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RESEARCH PAPER

Direct identification of fungi associated with indoor and outdoor ornamental plants by utilizing PCR assay

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A B S T R A C T:

Abstract:
Infection by phytopathogenic fungal species can result in significant losses in ornamental plants. For accurate diagnosis of plant diseases, DNA-based techniques have become dependent method. This study aimed to elaborate Polymerase chain reaction (PCR) for rapid detection of fungal pathogens causing diseases on ornamental plants. In this study, specific primers were used to screen suspected plant diseases. The results revealed that *Alternaria*, *Cylindrocladium*, *Myrothecium*, *Sclerotium*, *Cercospora*, and *Bipolaris*, were the most abundant fungi that infect indoor or outdoor ornamental plants. From which, *Alternaria alternata*, *Cylindrocladium* sp., and *Myrothecium* sp. were found to be the most frequent fungi on foliage of these plants.

KEY WORDS:
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1. INTRODUCTION:

Plant diseases, weeds, and environmental conditions are currently the most serious threats to agricultural productivity, especially in developing countries. They account for 31 to 42 percent of crop losses worldwide (Van Den Bosch et al., 2006). Agriculture losses due to plant diseases only (biotic and abiotic causes) are estimated to be roughly 35 percent of the annual production globally (Agrios, 2004). Fungi are the most important infectious pathogens in plants, causing diseases and/or toxic compounds to human and animal (Authority, 2010). Many fungal infections can survive in the soil as saprophytes feeding on dead organic debris, or as dormant spores resistant to extreme climatic conditions such as heat or dehydration (Schippers and Gams, 1979).

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Phytopathogenic fungi are an important infectious agent in plants, as more than 70% of important crop diseases caused by fungi (Agrios and Pichat, 2005); (Larrañaga et al., 2012).
Ornamental plants have worldwide importance due to their vital role in different communities. Besides having aesthetic pleasure, they enhance the environment and provide better quality to our life (Savé, 2007). During production, storage, transport, and end-consumer usage, pathogens such as fungi, bacteria, and viruses can be fatal to ornamentals as they cause diseases in ornamental crops. Fungi play a crucial role in ornamental production and maintenance and the diseases that they cause are considered worldwide threats to ornamental plants and the losses due to these detrimental biotic pathogens are very high (Shuping and Eloff, 2017).
A major problem in the identification of biotic causal agents is the inability of some infectious pathogens to grow on artificial media. Some fungi like powdery and downy mildew-causing agents require a living host to grow. Furthermore,

classical pathogen diagnostic depends on symptom observation, cultural characteristics, and microscopy, although are reliable they are laborious, time-consuming, and need experienced people (Punja et al., 2007). Therefore, minimizing the losses and make an urgent decision to cure the problem and make a proper disease control**,** need a quick diagnostic of the diseases. Additionally, in cases where the plant pathogen is difficult or impossible to grow on artificial media, alternative methods such as the use of PCR may be used for their detection (Riley et al., 2002). Rapid detection of crop pathogens are indispensable for improving any plant yield. Disease prevention and disease management programs also depend on the speed of identification of causal pathogens (Feau et al., 2018). PCR technique has been used to quickly detect, characterize, and identify a wide variety of organisms. One of the most important steps in a PCR diagnostics investigation is the production of specific PCR primers that helps to identify the pathogen to the species level (Ma and Michailides, 2007). Additionally, the molecular methods are sensitive that can detect the minimum amount of plant pathogens in host plants which are sometimes at an early stage or even at the pre-symptomatic stage of the diseases (Jafari et al., 2017). Early detection of plant pathogens is an imperative demand for any crop production in general and ornamental plants in particular. Because most ornamental plants are of high value and cannot tolerate a minimum amount of diseases and the visibility of symptoms affect the aesthetic appearance of the ornamental plant and is considered a belittled character (Skolik, 2020). This study aimed to develop a specific and sensitive identification method for fungal pathogen**s** of ornament plants.

2.MATERIALS AND METHODS

2.1Sample collection

Samples of 18 herbaceous ornamental plants were collected in Erbil city. These included indoor and outdoor ornamental plants of which some were annual plants and perennials. The plant specimens included the whole plant or a portion of the plant such as roots, stems, leaves, flowers. The samples were showing characteristic visible symptoms like spots, blights, wilts, rots, and signs in some cases. The diseased plant samples were placed in clean nylon bags and sealed**,** then kept in a cool box until the arrival at the laboratory where the bags were kept in the refrigerator for later use for description and isolation. The list of ornamental plants covered in this study is shown in table 2.1.

