Aspergillus spp. associated with Aspergillus vine canker in Mexico

Rufina Hernandez-Martinez (ruhernan@cicese.mx)
Centro de Investigacion Cientifica y de Educacion Superior de Ensenada Division de Biologia Experimental y Aplicada https://orcid.org/0000-0002-0914-3732

Research Article

Keywords: Aspergillus section Nigri, grapevine trunk diseases, virulence, fungi

Posted Date: June 29th, 2023

DOI: https://doi.org/10.21203/rs.3.rs-3077304/v1

Abstract

The genus *Aspergillus* encompasses species with high economic and social impact. Species belonging to section Nigri known as black aspergilli, have predominantly been isolated from soil and plants. Fungi associated with Aspergillus vine canker infect vigorous new shoots and canes during plant training, entering through wounds. Recently, grapevines exhibiting symptoms resembling Aspergillus vine canker have been observed in Mexico. Thus, this study aimed to investigate these diseased plants. Woody samples from symptomatic plants were surface sterilized with alcohol and fire and then placed onto Potato Dextrose Agar (PDA) medium. Seventeen isolates displaying morphology similar to the genus *Aspergillus* were obtained. Colony and microscopic characteristics were observed on Czapek Yeast Extract Agar (CYA) and Malt Extract Agar (MEA). Phylogenetic analysis using calmodulin (*CaM*) and β-tubulin (*BenA*) gene markers, revealed the presence of *Aspergillus niger*, *A. tubingensis*, and *A. welwitschiae* species. In pathogenicity assays, *A. niger* A10BCMX, *A. niger* A8SMX, and *A. tubingensis* A13SMX exhibited the highest virulence, resulting in lesions up to 2 cm in length after 50 days of inoculation. Powdery black conidia were found within the wound, specifically in the cambium. In addition to necrotic lesions, mycelia and black conidia were observed on the leaves. This study represents the first report of *Aspergillus* species associated with Aspergillus vine canker in Mexican vineyards and the first report worldwide of *A. welwitschiae* associated with this disease.

Introduction

The genus *Aspergillus* comprises a diverse group of species with high economic and social impact. They are classified into six subgenera, 27 sections, and 446 species (Houbraken et al 2020). The species belonging to section Nigri, known as black aspergilli, are ubiquitous saprophytes particularly present in tropical and subtropical regions (Leong et al., 2004). These fungi are important in the food, medical, and biotechnology industries, and they have mainly been isolated from soil and plants (Gams *et al.*, 1985; Varga et al., 2011; Xanthopoulou et al., 2019). The most common species in section Nigri include *Aspergillus acidus*, *A. brasiliensis*, *A. carbonarius*, *A. niger*, and *A. tubingensis* (Houbraken et al 2020).

These species commonly cause Aspergillus rot in several crops, which is one of the most important post-harvest diseases that affect grapes. Several species of *Aspergillus* have been isolated from grapevines, as they are part of the epiphytic flora of the grapes and could be present in the soil and the plant throughout the complete production cycle. Factors such as warm temperatures, damage caused by insect pests, rainfall, or fungal infections facilitate their entry into the grapes (Mondani et al., 2020). Aspergillus rot alters the sanitary quality of grapes, causing them to turn brown and be covered with black spores (Leong et al., 2004; Steel et al., 2013). Some species of *Aspergillus* are known for the secretion of mycotoxins, mainly ochratoxin A (OTA), which is very toxic and has nephrotoxic, carcinogenic, and immunosuppressive properties (Arfaoui et al 2019). Due to the accumulation of OTA, *Aspergillus niger*, *A. aculeatus*, and *A. carbonarius* are the most toxigenic for grapes (Welke 2019).

The first detection of *Aspergillus niger* causing grapevine canker disease was in the San Joaquin Valley, CA in 1989, affecting excessively vigorous young 'Red Globe' grapevines. In Coachella Valley, California similar canker symptoms were observed on 'Red Globe', 'Crimson Seedless', 'Chardonnay', and 'Grenache' (Michailides et al. 2002). In Europe *A. niger* causing Aspergillus vine canker was reported for the first time in southeastern Sicily, Italy (Vitale et al., 2008). In 2012, *A. niger*, *A. tubingensis*, and *A. carbonarius* were identified in the same region (Vitale et al., 2012). Aspergillus vine canker is relatively rare, and its symptoms include drops of reddish sap at the infection site, swollen and spongy trunk, discolored and necrotic tissue, and black spores within the canker. The fungus infects new shoots and canes during vine training, primarily through pruning wounds (Michailides et al. 2002; Sharma, 2012; Vitale et al., 2012).

Recently, grapevines with similar symptoms to Aspergillus vine canker have been observed in Mexico. This study aims to characterize the *Aspergillus* species associated with those plants.

Materials and methods

Fungal isolation

From 2020 to 2022, grapevines in vineyards established in Baja California, Sonora, and Guanajuato showed symptoms of canker discoloration, spongy tissue, and black spores within the canker (Fig. 1). Samples were collected from cultivars 'Cotton Candy', 'Sweet Celebration', 'Cabernet Sauvignon', 'Merlot', and 'Chardonnay' and were taken to the plant pathology laboratory at CICESE for examination. Small pieces of symptomatic plant tissue were surface-sterilized using 95% ethanol and fire. Then, they were placed onto Potato Dextrose Agar (PDA) plates, supplemented with 25 $mg{\cdot}mL^{-1}$ chloramphenicol to avoid bacteria growth. The plates were incubated at 30°C until fungal growth was observed. Fungal colonies with similar morphology to the genus *Aspergillus* were the most predominant. To obtain pure cultures, hyphal tips from each colony were individually transferred to PDA plates, and each isolate was preserved at 4°C in 20% glycerol.

Morphological characterization

For morphological characterization, a mycelium plug of each fungal isolate was inoculated on the recommended media for *Aspergillus*, PDA, Czapek Yeast Autolysate Agar (CYA), and Malt Extract Agar (MEA). The plates were incubated at 30°C for seven days. After, colony pigmentation, elevation, shape, margin, and reverse color on the plate were recorded, along with the formation of aerial mycelium and sporulation. Microscopic characteristics were observed from cultures on CYA after 7–10 days using a light microscope (AxioVert200 Zeiss). Images of the spores and conidiophores were captured using a camera AxioCam HRc Zeiss and analyzed using AxioVision 4.8.2. and ImageJ 1.49v. The shape, texture, ornamentation, and size of the conidia, as well as the shape of the head, vesicle shape and diameter, stipe, and the color of conidiophores were described.

