Hasnain A *et al.* (2023)
Notulae Botanicae Horti Agrobotanici Cluj-Napoca
Volume 51, Issue 2, Article number 13167
DOI:10.15835/nbha51213167
Research Article

Molecular study of pathogenic and saprophytic fungal species on infected parts of *Malus pumila* L. of district Qilla Abdullah, Balochistan, Pakistan

Ali HASNAIN[1], Muhammad M. KAKAR[1], Nousheen YOUSAF[1], Shaukat ALI [2], Aisha TAHIR[3], Zahid MEHMOOD[1], Arooba JOHN[1], Amna EJAZ[1], BINYAMEEN[1], HARMA[1], Maryam IQBAL[1], Hafiz N. ANJUM[1], Tantri ANGGRAENI[4], Zafar IQBAL KHAN[5], Hsi-Hsien YANG[6]*, Muhammad U.F. AWAN[1]*

[1]*Department of Botany, Government College University, Lahore 54000, Pakistan; imalichoudhary@gmail.com; asilkhan580@gmail.com; drnousheenyousaf@gmail.com; zahidmehmood9037@gmail.com; Aroobajohn5@gmail.com; amnaejaz67@gmail.com; meen3014@gmail.com; harmashah9@gmail.com; maryamiqbalgcu@gmail.com; mnavid266@gmail.com; dr.umerfarooqawan@gcu.edu.pk (*corresponding author)*
[2]*Department of Zoology, Government College University Lahore, 54000, Pakistan; dr.shaukatali@gcu.edu.pk*
[3]*Department of Biochemistry, University of Health Sciences, Lahore, Pakistan; dr.aisha@uhs.edu.pk*
[4]*Indonesian Agency for Agriculture Instrument Standardization for Sweetener and Fiber Crops Raya Karangploso KM. 4, Malang, East Java 65152, Indonesia; tantridyah@pertanian.go.id*
[5]*Department of Botany, University of Sargodha, Sargodha, Pakistan; zafar.khan@uos.edu.pk*
[6]*Department of Environmental Engineering and Management, Chaoyang University of Technology, (R.O.C.) Taiwan; hhyang@cyut.edu.tw (*corresponding author)*

Abstract

Apple (*Malus pumila* L) of the family *Rosaceae,* most cultivated fruit in temperate regions of the world and is used fresh or processed. The apple production is affected by several pathogens including fungi. The present study was designed to identify disease-causing agents that reduce fruit production in the district Qilla Abdullah of Balochistan, Pakistan, which is the main apple production area of the province. Three varieties of apple: 'Tur-Kulu' ('Red Delicious'), 'Shin-Kulu' ('Golden Delicious'), and 'Kaja' were selected. Infected leaf samples were collected from eight different sites of tehsil Gulistan, district Qilla Abdullah. The cultures of fungal micro-flora were grown on two media, potato dextrose agar (PDA), and malt extract agar (MEA) followed by incubation for one week. The resulting colonies were observed under a microscope and identified based on morphological characters. Predominant fungal species was identified through ITS marker and PCR amplification. The isolated pathogens belonged to Zygomycota and Ascomycota divisions. The pathogens found were *Aspergillus niger, A. oryzae, A. terrus, Colletotrichum gloeosporioides*, *Fusarium oxysporum, Mucor* spp.*, Penicillium expansum*, and one species of *Absidia* as well as *Rhizopus. Colletotrichum gloeosporioides* were predominantly found in all varieties. Morphological and phylogenetic analysis confirmed the identity of

Received: 13 Apr 2023. Received in revised form: 10 Jun 2023. Accepted: 23 Jun 2023. Published online: 28 Jun 2023.
From Volume 49, Issue 1, 2021, Notulae Botanicae Horti Agrobotanici Cluj-Napoca journal uses article numbers in place of the traditional method of continuous pagination through the volume. The journal will continue to appear quarterly, as before, with four annual numbers.

Colletotrichum gloeosporioides. As a result of this study, the predominant pathogen species *Colletotrichum gloeosporioides* is one of the causes of leaf infection in apple varieties.

Keywords: *Colletotrichum gloeosporioides*; CTAB method; fungal pathogen; ITS marker

Introduction

Fruits are the most important food and source of essential vitamins for human beings (Sanzani *et al*., 2016). Apple (*Malus domestica* Borkh.) is one of the important ancient planted fruit crops in the world (Cornille *et al.,* 2014; Duan *et al*., 2017). Initially it was found as wild fruit in central Asia and then spread all over Europe in pre-historical times. Apple's nutritional values make it unique from other fruits. Due to their excessive dietary values, the demand of apples is increased all over the world. It is the 3rd most cultivated fruit in the world with an estimated production of 86 million metric tons (FAO, 2023).

Balochistan is a province of Pakistan and has the largest area among all provinces. It is also called "The Fruit Basket of Pakistan" due to the production of fruits. Balochistan provides 90% of fruits all over the country, with 34% production of apples (Samad, 2023). Apples are the fourth most important deciduous fruit grown widely in Balochistan. 'Shin-Kulu' ('Golden Delicious') and 'Tur-Kulu' ('Red Delicious') are famous varieties of apples in Balochistan due to their attractive colour and taste (Noonari *et al*., 2015). The most suitable climatic conditions and soil for apple growth prevail in the hilly areas of north Balochistan and KP province. The total area of Balochistan which is under cultivation is about 119.8 thousand hectares with an annual production of 427.9 thousand tons of apples (Khan *et al*., 2019). Most apples are grown in Qilla Saifullah, Quetta, Kalat, Qilla Abdullah, Ziarat, Zhob, Pishin, and some other districts of Balochistan (Nisar *et al*., 2011).

