

Molecular Detection and Pathogenicity of *Alternaria alternata* and *Cladosporium herbarum* Causing Grape Bunch Rot Disease

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Abstract

Grape bunch rot ranks as one of the most devastating diseases damaging grape production and postharvest quality globally. In the present research, we utilized two fungal infections linked with bunch rot of grapes (*Alternaria alternata* and *Cladosporium herbarum*) on two grape cultivars (Halwany and Thompson) cultivated in Duhok province, Kurdistan Region, Iraq. Infectious berries representing diverse vineyards were utilized for fungus isolation. Fungal isolates were identified depending on culture and morphological features that grown on potato dextrose agar (PDA). In addition, molecular techniques such as PCR amplification and sequencing of 28S and 5.8S ribosomal RNA gene areas were used. Morphological study confirmed the existence of *Alternaria alternata* and *Cladosporium herbarum* as the predominant species. Molecular techniques generated certain bands of 600 and 1200 bp for the ITS and LSU sections, respectively, and BLAST findings revealed that the highest query sequence was 100% identical to each fungus in GenBank. Pathogenicity assays indicated both of the fungi were pathogenic. *Alternaria alternata* produced most serious lesions on the two cultivars Thompson and Halwany, having lesion diameters of 1.16 cm and 0.82 cm, respectively, which were followed by *Cladosporium herbarum* (0.70 and 0.63 cm). The findings confirmed *A. alternata* and *C. herbarum* as major causes of grape bunch rot in the region and it is the first record of this disease in Iraq.

Keywords: Grape bunch rot, PCR detection, Cultural characters, Pathogenicity test.

Introduction

Vitis vinifera L. (grapevine) represents one of the world's oldest and most important fruit crop genera, having over 100 species [1]. *Vitis* L. constitutes one of the globe's most nutritious and economically important fruit species. Grapes, due to their soft tissue and non-climacteric character, are extremely susceptible to pathogen infection during postharvest handling [2]. Around thirty fungal infections

can harm grapevines globally, according to numerous studies [3]. *Botrytis cinerea*, *Aspergillus carbonarius*, *Penicillium expansum*, *Cladosporium herbarum*, *Alternaria alternata*, *Guignardia bidwellii*, *Rhizopus stolonifera*, and *Greeneria uvicola* are some of the well-known and harmful postharvest pathogenic fungi in grapes [4].

Avoiding major outbreaks and production and quality losses in vineyards requires quick and precise disease detection. However, a therapy is

not always necessary just because a virus or disease is present. The weather, the existence of inoculum (disease history), and the vines' vulnerability are the main factors that determine how severe a disease is from year to year. This implies that an infection may be catastrophic one year and negligible the next. As a result, the steps that should be done to avoid losses may change from season to season. In Chilean vineyards, Cladosporium rot (*Cladosporium* spp.) is a prevalent disease, and *C. herbarum* was thought to be the main invader [5]. Because of late harvests, the disease incidence frequently exceeds 50% of clusters. In order to achieve the phenolic maturity that ensures roma and flavor development required for the best possible wine quality, this wait is required. Because of these circumstances, red cultivars are sometimes picked when the grapes are half senescent, increasing the likelihood and severity of Cladosporium rot, which lowers production and degrades wine quality. There are numerous saprotrophic and pathogenic species in the global genus *Alternaria*. They are present in a wide variety of goods and materials. In the outdoors, a variety of species are plant pathogens. All of the plant's aerial components stem, leaves, fruits, pods, and heads are impacted by fungi [6]. During the summers of 2007 and 2008, *Alternaria* bunch rot was seen in various Slovakian grape-growing regions. The infection began to spread in July, shortly before berry-touch, and persisted until berries ripened in the majority of vineyards. The fruit eventually turned necrotic and dropped from bunches. *A. alternata* was the source of the disease, which resulted in large losses in yield [7].

The purpose of the present research was the morphological and molecular identification of grape bunch rot causal agents *Cladosporium herbarum* and *Alternaria alternata*.