2.2DNA extraction of plant tissue

DNA extracted from plants showing a variety of symptoms and listed in table 2.1**.** Genomic DNA was extracted using a DNA extraction kit (BETA BAYERN /Germany) following the procedure of the manufacturer manual as following:

- Plant material ground in liquid nitrogen with a mortar and pestle, then 50mg was added to Eppendorf tube contained 20μL of proteinase k solution (20 mg/ml), and 200 μL of Lysis solution. Then mixed by vortexing, and incubated at 65 °C for 30 min with continuous inverting every 5 minutes.
- 100 μL of precipitation solution was added to the solution, mix well by gentle inverting, and incubated for 3 minutes at 4 °C.
- The tubes were centrifuged for 5 minutes at 13,000 rpm then 250 μL of the clear lyses (supernatant) were transferred to new 1.5 ml micro-centrifuge tubes and the sediments are discarded.
- 200 μL of the binding solution was added then vortexed for 15 seconds until thorough mixing.
- 200 μl of absolute ethanol (99%) was added and pulse-vortexed for 15 seconds. Then, using a 2 ml collection tube, the Lysate transfer to the upper reservoir of the spine column without wetting the rim and then centrifuged at 13,000 rpm for 1 minute.
- Washing solution 1 (500 μL) was added to the spin column then centrifuged at 13,000 rpm for 1 minute with the collection tube attached.
- 500 μL of washing solution 2 was added to the spin column and followed centrifuging at 13,000 rpm for 1 min with the collection tube attached then followed a second round of centrifuging at the same speed and time to dry the spine column.
- The spine columns were transferred to the new 1.5 ml microcentrifuge tube and 100 μL of elution solution was added to the spine column with a micro-centrifuge tube and left to stand for at least 1 min.
- Genomic DNA was eluted by centrifugation at 13,000 rpm for 1 min.

Table 2.1 Indoor and outdoor ornamental plants included in the study.

No.	Common name	Scientific name	Place of collection
1	Rosa	*Rose*	Zhian- outdoor
2	Yucca elephanties	*Yucca gigantea*	Girdarasha- indoor
3	Areca palm	*Dypsis lutescens*	Zanko- indoor
4	Areca palm	*Dypsis lutescens*	Zanko- indoor
5	Weeping fig, benjamin fig	*Ficus benjamina*	Zanko- indoor
6	Swiss cheese plant	*Monstera deliciosa*	Zanko- outdoor
7	Spider plant	*Dracaena reflexa*	Grdarasha- indoor
8	Laceleaf	*Anthurium andraeanum*	Roshnberi- indoor
9	Peace lily	*Spathiphyllum wallisii*	Zanko- indoor
10	elephant ears	*Alocasia*	Zanko- indoor
11	Dwarf umbrella	*Schefflera arboricola*	Zanko- indoor
12	Ficus 'Flash'	*Ficus microcarpa hillii*	Shaneder-outdoor
13	Rubber fig	*Ficus elastic*	Ronaki- outdoor
14	Mexican grass tree	*Dasylirion longissimum*	Ronaki- outdoor
15	Nippon lily, sacred lily, and Japanese sacred lily	*Rohdea japonica*	Ronaki- outdoor
16	golden pothos	*Epipremnum aureum*	Hewlere nwe
17	Silhouette Plant, Madagascar Dragon Tree	*Dracaena reflexa*	Rasty- indoor
18	ZZ Plant, Aroid Palm	*Zamioculcas zamiifolia*	Zhian- indoor

2.3DNA amplification

Genus-specific and species-primers were used to detect the associated fungi on ornamental plants as shown in Table (2.1). Primers were either from published papers or were designed, using primer 3 online program, from published gene sequences of fungi common on ornamental plants. These genes are found on National Center for Biotechnology Information (NCBI) and their accession numbers listed in Table (2.2), are used as reference sequences to design primers to amplify the respective genes. DNA was amplified using a thermocycler (MJ RESEARCH/ Germany) in 25 µl (reaction volume) in PCR tubes using PCR master mix (AMPLIQON / Germany). The master mix was composed of: 12.5 µl of 2x PCR master mix, 1.5 µl of each forward primer, 1.5 µl of each reverse primer, 3 µl of DNA template and 6.5 µl DNase free Water to complete the volume to 25 µl.