DNA extraction and PCR amplification

The *Aspergillus* isolates were cultivated in Potato Dextrose Broth (PDB) at 30°C for three days, and mycelium was recovered through filtration. Genomic DNA extraction was performed using the CTAB protocol (Wagner et al. 1987). To characterize *Aspergillus* spp., the phylogenetic markers for calmodulin (*CaM*), and β-tubulin (*BenA*) were employed. The primers Bt2a (GGT AAC CAA ATC GGT GCT GCT TTC) and Bt2b (ACC CTC AGT GTA GTG ACC CTT GGC) (Glass and Donaldson, 1995) were used for amplifying *BenA*; while CMD5 (CCG AGT ACA AGG ARG CCT TC) and CMD6 (CCG ATR GAG GTC ATR ACG TGG) (Hong et al., 2005) were used for amplifying *CaM*. The PCR reactions were conducted in a final volume of 25 µL, with each tube containing 2.5 µL of 10X PCR buffer with 15 mM $MgCl_2$, 0.5µL of 20 mM dNTPs, 2.5 of 25 mM $MgCl_2$, 0.625 µL of 10 µM of each primer, 0.25 µL of bovine serum albumin (BSA), 0.125 µL of Taq DNA polymerase (DreamTaq DNA polymerase, Thermofisher) at 5 units·μL^{-1}, 1 µL of 30 ng·μL^{-1} template DNA, and 16.875 µL of ultra-pure water. The PCR amplification was carried out in a Bio-Rad T-100 thermal cycler following the next conditions. For *BenA*, an initial cycle of 95°C for 3 min, followed by 35 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 1 min. For *CaM*, an initial cycle of 94°C for 2 min, followed by 35 cycles of 94°C for 1 min, 58°C for 1 min, and 72°C for 1 min and 30 s. Both programs had a final cycle of 72°C for 10 min. PCR products were observed by electrophoresis and were purified using the GeneJet PCR purification kit (Thermo Scientific). Then, the purified products were sequenced by Eton Bioscience Inc.

Phylogenetic analysis

For phylogeny analysis, the obtained sequences were verified using BioEdit v.7.0.5.3 (Hall 1999) and deposited in GenBank (https://www.ncbi.nlm.nih.gov/genbank/). A BLASTn was conducted to find the sequences with the highest similarity (Table 1). Sequence alignment was performed using ClustalW with pairwise alignment parameters: gap opening 10, gap extension 0.1, and multiple alignment parameters: gap opening 10, gap extension 0.2; transition weight was set to 0.5, and delay divergent sequences were delayed by 25%) (Thompson et al. 1994). Manual adjustments were made to the CaM and BenA alignments where necessary, and they were imported into BioEdit v.7.0.5.3 to obtain the concatenated matrix. The best model of nucleotide substitution was selected based on the Akaike Information Criterion (AIC). Maximum Likelihood (ML) analysis and Maximum Parsimony (MP) analysis were performed using MEGA-X (Kumar et al., 2018) with the concatenated sequence alignment. The ML analysis employed the T92 + G model (Kimura 1980), and parameters for ML were set to the Bootstrap method with 1000 replicates. Initial tree(s) for the heuristic search were automatically obtained using the Maximum Parsimony method. Gaps were treated as missing data. The tree was visualized in MX: Tree Explorer (Kumar et al., 2018).

Table 1
List of isolates and GenBank accession numbers of *Aspergillus* spp. used in this study for phylogenetic analysis.

Specie	Isolate	Host	Origin	GenBank accession number	
				CaM	*BenA*
Aspergillus aculeatus	NRRL 5094	Tropical soil	Unknown	EF661148	EF661083
A. aculeatinus	CBS 121060	Coffee bean	Thailand	EU159241	EU159220
A. brasiliensis	EGE-KL-T96	dried figs	Turkey	KR064380	KR064512
A. brasiliensis	CBS 101740	Soil	Brazil	FN594543	FJ629272
A. carbonarius	NRRL 369	Paper	USA	EF661167	EF661099
A. carbonarius	DTO:179-F4	Indoor house dust	South Africa	KJ775280	KP329846
A. costaricaensis	CBS 115574	Soil	Costa Rica	FN594545	FJ629277
A. ellipticus	NRRL 5120	Soil	Costa Rica	EF661170	EF661122
A. eucalypticola	53A2	Soil	India	EU482433	EU482435
A. flavus	CBS 100927	Cellophane	South Pacific Islands	EF20206	AY819992
A. heteromorphus	NRRL 4747	Culture contaminant	Brazil	EF661169	EF661103
A. ibericus	NRRL 35644	Food, fruit (grapes)	Portugal	EF661163	EF661102
A. japonicas	CBS 114.51	Unknown	Unknown	FN594551	HE577804
A. lacticoffeatus	CBS 101883	Coffea robusta, surface sterilized beans	Sumatra	FN594552	AY8199983
A. luchuensis	KACC 46772	awamori-koji	Japon	JX500071	JX500062
A. neoniger	CBS 115656	sulphur sponge, Porifera	Venezuela	FJ491700	FJ491691

Specie	Isolate	Host	Origin	GenBank accession number	
				CaM	*BenA*
A. niger	NRRL 326	Tannin-gallic acid fermentation	USA	EF661154	EF661089
A. niger	CD1200	*Homo sapiens*	Italy	KX231826	KX231821
A. niger	**A2BCMX**	**V. vinifera**	**Mexico**	OQ924284	OQ631061
A. niger	**A5SMX**	**V. vinifera**	**Mexico**	OQ924287	OQ631064
A. niger	**A6SMX**	**V. vinifera**	**Mexico**	OQ924288	OQ631065
A. niger	**A7SMX**	**V. vinifera**	**Mexico**	OQ924289	OQ631066
A. niger	**A8SMX**	**V. vinifera**	**Mexico**	OQ924290	OQ631067
A. niger	**A9SMX**	**V. vinifera**	**Mexico**	OQ924291	OQ631068
A. niger	**A10BCMX**	**V. vinifera**	**Mexico**	OQ924292	OQ631069
A. niger	**A11SMX**	**V. vinifera**	**Mexico**	OQ924293	OQ631070
A. niger	**A14GMX**	**V. vinifera**	**Mexico**	OQ924296	OQ631073
A. piperis	DTO 418-E4	dried food	Nigeria	MN882818	MN882790
A. piperis	CMV011A9	Zea mays	South Africa	MK451493	MK451187
A. sclerotiicarbonarius	CBS 121057	Coffee bean	Thailand	FN594556	EU159229
A. sclerotioniger	CBS 115572	Arabica coffee green	India	FN594557	FJ629304
A. tubingensis	CBS 134.48	Unknown	Unknown	FN594558	FJ629305
A. tubingensis	NRRL 4875	Unknown	Unknown	EF661151	EF661086
A. tubingensis	**A1BCMX**	**V. vinifera**	**Mexico**	OQ924283	OQ631060
A. tubingensis	**A3BCMX**	**V. vinifera**	**Mexico**	OQ924285	OQ631062
A. tubingensis	**A4BCMX**	**V. vinifera**	**Mexico**	OQ924286	OQ631063
A. tubingensis	**A12SMX**	**V. vinifera**	**Mexico**	OQ924294	OQ631071
A. tubingensis	**A13SMX**	**V. vinifera**	**Mexico**	OQ924296	OQ631072
A. tubingensis	**A16BCMX**	**V. vinifera**	**Mexico**	OQ924298	OQ631075