Materials and Methods

Fungal cultures

In commercial vineyards in the province of Duhok, symptomatic ripe to overripe berries were used to isolate *Alternaria alternata* and *Cladosporium herbarum*. Berries with symptoms were surface disinfected with 1% (w/v) sodium hypochlorite for three minutes, washed twice using sterilized distilled water, and then dried on sterilized filter paper. Sanitized tissue pieces (5mm) that were removed from the lesion borders were placed on sterilized potato dextrose agar media (PDA) and cultured for 7–14 days at $25 \pm 2^\circ\text{C}$. The spores were collected by adding 10 milliliters of sterile distilled water to PDA plates that had been inoculated and grown at 25°C for seven to fourteen days in an automated incubator. A hemocytometer was used to adjust the spore concentration to the necessary amount (1×10^6 spores $\cdot \text{mL}^{-1}$) after the collected conidial suspension was filtered through sterile masculine cloth [8]. The original parameters for recognizing colonies of *C. herbarum* and *A. alternata* were the appearance, shape, and coloration of the colonies on PDA, together with the morphology of conidiophores and conidia. Morphological analysis, PCR amplification, and signature gene sequencing were used to identify *C. herbarum* and *A. alternata* [9&10].

Morphological identification

By examining their morphological features, including as colony appearance and conidia morphology, the cultures that resembled *Alternaria alternata* and *Cladosporium herbarum* were identified. The fungal pathogen *Alternaria alternata* morphological characteristics were examined through the culture growth on PDA for 7–14 days at 25°C . Findings on the morphological characteristics of mycelium, conidiophores, conidia, and chlamydospores, as stated by [11].

In order to identify *Cladosporium herbarum*, morphological traits were identified using the standardized methods of [12]. Conidia and conidiophores were seen morphologically using

colonies cultivated on PDA plates for 7–14 days

Molecular identification

DNA Extraction

The DNA was extracted from the mycelium of *Altranria alternata* (isolate No.AR1) and *Cladosporium herbarum* (Isolate No. CR1). The Jena Bioscience plant and fungus DNA preparation Kit (Jena Bioscience GmbH.07749 Jena Germany) was utilized in order to obtain genomic DNA of isolated fungi.

Polymerase Chain Reaction (PCR) Amplification

The primers that produced by Micro-gene Company (South Korea) are specific to utilize the RNA (rRNA) sequences (Table 1). PCR reaction was carried out in a complete volume of 50 µl of reaction mixture containing; 2x Taq DNA Polymerase M-aster Mix (AMPLIQON A/S Stenhuggervej 22), 10 Picomol (pmol) primers, DNase free water and template DNA by Bioresearch PTC-200 Gradient thermal cycler.

DNA sequencing

Table1: Pair of primers used in rRNA gene sequence from both fungi .

Primer code	Sequence 5'-3'	Amplicon size(bp)	PCR Condition	Reference
LSU-F (28S rRNA)	5'- ACCCGCTGAACTTA AGC -3'	1200	95°-5 min; 95°-40 sec, 58°-40 sec, 72°-1 min; 72°-10 min; 4°	Van Tuinen et al 1998 [13]
LSU-R (28S rRNA)	5'- CGCCAGTTCTGCTT ACC -3'			
ITS1 -F (5.8S rRNA)	5'- TCCGTAGGTGAACC TGCGG-3'	600	95°-5 min; 95°-1 min, 55°-1 min, 72°-1 min; 72°-10 min; 4° ∞	White et al 1990 [14]
ITS4 -R (5.8S rRNA)	5'- TCCTCCGCTTATTG ATATGC-3'			

at 25°C

The ABI Prism Terminator Sequencing Kit (Applied Bio system) at Macrogen Company of Korea was used for sequencing a PCR result of fungal specimens of 28S and 5.8 rRNA partial genes. Finch TV application was used to modify rRNA chromatograms and verify base calls.

Sequence alignment and submission

The NCBI (National Center for Biotechnology Information) web page offers the Basic Local Alignment Search Tool (BLAST), a search engine that uses the sequence alignment method (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) to compare and align testing or query sequences against various biological sequences to find additional matches with fungi species. A portion of the samples' 5.8S rRNA gene sequence has been uploaded to the National Center for Biotechnology Information (NCBI).