Due to its high nutritional value, apple is widely grown in the world. Its large cultivation in this area makes apples a host reservoir for many microorganisms (Abdelfattah *et al*., 2016; Liu *et al*., 2018). The production of apples is affected by different threats from pathogens and their toxins, such as mycotoxins (Fisher *et al*., 2012). Powdery mildew (*Podosphaera leucotricha*), bitter rot (*Colletotrichum acutatum* complex, *Colletotrichum gloeosporioides* complex), apple scab (*Venturia inaequalis*), black rot (*Botryosphaeria obtuseco*), and white rot (*Botyrosphaeria dothidea*), cedar-quince rust (*Gymnosporangium juniperi-virginianae, Gymnosporangium clavipes, Gymnosporangium globosum*), and sooty blotch/flyspeck (*Geastrumia polystigmatis, Zygophiala jamaicensis*) are fungal diseases with their causative agents that cause the most serious damage in apples (Holb, 2009; Nicole, 2019). Previous studies indicated that fungi caused premature fruit drops that become the source of 50% losses in the yield of apples (Raja *et al*., 2017). *Alternaria alternata* produce mycotoxins which are responsible for the core rot and mouldy core of apples in some regions of the world (El-Mohamedy and El-Sayed, 2015).

Several fungi, including *Alternaria alternata, Cladosporium cladosporioides, Fusarium semitectum,* and *Penicillium* spp., produce mycotoxins which were responsible for apple fruit drops and also harmful to human beings. But amongst all pathogens, *Alternaria alternata* is responsible for fruit drops in apples (Youssef and Roberto, 2020). The *Alternaria* spores could be carried into the apple cavity by mites (Van der Walt *et al*., 2011). Fungi cause the most severe diseases in plants. When the fruit is growing on a plant or stored, the fungi grow on it and produce mycotoxins that are dangerous for consumption by human beings. The *Monilinia* and *Penicillium* species are also reported to cause spoilage of fruit in apples (Alwakeel, 2013).

The aim of this study was identification of predominant fungal pathogens based on their morphological and molecular characteristics that affect the growth and yield of apples in the district Qilla Abdullah of Balochistan province of Pakistan.

Materials and Methods

Study area and sample collection

Tehsil Gulistan, district Qilla Abdullah, Balochistan was selected for the research purpose. The main reason for selecting the site was its richness in apple production. The diseased plant parts of various apple varieties were collected from eight different sites. These sites were Adrhamanzai, Batayzai, Habibzai, Imranzai, Kolazai, Masiyzai, Mayzai, and Slumankhail. Three varieties of apple: 'Tur-Kulu' ('Red Delicious'), 'Shin-Kulu' ('Golden Delicious'), and 'Kaja' (Figure 1) were selected based on infection prevalence on leaves, branches, and fruits.

(a) (b) (c)

Figure 1. Selected fruit varieties of apples: (a) 'Tur-Kulu', (b) 'Shin-Kulu', and (c) 'Kaja' were used for the study

Identification of disease

The infected leaves, branches, and fruits from apple trees were collected in zipper bags. Two methods i.e., direct identification and culturing methods were applied to investigate causative agents of infection (Skyes and Rankin, 2014).

Direct identification

Some diseases were directly identified by assessing the physical condition of diseased plant parts. The infection was also studied using a microscope. The spores and mycelia from infected parts of plants were placed on a slide containing a trypan blue stain. The materials were spread on the slide, a cover slip was placed and the slide was observed under a microscope.

Culturing method

Preparation of media

The choice of culture medium depends on the type of microorganism to be isolated for identification purposes.

Fungal culture media

The most direct and often the most convincing way to establish a diagnosis of a fungal infection is to culture the fungus from an infectious sample. Two types of fungal media, malt extract agar (MEA) and potato dextrose agar (PDA) were used to culture fungus (Black, 2020). Saline suspension of specific spores from diseased plant parts was collected and spread on the media plates under sterile conditions. Agar media plates

and MEA, and PDA plates were incubated at 37 °C and 28 °C, respectively for a week. The fungal colonies were observed and the pure cultures were maintained.

Identification of fungal pathogens

For slide preparation, different colonies from culture plates were picked using a sterilized inoculating loop, macerated finely onto slides containing a drop of trypan blue, and then covered with coverslips. Slides were observed using a light microscope at different magnifications (4X, 10X, and 40X). Fungal species were identified on basis of their morphological characters (Shamly *et al*., 2014).

Molecular identification of predominant pathogen fungi

DNA extraction and quantification

Molecular identification of predominant pathogens was done by taking fungus samples from the infected part of all varieties of plants through isolation of Genomic DNA, by using the CTAB method as described by Gardes and Bruns (1993) with some modifications the extracted DNA was visualized by agarose gel electrophoresis (Lee *et al*., 2012) and quantified on a double beam spectrophotometer Morris (2015).

Amplification and sequencing of fungal ITS region

For the amplification of the fungal Internal Transcribed Spacer (ITS) region, ITS-1 (forward primer) GCTGCGTTCTTCATCGATGC and ITS-4 (reverse primer) TCCTCCGCTTATTGATATGC were used (White *et al*., 1990). The total volume of the PCR reaction mixture was 20 µL comprising PCR Master Mix: 12 µL, DNA template: 1 µL, forward Primer: 0.5 µL, reverse primers: 0.5 µL, and dd. water 6 µL. The Amplified product of PCR was examined by 1% of agarose gel electrophoresis. The PCR product was sequenced by Capillary Electrophoresis Sequencing on ABI 3730xl System.

Sequence alignment and phylogenetic analysis

The obtained sequences were read, aligned, and edited to get a consensus sequence on BioEdit (*https://bioedit.software.informer.com/7.2/*). The new sequence generated in this study was compared with the closely related sequences of other *Colletotrichum* species, retrieved from GenBank. All the sequences of the ITS region were aligned using MUSCLE alignment software which generated a final dataset of 571 positions. For phylogenetic analyses, the tree was constructed based on Maximum Likelihood (ML) method. The Tamura-Nei model was chosen with the lowest BIC scores (Bayesian Information Criterion) (Tamura and Nei, 1993). Maximum likelihood analyses were performed with MEGA11 with 100 replicates (Tamura *et al*., 2021). Less than 50% of bootstrap support on the nodes was considered insignificant and was not shown.

Results

Direct identification of samples

The direct identification method identified five types of disease symptoms in the leaves of apple trees. Figure 2 shows that (a) is necrosis; (b) shows leaf blight infection (c) is canker infection (brown stem), while (d) illustrates frog eye direction infection (e) depicts anthracnose infection.