PCR was performed under the following conditions: initial preheat for 3 min at 95ºC, followed by 35 cycles at 95ºC for 15 seconds, annealing temperature was ranged between 55-62ºC for 30 seconds followed by final amplification step 72ºC for 15 min (Fang and Ramasamy, 2015).

Table 2.2 primers used for PCR identification of fungi from ornamental plants.

	Primer	Target fungus	Primer sequence	Size (bp)	Source
1	Aa-F Aa-R	*Alternaria* sp.	5-TTTGGGCGGTAAGATAAAGG-3 5-CACCCAAACACTCGCATAGA-3	183	Designed from, GenBank: KX228346.1
2	BC-F BC-R	*Botrytis cinerea*	5-ACCCGCACCTAATTCGTCAAC-3 5-GGGTCTTCGATACGGGAGAA-3	604	(Rigotti et al., 2006)
3	Bp-F Bp-R	*Bipolaris* sp.	5-TTTTGCGCACTTGTTGTTTC-3 5-ATTTCGCTGCGTTCTTCATC-3	165	Designed from ITS region, GenBank: NR-147489.1
4	Cc-F Cc-R	*Cylindricladium clavatum*	5-TGCTGCTCTAAATGGGAGGT-3 5-GATGATCAACCAAGCCCAAG-3	172	From LSU rRNA gene, GenBank: MH872158.1
5	Cs-F Cs-R	*Cercospora* sp.	5-ACCTTGATGCAGAGGAGGAA-3 5-GGTTGTGATCAGGGAAAGGA-3	230	DNA-designed from, GenBank: MH511937.1
6	Mth-F Mth-R	*Myrothecium roridum*	5-CCCAAACCCTTTGTGAACCT-3 5-ATTTCGCTGCGTTCTTCATC-3	210	Designed from ITS 1, 5.8S, GenBank: MN856232.1
7	Sr-F Sr-R	*Sclerotium rolfsii*	5-TTTGTCAAGGCTTGGATGTG-3 5-GCGGGTAGTCCTACCTGATTT-3	228	Designed from ITS1, 5.8S, GenBank: MT560347.1
8	Pi1s1-F Pi2A-1-R	*Phytophthora infestans*	5-GCGTTGGGACTCCGGTCTGAGC-3 5-CGCAAGACACTTCACATCTGGG-3	350	(Jyan et al., 2002)
9	PuF PuR	*Pythium ultimum*	5-ATGATGGACTAGCTGATGAA-3 5-TTCCATTACACTTCATAGAA-3	407	(Kernaghan et al., 2008)

2.4Agarose gel Preparation

Agarose gels were prepared (1%) by suspending 1 g agarose powder in 1X Tris-Borate-EDTA(TBE) and heated in a microwave oven to dissolve it. The solution was mixed with Fluro Safe dye (Fisher Scientific USA) after cooling down to 60°C. after thorough mixing, the gel was poured into a plastic frame with inserting a comb to create wells. Loading dye (Bromophenol blue) was added to the DNA sample and then the wells were loaded with 5 µl of each template. The electrophoresis was carried out at 100V for 45 minutes until the dye has moved halfway between the wells and the gel's end and then photographed under ultraviolet (UV) illumination.

3.RESULTS

Direct fungal detection, using PCR, was achieved for 18 suspected diseased ornamental plant samples (Table 2.1). For this purpose, 9 species-specific primer pairs were used for screening the samples. The results of screening plant samples with primers specific for *Alternaria* detection revealed that all plant samples showing visible symptoms were affected with the fungus *Alternaria alternata* (figure 3.1). The figure shows positive reaction with a size of the bands of 183 base pairs (bp) and the strength of the bands across the ladder.

The results also showed a positive reaction (165 bp) to the specific primer for *Bipolaris* sp. In which the fungus was detected in 12 samples out of 18 which are *Yucca gigantea*, *Dypsis lutescens*, *Monstera deliciosa*, *Dracaena reflexa*, *Spathiphyllum wallisii*, *Alocasia amazonica*, *Schefflera arboricola*, *Ficus microcarpa hillii*, *Ficus elastica*, *Epipremnum aureum*, *Compacta dragon*, and *Zamioculcas zamiifolia*. However, the remaining samples were showed a negative reaction in which no bands were observed (fig 3.2.).