Pathogenicity tests

Matured grapes Halwany and Thomson seedless were grown in Duhok farms and used in our study to infect with *C. herbarum* and *A. alternata*. The grapes were chosen based on their berry color, maturity, similar size, and lack of damage or infection. A sterile hypodermic syringe was used to injure grape berries to a depth of 1 mm at three locations along the calyx end. Each fruit, with pedicel attached, was surface disinfested with 75% ethanol for 15 seconds. Berries were infected with 10 μ l of conidial suspension (1×10^6 conidia \cdot mL $^{-1}$) collected from a 10-day-old culture on PDA. To maintain 95% relative humidity, infected grapes were stored in plastic bags lined with thin films. The berries were incubated for 7-14 days at 25°C. The lesion diameter was calculated by averaging the diameters of the lesions formed surrounding each wound. An equal amount of wounded and uninjured berries treated with sterile water were left as a control [15]. In order to finish Koch's postulates, causative agents were reisolated and identified in all trials.

Statistical analysis

Data were analyzed using GenStat 12th edition. Data were subjected to analysis of variance (ANOVA) and pooled together after testing the homogeneity of variance ($P \leq 0.05$). Means of the treatments were compared by Duncan Multiple Range Test at 5% level.

Results and Discussion

Morphological characters of *A. alternata* :

Colony characteristics on potato dextrose agar (PDA) is black-olivaceous to black/greyish, exhibiting a dark grey-brownish appearance on the bottom and an abundance of mycelium on

the surface (Figure 1). Conidiophores could be either short or long, and they could grow alone or in clusters. Their shapes were straight or curved, pale olivaceous to olivaceous-brown, slightly enlarged at the tip, and had terminal scars that showed where the conidia were attached. On conidiophores, conidia develop in chains of ten or more.

They sized 25-37.5x 7.5-12.5 μ m, had a tapering apex with septa, and ranged in shape between obclavate to mainly ellipsoidal. They appeared bright olivaceous to dark brown in color. The conidium's length was two to four times more than its width. These characters matched the definitions found of [16&17].

Morphological identification of *C. herbarum* :

Cladosporium colonies on potato dextrose agar (PDA) had an olive-colored green top surface with black or greenish-black staining on the underside (Figure 2). *C. herbarum* was identified by its profuse aerial mycelium, septate conidiophores that develop laterally, and one or more swellings. Conidia were ellipsoidal, pigmented, rough, and sometimes septated, measuring 10-22.5 \times 5-7.5 μ m. This outcome was in agreement with the descriptions of [18&19].

Molecular characterization

PCR amplification of partial rRNA genes

The 28S and 5.8S primers that are specific to genes were developed utilizing the fungal ribosomal RNA sequences. A band with the 600 bp and 1200 bp size were observed using the two primers. A 1.5% Agarose gel was used to electrophorese and visualize the PCR result (Figure 3).

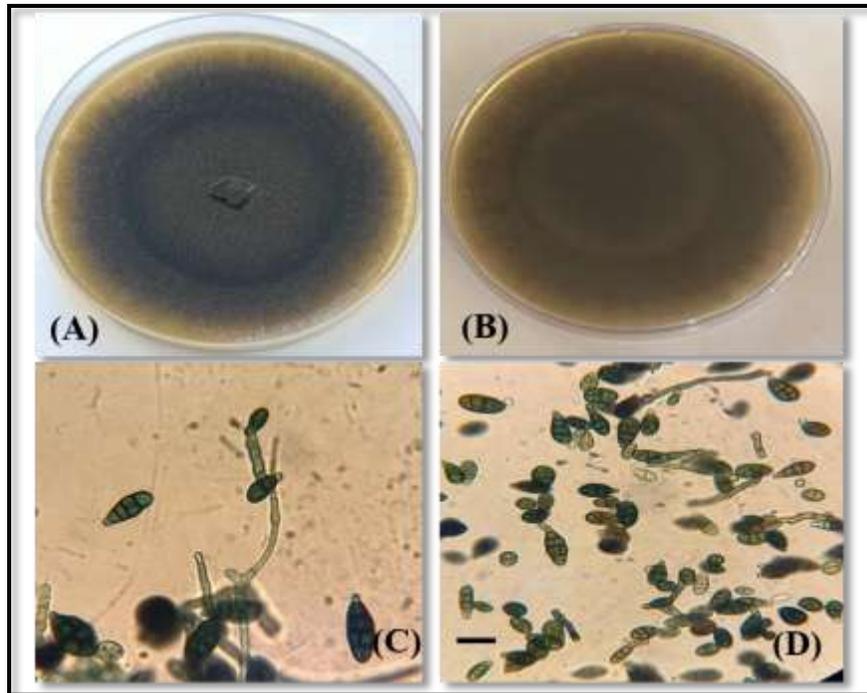


Figure (1): *Alternaria alternata*, (A) Colony surface on PDA; (B) Colony reverse; (C) Conidiophores; (D) Conidia. Scale bar: D= 30 μ m