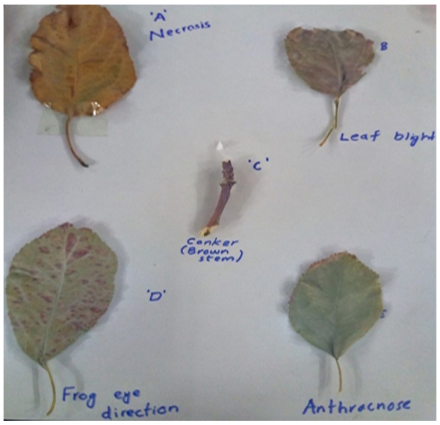

Figure 2. Disease identification of apple leaves on basis of physical appearance: (a) Necrosis; (b) Leaf blight infection; (c) Canker infection (brown stem); (d) Frog eye direction infection; (e) Anthracnose infection

Identification of fungi responsible for apple diseases

The fungi isolated from leaves and branches were identified from infectious sites based on colony morphology and microscopy. Colony features (color, shape, size, and hyphae) were observed under a stereomicroscope. Microscopic characters such as conidia, spore, hyphae, and other structures were observed using a lactophenol cotton-blue stained slide mounted with a small portion of the spore and mycelium (Gaddeyya *et al*., 2012). The identification keys of Frisvad and Samson (2004) were used to classify isolates belonging to different genera.

Variety 1. 'Shin-Kulu' ('Golden Delicious')

Aspergillus niger

Colonies were black having light brown conidia, round with spherical to globose vesicles, rough texture, irregular margins, 40-50 mm in one week; one conidial head on the tip of aseptate conidiophores; conidial head bearing spores were observed; conidia were dark black and globose as shown in the Figure 3 (a).

Mucor sp. 1

Colonies were cottony to fluffy in appearance, white to yellow; erect sporangiophore and the spores were present in the sporangium, colour of sporangium was dark green at the top, with light green at the center as shown in Figure 3 (b).

Colletotrichum gloeosporioides

Colonies were gray to black, regular, cottony, and raised, smooth margins, white aerial mycelium covering the whole surface of the plate, hyaline conidiophore, conidia cylindrical with a round end, hyphae hyaline, as shown in Figure 3 (c).

Fusarium verticilliodes

Colonies were white to creamy and pink, rough texture, irregular margins; conidia were elongated and kidney shaped in sporodochium as shown in Figure 3 (d).

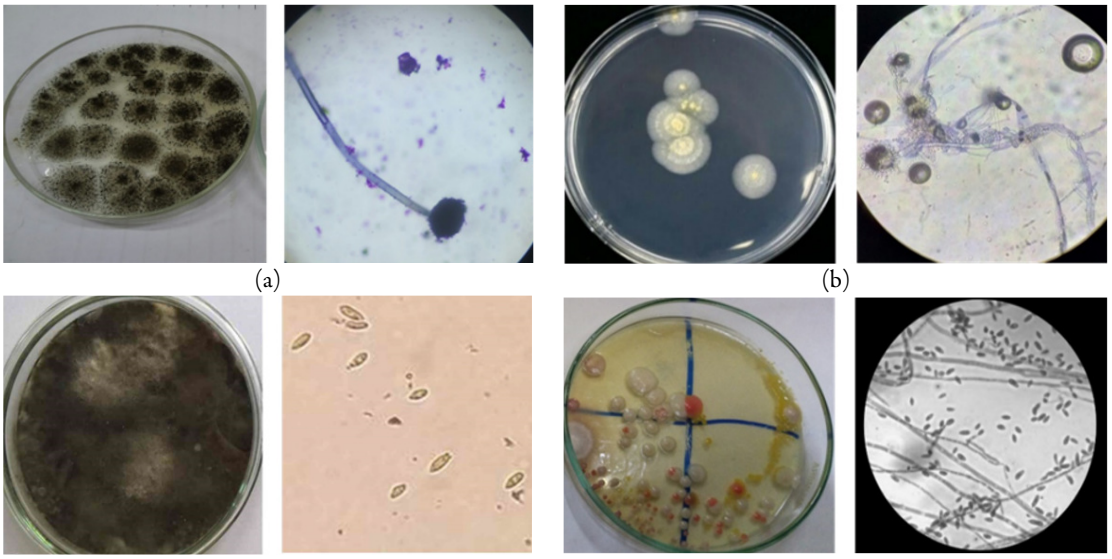

Figure 3. Isolated fungal colonies from variety 'Shin-Kulu' ('Golden Delicious'): (a) *Aspergillus niger* colonies were visible as small colonies of black conidia and micrograph of *Aspergillus niger*; (b) White colonies of Mucor sp. 1 and micrograph of asexual form; (c) Black colonies of *Colletotrichum gloeosporioides* and micrograph of conidia; (d) Colonies of *Fusarium verticilliodes* and micrograph of conidia & hyphae

Variety 2: 'Tur-Kulu' ('Red Delicious')

Rhizopus microsporus

Colonies were white to greyish black brown, rough texture, uneven, irregular, filamentous cottony and fluffy growth; well-developed rhizoid; hyaline to globose, light brown sporangium, round and terminal as shown in Figure 4 (a).

Penicillium expansum

Colonies white and greyish; bearing conidiophores; having brush-like conidia as shown in Figure 4 (b).

Absidia sp.

Colonies were cottony white, hyphae were septate and long; sporangiophores were alone and branched; columella was present; conidia were spherical and long in structure as shown in Figure 4 (c).

Aspergillus oryzae

Colonies were greyish brown; rough texture; circular and irregular margins; vesicles possessed stigmata bearing conidia; conidia were yellow with septate conidiophores; hyaline; thick-walled hyphae as shown in Figure 4 (d).

Colletotrichum gloeosporioides

Colonies were yellowish white, white aerial mycelium, hyphae hyaline, conidia cylindrical with a round end, hyaline conidiophore, as shown in Figure 4 (e).

