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Research Article

Isolation, Morphological Characterization, and Phylogenetic Identification of *Botrytis cinerea* Associated with Postharvest Gray Mold of Apple Fruits

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Abstract:

Apple (*Malus domestica* Borkh L., Family Rosaceae) is among the most popular fruits worldwide. Apple fruits are attacked by several fungal pathogens both pre- and post-harvest. The post-harvest fruit rot of apples is one of the most threatening diseases in apple cultivation. In the current study, we aimed to isolate, characterize, and identify the fungal phytopathogens associated with postharvest rot of apple fruits. Herein, we isolated eight genera from rotted Anna apple fruits during the 2018 season from orchards, wholesale, and retail markets as well as refrigerators. Then, the different fungal pathogens were identified according to their cultural and microscopic characteristics as *Penicillium expansum*, *Alternaria alternate*, *Botrytis cinerea*, *Aspergillus niger*, *Mucor* sp., *Rhizopus stolonifer*, *Stemphyllum* sp., and *Trichothecium roseum.* In this study, investigations were made into the pathogenicity tests of the highest-frequency fungi. Pathogenicity tests on apples (Anna cv.) revealed that *B. cinerea* was the highest virulent fungus. Additionally, a simple linear regression (SLR) analysis was carried out to better understand the relationship between the diameter of the infected area caused by post-harvest pathogens and storage time. Therefore, *B. cinerea* was identified by molecular testing using internal transcribed spacer (ITS) sequencing and phylogenetic analyses. Postharvest gray mold, caused by *Botrytis cinerea*, is one of the most important diseases and seriously affects apple fruit quality, resulting in huge economic losses. Proper measures should be adopted to protect apple fruits from fungal decay.

1. Introduction

Apple (*Malus domestic*) is the third most-produced fruit crop worldwide after bananas and watermelons (Calvo-Castro et al., 2022). It is one of the most reachable and widely consumed fruits (Calvo-Castro et al., 2022). The global production of apples reached 93144358.17 tons and the harvested area was 4822226 hectares in 2021 (FAOSTAT, 2021). Egypt cultivated 33942 hectares of apples, yielding a total production of 793.30 thousand tons. Apple fruits are attacked by numerous viral, bacterial, and fungal phytopathogens pre- and post-harvest that cause significant economic losses in apple production (Patriarca, 2019). More than 90 phytopathogenic fungi species have been reported to be associated with fruit decay and rots of apples during storage (Jones and Aldwinckle 1990; Sutton et al. 2014). Several factors affect the relative importance of each pathogen including climatic storage conditions, cultivar, and harvest time (Børve et al., 2010; Weber, 2011; Sever et al., 2012; Børve et al., 2013)

Pre-harvest diseases of apples including, but not limited to, brooks fruit spot caused by *Mycosphaerella pomi*, sooty blotch sooty blotch caused by *Gloeodespomigena* sp., fly speck caused by *Zygophiala jamaicensis* and *Mycrotheria* spp., core rot caused by *Alternaria alternata* and other *Alternaria* species such as *A. mali* and *A. tenuis*, and Phacidiopycnis fruit rot caused by *Phacidiopycnis washingtonensi* (Jones and Aldwinckle 1990; Sutton et al., 2014). On the other hand, Pre-harvest diseases of apples including, but not limited to, bitter rot (Anthracnose) caused by *Colletotrichum gloeosporiodes*, blossom-end and lenticel rots caused by *Neonectria galligena*, blue mold caused by *Penicillium expansum*, brown rot caused by *Monilinia fructigena*, bull's eye rot (aka Gloeosporium Rot) caused by *Pezicula malicorticis* or *Neofabraea* spp., gray mold caused by *Botrytis cinerea*, pink mould caused by *Trichothecium roseum*, rubbery rot caused by *Phacidiopycnis washingtonensis*, wet-core rot caused by *Fusarium* spp., whiskers rot caused by *Rhizopus arrhizus*, and rot due to other saprophytic fungi that occur occasionally in injured fruits during storage, such as *Rhizopus nigricans*, *Aspergillus niger*, *A. flavus*, and *Pestalotia* sp. (Jones and Aldwinckle 1990; Sutton et al., 2014).

Additionally, *Aspergillus niger*, *Botrytis cinerea*, *Penicillium expansum*, *Mucor* sp., *Lasiodiplodia* sp., *Rhizopus stolonifer*, *Fusarium* sp., and *Alternaria alternate* are some fungi that might damage apples postharvest (Pétriacq et al., 2018). Moreover, researchers have identified several apple-associated pathogens responsible for gray mold, including *Cadophora luteo-olivacea* (Sernaite et al., 2020). Another study isolated and classified fungi associated with spoilage of post-harvest apple fruits including *A. niger*, *P. expansum*, *A. alternate*,

Fusarium sp., and *Rhizopus stolonifer* (Amiri, 2020). Apple post-harvest fruit rot is a significant issue that affects the quality and shelf life of apples. The disease is caused by various fungi, including *Botryosphaeria dothidea*, which is among the most prevalent pathogens responsible for apple ring rot. The disease is characterized by the decay of fruit tissues, leading to significant economic losses during storage (Carneiro et al., 2022).

Furthermore, international trading activities and the changing climate pose enhanced risks of the establishment of new pathogens and the spread of emerging postharvest diseases. For example, climate change enhanced the spread of *Diplodia seriata* (Syn. *Botryosphaeria obtuse*), a causal agent associated with pre-harvest rot of apples in northern Germany (Weber and Quast 2009; Weber, 2009). Likewise, high air temperatures may favor the development of pathogens better known from warmer regions, such as *Glomerella acutata* (Weber and Quast, 2009). Moreover, *Phacidiopycnis washingtonensis* was reported as a causal agent of new storage rot of apples in northern Europe (Weber, 2011). Likewise, *Rhizopus oryzae* was also reported recently causing postharvest soft rot of apple fruit in Saudi Arabia (Al-Dhabaan, 2018) and China (Khokhar et al., 2019).

