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DETECTION AND IDENTIFICATION OF MYCOBIOTA ASSOCIATED WITH RICE IN THREE DISTRICTS OF IRAQ

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A B S T R A C T

The reduction of yield, quality, and nutritional value of grain cereals by different fungal species is of great concern around the world. A comprehensive investigation was made on fungal mycobiota in rice from three provinces; Baghdad, Al-Najaf and Al-Qadisiyah of Iraq surveyed during the growing season 2014-2015. Fields were selected due to their location near the Euphrates and Tigris Rivers. Mycological analysis indicated that there were a difference rates in disease infection due to various fungal species. Predominant fungal species identified as *Bipolaris spicifera*, *Curvularia lunata*, *Fusarium* spp., *Nigrospora oryzae*, *Exserohilum rostratum*, *Alternaria* spp. and *Thanatephorus cucumeris*. Confirmation of the associated fungal species was further accomplished by analysis of their ribosomal DNA and internal transcribed spacer region sequences. The present study provides a detailed description of the associated fungal species, which may help in understanding the population dynamics and in developing effective control measures.

Keywords: Rice, Fungal mucobiota, Detection and Survey, Rice Diseases, Iraq.

INTRODUCTION

Rice (*Oryza sativa* L.) is considered as one of the oldest and most important source of carbohydrate and staple foods for the increasing world population. People in many countries including Bangladesh, Cambodia, Myanmar and Vietnam rely on rice for 70% of their daily calories (Zhang, 2007; Sheehy *et al*., 2008).

In the Iraqi context, land allocation for rice farming is second to only wheat which has the largest land space, and consider one of the main summer cereal crops (Kosai and Ali, 2010). It is the second most economically important crop by cultivated area. Rice is grown during the summer season in the Mesopotamia (Southern Iraq), between Euphrates and Tigris Rivers from June to end of November, rice planting inside the fields by using flood irrigation.

The U.S. Department of Agriculture (USDA) estimates Iraq's market year (MY) 2015/2016 rice production (crop year 2014-2015) at 110,000 metric tons, down 59 percent from 267,000 tons in 2013-2014. Harvested area is estimated at 48,000 hectares, down 50 percent, with rough rice yield at 3.44 tons per hectare, a decrease of 17.7percent from 2013-14. Currently, Iraqi imports rice from USA, Uruguay, Argentine, Brazil and India due to insufficient yield to meet demand.

Food price of Rice has been increasing and this too has been contributing to another 100 million people going hungry worldwide (Khush,2005; Sheehy et al.,2008; Skamnioti and Gurr,2009). This indicates incompatibility between human needs, income and the production of food to feed the increasing world population. The population density of the world will increase to 9.2 billion by 2050 and out of this about 86 % of these people are from undeveloped countries (Oerke and Dehne,2004; Skamnioti and Gurr,2009). This presents a significant task for scientists, breeders, regulators and farmers who have to ensure that progress in made to close the yield gap in existence now in the country.

Rice is subjected to many diseases such as rice blast caused by *Curvularia lunata* (Kamaluddeen and Abhilasha, 2013), *Pyricularia oryzae* (Brown leaf spot),

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Fusarium spp. (Khan and Sinha, 2005; Nur Ain Izzati *et al*., 2005), *Thanatephorus cucumeris* causes sheath blight disease (Khosravi *et al*., 2011), *Bipolaris spicifera* (Shabana *et al*., 2008, Manamgoda *et al*., 2014), *Alternaria* spp. (Schwarz *et al*., 2012), *Exserohilum rostratum* (Sami, 2006; Sharma *et al*., 2014), *Helminthosporium oryzae* (Vidhyasekaran *et al*., 1991), and bacterial blight caused by the causative agent *Xanthomonas oryzae*. In addition to these devastating diseases which are economically important, there are many other diseases such as rice tungro, rice spot, rice scald, rice smut, rice rot and rice dwarfing disease that cause losses in various regions of the world (Inger Genetic Resources 1996). Numerous fungal, bacterial, virul, and nematode species are responsible for these diseases (Mostapha, 2004; Agrios, 2005; Manandhar and Yami, 2008; Agrawal and Kootasthane, 2009). The degree of devastation caused by each of these diseases is different in different rice growing countries.

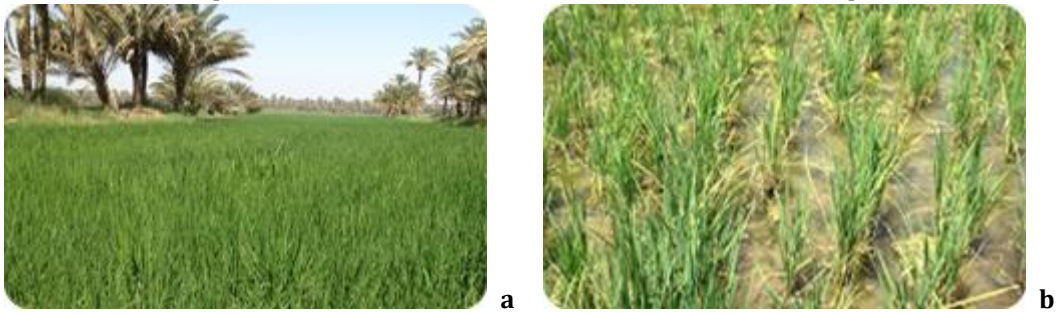
Plant pathogens in general and especially soil borne pathogens are difficult to control due to their adaptation to their ecological niche and their ability to impact a range of host species as they maintain a high rate of survival under environmental pressure conditions through the production of resistant spores propagules such as spores, chlamydospores and sclerotia (Vincent and Cochrane, 1971; Thabet, 2008).. The pathogens will only be further increased if the diseases and pests left unchecked and uncontrolled as these diseases and pests are able to reduce Rice yield between 50-70 % when they strike certain farms (Karthikeyan and Gnanamanickam, 2008).

Present study is aimed at detection of different pathogenic fungal species associated with infected rice plants in the fields from three provinces of Iraqi using different detection methods.