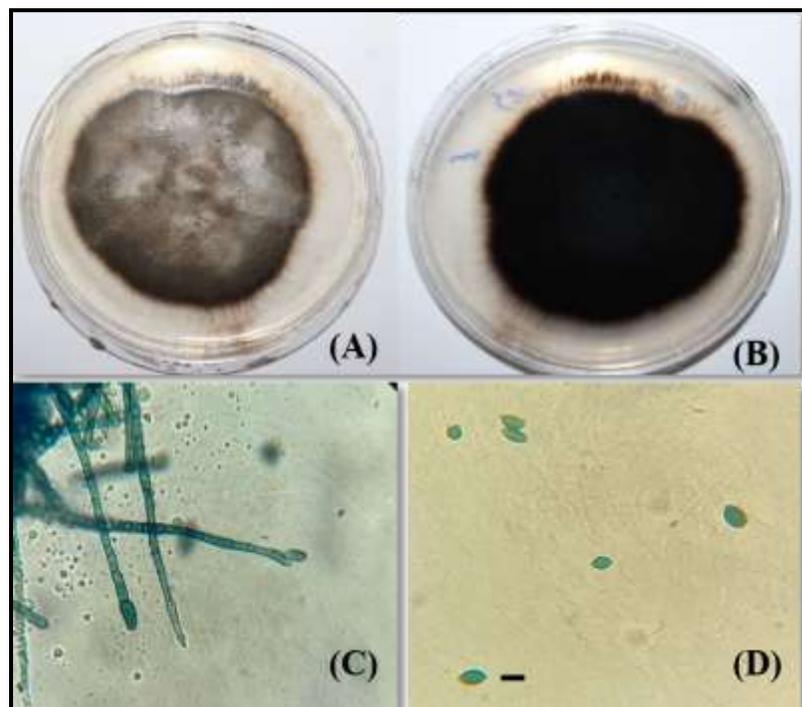


Figure (2): *Cladosporium herbarum*, (A) Colony forward on PDA; (B) Colony reverse; (C) Conidiophores; (D,E) Conidia. Scale bar: D= 15 μ m

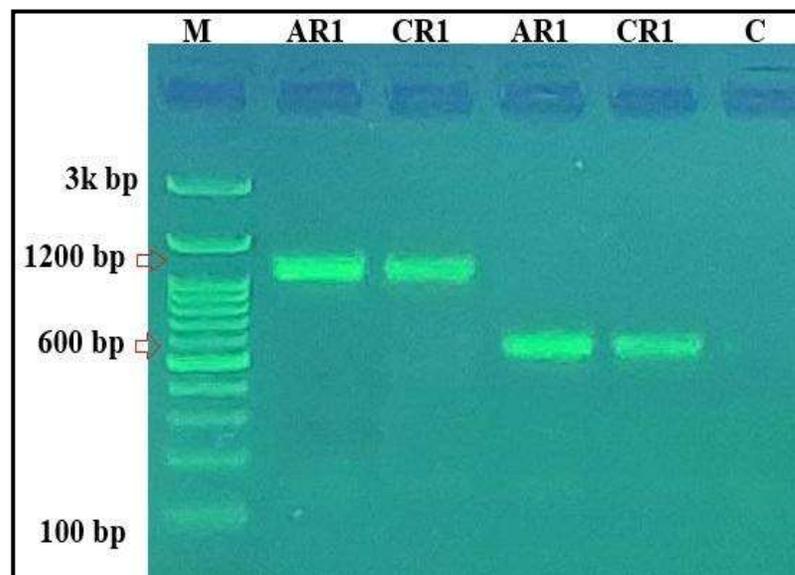


Figure (3) : PCR amplification of partial 28S rRNA;1200 bp and 5.8S rRNA are 600 bp from lanes of AR1 and CR1, M is ladder include 3k bp- 100bp bands and C is negative control