The fungus *Cylindrocladium clavatum* (*Calonectria clavata*) was detected on 17 samples out of 18 ornamental plants, using the specific primers for detection of the fungus (fig 3.3). The agarose gel showing PCR product (172 bp) for *Rosa*, *Yucca gigantea*, *Dypsis lutescens*, *Spathiphyllum wallisii*, *Ficus benjamina*, *Monstera deliciosa*, *Dracaena reflexa*, *Anthurium andraeanum*, *Spathiphyllum wallisii*, *Alocasia amazonica*, *Schefflera arboricola*, *Ficus elastica*, *Dasylirion longissimum*, *Epipremnum aureum*), but had a negative reaction towards *Ficus microcarpa hillii*.

The molecular identification using PCR for detection of *Cercospora* sp. was showed that 6 samples out of 18 were infected with the fungus, with the PCR product of 230 bp, (Figure 3.4). The infected ornamental plants were *Rose*, *Ficus benjamina*, *Dracaena reflexa*, *Ficus microcarpa hillii*, *Epipremnum aureum*, *Zamioculcas zamiifolia*.

The use of specific primers detected *Myrothecium roridum* in 14 samples out of 18 ornamental plants were collected for identification of fungal diseases. The infected plants were (Yucca *gigantea*, *Dypsis lutescens*, *Monstera deliciosa*, *Dracaena reflexa*, *Anthurium andraeanum*, *Spathiphyllum wallisii*, *Alocasia amazonica*, *Schefflera arboricola*, *Ficus microcarpa hillii*, *Ficus elastica*, *Dasylirion longissimum*, *Rohdea japonica*, *Epipremnum aureum*, *Zamioculcas zamiifolia*) and the four samples showing negative reaction were *Rose*, *Dipsis lutescens*, *Ficus benjamina*, and *Dracaena reflexa*. The size of PCR products (210 bp) is shown in fig (3.5).

The specific primer used to detect the fungus *Sclerotium rolfsi* in the samples, and the results showed that 11 samples out of 18 were positive. The infected plants were (*Dypsis lutescens*, *Dracaena reflexa*, *Spathiphyllum wallisii*, *Schefflera arboricola*, *Ficus microcarpa hillii*, *Ficus elastica*, *Dasylirion longissimum*, *Rohdea japonica*, *Epipremnum aureum*, *Zamioculcas zamiifolia*). A band size of 228 bp was observed across the DNA ladder (figure 3.6). However, other samples (*Rose*, *Yucca gigantea*, *Dypsis lutescens*, *Ficus benjamina*, *Monstera deliciosa*, *Anthurium andraeanum*, *Dracaena reflexa*), were clear of the fungus at the time of diagnosis.

Using the specific primers for identification of *Botrytis cinerea*, *Phytophthora*, and *Pythium*, no reactions and no bands were observed across the ladder for these fungi.