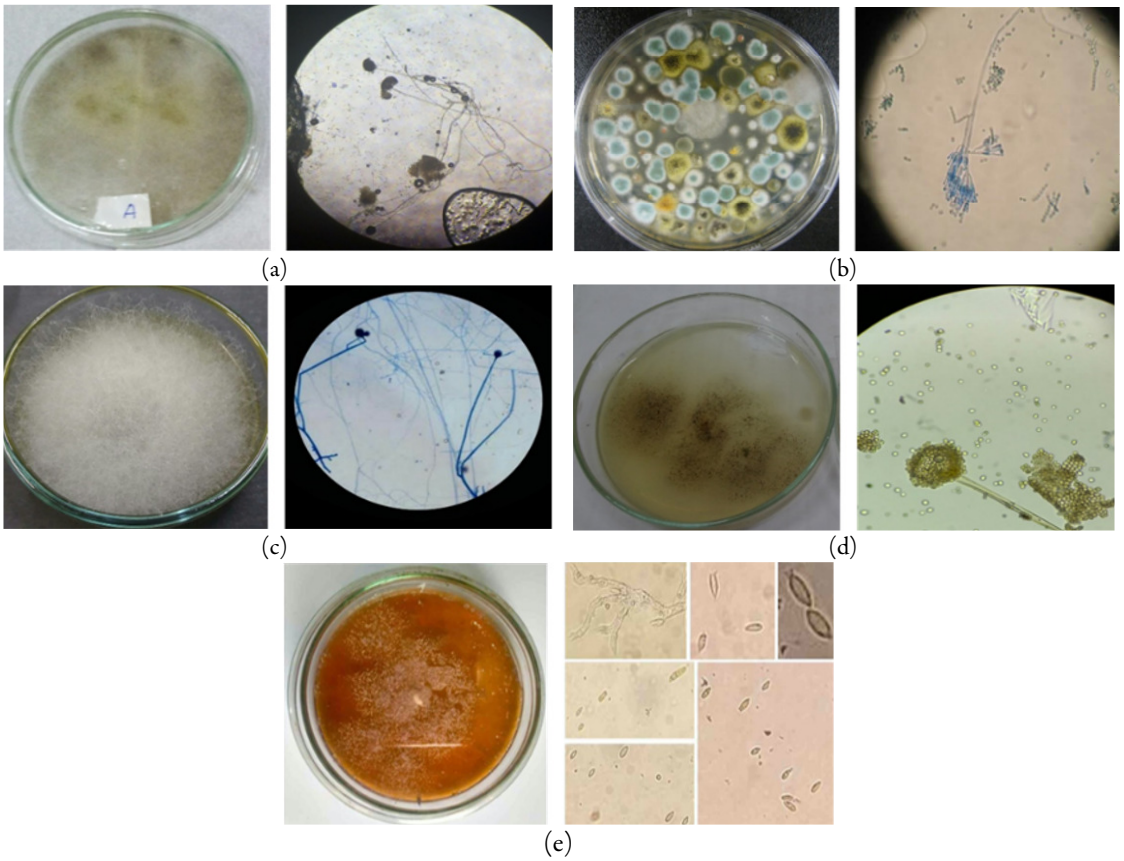

Figure 4. Isolated fungal colonies from variety 'Tur-Kulu' ('Red Delicious'): (a) Greyish black brown colonies of *Rhizopus microspores* and micrographs of asexual forms; (b) *Penicillium expansum* colonies and micrograph of asexual form; (c) *Absidia* sp. (cottony white) and micrograph of the asexual farm; (d) Grayish brown colonies of *Aspergillus oryzae* and micrograph of asexual form; (e) Colonies of *Colletotrichum gloeosporioides* and micrograph of asexual form

Variety 3: 'Kaja'

Aspergillus glaucus

Colonies were subglobose; echinate conidia arose from the conidial head; conidiophores were long; aseptate, and wide as shown in Figure 5 (a).

Fusarium oxysporum

Colonies hyphae were septate and non-pigmented (with trypan blue stain). There was an aspherical chlamydospore as shown in Figure 5 (b).

Mucor sp. 2

Colonies were whitish cottony black; columella; aerial hyphae, and the sporangiospores in sporangium as shown in Figure 5 (c).

Aspergillus terreus

Colonies were black-brown, hair-like soft tufts and stipe, coarsely roughened texture, irregular, granular, hard, sticky margins, thick and double-walled hyaline conidiophore as shown in Figure 5 (d).

Colletotrichum gloeosporioides

Colonies were Greyish to white, regular, smooth borders, hyaline conidiophore, conidia round ended; as shown in Figure 5 (e).