MATERIALS AND METHODS

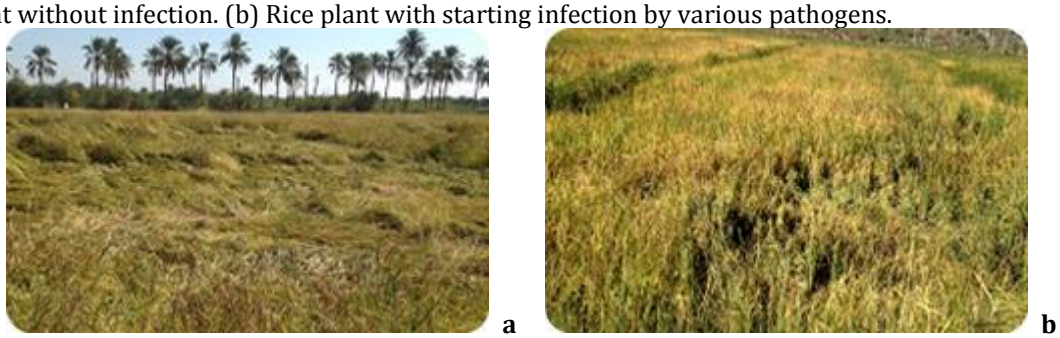
Isolation of Causal Organisms from Rice Plant: Samples were collected during the year, 2014 from infected rice plants in three Provinces (Baghdad, Al-Najaf and Al-Qadisiyah). 50 replicate of each collection were made from each province. The first collection was done in the end of July and the second collection was taken in the end of October.

Infected plant tissues such as; root, stem, leaves and panicle were used to recover fungal pathogens on Potato Dextrose Agar (PDA) at pH; 5.5 and supplemented with antibiotics (100 µg mL^{-1} of streptomycin or rose bengal), and incubated at 28±2 °C. The growth patterns of the isolated fungal species were observed for 5-7 days. Pure cultures of mycelium and spores of the fungi were examined macroscopically and microscopically (Micros, Austria, MC 400, Deutsch. English) at 100x magnification. All the fungi were maintained on PDA media, besides their identities verified using ITS-rDNA analysis. Preliminary screening of rice pathogens identified 23 fungi as the candidate to infection rice plants.



a **b**

Figure 1. Rice plant under field conditions (after two months of planting).
(a) Rice plant without infection. (b) Rice plant with starting infection by various pathogens.



a **b**

Figure 2. Rice plant under field conditions (In the end of season).
(a) and (b) Rice plant with high infection by various pathogens.

Fungal Growth of Pathogens and DNA Extraction: For extraction of fungal genomic DNA, all pure fungal isolates were cultured in potato dextrose broth (PDB) media. 100 mL of PDB was inoculated with 10 mm mycelia plug of PDA in 250 mL flask and incubated at (28 ± 2^0C) for 5 days in rotary shaker at 150 rotations per min (rpm). CTAB method modified from Murray and Thompson (1980) protocol was used for extraction of the genomic rDNA of all isolates. 100mg of freshly growing fungal mycelium was finely harvested from PDB media and 1ml of preheated CTAB buffer, 2 µL of mercaptoethanol and 1µL of RNase were added. Mycelium was macerated and was taken in 2.0 ml micro centrifuge tube, vortexed and incubated in water bath at 65^0C for 1 hour, the contents were mixed by inverting the tubes for each 15 minutes followed by keeping for 5 minutes at room temperature, and centrifuged at 13,000 rpm. Equal volume 500µL of chloroform: isoamyl alcohol (24:1) was added and the process was repeated twice to avoid proteins. The aqueous layer was transferred to a fresh centrifuge tube and 800µl of isopropanol was added and incubated overnight at -20^0C. The pellet was washed with 1 mL of CTAB washing buffer incubated for 30 minutes and pellet was dried completely for 2-3 hours and dispensed with ddH_2O to rehydrate the genomic DNA. The concentration of DNA was visualized on 1% agarose gel, and then stored at -20 °C.

Agarose Gel Electrophoresis of Products: The concentration of total DNA was measured spectrophotometerically using NanoDrop (Nanospec cube, Germany). The quality and quantity of DNA and PCR products was determined by electrophoresis on 1.0 % (w/v) agarose gel (Vivantis, USA) in 1 X TAE (Tris hydroxymethyl Aminomethane-acetate-ethylenediamin-etetraacetic acid) buffer, and stained with 4 µL of ethidium bromide (EtBr) (0.5 µg mL^{-1}) solution. The gel was electrophoresed at 85 V for ~45 minutes to separate fragments between 300-650bp. Each sample was mixed with loading dye (6 X Ficoll dye) and loaded into the wells. A molecular weight ladder (0.1 µg $µL^{-1}$, AL-Musayyib Bridge Corporation, korea) was also included into one of the wells to enable size determination and visual quantification. The DNA bands in the gel were visualized under UV-Transilluminator (Cleaver Scientific, Ltd, UK) with camera Canon. The fragment size was determined by comparing with the molecular weight markers ladder (100bp DNA ladder, AL-Musayyib Bridge).

Amplification of rDNA Internal Transcribed Spacers (ITS) of Rice Pathogens: The ribosomal DNA (rDNA) of internal transcribed spacer region was amplified using a pair of specific primers (ITS-1 and ITS-4), custom synthesized at AL-Musayyib bridge and supplied as lyophilized product of desalted oligos. The forward and reverse primer pair used in this study are ITS1 -F-5'TCTGTAGGTGAACCTGCGG3' and ITS4-R-5'TCCTCCGCTTATTGATATGC3' (White *et al*., 1990; Chakraborty *et al*., 2010). The gene sequence of ITS was pasted into Primer 3 program, and primer pairs were recommended. Annealing temperature was set between 50-60 ºC and the GC content was set to between 40-60 % to increase annealing ability of the primer (Kalendar, 2006). The reactions were performed in a 25µL tubes with Taq promega (USA) under the following thermal cycling conditions: 94ºC initial denaturation cycle for 4 minutes followed by 35 cycles of 94ºC for 1 minutes, 50-60ºC for 1 minutes and 72ºC for 2 minutes, and then a final extension at 72ºC for 10 minutes. The PCR products were checked on a 1.0 % agarose gel to determine if the amplified product matched the predicted size of amplified product according to the Primer 3 Programme used.