Gray mold is a common postharvest disease on apples and pears wherever the fruits are grown worldwide. This disease can cause significant losses in both apples and pears during storage with losses as high as 20-50% (Lambrese 2018; Amiri, 2020). Gray mold is mainly caused by the necrotrophic phytopathogen *Botrytis cinerea* Pers., (teleomorph *Botryotinia fuckeliana* (de Bary) Whetzel). Although other *B. mali* has also been reported (O'Gorman et al. 2008; Mikani et al., 2008). *B. cinerea* infects and develops on damaged and senescing tissues, causing tissue death while exuding toxins and enzymes involved in reactive oxygen species (Petrasch et al., 2019)**.** Symptoms of gray mold on apples include a decayed area with a light brown to dark brown appearance across which the color remains similar. The decayed fruit is spongy, and diseased tissue does not separate from the healthy tissue as dies blue mold (Lambrese 2018; Amiri, 2020). Early symptoms of gray mold can be confused with those of speck rot on apple and Phacidiopycnis rot on pear.

To isolate, characterize, and identify these fungal pathogens, various methods can be used, such as morphological observation**,** molecular analysis**,** pathogenicity tests**,** and biochemical assays (Wang et al., 2023). In the current study, we aimed to isolate and characterize the fungal phytopathogens associated with postharvest rot of apple fruits based on their morphological features and pathogenicity. Furthermore, we aimed to confirm the identification of the most aggressive isolate(s) using molecular phylogenetics. We believe that updating our knowledge about the fungal phytopathogens associated with postharvest rot of apple fruits might lead to the development of an effective means to control this serious disease, and consequently reduce its economic losses.

2. Materials and Methods

2.1. Sample collection

Diseased apples of the cultivar "Anna" showing rots and molds symptoms (disease incidence: 10-30%) were collected from orchards, wholesale, and retail markets as well as refrigerators from Basioun city, Gharbia Governorate (30.9399° N, 30.8176° E) and Sakha, Kafr El Sheikh Governorate (31.0894° N, 30.9444° E) during the 2018 season. The signs observed were brownish curd and whitish gray to dark mycelia with abundant conidia on the infected apple fruits (Figure 1A). The diseased specimens were stored on plastic box and kept at 3±1°C and 90% relative humidity inside the refrigerator till used for the isolation of the causal pathogens.

2.2. Pathogens isolation

To isolate the fungal pathogens associated with apple rot diseases, infected apple fruits were cut into small pieces, surface sterilized with 0.3% sodium hypochlorite (NaOCl) for three min, then washed several times with sterile distilled water, and dried between sterilized filter papers to remove the extra water. Subsequently, small sterilized pieces (from the intermediate tissues between healthy and rotted ones) were directly cultured on Petri dishes containing 15 ml potato dextrose agar (PDA) medium supplemented with 25 $mg.mL^{-1}$ Ampicillin and then incubated at 25±2°C for 7 days and observed for fungal growth. For pure cultures, hyphal tips method (Brown, 1924) or single-spore cultures (Hansen, 1926) were used based on the pathogen's colony features. Purified cultures were transferred to fresh PDA medium and incubated at 25±2°C for 7 days, then identified at the Department of Agricultural Botany, Faculty of Agriculture Kafr El-sheik University (Barnett and Hunter, 1972).

2.3. Morphological characterization of fungal pathogens associated with rotted apple fruits

The developed purified fungal cultures were firstly identified based on their cultural morphology characteristics (size, form, elevation, margin/border, surface, opacity, color, and texture) of each individual colony of fungi growing on PDA in a Petri dish. Briefly, a mycelial plug (approximately 5 mm in diameter) was collected from areas of active growth near the edges of 5-days-old cultures, transferred to PDA, and incubated at 25±2°C in the dark for 5 days, then the diameters of developed colonies were measured and recorded using the criss-cross method (Tao et al., 2011) and other colony features were assessed visually.

Subsequently, to confirm the identification of these isolates, all isolated fungal pathogens were microscopically examined and identified based on the morphological features of their mycelia, conidiophores, conidia (size, shape, color, and septation), and other noticed structures (Barnett and Hunter, 1972). Briefly, for detailed microscopic examination, sporulating mycelium was mounted in 20% lactophenol on glass slides and examined using light microscope.

2.4. Pathogenicity test

Healthy mature fruits of apple cultivar "Anna" were selected, washed by dipping in tap water for 10 min, and then dried at room temperature during the 2018 season. The fruits were surface sterilized in 0.3 % sodium hypochlorite for three minutes and then washed several times in sterilized distilled water. Since *Penicillium expansum*, *Alternaria alternate*, and *Botrytis cinerea* were the highest frequency fungi isolated from the fruits, pathogenicity tests were performed by inoculating a 5-mm fungal plug into a hole (5 mm diameter and 4 mm deep) made into the fruit skin using a cork borer. The fungal inoculum was taken from the growing margin of 7 days old PDA cultures of the tested fungi. Each treatment consisted of three replicates with four fruits/replicate. Check treatment was similarly treated but without fungal inoculation. The inoculated fruits were placed in opened polyethylene bags and stored at 25±2 °C and 65% relative humidity (RH). Disease development of infected fruit was estimated as the diameter of the external rot for each pathogen (Moline and Locke, 1993). As *Botrytis cinerea* was the highest virulent fungus, it was selected for further studies.