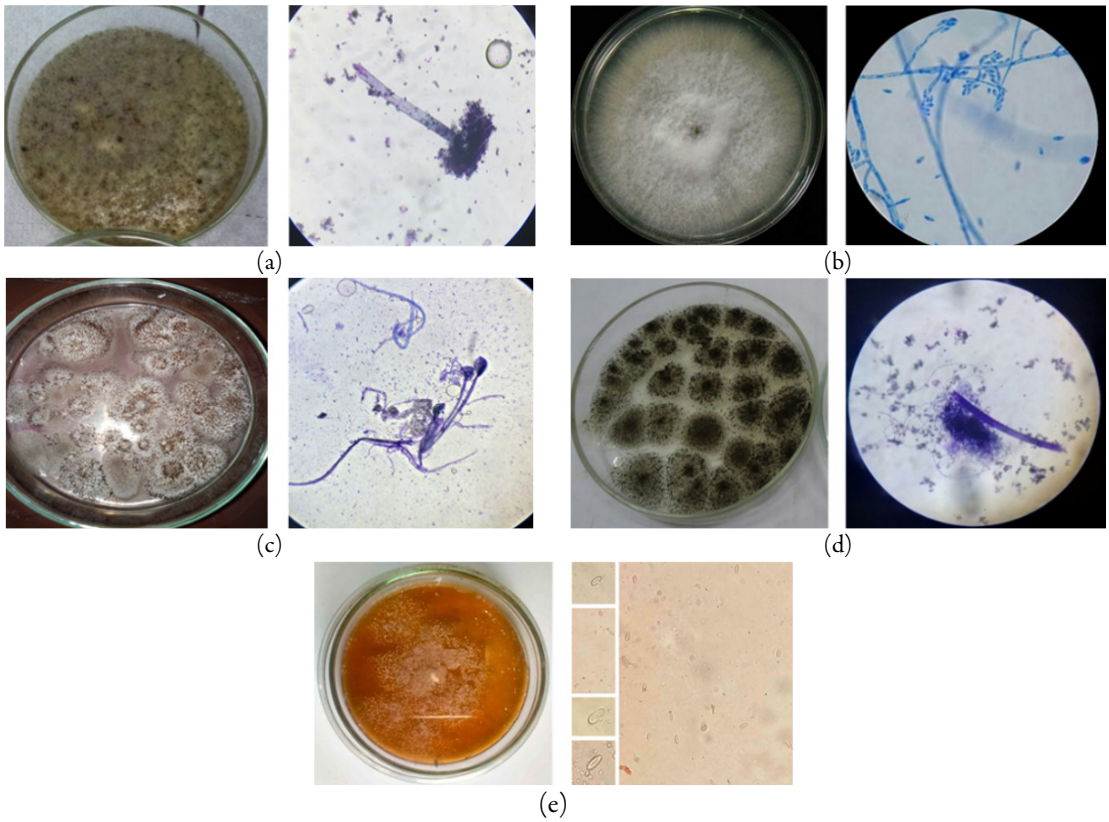

Figure 5. Isolated fungal colonies from variety Kaja: (a) Yellow-brown growth (Yellow brown and white colonies are present) and micrographs of *Aspergillus glaucus*; (b) Culture showing a whitish colony of *Fusarium oxysporum* and micrograph of conidia and conidiophore; (c) Whitish cottony and black colonies of *Mucor* sp. 2 and micrograph of asexual form; (6d) Black colonies of *Aspergillus terreus* and micrograph of asexual form; (e) Colonies of *Colletotrichum gloeosporioides* and micrograph of asexual form

The apple trees of Qilla Abdullah were infected by different infections. Fungal infection symptoms were caused by members of Ascomycota, and Zygomycota, and were identified in diseased apple parts in the current study. Various fungal species such as *Absidia* sp., *Aspergillus oryzae, A. niger, A. terrus, A. glaucus, Colletotrichum gleosporoides*, *Fusarium verticilloides, Fusarium oxysporum*, *Penicillium expansum, Mucor* sp.1, *Mucor* sp. 2, and *Rhizopus microsporus* were isolated and identified from diseases apple trees as given in Table 1.

Table 1. Different pathogenic and saprophytic fungi isolated from infectious parts of apple trees

Isolated fungi	Variety		
	'Shin kulu' ('Golden Delicious')	'Tur kulu' ('Red Delicious')	'Kaja'
Aspergillus niger	Yes	Nil	Nil
Aspergillus glaucus	Nil	Nil	Yes
Aspergillus oryzae	Nil	Yes	Nil
Aspergillus terreus	Nil	Nil	Yes
Fusarium oxysporum	Nil	Nil	Yes
Fusarium verticilliodes	Yes	Nil	Nil
Mucor sp. 1	Yes	Nil	Yes
Mucor sp. 2	Nil	Nil	Yes
Absidia sp.	Nil	Yes	Nil
Penicillium expansum	Nil	Yes	Nil
Rhizopus microspores	Nil	Yes	Nil
Colletotrichum gleosporoides	Yes	Yes	Yes

Molecular identification

Genomic DNA extraction and quantification

DNA from the predominant pathogenic fungus was extracted from samples obtained from three apple varieties (T1, T2, and T3) by the CTAB method with some modifications. DNA quantification was done by using UV/VIS spectroscopy at the absorbance of 260 and 280 nm respectively.

Amplification of ITS region

ITS primers were used for PCR amplification the size of the PCR product was 560bp which indicated that ITS regions were amplified. The result of the gel is shown in Figure 6.

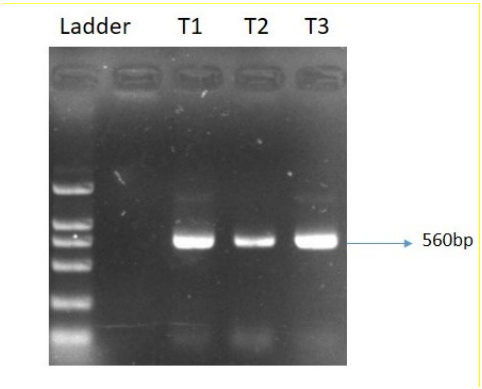

Figure 6. The result of ITS markers on genomic DNA of *Colletotrichum* spp

Molecular identification by BLAST

The cleaned and edited sequence of fungal ITS region was BLAST searched on the NCBI website for closely related sequence homology. The query sequence was matched with *Colletotrichum* sp., indicating that the predominant pathogen infecting all three apple verities was *C. gloeosporioides*. Figure 7 shows a rectangle cladogram of the neighbour joining distance tree of results obtained from BLAST in which nearest clade of unknown sequence comprised of *C. gloeosporioides.*

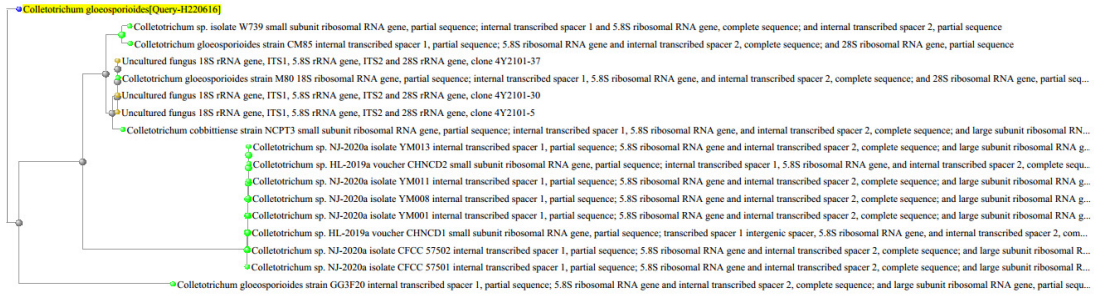

Figure 7. Cladogram acquired from BLAST showing sequence homology of unknown fungal species with *Colletotrichum gloeosporioides*