RESULTS AND DISCUSSION

Microscopic Evaluation of Rice Pathogens: The results of the microscopic analysis identified the twenty-three (23) fungal isolates from three locations and the results are furnished in table 1. These isolates were identified as *B. spicifera* (*H. oryzea*), *C. lunata*, *Fusarium* spp., *E. rostratum*, *N. oryzae, T.cucumeris* and *Alternaria* spp. Figure (3-25). The lesions attributed by these pathogens infections were clear on the culm, culm nodes, panicle, and neck node, where the inner nodal infection of the culm occurs in a banded pattern.

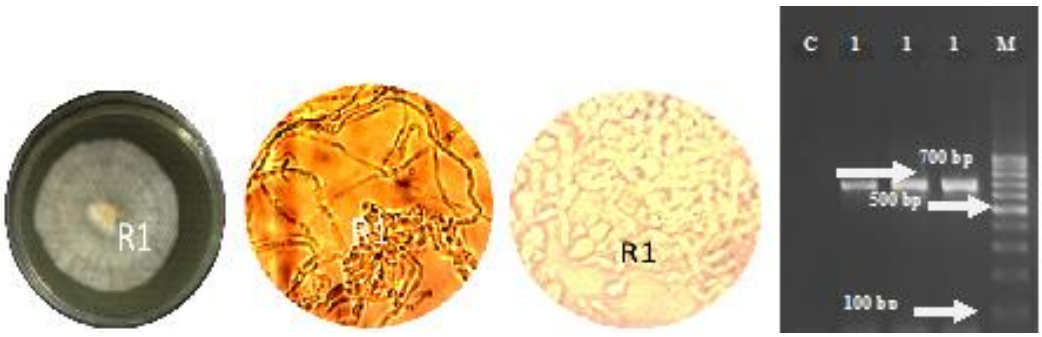
PCR Amplification of ITS-rDNA Sequences of Rice Pathogens: The amplified products of the ITS-rDNA region of all 23 fungi confirmed their identity. The PCR products obtained with the specific ITS primers used are presented in Figure 3-25. Since the same ITS primers were used for all the amplification.

Sheath blight by *Thanatephorus cucumeris* ((Frank) Donk), (anamorph: *Rhizoctonia solani* Kühn) fungus which is the most important disease influencing rice production in many rice-growing countries in the world. The fungus attack the plant in any stage from seedlings, to late developmental stage and can survives in the soil and attack roots and lower stems of plants.

This pathogen also attacks numerous vegetable plants such as potatoes, legumes, soybeans, sorghum, barley, cotton,wheat and corn (Vijayan and Nair, 1985; Justesen *et al*., 2003; Najeeb *et al*., 2008; Khosravi *et al*., 2011). In this study we identified 7 isolates of *T. cucumeris* (teleomorph) from three different locations as the causative agent on rice plant as we can see from Figure 3 to 8.

Table 1. Fungal isolates used in this study according to host plant and geographic origin.

Fungal Isolates		Host Plant	Geographic origin
June*	October*		
-	*T.cucumeris* R1	Rice	Tewatha / Baghdad
T.cucumeris R2	-	Rice	Al-Mhanaweia/ Al-Qadisiah
-	*T. cucumeris* R4	Rice	Mashkhab / Al-Najaf
-	*T.cucumeris* R10	Rice	Tewatha / Baghdad
-	*T.cucumeris* R12	Rice	Mashkhab / Al-Najaf
T.cucumeris R14	-	Rice	Al-Mhanaweia/ Al-Qadisiah
-	*F.solani* R3	Rice	Mashkhab / Al-Najaf
F.oxysporum R5	-	Rice	Al-Mhanaweia/ Al-Qadisiah
-	*F. oxysporum* R6	Rice	Mashkhab / Al-Najaf
-	*F.solani* R8	Rice	Mashkhab / Al-Najaf
F.solani R11	-	Rice	Al-Mhanaweia/ Al-Qadisiah
-	*F.solani* R13	Rice	Mashkhab / Al-Najaf
-	*F.solani* R16	Rice	Mashkhab / Al-Najaf
F.verticillioides R17	-	Rice	Al-Mhanaweia/ Al-Qadisiah
-	*N. oryzae* R9	Rice	Mashkhab / Al-Najaf
-	*C. lunata* R7	Rice	Mashkhab / Al-Najaf
C.lunata R21	-	Rice	Al-Mhanaweia/ Al-Qadisiah
B. spicifera R15	-	Rice	Al-Mhanaweia/ Al-Qadisiah
-	*E.rostratum* R19	Rice	Mashkhab / Al-Najaf
A.alternata R18	-	Rice	Tewatha / Baghdad
-	*A. alternate* R20	Rice	Mashkhab / Al-Najaf
A.tenuissima R23	-	Rice	Al-Mhanaweia/ Al-Qadisiah
-	*A. tenuissima* R24	Rice	Mashkhab / Al-Najaf



a b c d

Figure 3. Observation of Rice pathogen under microscope. (a) *Thanatephorus cucumeris* R1: teleomorph [(A.B. Frank) Donk] Alternate State of (Anamorph: *Rhizoctonia sola ni* Kühn). (b and c) Branches, Basidia and basidiospore. (d) 100bp Ladder (AL-Musayyib bridge). (e) Agarose gel electrophoretic (1%) profile of DNA genomic extraction and amplification of ~700bp PCR fragment. Lanes 1 -1: Genomic DNA; Lane M: Ladder 100bp (AL-Musayyib bridge); Lane C: Control.

a b c d

Figure 4. Observation of Rice pathogen under microscope. (a) *Thanatephorus cucumeris* R2: teleomorph [(A.B. Frank) Donk] Alternate State of (Anamorph: *Rhizoctonia solani* Kühn).(b and c) Branches, Basidia and basidiospore. (d) Agarose gel electrophoretic (1%) profile of DNA genomic extraction and amplification of ~550bp PCR fragment. Lanes 2-2: Genomic DNA; Lane M: Ladder 100bp (AL-Musayyib bridge); Lane C: Control.