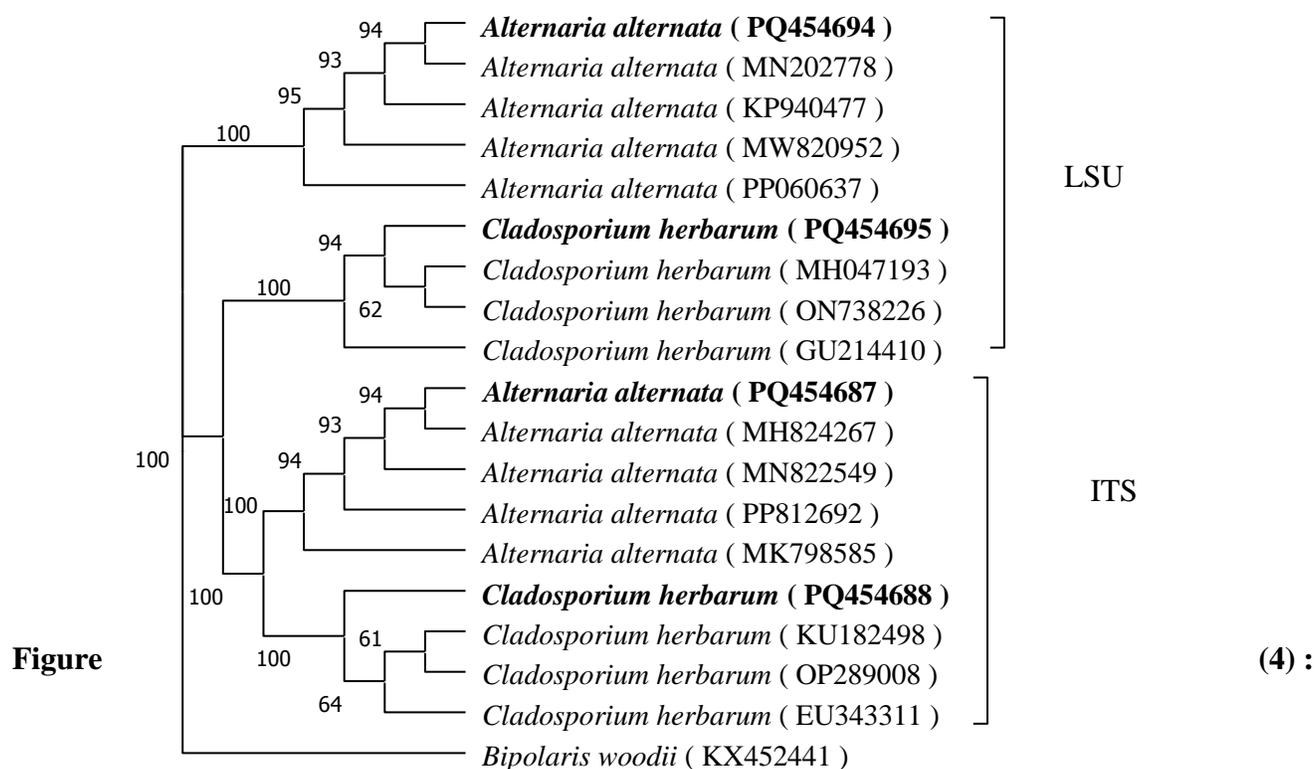
Molecular Identification and submission of Fungi species in Gen-bank

The BLAST tool in Gen Bank (<http://blast.ncbi.nlm.nih.gov/>) provided the partial rRNA sequence specimen with sizes of 1200 and 600 bp, which was utilized in comparing our amplified sequence against other recorded species of fungal sequences. The top query sequence was completely unique with each fungus, according to the BLAST findings.

Phylogenetic inferences

In accordance with a comparison of DNA sequences, the phylogenetic tree shows the

genetic links among isolates of *Cladosporium herbarum* and *Alternaria alternata* (Figure 4). Evolutionary divergence between the fungal strains is reflected in the tree building, which is probably based on a distance-based method maximal parsimony. The sequences are clearly divided into two different clades, one for each species, according to the tree. Similar to how all *C. herbarum* isolates form a cohesive cluster, all *A. alternata* isolates group closely together. This unique branching arrangement shows that the genetic marker (probably ITS or LSU) is useful for differentiating among those highly related fungal taxa and validates the taxonomic identity of the two species.