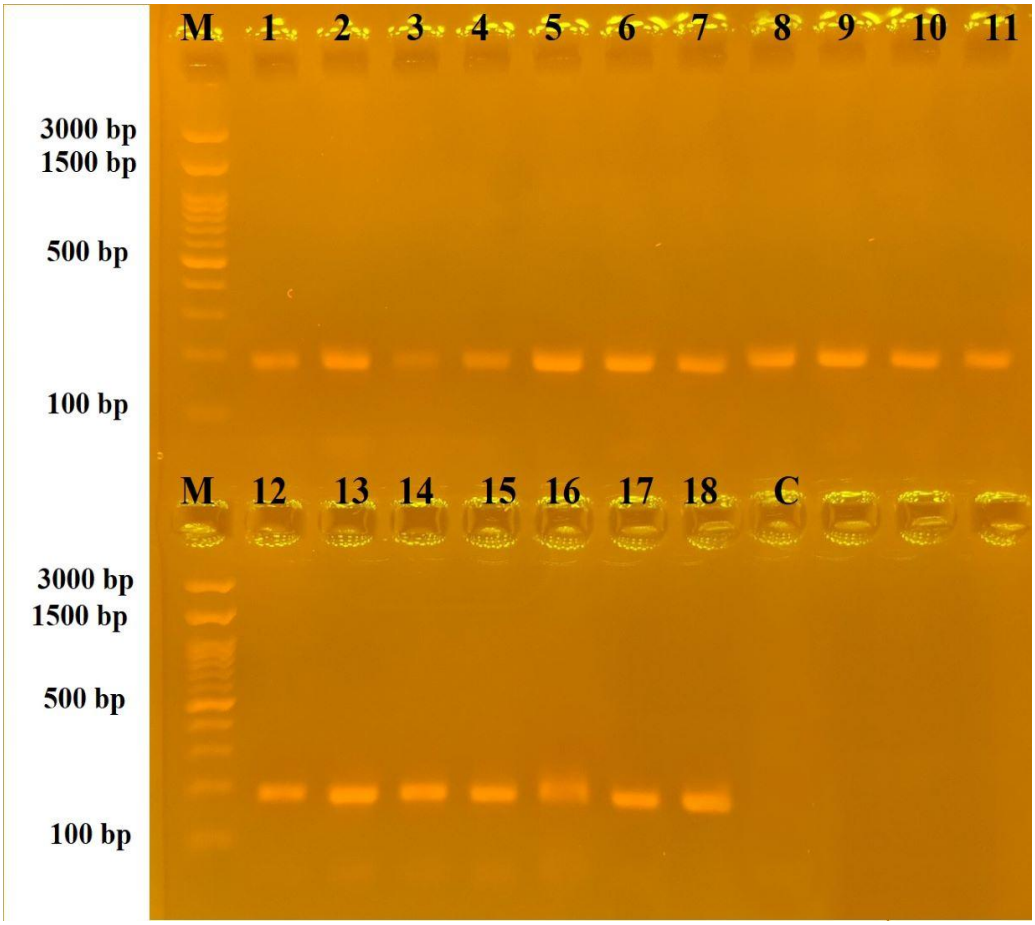

Figure 3.1: Extracted DNA of 18 plant samples were amplified with primer pairs Aa-F and Aa-R, the specific primer for detection of *Ailternaria* spp., where: M: molecular size marker, 1-18 are plant samples, and C is the no template control. Bands of 183 bp represent positive reactions.

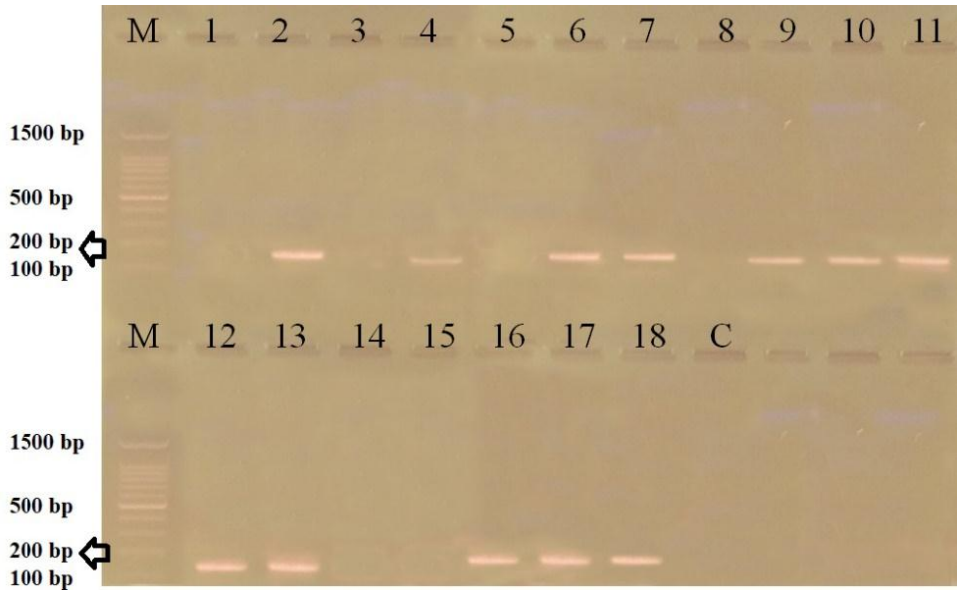

Fig.3 .2 Polymerase chain reaction amplification products using specific primer for *Bipolaris* sp. Where samples 2, 4, 6, 7, 9, 10, 11, 12, 13, 16, 17, and 18 were showed positive reaction and 1, 3, 5, 8, 14, and 15 were negative, and C is the negative control.