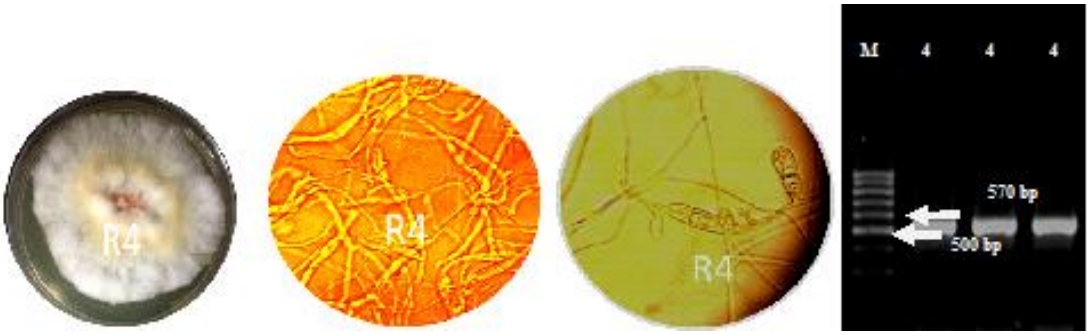


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Figure 5. Observation of Rice pathogen under microscope. (a) *Thanatephorus cucumeris* R4. (b and c) Branches, Basidia and basidiospore. (d) Agarose gel electrophoretic (1%) profile of DNA genomic extraction and amplification of ~570bp PCR fragment. Lanes 4-4: Genomic DNA; Lane M: Ladder 100bp (AL-Musayyib bridge); Lane C: Control.

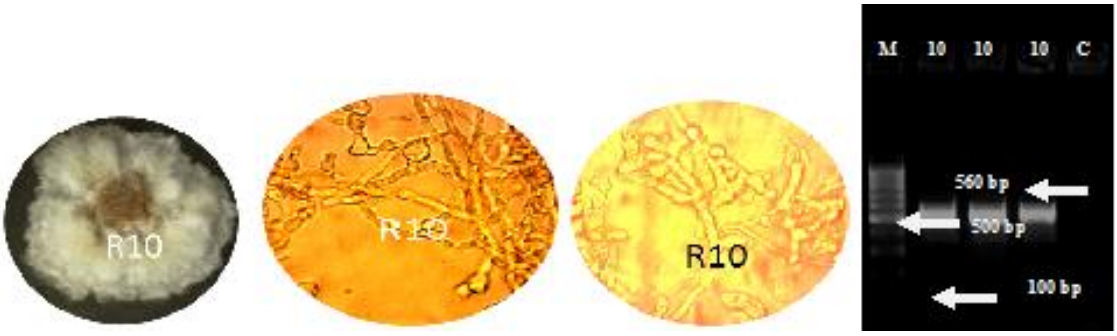

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Figure 6. Observation of Rice pathogen under microscope. (a) *Thanatephorus cucumeris* R10. (b and c) Branches, Basidia and basidiospore. (d) Agarose gel electrophoretic (1%) profile of DNA genomic extraction and amplification of ~560bp PCR fragment. Lanes 10 -10: Genomic DNA; Lane M: Ladder 100bp (AL-Musayyib bridge); Lane C: Control.

a b c d

Figure 7. Observation of Rice pathogen under microscope. (a) *Thanatephorus cucumeris* R12. (b and c) Branches, Basidia and basidiospore. (d) Agarose gel electrophoretic (1%) profile of DNA genomic extraction and amplification of ~550bp PCR fragment. Lanes 12 -12: Genomic DNA; Lane M: Ladder 100bp (AL-Musayyib bridge); Lane C: Control.

a b c d

Figure 8. Observation of Rice pathogen under microscope. (a) *Thanatephorus cucumeris* R14: teleomorph [(A.B. Frank) Donk] Alternate State of (Anamorph: *Rhizoctonia solani* Kühn). (b and c) Branches, Basidia and basidiospore. (d) Agarose gel electrophoretic (1%) profile of DNA genomic extraction and amplification of ~550bp PCR fragment. Lanes 14 -14: Genomic DNA; Lane M: Ladder 100bp (AL-Musayyib bridge); Lane C: Control.

Figure 9 to 16 are the evidences of the identified five isolates of *Fusarium solani* (Figure 9,12,13,14 and 15), two isolates of *Fusarium oxysporum* (Figure 10 and 11), and one isolate of *Fusarium verticillioides* (Figure 16) as causal pathogens of rice plants.

Fusarium spp. was described for the first time by Von Martius (1842) as *F. solani* from rotten tubers of potato. Marasas *et al*.,(1984) and Desjardins (2006) have reported that *Fusarium* species are producing mycotoxins that contaminated the food, the consumption of which may lead to various serious human and animal diseases (Morgavi and Riley, 2007). *Fusarium* spp., is an important causal agent of several crop diseases, such as root and fruit rot of *Cucurbita* spp., root rot stem of pea, sudden death syndrome of soybean, foot rot of bean and dry rot of potato, and the destructive effects of this disease on rice (Maryam *et al*.,2013), and cereal grains continue to date (White, 1990; Goswami and Kistler, 2004; Nur Ain Izzati, 2011).

a b c d

Figure 9. Observation of Rice pathogen under microscope. (a) *Fusarium solani* R3. (b and c) Branches, hyphae and chlamydospores. (d) Agarose gel electrophoretic (1%) profile of DNA genomic extraction and amplification of ~550bp PCR fragment. Lanes 3-3: Genomic DNA; Lane M: Ladder 100bp (AL-Musayyib bridge); Lane C: Control.