Evolutionary analyses were carried out in MEGA.11 blast display phylogenetic placement of isolated fungal specimen according to ITS and LSU sequences employing maximum parsimony accessible in GenBank sequences. Examined Iraqi isolates in bold. *Bipolaris woodie* utilized to root a tree

Pathogenicity tests

The entire of those infected isolates caused lesion lengths that were significantly different than those in the untreated plants, according to the pathogenicity outcomes displayed in (Table 2). The largest necrotic lesions (1.16 cm) and (0.82 cm) within the infected cultivar grape were formed by *Alternaria alternata*, the most virulent fungal species among the two cultivars Thompson and Halwany, which was followed

by *Cladosporium herbarum* (0.7 and 0.63 cm) on both cultivars.

The Thompson cultivar (0.62 cm) suffered greater damage by fungi than the Halwany cultivar (0.48 cm), despite the fact that the impact of fungi on tested cultivars revealed that the two cultivars were harmed with no discernible difference among them. On both cultivars, *Alternaria alternata* was a more aggressive fungus (0.99 cm) than *Cladosporium herbarum* (0.67 cm).

Table 2: Effect of *A. alternata* and *C. herbarum* on grape berries of both Thompson seedless and Halwany cultivars

Fungi Grape	<i>Alternaria alternata</i>	<i>Cladosporium herbarum</i>	Control	Cultivar Effect
Thompson	1.16 a	0.7 b	0 c	0.62 a
Halwany	0.82 b	0.63 b	0 c	0.48 a
Pathogen Effect	0.99 a	0.67 b	0 c	*****

-Means of different letters differ significantly depending on Duncan's Multiple Range test ($P \leq 0.05$)

-Each mean represents three replications

A fungus *Alternaria* causes grape bunch rot throughout withering, which results in passito-style wines [20]. Upon grapes as well as other fruits, strains of *Alternaria alternata* have been observed to create the mycotoxins alternariol and alternariol methyl ether; however, it is unclear how much of an issue this is for wine grapes [21]. Whereas *Alternaria* species are mostly found as endophytes on grapes berries, they may additionally trigger losses to crops beneath severe disease prevalence circumstances since they are opportunistic pathogens [22 & 23]. This fungal infection damages berries, rachides, and pedicels in grapes by causing bunch rot [24]. Throughout grape maturation in the field or post-harvest processing, species like *Alternaria alternata*, *Alternaria arborescens*, and *Alternaria tenuissima* have been isolated from grapes on a regular basis [25,26 & 27]. In South Africa, after-harvest decay of fruit in grapes was discovered linked to *A. alternata*. According

to [28 & 29] *C. cladosporioides* and *C. herbarum* constituted the pathogens that caused Cladosporium rot in grapevines. According to [15], infection incidence increased in contaminated grapes following one day. On contaminated grapes, velvety olive to brown colonies were seen (Fig.5). As a result, grapes deteriorated and lesion diameter grew in a positive correlation with incubation period. Extracellular secretions were the cause of the physiological changes seen in grape tissues. Excessive concentrations of hydrolytic enzymes, such as cellobiohydrolase and β -glucosidases, were released by pathogenic fungi, which compromised tissue integrity and allowed the fungi to absorb resources from the host tissue, resulting in soft rot surrounding the area of infection [30].

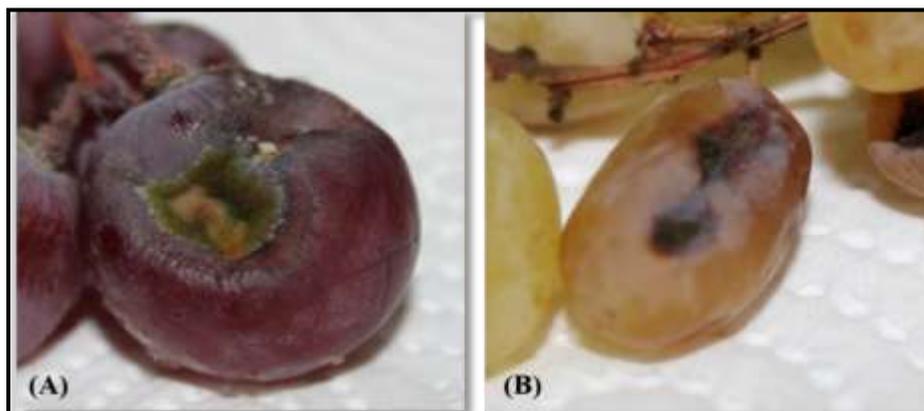


Figure (5) : Bunch rot disease on Halwany (A) and Thomsan (B) Cultivars caused by *Alternaria alternata*