2.5. Molecular identification of *B. cinerea*

To confirm the identification of the most aggressive isolate (*B. cinerea).* Briefly, this isolate was grown on a sterilized PDA and incubated at 25 ± 2 ◦C for 5 days. Subsequently, the mycelium was collected and filtered using cheesecloth, washed twice with sterilized deionized water, and dried using filter paper. Approximately 0.1 g of the mycelium was ground to a fine powder using liquid nitrogen. The total DNA of the pathogenic fungus was extracted using a Quick-DNA ™ fungal miniprep kit according to the manufacturer's instructions and then purified, and the targeted sequences of the ITS region (ITS-5.8SrDNA) were amplified using PCR. The purified PCR products were sent for sequencing (Aoke Dingsheng Biotechnology Co., Beijing, China). Sanger sequencing was used to perform the two-directional sequencing of the ITS-5.8S rDNA sequences. DNABASER software (Heracle BioSoft S.R.L., Arges, Romania) was used to process and assemble consensus sequences. Subsequently, a Nucleotide-Nucleotide Basic Local Alignment Search Tool (BLASTn) was used to compare the assembled sequence with the most recent available data in GenBank and the national center for biotechnology information website (NCBI, http://www.ncbi.nlm.nih.gov/gene/; accessed on 28 October 2023).

2.6. Evolutionary analysis and phylogenetic tree of *B. cinerea*

The evolutionary history was inferred by using the Maximum Likelihood method and Tamura-Nei model (Tamura and Nei, 1993). The tree with the highest log likelihood (-15119.71) is generated. Initial tree for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Tamura-Nei model, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. In addition to the sequence of *Botrytis cinerea* – isolate AYA2020 large subunit ribosomal RNA gene (GenBank Accession No. OR730908; 577 bp), the evolutionary analysis involved another 20 nucleotide sequences retrieved from the most recent available data in NCBI GenBank (Table 1). Codon positions included were $1^{st}+2^{nd}+3^{rd}$+Noncoding. There was a total of 899 positions in the final dataset. Evolutionary analyses were conducted in MEGA11 (Tamura et al., 2021).

2.7. Statistical analysis

Data were statistically analyzed according to the analysis of variance technique (ANOVA) followed by post hoc pairwise comparisons using the Tukey honestly significant difference test were used to compare variances between means of different treatments (HSD; $p \leq 0.05$). Moreover, a simple linear regression (SLR) analysis was carried out to better understand the correlation between the diameter of the infected area caused by *B. cinerea* and time.

3. Results

3.1. Fungal genera associated with postharvest gray mold of apple fruits

Generally, eight fungal genera were isolated from rotted apple fruits (Figure 1B), which were collected from local markets and commercial farms at different locations. Based on their cultural morphology (Table 2) and microscopic characteristics (Table 3), they identified as *Alternaria alternate*, *Aspergillus niger*, *Botrtis cinerea*, *Mucor* sp., *Penicillium expansum*, *Rhizopus stolonifer*, *Stemphyllum* sp., and *Trichothecium roseum*. It worth mentioning that *P. expansum* was the most dominant pathogen with frequency up to 28 %, followed by *A. alternate* (24 %), *B. cinerea* (19 %) and *A. niger* (9%), whereas the frequency of remaining fungal genera did not exceed 5 % for each pathogen (Figure 1B).

3.1.1. Cultural morphology characteristics

Generally, cultural morphological observations showed that most of the examined cultures produced filamentous, flat, filiform at the border, with smooth surface, fluffy, dark, or translucent colonies when grown on PDA media at 25±2°C in the dark for 5 days (Table 2) with slight differences between isolated fungal genera. The macroscopic features of the isolated genera were as follows:

Table 1. Sequences from different fungal genera that produce significant Alignment with large subunit ribosomal RNA (26S) of internal transcribed spacer (ITS) region from *Botrytis cinerea* [a,b]

Description	Max Score	Total Score	Query Cover (%)	E va-lue	Identity (%)	Accession Length (bp)	Accession
Botrytis cinerea isolate 9 large subunit ribosomal RNA gene, partial sequence	1027	1027	94	0.0	99.82	560	MW550268.1
Botryotinia fuckeliana strain 3318 28S ribosomal RNA gene, partial sequence	1040	1040	95	0.0	100.00	574	DQ666677.1
Botrytis sp. strain BMXTX3 large subunit ribosomal RNA gene, partial sequence	1037	1037	97	0.0	99.13	581	OQ061470.1
Botrytis sp. strain BMXTX2 large subunit ribosomal RNA gene, partial sequence	1037	1037	97	0.0	99.13	581	OQ061469.1
Botrytis cinerea strain SQ02-3-2 large subunit ribosomal RNA gene, partial sequence	1048	1048	96	0.0	99.82	590	MN561689.1
Botrytis cinerea strain LEst01 28S ribosomal RNA gene, partial sequence	1053	1053	97	0.0	99.83	591	KC292909.1
Botryotinia fuckeliana 28S ribosomal RNA gene, partial sequence	1053	1053	97	0.0	99.83	591	AF250919.1
Botrytis cinerea strain SQ01-501 large subunit ribosomal RNA gene, partial sequence	1048	1048	96	0.0	99.82	593	MN561690.1
Botrytis caroliniana strain ATCC MYA-4858 28S ribosomal RNA gene, partial sequence	1033	1033	95	0.0	99.82	593	KC171323.1
Botrytis cinerea strain ATCC 11542 28S large subunit ribosomal RNA gene, partial sequence	1035	1035	95	0.0	99.82	594	KU729179.1
Botrytis cinerea strain SQ02-3-1 large subunit ribosomal RNA gene, partial sequence	1053	1053	97	0.0	99.83	595	MN561691.1
Botrytis cinerea strain ATCC 90480 26S ribosomal RNA gene, partial sequence	1035	1035	95	0.0	99.82	595	KP780471.1
Botrytis cinerea isolate HP128 28S ribosomal RNA gene, partial sequence	1031	1031	95	0.0	99.65	597	KT323330.1
Sclerotinia sclerotiorum strain FZ001 large subunit ribosomal RNA gene, partial sequence	1011	1011	97	0.0	98.43	613	EU926159.1
Botrytis sp. LH-2021a isolate XJAU-HC257-1 large subunit ribosomal RNA gene, partial sequence	1053	1053	97	0.0	99.65	619	OK490508.1
Sclerotinia sclerotiorum gene for 28S ribosomal RNA, partial sequence	1009	1009	97	0.0	98.43	874	LC429380.1
Botrytis aclada culture CBS:103.23 strain CBS 103.23 large subunit ribosomal RNA gene, partial sequence	1053	1053	97	0.0	99.83	884	MH866244.1
Botrytis paeoniae culture CBS:132.53 strain CBS 132.53 large subunit ribosomal RNA gene, partial sequence	1053	1053	97	0.0	99.83	885	MH868667.1
Botrytis cinerea strain GLMC 635 large subunit ribosomal RNA gene, partial sequence	1053	1053	97	0.0	99.83	886	MT156168.1
Botrytis cinerea isolate SICAUCC 19-0002 large subunit ribosomal RNA gene, partial sequence	1053	1053	97	0.0	99.83	899	MN148531.1