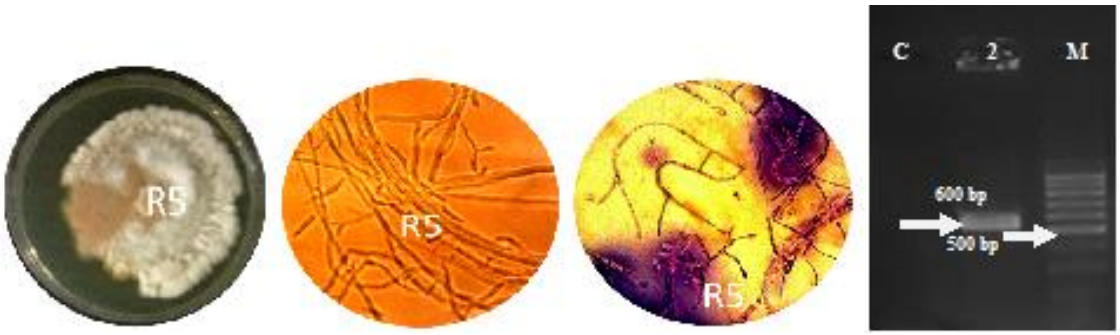

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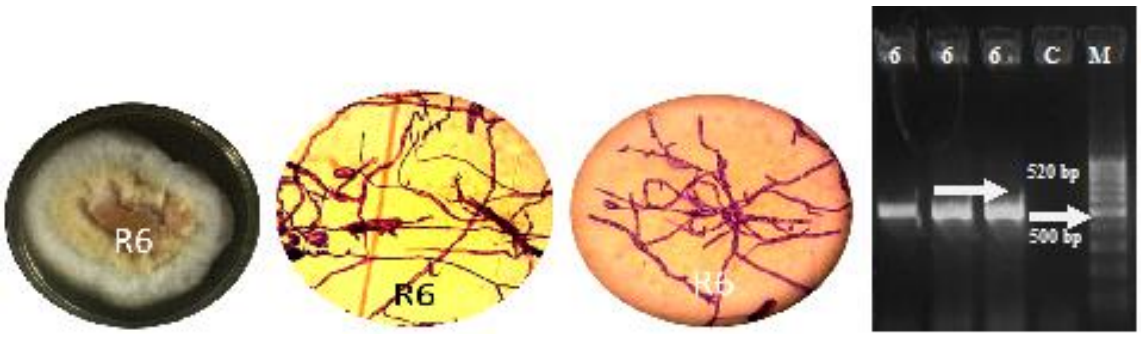
Figure 10. Observation of Rice pathogen under microscope. (a) *Fusarium oxysporum* R5. (b and c) Branches, hyphae, chlamydospores and swollen monophialides. (d) Agarose gel electrophoretic (1%) profile of DNA genomic extraction and amplification of ~600bp PCR fragment. Lanes 5: Genomic DNA; Lane M: Ladder 100bp (AL-Musayyib bridge); Lane C: Control.



a b c d

Figure 11. Observation of Rice pathogen under microscope. (a) *Fusarium oxysporum* R6. (b and c) Branches, hyphae and chlamydospores. (d) Agarose gel electrophoretic (1%) profile of DNA genomic extraction and amplification of ~520bp PCR fragment. Lanes 6-6: Genomic DNA; Lane M: Ladder 100bp (AL-Musayyib bridge); Lane C: Control.

a b c d

Figure 12. Observation of Rice pathogen under microscope. (a) *Fusarium solani* R8. (b and c) Branches, hyphae and chlamydospores. (d) Agarose gel electrophoretic (1%) profile of DNA genomic extraction and amplification of ~550bp PCR fragment. Lanes 8: Genomic DNA; Lane M: Ladder 100bp (AL-Musayyib bridge); Lane C: Control.

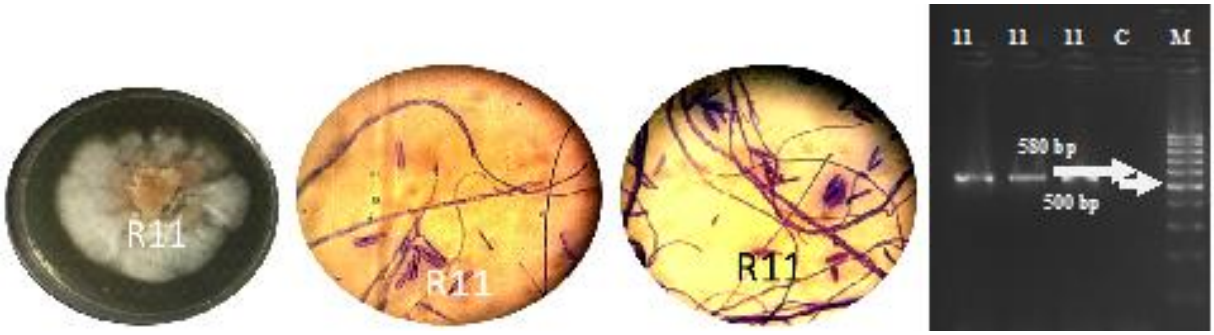


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Figure 13. Observation of Rice pathogen under microscope. (a) *Fusarium solani* R11. (b and c) Branches, hyphae, chlamydospores and swollen monophialides. (d) Agarose gel electrophoretic (1%) profile of DNA genomic extraction and amplification of ~550bp PCR fragment. Lanes 11 -11: Genomic DNA; Lane M: Ladder 100bp (AL-Musayyib bridge); Lane C: Control.

a b c d

Figure 14. Observation of Rice pathogen under microscope. (a) *Fusarium solani* R13. (b and c) Branches, hyphae and chlamydospores. (d) Agarose gel electrophoretic (1%) profile of DNA genomic extraction and amplification of ~550bp PCR fragment. Lanes 13 -13: Genomic DNA; Lane M: Ladder 100bp (AL-Musayyib bridge); Lane C: Control.

a b c d

Figure 15. Observation of Rice pathogen under microscope. (a) *Fusarium solani* R16: (b and c) Branches, hyphae and chlamydospores. (d) Agarose gel electrophoretic (1%) profile of DNA genomic extraction and amplification of ~550bp PCR fragment. Lanes 16 -16: Genomic DNA; Lane M: Ladder 100bp (AL-Musayyib bridge); Lane C: Control.

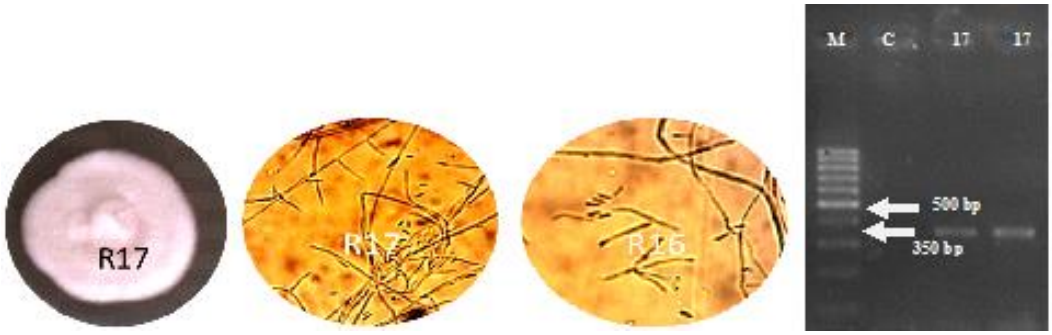

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Figure 16. Observation of Rice pathogen under microscope. (a) *Fusarium verticillioides* R17. (b and c) Branches, hyphae and chlamydospores. (d) Agarose gel electrophoretic (1%) profile of DNA genomic extraction and amplification of ~350bp PCR fragment. Lanes 17 -17: Genomic DNA; Lane M: Ladder 100bp (AL-Musayyib bridge); Lane C: Control.