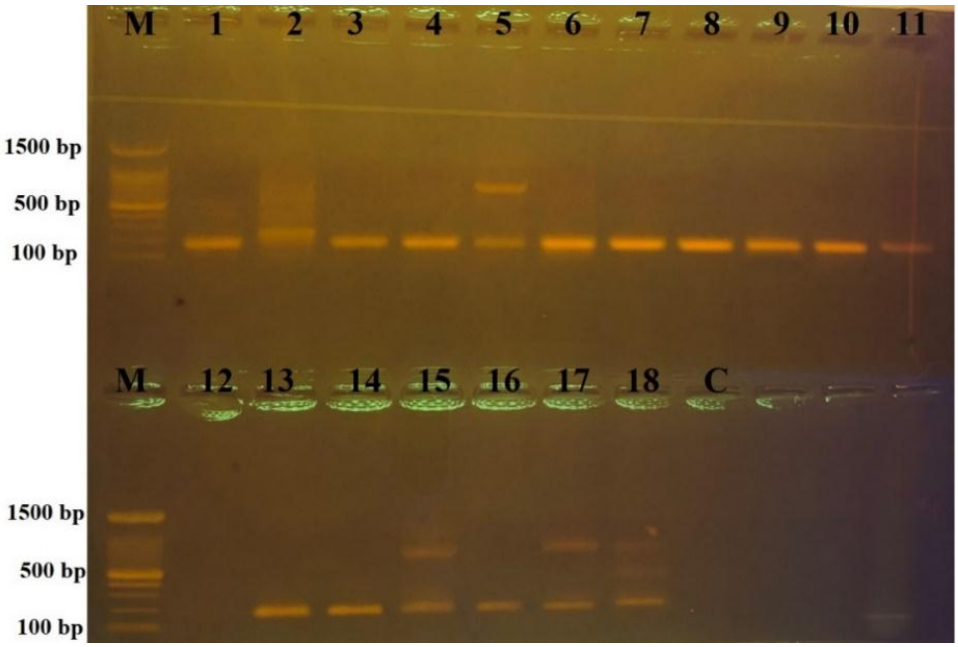

Figure (3.3) Polymerase chain reaction amplification products using specific primer for *Cylindrocladium clavatum* (*Calonectria clavata*), the positive reaction of 172 bp was detected for 17 samples out of 18 ornamental plants.

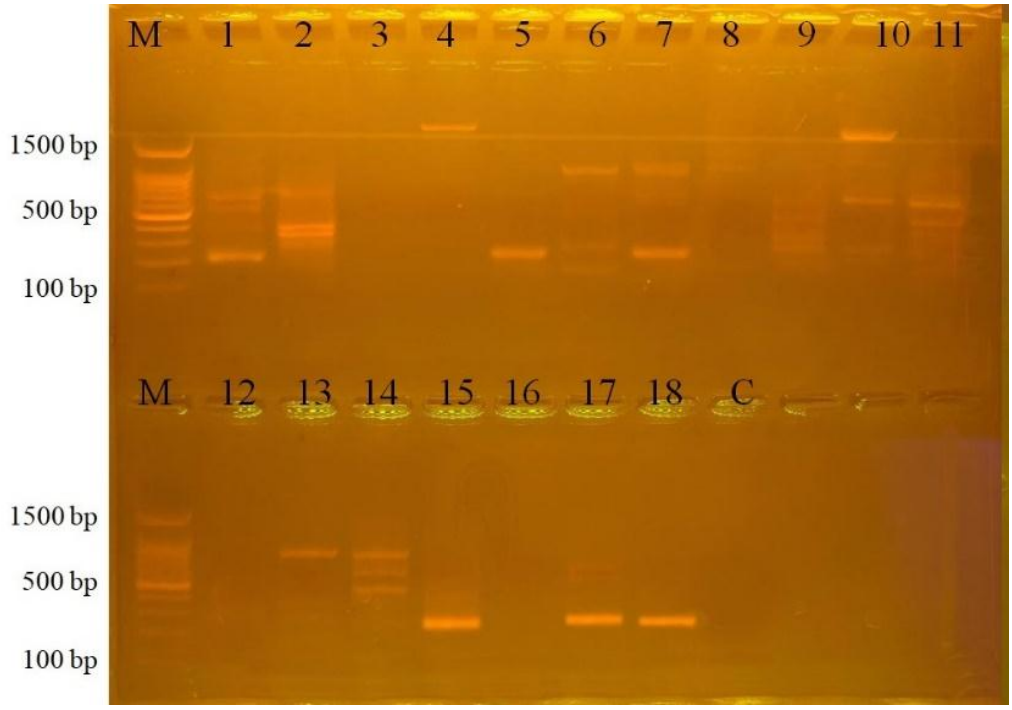

Figure (3.4) Polymerase chain reaction amplification products using specific primer for *Cercospora* sp., 6 samples out of 18 (1, 5,7, 15, 17, and 18) were infected with the fungus, the 230 bp product is shown across the DNA marker.