a The listed fungal genera were identified using the nucleotide-nucleotide BLAST (BLASTn) using *Botrytis cinerea* isolate AYA-2020 (GenBank accession no. OR730908; 577 bp) as a query sequence against available data in GenBank, national center for biotechnology information website (NCBI, http://www.ncbi.nlm.nih.gov/gene/).

b listed fungal genera were used to generate the phylogenetic tree presented in Figure 3.

Table 2. Cultural morphology characteristics of fungal phytopathogens associated with post-harvest rot and mold diseases of apple fruits [a].

Pathogen	Cultural morphology characteristics							
	Size [b] (cm)	Form	Elevation	Margin	Surface	Opacity	Color	Texture
Alternaria alternate	6.5	Irregular	Raised	Undulate	Lobate	Dark	White turns dark brownish	Fluffy
Aspergillus niger	8.4	Filamentous	Flat	Filiform	Smooth	Dark	White turns black	Velvety
Botrytis cinerea	8.5	Irregular	Flat	Undulate	Smooth	Translucent	White turns dark brownish or gray	Fluffy
Mucor sp.	8.8	Filamentous	Flat	Filiform	Smooth	Dark	White turns black later.	Cottony to fluffy
Penicillium expansum	7.9	Filamentous	Flat	Filiform	Smooth	Dark	Blue to greenish olive	Fluffy
Rhizopus stolonifer	8.7	Rhizoid	Flat	Filiform	Cottony	Dark	White turns dark gray or black	Fluffy
Stemphyllum sp.	6.8	Filamentous	Flat	Filiform	Rough	Translucent	Light-brown to olivaceous-black	Fluffy
Trichothecium roseum	6.1	Circular	Umbonate	Undulate	Flat and granular	Opaque	Initially white, turns light pink or orange	Powdery

[a] Colonies grown on PDA at 20±2°C in the dark for 5 days.

[b] Diameter of developed colonies using the criss-cross method (Tao et al. 2011).

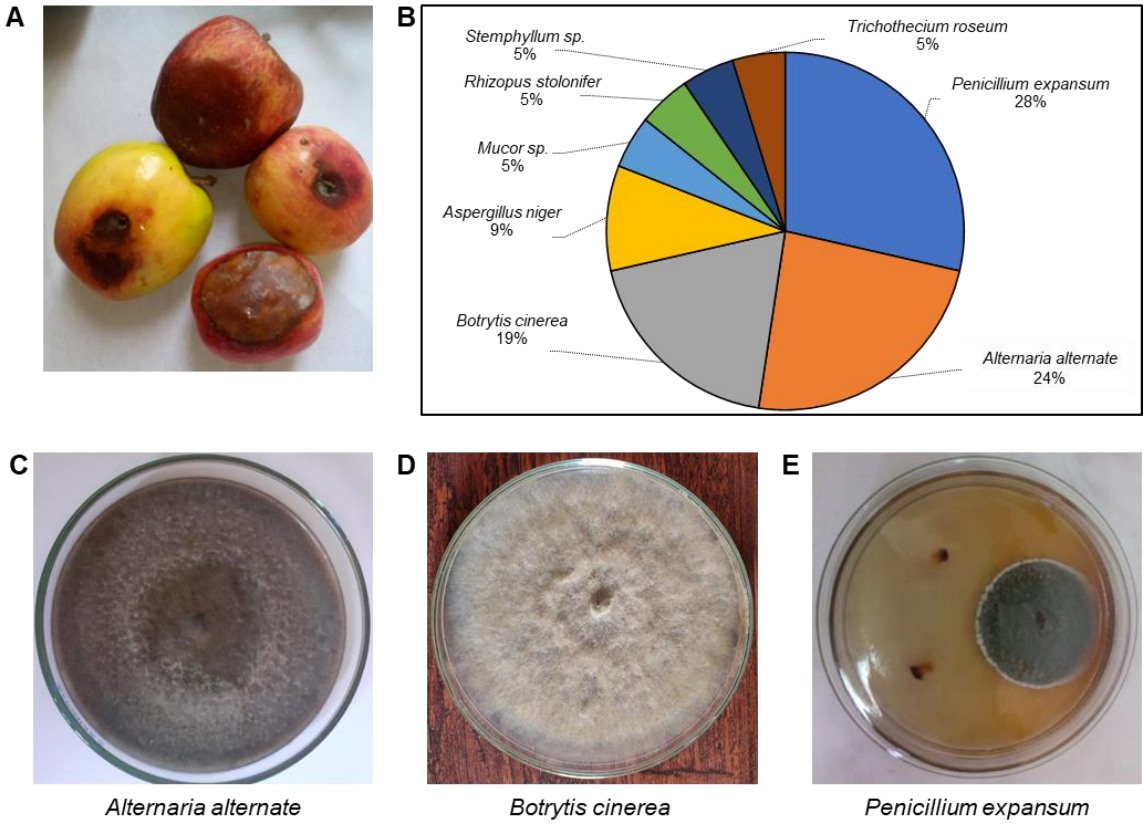

Figure 1. Isolation and identification of the pathogens associated with rotted apple fruits. (A) Diseased apples of the cultivar "Anna", showing rots and molds symptoms (disease incidence: 10-30%), collected from orchards, wholesale, and retail markets as well as commercial refrigerators. (B) Percentage of the fungal genera isolated and identified from rotten apple fruits collected from local markets and commercial farms. (C, D, and E) Cultural morphology of, *Alternaria alternate*, *Botrytis cinerea*, an. *Penicillium expansum*, respectively.