Specie	Isolate	Host	Origin	GenBank accession number	
				CaM	*BenA*
A. tubingensis	**A17BCMX**	**V. vinifera**	**Mexico**	OQ924299	OQ631076
A. uvarum	ITEM 4834	Grapes	Italy	AM745755	AM745751
A. vadensis	CBS 113365	dead plant tissue	Unknown	FN594560	AY585531
A. welwitschiae	CBS 139.54	Welwitschia mirabilis, female inflorescence, stored 2 years	Namibia	KC480196	MN969369
A. welwitschiae	PW4587	Unknown	Unknown	MT415546	MT415524
A. welwitschiae	**A15GMX**	**V. vinifera**	**Mexico**	OQ924297	OQ631074
Isolates from this study are highlighted in bold.					

Pathogenicity assays

The pathogenicity of the *Aspergillus* isolates was evaluated using two methods. First, selected *Aspergillus* isolates were grown on PDA for three days, and conidiospores were collected using a cotton swab. A suspension of $1x10^6$ conidia/mL was prepared and used to inoculate 'Merlot' grapevine leaves. For this purpose, 10 µL of the suspension was placed at the union of the petiole with the lamina on the abaxial side of the leaf, and punctured until the conidia suspension was absorbed by capillarity. Sterile water was used to inoculate control leaves. Ten leaves were used per treatment. The inoculated leaves were placed in a humid chamber and incubated at room temperature for ten days. Afterward, the presence of necrotic spots, mycelium, and spores was evaluated.

Subsequently, a pathogenesis assay was performed on 'Merlot' grapevine plants using woody tissue. For inoculation, a mycelial plug of each selected Aspergillus isolate was placed in a wound made with a 2 mm diameter drill bit in the stem. Control plants were inoculated with a sterile PDA plug. The wounds were then covered with Parafilm® to protect them from dryness. Each treatment consisted of 10 plants. The grapevine plants were kept in a greenhouse under semi-controlled conditions with an average temperature of 35°C during the day and 25°C during the night for 50 days. Afterward, samples were taken, and the presence of necrotic lesions and *Aspergillus* conidia was evaluated.

To fulfill Koch's postulates, tissue from all infected plants was recovered, inoculated onto PDA plates, and incubated at 30°C until fungal growth was observed. Statistical analysis to determine significant differences in pathogenicity was performed using one-way ANOVA, followed by a post hoc Fisher LSD analysis with $\alpha < 0.05$, using STATISTICA 8.0.

Results and discussion

Morphological and phylogenetic characterization of fungal isolates

Symptoms of Aspergillus vine canker, such as discoloration, necrosis, spongy tissue, cankers, and abundant black conidia, were observed on collected grapevine plants (Fig. 1). In total, seventeen fungal isolates were obtained, which showed similar morphology to *Aspergillus* spp. Most of them had black colonies with a white edge, and some have black colonies with a yellow edge. The species in the section Nigri are very similar and it is difficult to differentiate them due to their morphological characteristics. No sclerotia formations were observed in any of the *Aspergillus* isolates. While sclerotia production has been observed in some *A. niger* and *A. tubingensis* isolates, it has never been described in *A. welwitschiae* (da Silva et al., 2020).

On PDA medium most of the isolates presented black to blackish-brown sporulation and granular colony growth. On MEA medium, all isolates showed barely visible white mycelium and granular to floppy colony growth. *Aspergillus welwitschiae* exhibited less sporulation, while *A. tubingensis* displayed greenish sporulation with whitish underside. On CYA medium *A. niger* exhibited higher sporulation, and *A. tubingensis* displayed an orange colony with a white edge, which could indicate OTA production (Fig. 2) (Arfaoui et al., 2019; Welke, 2019).

OTA is one of the most toxic secondary metabolites produced by several *Aspergillus* spp. such as *A. carbonarius*, *A. niger*, *A. luchuensis* and *A. tubingensis* (Laaziz *et al.*,2022). This toxin has been detected in agricultural and food products, including grapes, and derived products (Leong et al., 2006; Perrone et al., 2006; Selouane et al., 2009), and poses a hazard to human health due to its nephrotoxic, carcinogenic, and immunosuppressive properties (Arfaoui et al., 2019).

In the microscopic observations, *Aspergillus niger*, *A. tubingensis*, and *A. welwitschiae* exhibit globose and biseriate conidiophores with metulae and phialides covering the entire spherical vesicle, resulting in the typical radiate shape. The Stipes are thick-walled, smooth, and hyaline. The *Aspergillus* isolates displayed pale green to dark-brown conidia, both smooth and ornate. Dark-brown to black conidial aggregates were observed in *A. niger* and *A. tubingensis* (Fig. 3). However, the morphological characteristics alone are insufficient to differentiate between species of the Nigri section, making molecular characterization essential (D'hooge et al., 2018).

The phylogenetic analysis of *Aspergillus* sequences based on *CaM* and *BenA* included 45 taxa, corresponding to 21 species. The *CaM* and *BenA* sequences were approximately 540 bp and 520 bp, respectively. The combined dataset alignment comprised 1125 nucleotides, including gaps (553 nucleotides of *CaM* and 572 nucleotides of *BenA*). *Aspergillus flavus* (CBS 100927) was used as the outgroup taxon to root the tree. Maximum likelihood analysis using the Neighbor-Joining method and using Tamura 3-parameter model of pairwise distances estimated a tree with the highest log likelihood of -3353.88. According to the phylogenetic tree, the *Aspergillus* isolates from Mexico were identified as three different species: *A. niger*, *A. tubingensis*, and *A. welwitschiae* (Fig. 4). *Aspergillus niger* was the most

frequently found species with nine isolates in Baja California and Sonora. Seven isolates were identified as *A. tubingensis*, which was found in Baja California, Sonora, and Guanajuato. *Aspergillus welwitschiae* was only found in Guanajuato. In Baja California, the main species identified was *A. tubingensis*, while in Sonora, it was *A. niger*.