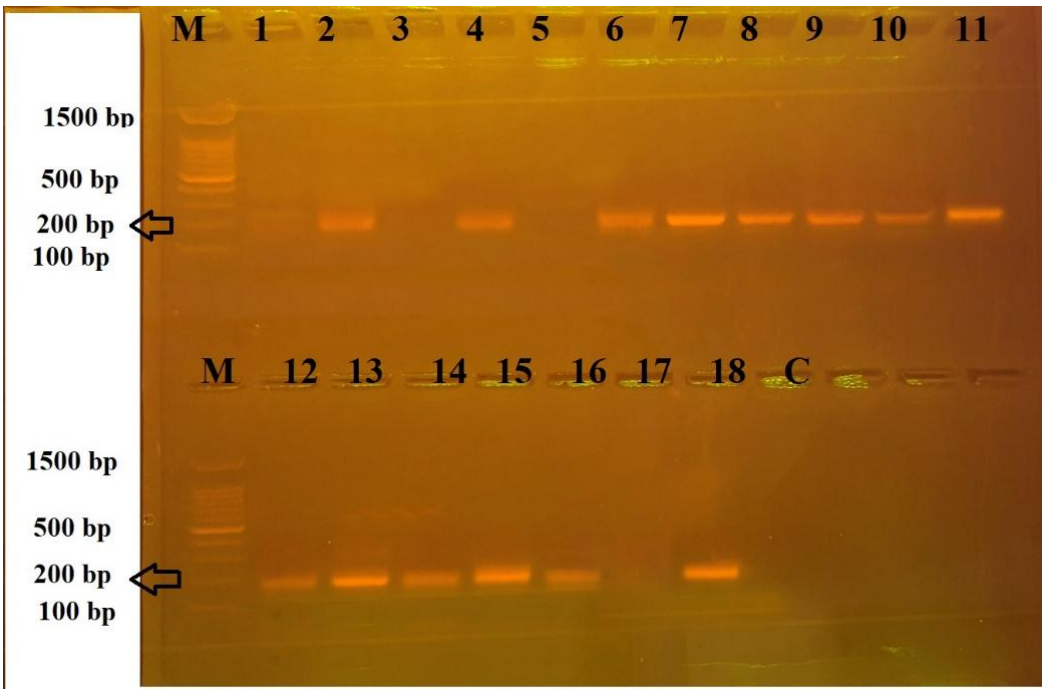

Fig (3.5) Polymerase chain reaction amplification products using specific primer for *Myrothecium roridum* on 18 samples. The reactions were positive on 14 suspected samples with showing a band of 210 bp across the DNA marker.