Phylogenetic analysis

This study generated 1 new sequence which has been submitted to GenBank (OR091360). The ITS dataset included 32 taxa and 571 sites, that comprised of 410 conserved, 157 variable, and 77 Parsimony informative sites. Phylogram included 31 in group taxa (all belong to genus *Colletotrichum*) and 1 out group taxon (*Monilochaetes infuscans*, NR155365). Our sequence of *Colletotrichum gloeosporioides* is clustered with the other collection of the same species (MN170560) in a well resolved clade (Figure 8) therefore, confirms its identity.

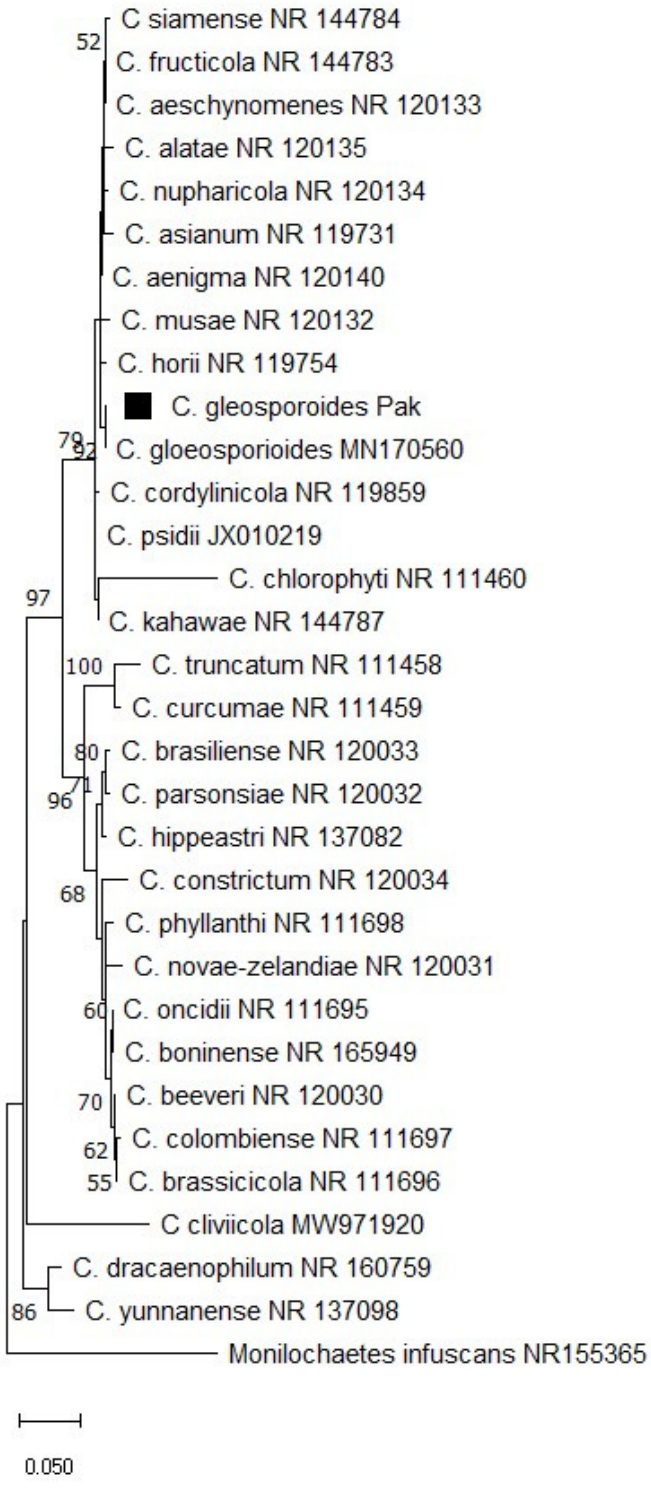

Figure 8. ITS-based phylogram of *Colletotrichum gloeosporioides* with allied taxa by Maximum Likelihood method. The tree with the highest log likelihood (-2251.54) is shown

Discussion

The apple is one of the most important fruit species, cultivated in the temperate regions of many countries, which has fruits with a pleasant and aromatic taste, rich in nutrients. Apples are an important source of vitamins, minerals, and carbohydrates, having an important nutritional value and being beneficial for human health (Patocka *et al*., 2020; Fotirić Akšić *et al*., 2022; Sestras and Sestras, 2023). Pakistan produced 589,171 tons annually and ranked 25 in apple-producing countries, while China is the largest apple producer with 40,393,000 tons of production. As apple trees are susceptible to different diseases which decreases the yield and quality of apples. In northern Balochistan, about 60% of the fruit trees have dried and the remaining is unlikely to survive due to the declining water and diseases. In recent years, in Quetta, Qilla Abdulah, Loralai, Qilla Saifullah, and Pishint, the yield of apples is affected by different diseases. It is estimated that the production of apples has reduced by at least 50% due to many reasons such as the impact of climate change, the shortage of water, fungal diseases, and pest attacks. Fungal diseases can cause yield losses in apple orchards and affect fruit quality (Siddique *et al*., 2007).

Penicillium expansum affects the bulbs and fruits of apple plants. Symptoms appear as oval, soft yellow-brown watery spots on the fruits (Sandor, 2008). *P. expansum* is one of the main pathogens causing the decay of fruits and vegetables. Postharvest fungal diseases of apples are mainly caused by *P. expansum* and *Rhizopus* sp. Both caused soft rot, a postharvest disease in apple trees (Kwon *et al*., 2011). *Fusarium* sp. has caused wilt disease in apples resulting in yellow leaves with interveinal chlorosis. Hence leaves fall off thus causing wilting of apple leaves in Qilla Abdullah, Balochistan.