Pathogenicity test

The pathogenicity test conducted on leaves revealed that *A. niger* A10BCMX, and *A. tubingensis* A13SMX caused necrotic lesions, with visible mycelia and black conidia (Fig. 5). In the woody tissue test, *A. niger* A10BCMX and A8SMX, and *A. tubingensis* A13SMX demonstrated the highest virulence (Fig. 6), resulting in lesions measuring up to 2 cm in length after 50 days (Table 2). Powdery black conidia were found within the wound, specifically in the cambium region. The control plants exhibited non-necrotic lesions, with green tissue indicating tissue regeneration at the wound site. All *Aspergillus* isolates were successfully recovered from the inoculation site three days after incubation at 30°C on PDA plates, thus confirming Koch's postulates. Similarly, in a study conducted by Vitale et al. in 2012, using grapevine plants 'Italia', lesions ranging from 0.96 cm to 1.6 cm (average 1.6 cm) were caused by *A. tubingensis*, *A. niger*, and *A. carbonarius* 50 days-post-inoculation, but no significant differences in virulence were observed among the isolates.

Table 2
Length of necrotic lesions caused by *Aspergillus* isolates in woody tissue of 'Merlot' grapevine at 50 days-post-inoculation.

Treatment	Lesion ± SD* (cm)
Control	0.32 ± 0.12 a
Aspergillus tubingensis	
A1BCMX	1.13 ± 0.57 bc
A4BCMX	1.07 ± 0.12 bc
A13SMX	1.35 ± 0.65 c
Aspergillus niger	
A2BCMX	0.77 ± 0.26 a
A6SMX	1.14 ± 0.29 bc
A8SMX	1.58 ± 0.83 c
A10BCMX	1.63 ± 0.91 c
A11SMX	1.07 ± 0.27 bc
A14GMX	1.00 ± 0.32 bc
A16BCMX	1.10 ± 0.47 bc
Aspergillus welwitschiae	
A15GMX	1.18 ± 0.53 bc

*SD = standard deviation.

The same letter indicates that there are no statistically significant differences, with $\alpha < 0.05$ based on Fisher's analysis ($\alpha < 0.05$).

In conclusion, we have identified three *Aspergillus* species, namely *A. niger*, *A. tubingensis*, and *A. welwitschiae*, isolated from Baja California, Sonora and Guanajuato, Mexico. In the leaf assay, the isolates *A. niger* A10BCMX and *A. tubingensis* A13SMX exhibited the highest virulence. Meanwhile. In the woody tissue assay, the isolates *A. niger* A8SMX, *A. niger* A10BCMX, and *A. tubingensis* A13SMX demonstrated the greatest virulence and produced black conidia in the infected plants. To our knowledge, this is the first report of *Aspergillus* species associated with Aspergillus vine canker in vineyards of Mexico, and the first report worldwide of *A. welwitschiae* associated with this disease.

Declarations

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Edelweiss Airam Rangel-Montoya and Rufina Hernandez-Martinez. The first draft of the manuscript was written by Edelweiss Airam Rangel-Montoya and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Funding: This research received no external funding.

Financial interests: The authors declare they have no financial interests.

Conflicts of Interest: The authors declare no conflict of interest.

Data Availability Statement: Not applicable.

Acknowledgments: Edelweiss Airam Rangel-Montoya was supported during the postdoctoral program from Consejo Nacional de Humanidades, Ciencias y Tecnologías (CONAHCYT-México).

References

1. Arfaoui, M., Vallance, J., Bruez, E., Rezgui, A., Melki, I., Chebil, S., Sadfi-Zouaoui, N., & Rey, P. (2019). Isolation, identification and in vitro characterization of grapevine rhizobacteria to control ochratoxigenic *Aspergillus* spp. on grapes. *Biological Control*, *129*, 201-211. https://doi.org/10.1016/j.biocontrol.2018.10.019
2. D'hooge, E., Becker, P., Stubbe, D., Normand, A. C., Piarroux, R., & Hendrickx, M. (2019). Black aspergilli: A remaining challenge in fungal taxonomy? *Medical mycology*, *57*(6), 773-780. https://doi.org/10.1093/mmy/myy124
3. da Silva, J. J. D., Iamanaka, B. T., Ferranti, L. S., Massi, F. P., Taniwaki, M. H., Puel, O., Lorber, S., Frisvad, J. C., & Fungaro, M. H. P. (2020). Diversity within *Aspergillus niger* clade and description of a new species: *Aspergillus vinaceus* sp. nov. *Journal of Fungi*, *6*(4), 371. https://doi.org/10.3390/jof6040371
4. Gams, W., Christensen, M., Onions, A. H., Pitt, J. I., Samson, R. A. (1986). Infrageneric Taxa of *Aspergillus*. In Samson, R. A., Pitt, J. I. (Eds) *Advances in Penicillium and Aspergillus Systematics. NATO ASI Series* (vol 102, pp 55–62) Springer, Boston, MA. https://doi.org/10.1007/978-1-4757-1856-0_5
5. Glass NL, Donaldson GC (1995). Development of premier sets designed for use with the PCR to amplify conserved genes from filamentous Ascomycetes. *Applied and Environmental Microbiology, 61*(4), 1323–1330. https://doi.org/10.1128/aem.61.4.1323-1330.1995
6. Hall, T. A., (1999). BioEdit: a user-friendly biological sequence alignment editor and analysisprogram for Windows 95/98/NT. *Nucleic acids symposium series, 41*, 95–98.
7. Hong, S. B., Go, S. J., Shin, H. D., Frisvad, J. C., & Samson, R. A. (2005). Polyphasic taxonomy of *Aspergillus fumigatus* and related species. *Mycologia, 97*, 1316–1329. https://doi.org/10.1080/15572536.2006.11832738