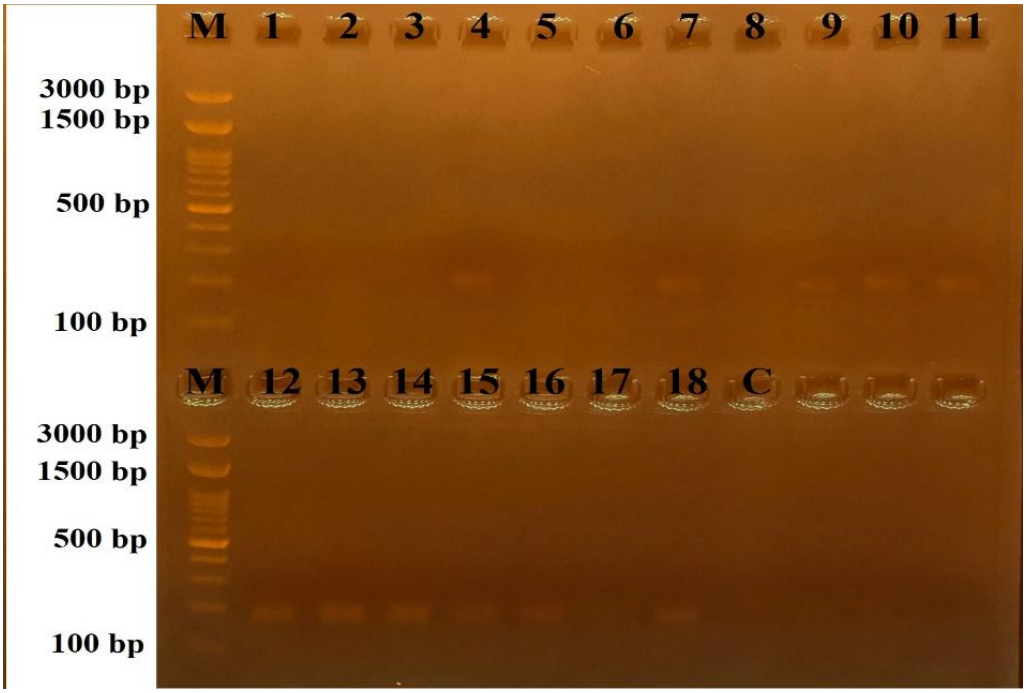

Fig 3.6 Polymerase chain reaction amplification products using specific primers for detection of *Sclerotium rolfsi* in 18 samples. Bands (228 bp) across the DNA marker represent a positive reaction to 11 samples (4, 7, 9, 10, 11, 12, 13, 14, 15, 16, and 18).

4.DISCUSSION

Early detection of plant diseases can ease the control of diseases through appropriate management strategies. Polymerase chain reaction (PCR) as a molecular technique is used for the identification of plant diseases that require detailed sampling and processing procedures. Direct pathogen detection from the suspected plants by PCR can, therefore, reduce losses, time and ultimately saving the crop. Molecular technique was used by McCuiston et al. (2007) to detect the nematode *Aphelenchoides fragariae* directly in host plant tissues using the species-specific primers. It was also used to detect phytoplasmas, the phloem-limited plant pathogenic prokaryotes in plants (Gundersen and Lee, 1996), for viruses and viroid infecting ornamental plants (Bostan et al., 2004, Boonham et al., 2002, Pallás et al., 2018), to detect bacteria (Jock et al., 2000), Oomycetes in ornamental nursery stock (Puertolas et al., 2021), and many fungal pathogens from ornamental plants (Matić et al., 2019, Hariharan and Prasannath, 2021, Luchi et al., 2020).

Similar to our results, other researchers found that *Alternaria*, *Myrothecium*, *Sclerotium*, *Cylindrocladium*, *Cercospora* and *Bipolaris*, have a wide host range of leafy ornamental plants (Chase, 1997, Matić et al., 2020, Mmbaga et al., 2010, Billah, 2017, Hirooka et al., 2008, Mmbaga et al., 2015b). *Alternaria alternata* is particularly important on foliage ornamental plants and the results of the current study, using PCR directly from samples, confirmed that all 18 tested samples were infected with this facultative pathogen. Molecular as an accurate and rapid tool was also used by other researchers to diagnose diseases caused by *Alternaria*. It was used to detect the pathogen on Lilac (*Syringa* spp.) (Shi et al., 2005), and on buttonwood plants (*Conocarpus erectus* L.) (Abbas et al., 2021). *Cylindrocladium* and *Myrothecium* on the other hand were also common on leafy ornamental plants tested with showing positive reactions of 17 and 14 out of 18, respectively. *Bipolaris* the cause of Helminthosporium spots was also found by (Chase, 1997) on ornamental plants like *Crysalidocarpus*, *Aechmea*, *Cereus*, *Rhipsalidopsis*, *Calathea*, and *Ficus lyrata*. This necessitates rapid disease detection and controls the disease to minimize the losses. In the current study, we managed to detect *Bipolaris* on 12 samples out of 18. *Sclerotium rolfsi*, which causes southern blight disease, was detected directly with the aid of PCR on 11 samples out of 18. This fungus is common on several plant hosts including ornamental plants. Stutz et al. (2019) utilized

specific primers to diagnose the disease on ZZ plant (*Zamioculcas zamiifolia*) as it caused soft rot on the stems of the plant. The results showed that infections by *Cercospora*, compared to other fungi, was less frequent on ornamental plants tested. Other research indicates that the fungus can infect some ornamental plants. *Cercospora violae* was found on sweet or garden violet (*Viola odorata* L.) (Trkulja et al., 2015), isolated on leaf spots of *Hydrangea macrophylla* (Mmbaga et al., 2015a), and detected, using PCR, on the black-bindweed (*Fallopia convolvulus*) a fast-growing annual flowering plant (Bakhshi et al., 2012).

5.CONCLUSIONS

It can be concluded from the results of the current study that *Alternaria*, *Myrothecium*, *Sclerotium*, *Cylindrocladium*, *Cercospora* and *Bipolaris*, were the most prevalent fungi on the plants screened in this study. The polymerase chain reaction is a fast and accurate method to detect these suspected fungal pathogens early before worsening the situation of the infected plants and by which appropriate control methods can be achieved.

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