In the present study, the PCR assay was used to check the predominant fungus infecting all varieties of apples by amplifying and sequencing the ITS region. The phylogenetic analysis in the present studies find out that the pre-dominant fungi *Colletotrichum gloeosporioides* are found to infect all the selected varieties of apples. This research result was matched with the study in which C. *gloeosporioides* were detected with other *Colletotrichum* species like *C. godetiae* and *C. acutatum* causing bitter rot disease (Sanzani *et al*., 2012) infected the mangoes. Another study revealed the same result in which seven different *Colletotrichum* species including *C. gloeosporioides* species complex isolated from three different apple orchards, causing bitter rot in apples and strawberries in Belgium (Grammen *et al*., 2019).

Overall, isolated pathogens can cause hazardous diseases which reduce not only the apple yield but also affect the growth of apple trees. Among identified fungi, such as *P. expansum* and *Rhizopus* sp. also caused post-harvest diseases of apples further aggravating the attack of fungal diseases *C. gloeosporioides*.

Conclusions

The present research specified the apple varieties such as 'Golden Delicious' ('Tur-Kulu') and 'Red Delicious' ('Shin-Kulu') which are more susceptible to fungal diseases. There is a lack of research work on the identification and isolation of apple diseases. The current study isolated and identified fungal pathogens and associated fungi for the first time from the apple trees of Qilla Abdullah. The total abundance of isolated genera was 11, belonging to the division Ascomycota and Zygomycota. The symptoms of wilting were present in plants growing all over the district and the infected leaves showed purplish-yellow spots. The nine pathogenic fungi from apple trees suggest the presence of several other fungal pathogens that needs to be investigated. The molecular analysis showed that *C. gloeosporioides* is pre-dominant in all collected varieties and the most prominent fungal pathogen causing bitter rot disease in apples. Identification of the disease-causing agents and precautions before the spread of the disease is very essential, which helps in the diagnosis and early detection of the pathogen. It may be helpful to maintain check and balance in the planting material for the prevalence of any potential pathogen even before the crop is sown. This fungal taxon can be very detrimental to apple yields,

so preventive measures must be taken to reduce the possibility of the causative disease. Adequate antifungal sprays and awareness are warranted in all districts and provinces to ensure an increase in total apple fruit production.

Authors' Contributions

MMK and AH: collected samples and experimental work; H-HY, ZI: complied all data; NY, TA and AT: morphological and molecular identification; SAZM: final editing; AE, B, H, MI, HNA: DNA isolation and PCR; AJ: English language and grammar checking.

All authors read and approved the final manuscript.

Ethical approval (for researches involving animals or humans)

Not applicable.

Acknowledgements

The authors would like to thank the Department of Botany Government College University Lahore, Pakistan and the College of Science and Engineering, Chaoyang University of Technology, Taiwan (R.O.C.)

Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

References

Abdelfattah A, Wisniewski M, Droby S, Schena L (2016). Spatial and compositional variation in the fungal communities of organic and conventionally grown apple fruit at the consumer point-of-purchase. Horticulture Research 3. *https://doi.org/10.1038/hortres.2016.47*

Alwakeel SS (2013). Molecular identification of isolated fungi from stored apples in Riyadh, Saudi Arabia. Saudi Journal of Biological Sciences 20(4):311-317. *https://doi.org/10.1016/j.sjbs.2013.05.002*

Black WD (2020). A comparison of several media types and basic techniques used to assess outdoor airborne fungi in Melbourne, Australia. PLoS One 15(12):e0238901. *https://doi.org/10.1371/journal.pone.0238901*

Cornille A, Giraud T, Smulders MJ, Roldán-Ruiz I, Gladieux P (2014). The domestication and evolutionary ecology of apples. Trends in Genetics 30(2):57-65. *https://doi.org/10.1016/j.tig.2013.10.002*

Duan N, Bai Y, Sun H., Wang N, Ma Y, Li M, Wang X, Jiao C, Legall N, Mao L, Wan S (2017). Genome re-sequencing reveals the history of apple and supports a two-stage model for fruit enlargement. Nature Communications 15(8):1. *https://doi.org/10.1038/s41467-017-00336-7*

FAO (2023). Retrieved 2023 February 10th from: *http://www.fao.org/faostat/en*

Fisher MC, Henk D, Briggs CJ, Brownstein JS, Madoff LC, McCraw SL, Gurr SJ (2012). Emerging fungal threats to animal, plant and ecosystem health. Nature 484(7393):186-194. *https://doi.org/10.1038/nature10947*

Fotirić Akšić M, Dabić Zagorac D, Gašić U, Tosti T, Natić M, Meland M (2022). Analysis of apple fruit (*Malus* × *domestica* Borkh.) quality attributes obtained from organic and integrated production systems. Sustainability 14(9):5300. *https://doi.org/10.3390/su14095300*

Frisvad JC, Samson RA (2004). Polyphasic taxonomy of *Penicillium* subgenus *Penicillium*. A guide to identification of food and air-borne terverticillate Penicillia and their mycotoxins. Studies in Mycology 49(1):1-74.

Gaddeyya G, Niharika PS, Bharathi P, Kumar PR (2012). Isolation and identification of soil mycoflora in different crop fields at Salur Mandal. Advances in Applied Science Research 3(4):2020-2026.

Gardes M, Bruns TD (1993). ITS primers with enhanced specificity for basidiomycetes-application to the identification of mycorrhizae and rusts. Molecular Ecology 2(2):113-8. *https://doi.org/10.1111/j.1365-294X.1993.tb00005.x*

Grammen A, Wenneker M, Van Campenhout J, Pham KT, Van Hemelrijck W, Bylemans D, Geeraerd A, Keulemans W (2019). Identification and pathogenicity assessment of Colletotrichum isolates causing bitter rot of apple fruit in Belgium. European Journal of Plant Pathology 153(1):47-63.