8. Houbraken, J., Kocsubé, S., Visagie, C. M., Yilmaz, N., Wang, X. C., Meijer, M., Kraak, B., Hubka, V., Bensch, K., Samson, R. A., & Frisvad, J. C. (2020). Classification of *Aspergillus*, *Penicillium*, *Talaromyces* and related genera (Eurotiales): An overview of families, genera, subgenera, sections, series and species. *Studies in mycology*, *95*, 5-169. https://doi.org/10.1016/j.simyco.2020.05.002
9. Kimura, M. (1980). A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. *Journal of Molecular Evolution, 16*, 111– 120.
10. Kumar, S., Stecher, G., Li, M., Knyaz, C., & Tamura, K., (2018). MEGA X: Molecular Evolutionary Genetics Analysis across computing platforms. *Molecular biology and evolution, 35*, 1547–1549. https://doi.org/10.1093/molbev/msy096
11. Laaziz, A., El Hammoudi, Y., Qjidaa, S., Hajjaji, A., Hajjaj, H., Haesaert, G., & Bouseta, A. (2022). Activity of essential oils from *Syzygium aromaticum* and *Rosmarinus officinalis* against growth and ochratoxin A production by *Aspergillus tubingensis* and *Aspergillus luchuensis* from Moroccan grapes. *Phytopathologia Mediterranea, 61*(2), 299-310. https://doi.org/10.36253/phyto-12841
12. Leong, S. L., Hocking, A. D., & Pitt, J. I. (2004). Occurrence of fruit rot fungi (*Aspergillus* section Nigri) on some drying varieties of irrigated grapes. *Australian Journal of Grape and Wine Research*, *10*(1), 83-88. https://doi.org/10.1111/j.1755-0238.2004.tb00010.x
13. Leong, S. L. L., Hocking, A. D., Pitt, J. I., Kazi, B. A., Emmett, R. W., Scott, E. S. (2006). Black *Aspergillus* species in Australian vineyards: from soil to ochratoxin A in wine. In Hocking, A. D., Pitt, J. I., Samson, R. A., Thrane, U. (Eds.) *Advances in Food Mycology. Advances in Experimental Medicine and Biology* (vol. 571, pp. 153-171). Springer, Boston, MA.
14. Michailides, T. J., Peacock, W., Christensen, P., Morgan, D. P., & Felts, D. (2002). First report of Aspergillus vine canker of table grapes caused by *Aspergillus niger*. *Plant Disease*, *86*(1), 75-75. https://doi.org/10.1094/PDIS.2002.86.1.75A
15. Mondani, L., Palumbo, R., Tsitsigiannis, D., Perdikis, D., Mazzoni, E., & Battilani, P. (2020). Pest management and ochratoxin A contamination in grapes: A review. *Toxins*, *12*(5), 303. https://doi.org/10.3390/toxins12050303
16. Perrone, G., Mule, G., Susca, A., Battilani, P., Pietri, A., & Logrieco, A. (2006). Ochratoxin A production and amplified fragment length polymorphism analysis of *Aspergillus carbonarius*, *Aspergillus tubingensis*, and *Aspergillus niger* strains isolated from grapes in Italy. *Applied and Environmental Microbiology*, *72*(1), 680-685. https://doi.org/10.1128/AEM.72.1.680-685.2006
17. Selouane, A., Bouya, D., Lebrihi, A., Decock, C., & Bouseta, A. (2009). Impact of some environmental factors on growth and production of ochratoxin A of/by *Aspergillus tubingensis*, *A. niger*, and *A. carbonarius* isolated from Moroccan grapes. *The Journal of Microbiology*, *47*, 411-419. https://doi.org/10.1007/s12275-008-0236-6
18. Sharma, R. (2012). Pathogenecity of *Aspergillus niger* in plants. *Cibtech Journal of Microbiology*, *1*(1), 47-51.
19. Steel, C. C., Blackman, J. W., & Schmidtke, L. M. (2013). Grapevine bunch rots: impacts on wine composition, quality, and potential procedures for the removal of wine faults. *Journal of agricultural*

and food chemistry, *61*(22), 5189-5206. https://doi.org/10.1021/jf400641r

20. Thompson, J. D., Higgins, D. G., & Gibson, T. J. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic acids research, 22*, 4673–4680. https://doi.org/10.1093/nar/22.22.4673
21. Varga, J., Frisvad, J. C., Kocsubé, S., Brankovics, B., Tóth, B., Szigeti, G., & Samson, R. (2011). New and revisited species in *Aspergillus* section Nigri. *Studies in Mycology*, *69*(1), 1-17. https://doi.org/10.3114/sim.2011.69.01
22. Vitale, A., Castello, I., & Polizzi, G. (2008). First report of Aspergillus vine canker on table grapes caused by *Aspergillus niger* in Europe. *Plant Disease*, *92*(10), 1471-1471. https://doi.org/10.1094/PDIS-92-10-1471B
23. Vitale, A., Cirvilleri, G., Panebianco, A., Epifani, F., Perrone, G., & Polizzi, G. (2012). Molecular characterisation and pathogenicity of *Aspergillus* Sect. Nigri causing Aspergillus vine canker of table grapes in Italy. *European journal of plant pathology*, *132*, 483-487. https://doi.org/10.1007/s10658-011-9906-z
24. Wagner D.B., Furnier G.R., Saghai-Maroof M.A., Williams SM, Dancik B.P., Allard R.W., (1987). Chloroplast DNA polymorphisms in lodgepole and jack pines and their hybrids. *PNAS, 84*, 2097–2100. https://doi.org/10.1073/pnas.84.7.209
25. Welke, J. E. (2019). Fungal and mycotoxin problems in grape juice and wine industries. *Current Opinion in Food Science, 29,* 7-13. https://doi.org/10.1016/j.cofs.2019.06.009
26. Xanthopoulou, A., Ganopoulos, I., Tryfinopoulou, P., Panagou, E. Z., Osanthanunkul, M., Madesis, P., & Kizis, D. (2019). Rapid and accurate identification of black aspergilli from grapes using high-resolution melting (HRM) analysis. *Journal of the Science of Food and Agriculture*, *99*(1), 309-314. https://doi.org/10.1002/jsfa.9189