- *Alternaria alternate* produces an irregular (about 6.5 cm diameter), raised, undulated, lobate, dark, white turns into dark brownish, and fluffy colonies (Figure 1C).

- *Aspergillus niger* produces a filamentous (about 8.4 cm diameter), flat, filiform, smooth, dark, and velvety colonies which were initially white but rapidly changed to black after producing conidial spore.

- *Botrytis cinerea* forms an irregular (approximately 8.5-cm diameter), flat, undulate, smooth, translucent, fluffy colony that is white colored and turns into dark brownish or gray rapidly (Figure 1D).

- *Mucor* sp. has fast growing colonies that were filamentous (about 8.8 cm diameter), flat, filiform, smooth, dark, cottony to fluffy colony. From the front, the color is white initially and becomes grayish brown rapidly. From the reverse, it is whitish.

- *Penicillium expansum* produces a filamentous (about 7.9 cm diameter), flat, filiform, smooth, dark, and fluffy colonies that are greenish olive colored (Figure 1E).

- *Rhizopus stolonifer* produces a rhizoid (about 8.7 cm diameter), flat, filiform, cottony, dark, and fluffy colony that is white turns dark gray or black at the end.

- *Stemphyllum* sp. forms a filamentous (approximately 6.8-cm diameter), flat, filiform, rough, translucent, fluffy colony that is light-brown to olivaceous-black colored.

- *Trichothecium roseum* produces a 6.1-cm circular, umbonate, undulate, flat, and granular, opaque powdery colonies that are initially white and turn light pink or orange later.

3.1.2. Microscopic characteristics

In general, microscopic examination of eight isolated fungal genera showed that they are producing a septate mycelium, simple (nonseptate) or septate conidiophores, and form conidia as asexual reproductive phase which is typically like ascomycetes (Table 3). However, the other two fungal genera produce nonseptate or rarely septate mycelium, simple (nonseptate) sporangiophore that ends with sporangia which is typically like zygomycetes. The microscopic characteristics of fungal genera associated with postharvest gray mold of apple fruits as follows:

- *Alternaria alternate* produced profuse mycelial growth on PDA. Initially, the mycelium was hyaline then turned gray-green to brown dark, multi-celled, septate,

and irregularly branched. The conidiophore was pale olivaceous to olivaceous- brown, septate, arisen singly or in clusters, occasionally producing a zigzag appearance, and slightly swollen at the apex having terminal scars indicating the point of attachment of conidia. The conidia were born individually or in chains up to 10 or more on conidiophore. The conidia were olivaceous to dark brown colored, club-shaped with a beak, and multicellular with transverse and longitudinal septa (Table 3). The sexual stage was not observed.

- *Aspergillus niger* produces hyaline or smooth colored, septate hyphae. The conidiophores are protrusions from the hyphae and are also hyaline, but simple (nonseptate), and unbranched. The conidiophore vesicle produces sterile cells known as metulae which support the phialides on the conidiophores. The phialides produce globose conidia that have a rough texture, are dark brown to black colored, are single-celled, and are formed in chains (Table 3). The conidial heads appear radial. The sexual stage was not observed.

- *Botrytis cinerea* produced hyaline or light gray, septate hyphae on PDA. The conidiophores were also hyaline or light grey, septate, and erect tree-like branched at the apex. Conidia were hyaline or brightly colored, large oval or spherical, single-celled, are produced at the tips of erect conidiophores in head-like formation and attached singly on sterigmata (Table 3). Moreover, tiny sclerotia were observed on 30-day-old colonies as rings along the edge of the Petri dish. The sexual stage was not observed.

- *Mucor* sp. formed hyaline or light gray, coenocytic (nonseptate), or rarely septate hyphae, but neither stolons nor rhizoids were observed. Sporangiophores were hyaline or light grey, short, erect, simple (nonseptate) or rarely branched, and without basal rhizoids, that end with large, globose to spherical, multispored sporangia. Sporangia were hyaline, gray to black, globose to ellipsoidal, filled with sporangiospores which were round or slightly elongated and smooth-walled (Table 3). Columella are hyaline or dematiaceous and are hardly visible. The sexual stage was not observed.

- *Penicillium expansum* forms a hyaline, septate, smooth, or rough-walled hyphae and conidiophores. Conidiophores were mono-verticillate branched to several metulae that also branched into tightly packed phialides. Phialides are usually flask-shaped and carry chains of single-celled conidia. Conidia were ellipsoid or pyriform, white to beige (Table 3). The microscopic structure of metulae, phialides, and conidia form a broom-like or brush-like appearance. The sexual stage was not observed.

- *Rhizopus stolonifer* grows as filamentous, hyaline or light grey, branched, coenocytic (nonseptate), or rarely septate hyphae. This genus was characterized by the presence of stolons and brownish root-like rhizoids. It formed hyaline or light grey, smooth-walled, nonseptate, simple (unbranched) sporangiophores individually or in groups (2-3 sporangiophores) from nodes directly opposite the rhizoids. Sporangia are globose, spherical with a flattened base, gray to black, and supported by a large apophysate columella atop a long stalk (sporangiophores). Sporangiospores were single-celled, angular, subglobose to ellipsoidal forms inside sporangia (Table 3). The sexual stage was not observed.

- *Stemphyllum* sp. produced a pale brown to brown, septate hyphae. Conidiophores were also pale brown to brown, septate, dematiaceous, simple, or branched. Conidiophores have some vesicular swellings or nodes. Conidia are solitary, oblong, or subspherical, rounded at the

Table 3. Microscopic characteristics of fungal phytopathogens associated with post-harvest rot and mold diseases of apple fruits [a].