Holb IJ (2009). Fungal disease management in environmentally friendly apple production – A review. In: Lichtfouse E (Eds). Climate change, intercropping, pest control and beneficial microorganisms. Sustainable Agriculture Reviews, Vol 2. Springer, Dordrecht pp 219-292. *https://doi.org/10.1007/978-90-481-2716-0_10*

Khan N, Riaz A, Rahman Z, Mawa JU, Begum H (2019). Shelf life assessment of apple fruit coated with aloe vera gel and calcium chloride. Pure and Applied Biology 8(3):1876-89. *https://doi.org/10.19045/bspab.2019.80131*

Kumar S, Stecher G, Tamura K (2016). MEGA7: Molecular evolutionary genetics analysis version 7.0 for bigger datasets. Molecular Biology and Evolution 33(7):1870-1874.

Kwon JH, Kim J, Kim WI (2011). First report of *Rhizopus oryzae* as a postharvest pathogen of apple in Korea. Mycobiology 39(2):140-142. *https://doi.org/10.4489/MYCO.2011.39.2.140*

Lata R, Chowdhury S, Gond SK, White JF (2018). Induction of abiotic stress tolerance in plants by endophytic microbes. Letters in Applied Microbiology 66(4):268-276. *https://doi.org/10.1111/lam.12855*

Lee PY, Costumbrado J, Hsu CY, Kim YH (2012). Agarose gel electrophoresis for the separation of DNA fragments. Journal of Visualized Experiments 62:3923. *https://doi.org/10.3791/3923*

Liu J, Abdelfattah A, Norelli J, Burchard, E, Schena L, Droby S, Wisniewski M (2018). Apple endophytic microbiota of different rootstock/scion combinations suggests a genotype-specific influence. Microbiome 6(1):1-1. *https://doi.org/10.1186/s40168-018-0403-x*

Morris R (2015). Spectrophotometry. Current Protocols Essential Laboratory Techniques 11(1):2-11.

Muhammad A, Attiq-Ur-Rehman NK, Asghar M, Baqi A, Mohammad Hussain H (2019). Spectrophotometric determination of phenolic antioxidants in four varieties of apples (*Pyrus malus*) from Balochistan, Pakistan. Pure and Applied Biology 8(1):768-779. *https://doi.org/10.19045/bspab.2019.80019*

Nicole WG (2019). Fruit Diseases of Apple. Plant Pathology Fact Sheet. College of Agriculture, Food and Environment Cooperative Extension Service.

Noonari S, Memon MI, Wahid R, Peerzdo MB, Bhatti MA, Kalwar GY (2015). Economic analysis of apple orchards production in district Mastung Balochistan Pakistan. Economic Analysis 7(10). *https://doi.org/core.ac.uk/outputs/234626420*

Patocka J, Bhardwaj K, Klimova B, Nepovimova E, Wu Q, Landi M, Kuca K, Valis M, Wu W (2020). *Malus domestica*: A review on nutritional features, chemical composition, traditional and medicinal value. Plants 9(11):1408. *https://doi.org/10.3390/plants9111408*

Raja WH, UnNabi S, Kumawat KL, Sharma OC (2017). Pre harvest fruit drop: a severe problem in apple. Indian Farmer 4(8):609-614.

Riad EM, Ziedan ES (2015). First record of core rot disease on apple fruit cv. Anna 106 local cultivar in Egypt. Journal of Agricultural Technology 11(6):1371-1380.

Samad S (2023). Balochistan: The Fruit Basket of Pakistan. The Balochistan Point.

Sandor A (2008). Apple production, perennial crop support series Jalalabad, Afghanistan. Manual produced by Roots of Peace, USAID, Afghanistan. Plant Soil 7:12-15.

Sanzani SM, Schena L, De Cicco V, Ippolito A (2012). Early detection of *Botrytis cinerea* latent infections as a tool to improve postharvest quality of table grapes. Postharvest Biology and Technology 68:64-71.

Sanzani, SM, Reverberi M, Geisen R (2016). Mycotoxins in harvested fruits and vegetables: Insights in producing fungi, biological role, conducive conditions, and tools to manage postharvest contamination. Postharvest Biology and Technology 1(122):95-105.

Sestras RE, Sestras AF (2023). Quantitative traits of interest in apple breeding and their implications for selection. Plants 12(4):903. *https://doi.org/10.3390/plants12040903*

Shah NA, Afzal M, Ahmed M, Ahmad QB, Farooq A, Rehman FU (2011). Marketing of apple in Northern Balochistan. Sarhad Journal of Agriculture 27(4):617-24.

Shamly V, Kali A, Srirangaraj S, Umadevi S (2014). Comparison of microscopic morphology of fungi using lactophenol cotton blue (LPCB), iodine glycerol and congo red formaldehyde staining. Journal of clinical and diagnostic research 8(7):4535. *https://doi.org/10.7860/JCDR/2014/8521.4535*

Siddiqui, BN, Muhammad S, Ashraf I (2007). Awareness of plant protection measures and their adoption by apple growers in Balochistan. Pakistan Journal of Agriculture Science 44(3):259-265.

Sykes JE, Rankin SC (2014). Isolation in cell culture. Canine and Feline Infectious Diseases 2:6. *https://doi.org/10.1016/B978-1-4377-0795-3.00001-6*

Tamura K, Nei M (1993). Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. Molecular Biology and Evolution 10:512-526.

Tamura K, Stecher G, Kumar S (2021). MEGA 11: Molecular Evolutionary Genetics Analysis Version 11. Molecular Biology and Evolution *https://doi.org/10.1093/molbev/msab120*

Van der Walt L, Spotts RA, Ueckermann EA, Smit FJ, Jensen T, McLeod A (2011). The association of *Tarsonemus mites* (Acari: Heterostigmata) with different apple developmental stages and apple core rot diseases. International Journal of Acarology 1(37):71-84. *https://doi.org/10.1.1.893.9216&rep*

White TJ, Bruns T, Lee SJ, 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.

Youssef K, Roberto SR (2020). Premature apple fruit drop: associated fungal species and attempted management solutions. Horticulturae 6(2):31. *https://doi.org/10.3390/horticulturae6020031*

https://bioedit.software.informer.com/7.2/

https://blast.ncbi.nlm.nih.gov/Blast.cgi