Figures

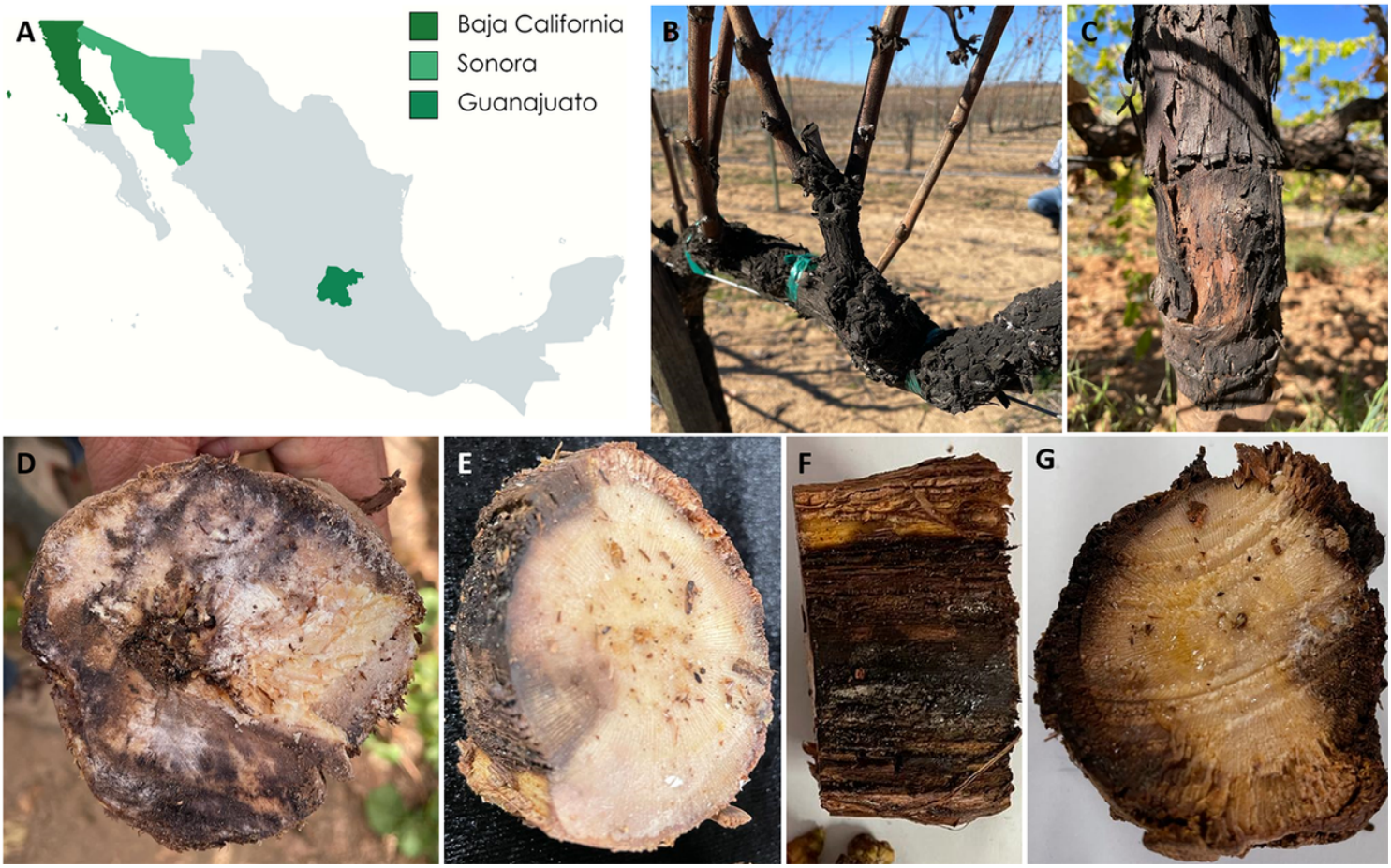

Figure 1

Symptomatic plants of Aspergillus vine canker observed in vineyards of Mexico. A) Sample sites in Baja California, Sonora, and Guanajuato vineyards. B-C) Grapevine plants exhibiting vascular necrosis, cankers, and wood necrosis. D-G) Different angles of woody tissue with blackened tissue.

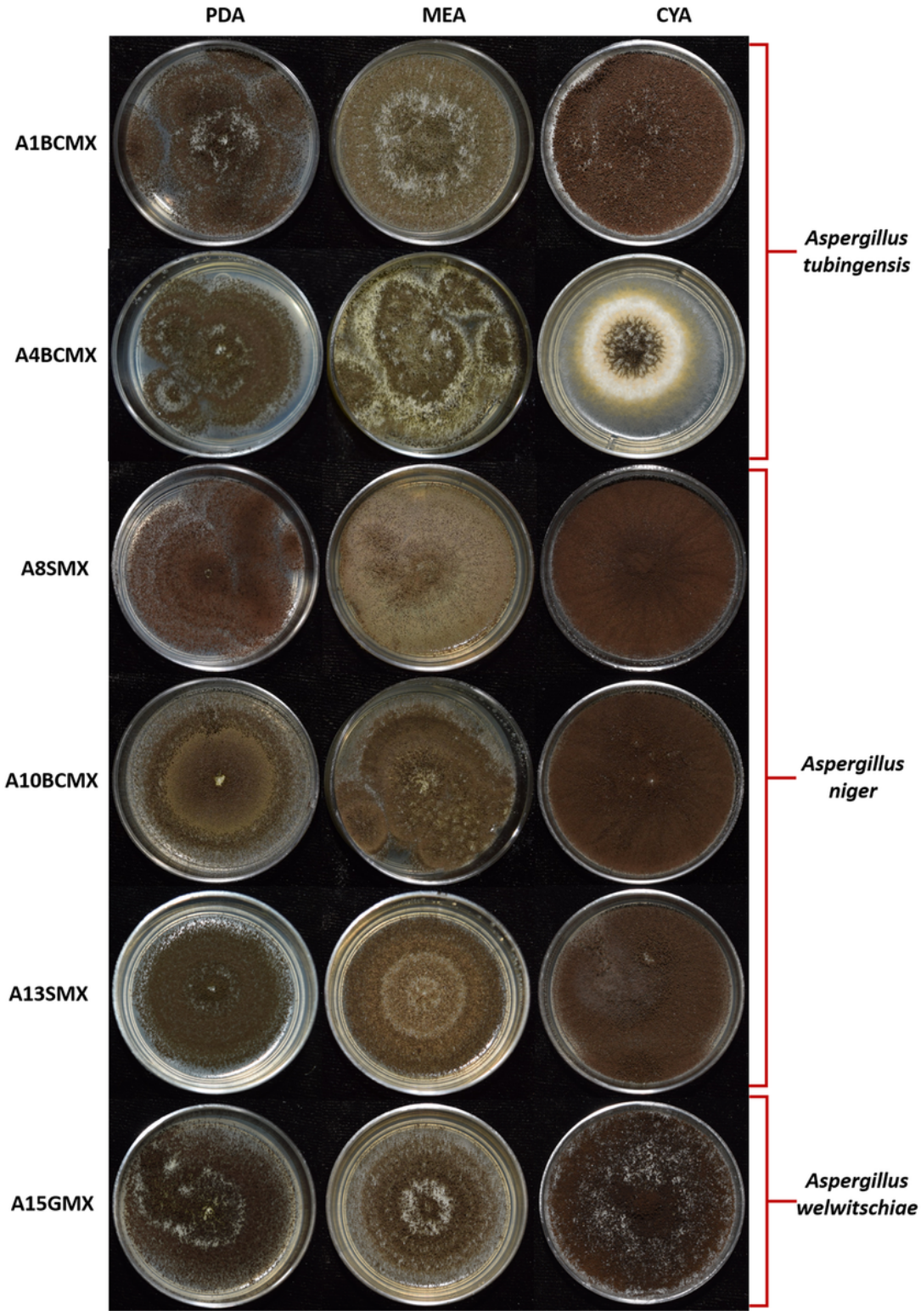

Figure 2

Colonies of *Aspergillus* isolates grown on different media at 30 ºC for 7 days in Potato Dextrose Agar (PDA), Czapek Yeast Autolysate Agar (CYA), and Malt Extract Agar (MEA).