Figure (6) : Bunch rot disease on Halwany(A) and Thomsan (B) Cultivars caused *C. herbarum*

Conclusions

The present study concludes that *A.alternata* and *C.herbarum* are causal agent of bunch rot in grape in Duhok province. It is the first record of this disease in Iraq. Accurate detection of these two fungi achieved through combination of morphological and molecular techniques. Pathogenicity test showed that both fungi are capable of infection with *A.alternata* showing

higher virulence than *C.herbarum* on the both used grape cultivars (Thompson and Halwany).

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References

- [1]. **Mullins MG, Bouquet A, Williams LA., 1992.** Biology of the grapevine. G. Mullins, ed. Cambridge University Press, Cambridge, U.K.
- [2]. **Vlassi, E., Vlachos, P. and Kornaros, M., 2018.** Effect of ozonation on table grapes preservation in cold storage. *Journal of Food Science and Technology*, 55(6), pp.20.
- [3]. **Wilcox WF, Gubler WD, Uyemoto JK. 2015.** Compendium of Grape Diseases, Disorders, and Pests, 2nd Ed. (pp. 39-45). American Phytopathological Society.
- [4]. **Steel, C.C., Blackman, J.W. and 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), pp.5189-5206.
- [5]. **Hewitt, W. B. 1994.** Berry rots and raisin rots. In R. C. Pearson & A. C. Goheen (Eds.), *Compendium of Grape Diseases* (pp. 26–28). American Phytopathological Society, St. Paul, MN.
- [6]. **Neeraj, B. and Verma, S., 2010.** Alternaria diseases of vegetable crops and new approaches for its control. *Asian Journal of Experimental Biological Sciences*, 1(3), pp.681-692.
- [7]. **Kakalíková, L., Jankura, E., & Šrobárová, A. (2009).** First report of Alternaria bunch rot of grapevines in Slovakia. *Australasian Plant Disease Notes*, 4(1), 68-69.
- [8]. **Li, D., Zhang, X., Gu, X., Zhang, Q., Zhao, L., Zheng, X., & Zhang, H., 2019.** The infection of grapes by *Talaromyces rugulosus* O1 and the role of cell wall-degrading enzymes and ochratoxin A in the
- evaluations. Their cooperation and assistance were crucial to this study's accomplishment. *Physiological and Molecular Plant Pathology*, 106, 263-269.
- [9]. **Ellis, M. B. 1971.** Dematiaceous Hyphomycetes. Commonwealth Mycological Institute, Kew, UK.
- [10]. **Solairaj, D., Legrand, N.N.G., Yang, Q. and Zhang, H., 2020.** Isolation of pathogenic fungi causing postharvest decay in table grapes and in vivo biocontrol activity of selected yeasts against them. *Physiological and Molecular Plant Pathology*, 110, p.101478.
- [11]. **Chowdhry, P.N. and Varshney, A., 2000.** Identification of different *Colletotrichum gloeosporioides* species. Manual on identification of plant pathogenic and biocontrol fungi of agricultural importance 14th September to 13th October, pp.73-78.
- [12]. **Schubert, K., Groenewald, J.Z., Braun, U., Dijksterhuis, J., Starink, M., Hill, C.F., Zalar, P., De Hoog, G.S. and Crous, P.W., 2007.** Biodiversity in the *Cladosporium herbarum* complex (Davidiellaceae, Capnodiales), with standardization of methods for *Cladosporium* taxonomy and diagnostics. *Studies in Mycology*, 58, pp.105-156.
- [13]. **Van Tuinen, D., Jacquot, E., Zhao, B., Gollotte, A., & GIANINAZZI-PEARSON, V. (1998).** Characterization of root colonization profiles by a microcosm community of arbuscular mycorrhizal fungi using 25S rDNA-targeted nested PCR. *Molecular Ecology*, 7(7), 879-887.
- [14]. **White, T. J., Bruns, T., Lee, S. J. W. T., & Taylor, J. 1990.** Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. *PCR protocols: a guide*