Pathogen	Mycelium		Conidiophore/Sporangiophore			Spores			Other structure
	Color	Septation	Color	Septation	Branching	Shape	Color	Septation	
Alternaria alternate	Gray-green to brown dark	Septate	pale olivaceous to olivaceous-brown	Septate	Occasionally producing a zigzag appearance	Club-shape with a beak	Olivaceous to dark brown	Multicellular with transverse and longitudinal septations	No
Aspergillus niger	Hyaline	Septate	Hyaline	simple (non-septate)	Unbranched	Globose	Dark brown to black	Single-celled forms in chains	No
Botrytis cinerea	Hyaline or Light grey	Septate	Hyaline or Light grey	Septate	Erect tree-like branched	Oval or spherical	Hyaline	Single-celled in head-like formation	Tiny sclerotia
Mucor sp.	Hyaline or Light grey	Unsepted or rarely septated	Hyaline or Light grey	Simple (Nonseptate)	Unbranched	Round	Gray to black	Single-celled forms inside sporangia	Sub-terranean hyphae but no rhizoids
Penicillium expansum	Hyaline	Septate	Hyaline	Septate	Mono-verticillate branched	Ellipsoid or pyriform	White to beige	Single-celled forms in chains	Broom-like
Rhizopus stolonifer	Hyaline or Light brown	Unsepted or rarely septated	Hyaline or Light grey	Simple (Nonseptate)	Unbranched	Angular, subglobose to ellipsoidal	Gray to black	Single-celled forms inside sporangia	Rhizoids
Stemphyllum sp.	Pale brown to brown	Septate	Pale brown to brown	Septate	Simple or branched	Oblong or subspherical, rounded at the tips	Pale to mid-brown	Multicellular with transverse and longitudinal septations	Vesicular swellings or nodes
Trichothecium roseum	Hyaline	Septate	Hyaline	Septate near the base	Erect, unbranched	Ellipsoidal to pyriform	Hyaline, and thick-walled	Young conidia are aseptate but mature ones are double-celled	Zigzag patterned chained conidia

[a] Colonies grown on PDA at 20±2°C in the dark for 5 days. for detailed microscopic examination, sporulating mycelium was mounted in 20% lactophenol on glass slides and examined using light microscope.

tips, pale to mid-brown, rough- or smooth-walled, and multicellular with transverse and longitudinal septa with a typical constriction at the central septum (Table 3). The sexual stage was not observed.

- *Trichothecium roseum* formed hyaline and septate hyphae, conidiophores, and conidia. Conidiophores were long, septate near the base, erect, unbranched, arise individually or in loose groups, and bear clusters of zigzag patterned chained conidia at the tip. Conidia were ellipsoidal to pyriform, hyaline to lightly colored, smooth, and slightly thick-walled, young conidia are aseptate, but mature ones are double-celled with the apical cell being larger than the curved basal cell (Table 3). The sexual stage was not observed.

3.2. The most aggressive post-harvest pathogen on apple fruit

The pathogenicity of the three highest frequency (most abundant) fungal genera including, *P. expansum*, *A. alternate*, and *B. cinerea* were tested on healthy mature fruits of the susceptible apple cultivar "Anna". Although all *P. expansum*, *A. alternate*, and *B. cinerea* were pathogenic on apple fruits (Figure 2A), *B. cinerea* was the most aggressive pathogen (Figure 2A and 2B). It is worth mentioning that the diameter of the infected area caused by *B. cinerea* was significantly higher than those caused by the other two pathogens (*P. expansum* and *A. alternate*) started from 4 days post-inoculation (dpi) and progressively increased to reach its highest peak (20 cm) at 12 dpi. Furthermore, to better understand the relationship between the diameter of the infected area caused by post-harvest pathogens and storage time, a simple linear regression (SLR) analysis was carried out (Figure 2C). SLR showed that there was a strong positive correlation between the diameter of the infected area caused by *B. cinerea* and time ($y = 1.7708x - 2.2348$, $R^2 = 0.9505$; Figures 2B and 2C).

3.3. Molecular identification of *B. cinerea*

B. cinerea isolate was found to be the most virulent on apple fruits in pathogenicity tests. This isolate was initially identified according to its cultural, morphological, and microscopic characteristics. As mentioned above, *B. cinerea* isolate produced dense fluffy cottony white growth and that were covered by small round or irregular-shaped dark brown sclerotia. From the front, the color is white initially and turns dark brownish or gray rapidly (Figure 3A). From the reverse, it is whitish (Figure 3B) when grown on a PDA after 5 days post incubation at 25 ±2 ºC. Moreover, it produces hyaline hyphae and conidiophores which were septate, and erect tree-like branched at the apex (Figure 3C). Collectively, the pathological and morphological characteristics proposed that the isolated fungus was *B. cinerea*, the causal agent of gray mold disease of apples.

Moreover, the identification of the most aggressive isolate, *B. cinerea*, was further confirmed based on the sequence of large subunit ribosomal RNA of the internal transcribed spacer (ITS) region (Figure 3D). Briefly, the query sequence showed a high similarity with the large subunit ribosomal RNA gene of *B. cinerea* -strain GLMC 635 (GenBank Accession No. MT1568.1; Figure 3D). The new sequence was deposited in the NCBI database and under the name *Botrytis cinerea* – isolate AYA2020 large subunit ribosomal RNA gene (GenBank Accession No. OR730908; 577 pb).