One isolate of *N.oryzae* was identified (Figure 17) as destructive agent of rice plant, and causes symptoms such as produce a colonies on infected leaves, and the lesions turn to grayish brown and produce single-celled conidia which carry by conidiophore, and causes grain spots on rice. In addition, we obtained two isolates of *C. lunata* (Figure 18 and 19), one isolate of *B. spicifera* (or *H.oryzea*) (Figure 20), and one isolate of *E. rostratum* (Figure 21). These diseases are the major economically important fungal diseases in the rice growing areas (Sami,2006; Kosai and Ali, 2010; Ali and Alwan, 2012). These pathogens attack the rice from seedling to milky stage in major field.

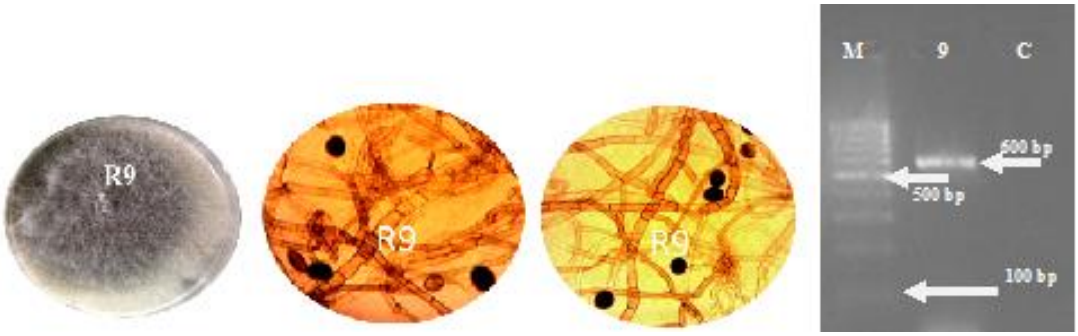

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Figure 17. Observation of Rice pathogen under microscope. (a) *Nigrospora oryzae* R9. (b and c) Branches, hyphae and conidia. (d) Agarose gel electrophoretic (1%) profile of DNA genomic extraction and amplification of ~600bp PCR fragment. Lanes 9: Genomic DNA; Lane M: Ladder 100bp (AL-Musayyib bridge); Lane C: Control.

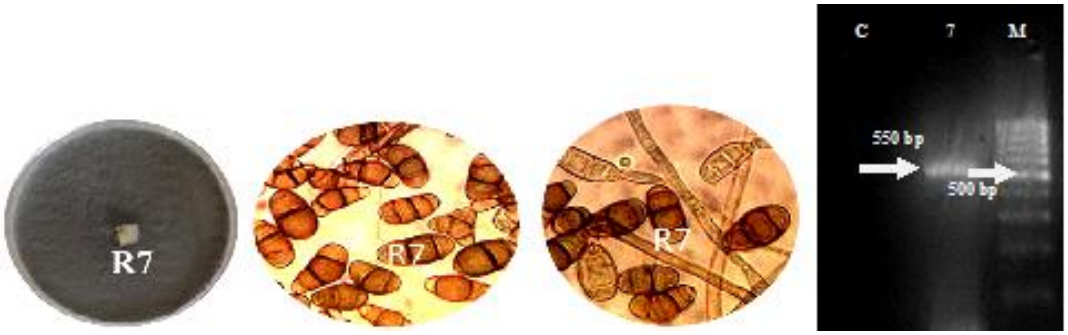

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Figure 18. Observation of Rice pathogen under microscope. (a) *Curvularia lunata* R7. (b and c) Branches and conidia. (d) Agarose gel electrophoretic (1%) profile of DNA genomic extraction and amplification of ~550bp PCR fragment. Lanes 7: Genomic DNA; Lane M: Ladder 100bp (AL-Musayyib bridge); Lane C: Control.

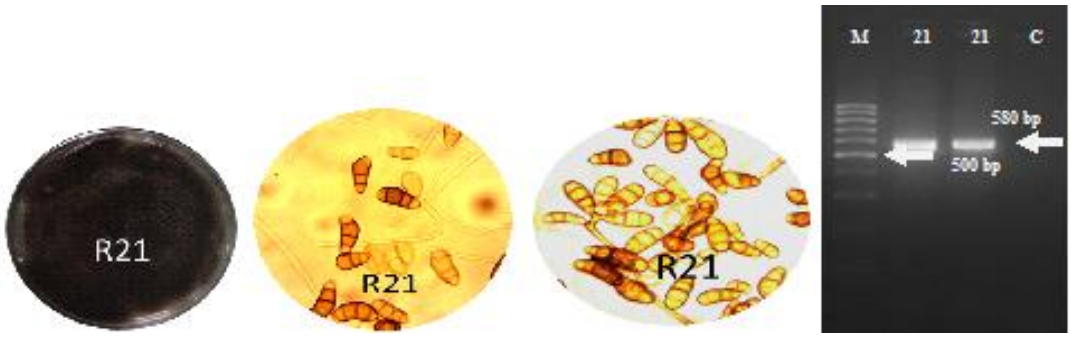

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Figure 19. Observation of Rice pathogen under microscope. (a) *Curvularia lunata* R21. (b and c) Branches and conidia. (d) Agarose gel electrophoretic (1%) profile of DNA genomic extraction and amplification of ~580bp PCR fragment. Lanes 21 -21: Genomic DNA; Lane M: Ladder 100bp (AL-Musayyib bridge); Lane C: Control.

a b c d

Figure 20. Observation of Rice pathogen under microscope. (a) *Bipolaris spicifera* R15 (*Helminthosporium oryzae*). (b and c) Branches and conidia. (d) Agarose gel electrophoretic (1%) profile of DNA genomic extraction and amplification of ~580bp PCR fragment. Lanes 15 -15: Genomic DNA; Lane M: Ladder 100bp (AL-Musayyib bridge); Lane C: Control.