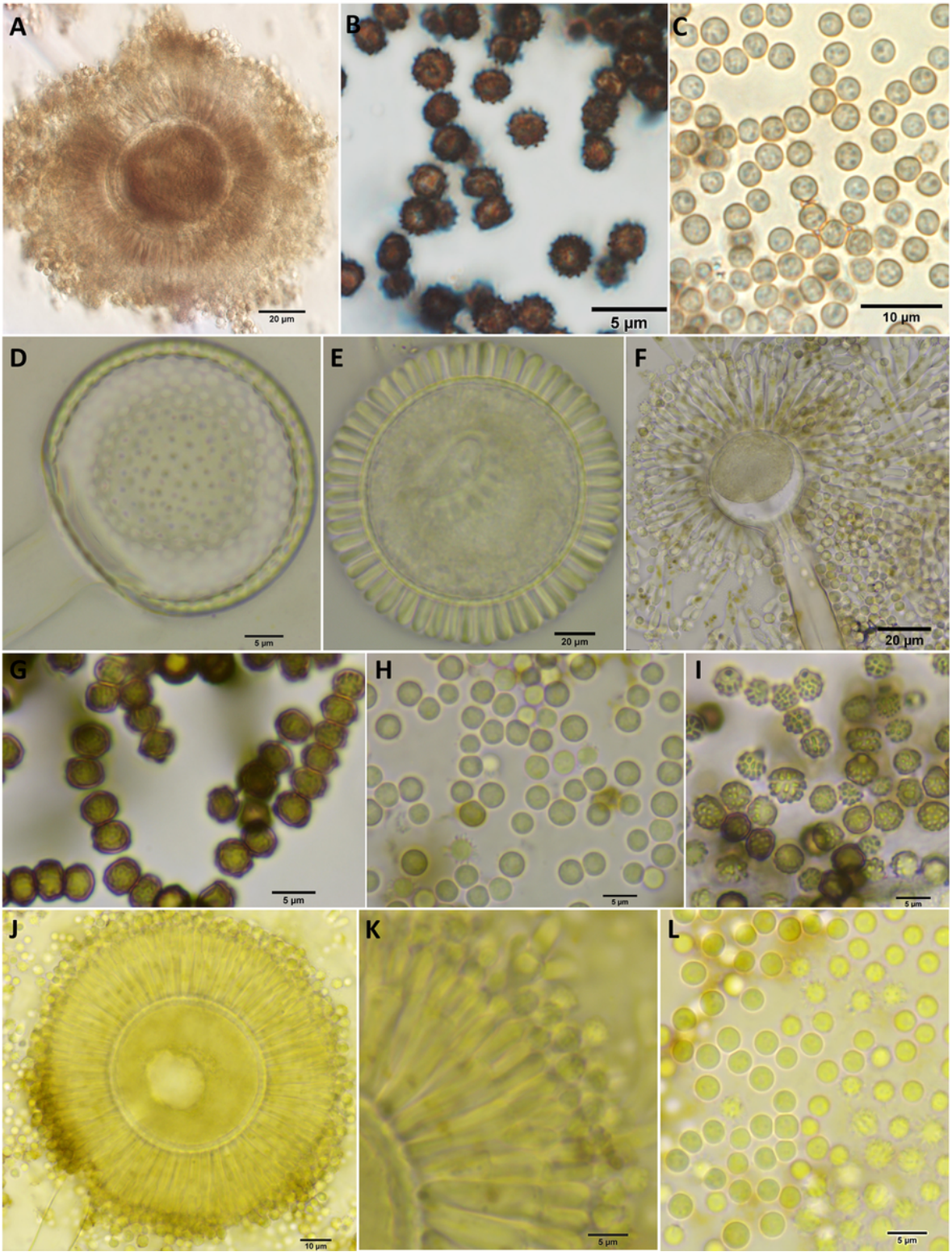

Figure 3

Microscopic characteristics of *Aspergillus*isolates grown on CYA at 30 ºC for 7 days. A-C) *Aspergillus tubingensis*. A) conidiophore, B) dark brown to black ornate conidial aggregates, C) smooth conidia. D-I) *Aspergillus niger*. D) conidial development, spherical vesicle, E) conidial development with metulae, F) conidiophore, G) dark brown to black conidial aggregates H) smooth conidia I) smooth and ornate

conidia. J-L) *Aspergillus welwitschiae*: J) conidiophore, K) metulae, phialides and conidia L) smooth and ornate conidia.