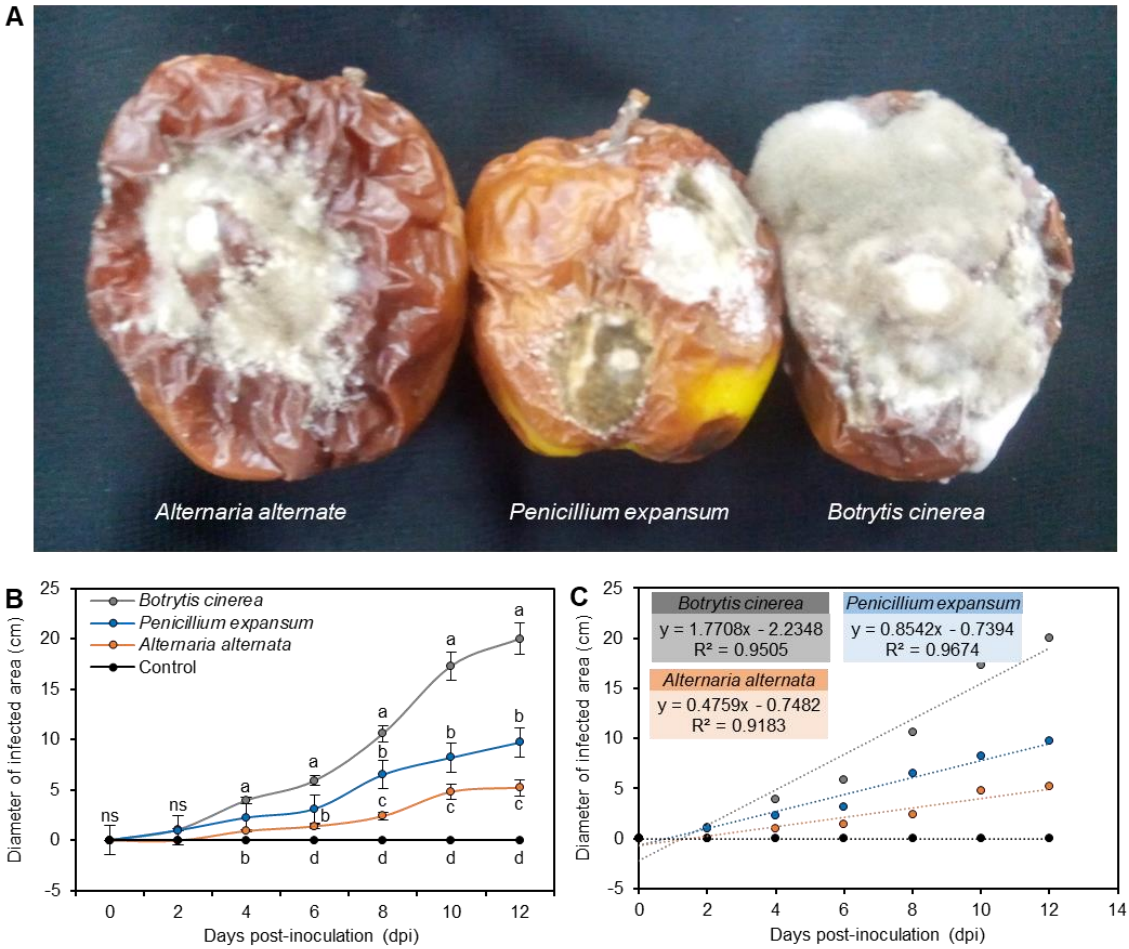

Figure 2. Pathogenicity of the most dominant fungal genera associated with postharvest rot and mold of apple on healthy mature fruits of the susceptible apple cultivar "Anna". (A) Pathogenicity of *Alternaria alternate*, *Penicillium expansum*, and *Botrytis cinerea*, on apple fruits stored at 25±2 °C and 65% relative humidity for 12 days post-inoculation (dpi). **(B)** Development of postharvest rot and mold of apple after inoculation with *A. alternate*, *P. expansum*, and *B. cinerea* and storage for 12 dpi at 25±2 °C and 65% relative humidity. Round dots denote the means, whereas the whiskers donate the standard deviations (means ± SD). Different letters signify statistically significant differences among isolates using Tukey's Honestly Significant Difference (HSD) Test ($p < 0.05$). **(C)** Simple linear regression between the diameter of the infected area (cm) and time post-inoculation (days) of the most dominant fungal genera (C).

4. Discussion

Fungal postharvest rot of apple fruits is a common problem that causes significant losses and reduces the quality of the fruits. Post-harvest losses reached 10% to 40% of all agricultural product losses worldwide according to assessments (Enyiukwu et al., 2014). In the current study, eight genera were found to be associated with the postharvest decay of apple fruit, including *Penicillium expansum*, *Alternaria alternate*, *Botrtis cinerea*, *Aspergillus niger*, *Mucor* sp., *Rhizopus stolonifer*, *Stemphyllum* sp., and *Trichothecium roseum.* In agreement with these findings, *B. cinerea*, *P. expansum*, *Mucor* sp., *A. alternate*, *Colletotrichum* sp., *Lasiodiplodia* sp., *R. stolonifer*, *Fusarium* sp., and *A. niger* were reported previously to cause postharvest damage to apple fruits (Pétriacq et al., 2018). In addition to pervious study, some of the fungal pathogens that cause postharvest rot of apple fruits are *Botryosphaeria dothidea*, *P. expansum*, *Colletotrichum gloeosporioides***,** and *Monilinia fructicola* (Wang et al., 2023). Some previous studies found and identified numerous postharvest pathogens which can cause the decay of apple fruits and belong to the genera of bull's eye rot (*Neofabraea* spp.), blue mold (*P. expansum*), brown rot (*Monilinia* spp.), *Alternaria* spp. and gray mold (*B. cinerea*) predominated (Głos et al., 2022). Collectively, the studies showed that the commonly fungal species isolated from the infected apple fruits at all stages were identified as follows *B. cinerea*, *P. expansum*, *A. Alternate* and *Monilinia* spp. and they were considered as the main pathogens with the highest pathogenicity and virulence on apple fruits.

Previous study isolated and identified the fungal pathogen causing strawberry fruits rot as *B. cinerea* through classical fungal taxonomy and molecular characterization based on their internal transcribed spacer (ITS) region of ribosomal DNA (rDNA) (Sultana et al., 2020).