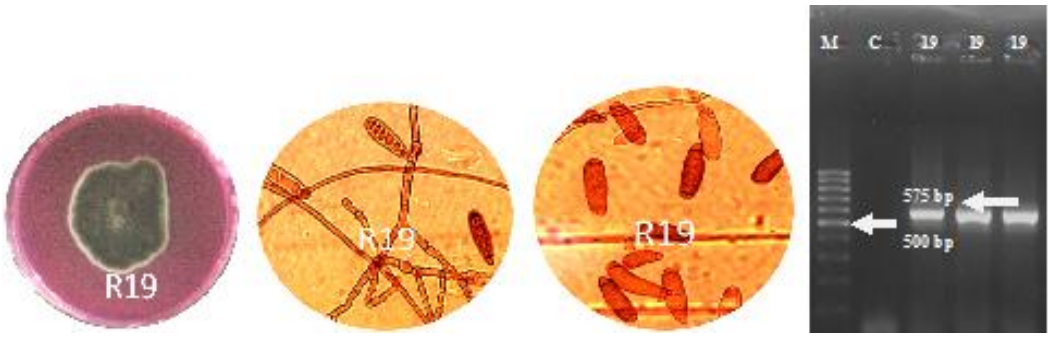

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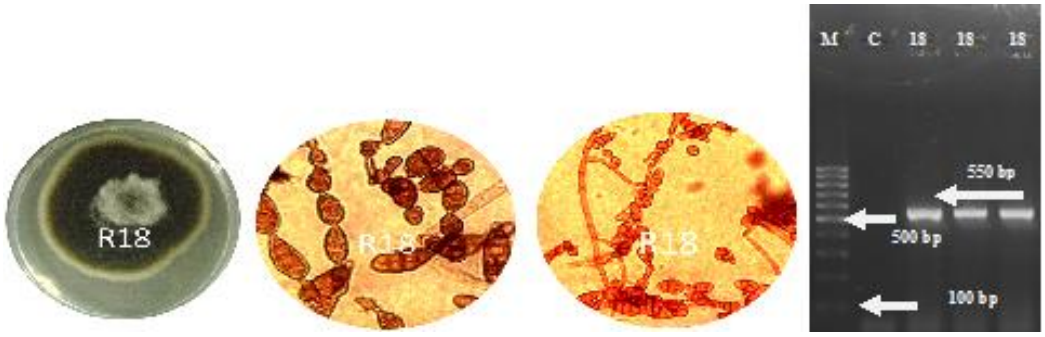
Figure 21. Observation of Rice pathogen under microscope. (a) *Exserohilum rostratum* R19. (b and c) Branches and conidia. (d) Agarose gel electrophoretic (1%) profile of DNA genomic extraction and amplification of ~575bp PCR fragment. Lanes 19 -19: Genomic DNA; Lane M: Ladder 100bp (AL-Musayyib bridge); Lane C: Control.

Two isolates of *Alternaria alternata* (Figure 22 and 23) and two isolates of *Alternaria tenuissima* (Figure 24 and 25) were iddentified. This finding was in accordance with the previous morphological characterization that found *Alternaria* spp. caused high reduction on Iraqi rice seeds germination (Salih *et al*., 2000), and infected seedlings (Iram and Ahmad, 2005).

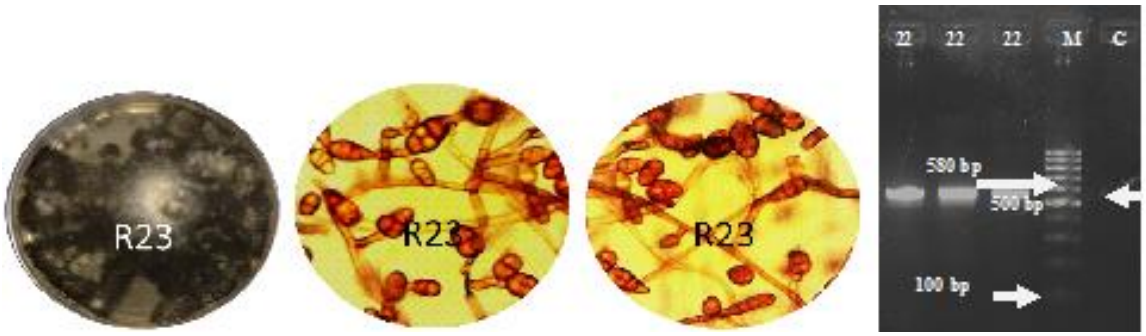


a b c d

Figure 22. Observation of Rice pathogen under microscope. (a) *Alternaria alternata* R18. (b and c) Branches and conidia. (d) Agarose gel electrophoretic (1%) profile of DNA genomic extraction and amplification of ~550bp PCR fragment. Lanes 18 -18: Genomic DNA; Lane M: Ladder 100bp (AL-Musayyib bridge); Lane C: Control.

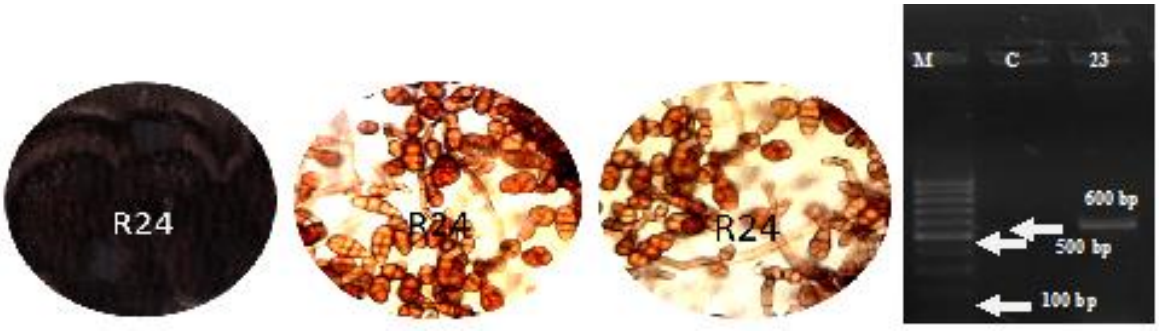
a b c d

Figure 23. Observation of Rice pathogen under microscope. (a) *Alternaria alternate* R20. (b and c) Branches and conidia. (d) Agarose gel electrophoretic (1%) profile of DNA genomic extraction and amplification of ~550bp PCR fragment. Lanes 20 -20: Genomic DNA; Lane M: Ladder 100bp (AL-Musayyib bridge); Lane C: Control.



a b c d

Figure 24. Observation of Rice pathogen under microscope. (a) *Alternaria tenuissima* R23. (b and c) Branches and conidia. (d) Agarose gel electrophoretic (1%) profile of DNA genomic extraction and amplification of ~580bp PCR fragment. Lanes 22-22: Genomic DNA; Lane M: Ladder 100bp (AL-Musayyib bridge); Lane C: Control.



a b c d

Figure 25. Observation of Rice pathogen under microscope. (a) *Alternaria tenuissima* R24. (b and c) Branches and conidia. (d) Agarose gel electrophoretic (1%) profile of DNA genomic extraction and amplification of ~600bp PCR fragment. Lanes 23: Genomic DNA; Lane M: Ladder 100bp (AL-Musayyib bridge); Lane C: Control.