- to methods and applications, 18(1), 315-322.
- [15]. **Solairaj, D., Legrand, N. N. G., Yang, Q., Liu, J., & Zhang, H. 2022.** Microclimatic parameters affect Cladosporium rot development and berry quality in table grapes. Horticultural Plant Journal, 8(2), 171-183.
- [16]. **Deshmukh, A. N. 1998.** Studies on leaf spot and fruit rot of chilli caused by *Alternaria alternata* (Fr.) Keissler (M.Sc. Agri. thesis). M.P.K.V., Rahuri.
- [17]. **Nagrale, D.T., Gaikwad, A.P. and Sharma, L., 2013.** Morphological and cultural characterization of *Alternaria alternata* (Fr.) Keissler blight of gerbera (*Gerbera jamesonii* H. Bolus ex JD Hook). Journal of Applied and Natural Science, 5(1), p.171.
- [18]. **Tashiro, N., Noguchi, M., Ide, Y. and Kuchiki, F., 2013.** Sooty spot caused by *Cladosporium cladosporioides* in postharvest Satsuma mandarin grown in heated greenhouses. Journal of General Plant Pathology, 79(2), pp.158-161.
- [19]. **Torres, D.E., Rojas-Martínez, R.I., Zavaleta-Mejía, E., Guevara-Fefer, P., Márquez-Guzmán, G.J. and Pérez-Martínez, C., 2017.** *Cladosporium cladosporioides* and *Cladosporium pseudocladosporioides* as potential new fungal antagonists of *Puccinia horiana* Henn., the causal agent of chrysanthemum white rust. PloS one, 12(1), p.e0170782.
- [20]. **Lorenzini, M. and Zapparoli, G., 2014.** Characterization and pathogenicity of *Alternaria* spp. strains associated with grape bunch rot during post-harvest withering. International journal of food microbiology, 186, pp.1-5.
- [21]. **Tournas, V.H. and Stack, M.E., 2001.** Production of alternariol and alternariol methyl ether by *Alternaria alternata* grown on fruits at various temperatures. Journal of food protection, 64(4), pp.528-532.
- [22]. **Mostert, L., Crous, P. W., & Petrini, O., 2000.** Endophytic fungi associated with shoots and leaves of *Vitis vinifera*, with specific reference to the *Phomopsis viticola* complex. specific reference to the *Phomopsis viticola* complex.
- [23]. **González, V. and Tello, M.L., 2011.** The endophytic mycota associated with *Vitis vinifera* in central Spain. Fungal diversity, 47(1), pp.29-42.
- [24]. **Swart, A.E., Lennox, C.L. and Holz, G., 1995.** Infection of table grape bunches by *Alternaria alternata*.
- [25]. **Romero, S.M., Comerio, R.M., Larumbe, G., Ritieni, A., Vaamonde, G. and Pinto, V.F., 2005.** Toxigenic fungi isolated from dried vine fruits in Argentina. International journal of food microbiology, 104(1), pp.43-49.
- [26]. **Serra, R., Lourenço, A., Alípio, P. and Venâncio, A., 2006.** Influence of the region of origin on the mycobiota of grapes with emphasis on *Aspergillus* and *Penicillium* species. Mycological research, 110(8), pp.971-978.
- [27]. **Polizzotto, R., Andersen, B., Martini, M., Grisan, S., Assante, G. and Musetti, R., 2012.** A polyphasic approach for the characterization of endophytic *Alternaria* strains isolated from grapevines. Journal of Microbiological Methods, 88(1), pp.162-171.
- [28]. **Briceño, E.X. and Latorre, B.A., 2008.** Characterization of *Cladosporium* rot in grapevines, a problem of growing importance in Chile. Plant disease, 92(12), pp.1635-1642.
- [29]. **Briceño, E.X. and Latorre, B.A., 2007.** Outbreaks of *Cladosporium* rot associated with delayed harvest wine

grapes in Chile. *Plant Disease*, 91(8), pp.1060-1060.

[30]. **Horbach, R., Navarro-Quesada, A.R., Knogge, W. and Deising, H.B., 2011.**

When and how to kill a plant cell: infection strategies of plant pathogenic fungi. *Journal of plant physiology*, 168(1), pp.51-62.