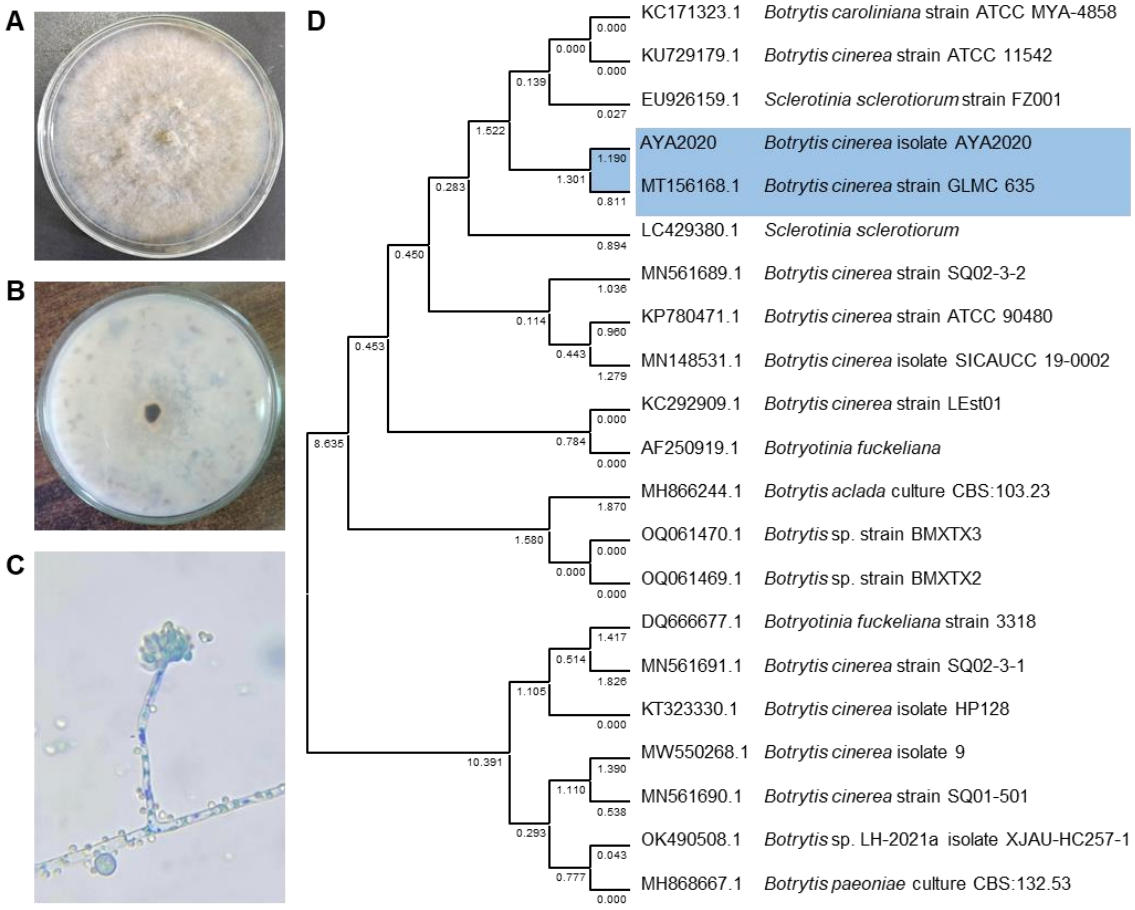

Figure 3. Evolutionary analysis and phylogenetic tree of *B. cinerea*. **(A and B)** The growth and morphological characteristics of the phytopathogenic fungus *B. cinerea* on potato dextrose agar (PDA) media from the top and the bottom of the Petri dish, respectively, after 5 days of incubation at 25±2°C. **(C)** Microscopic characteristics of *B. cinerea*, the causal agent of gray mold disease of apples.
(D) The evolutionary analysis with the Maximum Likelihood method and Tamura-Nei model using *Botrytis cinerea* – isolate AYA2020 large subunit ribosomal RNA gene (GenBank Accession No. OR730908; 577 pb) in comparison with 20 reference strains/isolates retrieved from the recent available data in NCBI GenBank (https://www.ncbi.nlm.nih.gov/).

Our results confirm that *B. cinerea* was the most aggressive post-harvest pathogen on apple fruits. A similar study demonstrated that *B. cinerea* is consistent most common postharvest disease of apple fruits, which is the most aggressive post-harvest pathogen on apple fruits (Mahmoud et al., 2019). Additionally, (Kim and Xiao, 2008) reported that gray mold accounted for 28% of the decayed apple fruit in commercial storage. It was previously reported that the most prevalent postharvest pathogen of apples is a gray mold fungus called *B. cinerea* (Sun et al., 2019).
Botrydial and botcinic acid, the two phytotoxins that *B. cinerea* produces, have been exposed to be necessary for full virulence (Breen et al., 2022). Remarkably, this isolate was much more aggressive on apple fruits than other genera therefore, we chose to use the PCR molecular method to confirm the identification of the isolate. This isolate belonged to the genus *B. cinerea* and shared 100% of its genetic makeup with *B. cinerea*, according to internal transcribed spacer (ITS) analysis. The authors suggested that the isolation and identification of these fungi could help develop effective strategies for controlling post-harvest fruit rot in apples (Amiri, 2020).

Conclusion

Infected apple fruits were collected from local markets and commercial farms at different locations during the 2018 season that showed brownish curd and whitish gray to dark mycelia with abundant conidia on the infected fruits. Generally, eight fungal genera were isolated from the infected fruits and identified based on their cultural morphology and microscopic characteristics, included *A. alternate*, *A. niger*, *B. cinerea*, *Mucor* sp., *P. expansum*, *R. stolonifer*, *Stemphyllum* sp., and *T. roseum*. Although *P. expansum* was the most dominant pathogen, *B. cinerea* showed the highest pathogenicity and virulence on apple fruits. Therefore, the identification of *B. cinerea* was further confirmed based on the sequence of ITS region. Molecular identification and phylogenetic tree showed that our isolate has high similarity with other previously published sequences of *B. cinerea*. These finding update our

knowledge about the fungal phytopathogens associated with postharvest rot of apple fruits and might lead to the development of an effective means to control this serious disease, and consequently reduce its economic losses.

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