The data presented in Figure 1-2 indicated that Iraqi rice was infected in field by various pathogens i.e. *T.cucumeris,Fusarium* spp., *B. spicifera* (*H. oryzea*), *C. lunata* and *Alternaria* spp.. The pathogens *T. cucumeris, E. rostratum*, *C.lunata*, *A. alternate* and *B. spicifera* (*H. oryzea*) are saprophytic fungi and cause different disease symptoms, and can complete a single cycle within a week under suitable conditions. *B. spicifera*, *E. rostratum*, *C.lunata* and *Alternaria* spp. produced clear symptoms within 72 hours post inoculation, in certain cases dark spots were observed 36 hours after treatment with pathogen (Brecht *et al*., 2007; Sousa *et al*., 2013). In the initial process of infection by *B. spicifera*, *E. rostratum*, *C. lunata* and *Alternaria* spp., the pathogens use many compounds exuded on its leaf surface that includes a complex mixture of hydrophobic materials that assist with host recognition, induction of spore germination and appressorium formation (Kolattukudy *et al*.,1995; Knogge, 1996).

The pathogens *B. spicifera*, *E. rostratum*, *C. lunata* and *Alternaria* spp.have specific cells named appressoria that exerts turgor pressure as a result of aggregation of many soluble materials including glycerol (Caracuel-Rios and Talbot, 2007; Jacobson, 2000; Ahmadpour *et al*.,2011). At the site of penetration, appressoria are often formed and develop high turgor pressure to support the penetration process**.** The penetrating hypha accumulates components of the cytoskeleton in the tip and secretes a variety of cell wall–degrading enzymes in a highly regulated fashion in order to penetrate the cuticle and the plant cell wall (Takeo and Kaoru, 1990). The appresorium is important in the penetration of host cell through the high intracellular, potential of melanin which is very important in establishing pathogenicity and virulence (Gao *et al*., 2015).

T. cucumeris causes disease symptoms such as stem canker on mature plants and causes basidia to appear on the surfaces, and infected roots will turn brown and die after a period of time (Figure 3-8). In this study we found *Fusarium* spp. infection of rice plants (Figure 9-16) was more frequent compared to *B. spicifera*, *E. rostratum*, *C. lunata*, *Alternaria* spp. and *T.cucumeris* due to that *Fusarium* spp. is a major pathogen of rice (Al-Jubouri, 2010), and thence causes severe loss of rice. This genus includes a wide heterogeneous group of fungi important for the food, drug industry, medicine and agriculture. Fusarium is a large genus of filamentous fungi, soil borne fungus, and has many species that are widely distributed in soil and in association with plants (Roncero *et al*., 2003). It has at least 50 subspecies lineages in this genus (Zaccardelli *et al*., 2008; Koenning, 2001). These results were in agreement with several studies that reported *Fusarium* spp. associated diseases was frequent and it's species have highly diverse and is responsible for severe economic losses due to reducing yield by at least 80 % in infected crops (Khan and Sinha, 2005; Nur Ain Izzati *et al*., 2005; Izham, 2008). This fungus have extremely broad host range and the high survival rate of resistant forms such as chlamydospores under different environmental conditions (Thabet, 2008). *Fusarium* spp. have been known to release secondary metabolites such as fusaric acid, trichothecenes, fumonisins or enniatins (Summerell and Leslie, 2011; Jens Laurids *et al*., 2013), and these infections reduce the quality of staple foods (Nelson *et al*., 1983).

Bipolaris spicifera, *Curvularia lunata*, *Fusarium* spp., *Nigrospora oryzae, Exserohilum rostratum, Alternaria* spp. and *Thanatephorus cucumeris* were detected as the causative agents by isolation from infected plants, and identification studies inclusive universal primer pairs ITS1 x ITS4 analysis of these pathogens by extracting the genomic DNA of all the isolates. The PCR product size of ITS of *Thanatephorus cucumeris* (Anamorph: *Rhizoctonia solani* Kühn) was within the range i.e. between 550 to 700bp (Figure 3d-8d) with slight variation in size of products as reported by (Sharon *et al*., 2006; Sharon *et al*., 2008). This study have also been succeed to get a PCR product between 350 to 600bp (Figure 9d-16d), with clear band on the gel obtained for the full ITS-rDNA region was amplified from our infected tissue by pathogenic *Fusarium* species, this is in agreement with the observations made by several authors (Abd-Elsalam *et al*., 2004; Jima, 2012). The amplification products of *N. oryzae*, *C. lunata, B. spicifera* and *E. rostratum* were approximately 550 to 600bp as presented in Figure 17d to 21d and in accordance with the previous findings (Manamgoda *et al*., 2012; Abass and Mohammed, 2014). Figure 22d to 25d provide some variation in PCR product size of amplified universal ITS of *Alternaria* species was about 550 to 600bp as mentioned by (Barry and Robert, 2000). These findings suggest that the ITS region of rDNA marker designed by White *et al*. (1990), can apply for effective diagnosis of many fungal pathogens. Moreover, it can give important information to investigate the polymorphism between fungal genera.

CONCLUSION

This study was detected and determined variation of pathogens exhibited by the isolation, through this we will be able to test their pathogenicity ability in pre and post emergence damping off, disease infected, and severity toward rice plant under greenhouse conditions. Moreover, we can gauge the significance of reduction disease caused by pathogens post biological control agent application, and will enable us to determine the level of devastation incurred by each pathogen prior to commencement of biological control experiments.

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