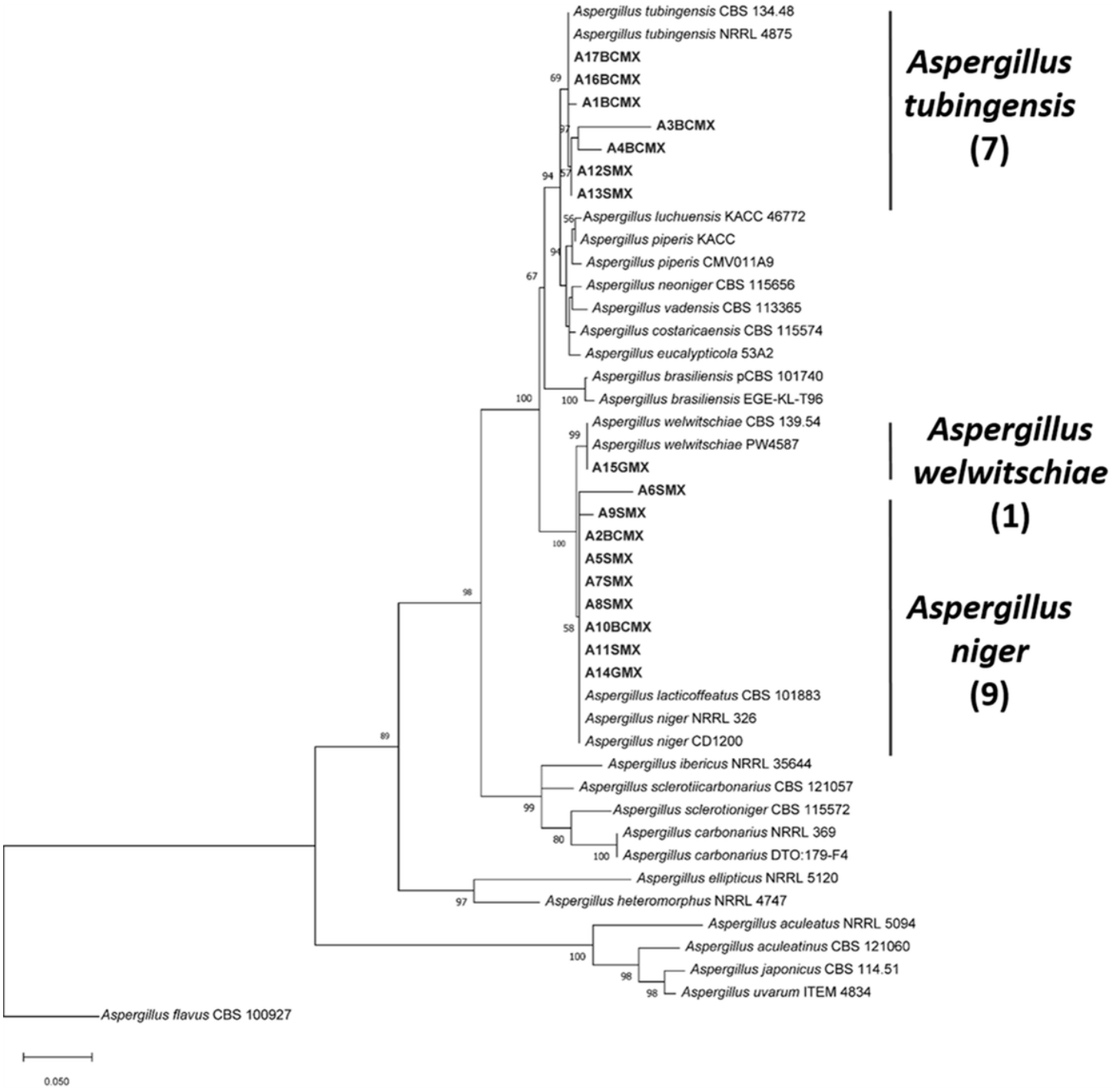

Figure 4

Phylogenetic analysis of *Aspergillus* spp. isolated from vineyards in Mexico. Maximum likelihood tree with the highest log likelihood (-3353.88) obtained from a concatenated dataset of *CaM* and *BenA* sequences. Bootstrap values from 1000 replicates greater than 50 are indicated at the node. The tree was rooted with *Aspergillus flavus* (CBS 100927). The isolates obtained in this study are highlighted in bold font. The number in brackets indicates the number of isolates obtained from each species.

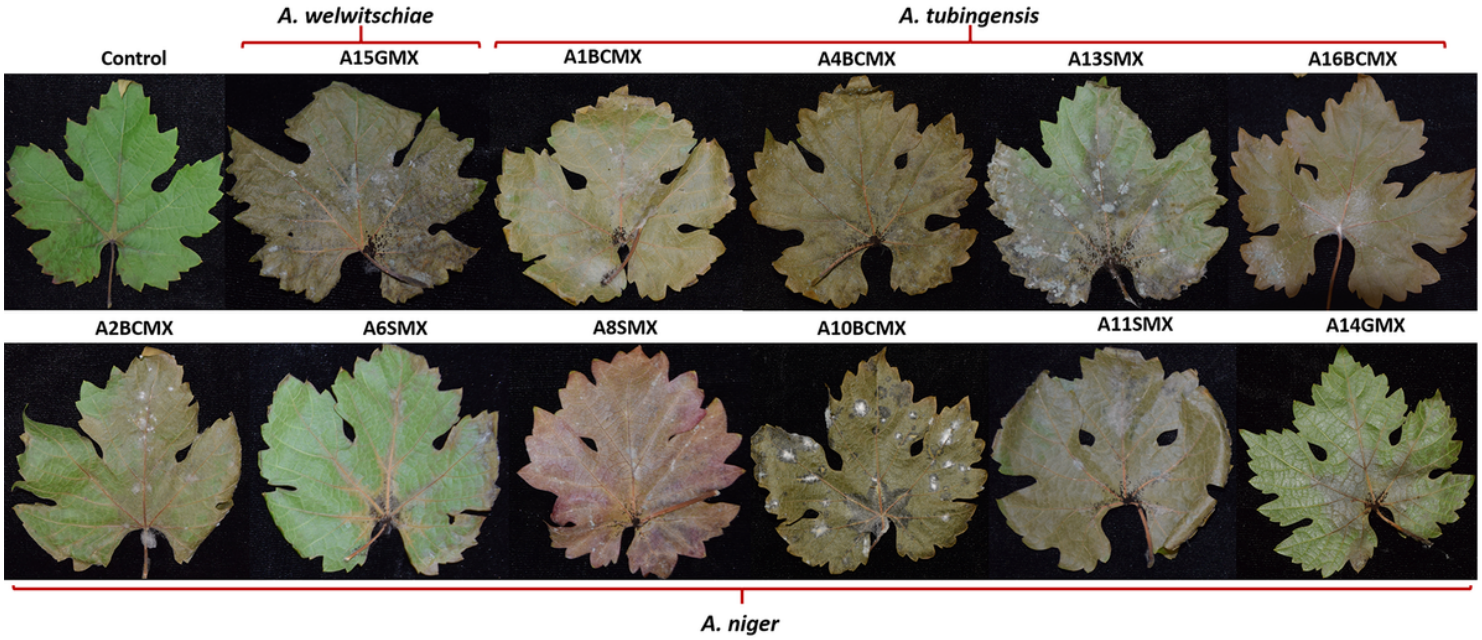

Figure 5

Pathogenicity test performed on 'Merlot' grapevine leaves by inoculating them with a suspension containing 1x10^6 conidia/mL of each *Aspergillus* isolate. The inoculated leaves were kept in a humid chamber at room temperature for 10 days. *Aspergillus niger* A10BCMX and *A. tubingensis* A13SMX induced necrotic lesions on the leaves and exhibited abundant mycelia and black conidia in the leaf tissue.

Figure 6

‘Merlot’ grapevine plants at 50 days-post-inoculation with *Aspergillus* isolates. Cross-section of the stem displaying necrotic lesions caused by the *Aspergillus*isolates, with the lesions observed in the xylem and phloem. B, length of necrotic lesions in woody tissue caused by Aspergillus isolates.