) showed a lower presence at setting in comparison to berries pea-size and berries harvest-ripe stages. An increase of *A. tubingensis* incidence from the first stages of berry development to the others (harvest or veraison) was described also during surveys in California (USA) (Palumbo et al. 2016, 2019).

Conversely, in the present survey, *A. welwistchiae* (detected at every sampling time) showed a higher presence at setting than at berries pea-size and berries harvest-ripe. *A. uvarum* (detected at every sample time except berries harvest-ripe) did not show significant variations across the different growth stages in which it was found. The dynamics of the three species across sampling times suggests that *A. tubingensis* increased from setting to berries harvest-ripe, while *A. welwistichiae* decreased and *A. uvarum* (even if not significantly) disappeared. Within each sampling time, *A. tubingensis* showed a significantly higher incidence than *A. welwistchiae* and *A. uvarum*.

Stereomicroscope observations conducted in samples placed in HC highlighted that flower debris was the substrate from which black aspergilli mainly developed at setting and berries pea-size. This is something that had been already described for *Botrytis cinerea*, another causal agent of bunch rot (Molitor et al. 2015). The results obtained in the present study suggest that also black aspergilli can cause bunch rot starting from flower debris colonization. Conversely, peduncles acted as substrates from which black aspergilli mainly developed at veraison. Only at berries harvest-ripe, an evidence of black aspergilli development was noticed from cracking in the berry surface, confirming that wounds on grape skin facilitated infection by black aspergilli (Lappa et al. 2018).

In conclusion, this study determined the presence of the three black aspergilli, *A. tubingensis*, *A. welwistchiae* and *A. uvarum*, associated with wine grapes cultivated in Umbria (central Italy). *A. tubingensis* was by far the predominant species. No *A. carbonarius* was found. Among the factors capable of influencing the occurrence of black aspergilli, vineyard location, farming system and grape growth stages were found to be those affecting the distribution of black aspergilli, in particular of *A. tubingensis*.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s42161-024-01780-3.

Funding Open access funding provided by Università degli Studi di Perugia within the CRUI-CARE Agreement.

Data availability All data generated or analyzed during this study is included in the published article (and its supplementary files).

Declarations

Ethical approval This study does not need any ethical approval.

Consent to participate Not applicable.

Consent for publication Not applicable.

Competing interests The author declares no competing interests.

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