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DETECTION OF ALTERNARIA ALTERNATA ISOLATED FROM GRAIN BY USING SPECIFIC PRIMER

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ABSTRACT

This work was concentrated on Alternaria alternata because they are Different pathotypes common pathogens of different plants having a high toxigenic potential for which a special and sensitive detection method is required and are particularly difficult to distinguish with conventional methods often do not distinguish between the isolates of the same species Therefore DNA based method was applied to find if the isolates differ significantly from each other.

KEYWORDS: Alternaria alternata, specific primer.

INTRODUCTION

Alternaria fungus is about one hundered species, it can be found in various places (Sadwosky,2002) it is ubiquitous and include plant pathogenic and saprophytic species which can damage crops in the field (Peever, 2004). Polyphagus nature and ability of them to produce toxic and carcinogenic materials indicates that it is potentially hazardous to human and animals health (Logerieco,1990), like Alternariol (AOH), Alternariol methyl ether (AME), and Tenuazonic acid (TeA) (Andersen, 2001, 2002).

The routine technique for detection and identification of *Alternaria* spp. Often requires culture isolation, morphological and physiological characterization (Simmon, 2007).

Recently, significant advances in fungal taxonomy & identification have come about through DNA methods which mainly based on the polymerase chain reaction (PCR) offer alternative approaches for detection of viable and not viable pathogenic microorganism in food. these methods have the advantages that the structure of DNA remains stable with physiological stages, and it is composition does not depend on culture condition (Pavon et.al., 2010).

The objective of this work was to detection and identification of the species *Alternaria alternata* by using developed biotechnology of PCR represented by specific primer.

MATERIALS & METHODS

1-Fungal isolates: isolates of *Alternaria alternata* where obtained from 20 samples of four types of grains (wheat, malt, rice, bean) (five for one) after sterilized it with sodium hypochlorite (6%) then cultured on Potato Dextrose Agar PDA (20 gm potato, 20 gm sucrose, 15 gm agar, 1000 ml d.w.) & Malt Extract Agar MEA(20 gm malt, 15 gm agar, 1000 ml d.w.), incubated (28°c – 7 days) then purification on PDA agar. this work were did in Microbiology lab, College of Science for Women, Babylon University, Iraq – Hilla.

2- DNA Extraction: in this study we used for extracted (DNA Extract Kit) supplied from (Promega, Madison Wi, USA) which include: DNA Rehydration solution, Protein precipitation solution m, Cell lysis solution, Nuclei lysis solution, RNase solution, in addition EDTA solution from BDH-Chem. LTD bool company, Lyticase enzyme from US biological CO. Isopropanol,Ethanol 70%. According to kit protocol DNA extracted of *Alternaria alternata* then stored at (2-8°c) until used in next step (Ciardo et al, 2010).

3- PCR Technique: in this step specific solution used which include:

1- T.E. buffer supplied from (Promega,Madison wi, USA).
2- Specific primer (for detection Alternaria alternate) supplied from Bioneer Co. / USA (Konstantinova et al., 2002) (table 1).

Table 1: show the specific primer used in the study.

Target sequences	Primer name	Sequence(5-3)	Fragment size bp
Alternaria alternata	AAF2	5′-TGC AAT CAG CGT CAG TAA CAA AT	340
	AAR3	5′- ATG GAT GCT TAG ACC TTT GCT GAT	

3- Master mix supplied from Bioneer Co. / USA, include:

a- Taq DNA polymerase.
b- (dNTPs) 250 mmole from (dTTP – dCTP – dGTP –dATP).
c- Tris – HCl (pH=9.0).
d- KCl.

In PCR step we used PCR apparatus by added specific primer with extract DNA into PCR tube which contain master mix with total volume 20 ml (table 2) according to master mix protocol.

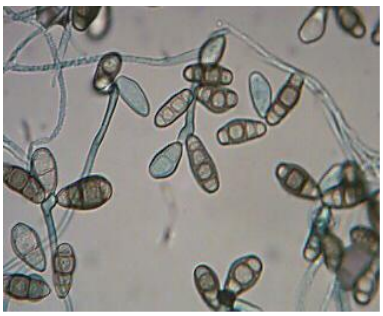
Table 2: total volume according to master mix protocol.

Solution	Volume
Master mix	5ul
DNA	5ul
Forword primer	2.5 ul
Reverse primer	2.5 ul
Deionized water	Complete volume to 20 ul
Total	20 ul

PCR amplification condition were: denaturation at 94°c for 2 min, annealing at 70°c for 40s, final extension for 5 min at 72°c followed by cooling at 4°c until recovery of the samples. After that amplification products were examined by electrophoresis in agarose gel (1gm agarose, 100ml T.B.E buffer, stained with 2ul Ethidium bromide according to (Sambrook, et. Al. 1989) the gels were viewed under UV light at 600 nm on a lumi imager system under UV light by UV transilluminator.

RESULT

This work was concentrated on *Alternaria alternata* because it is common pathogen of different plants and particularly difficult to distinguish with convential methods. The 20 isolates of *Alternaria alternata* as expected which obtained from four types of crops (wheat, malt, rice, bean)from local market / Hilla – Iraq.were identified by classical methods consist of cultured- isolated – purification-morphology (colony character) and microscopic examined, so we obtained 20 isolates of Alternaria have the same colony character (colour-edge – raised –touch) and under microscope revealed the conidia, hyphae. the usual question was wether the classical method (morphology & microscopically)is enough to revealed that these isolates for *Alternaria alternata*, therefore DNA based method was applied to explain this question.(Pic. No. 1,3,5,6,7,8,11,12,17,18,19,20 revealed isolates that belong to *Alternaria alternata* by using specific primer, a- hyphae, b- conidia)

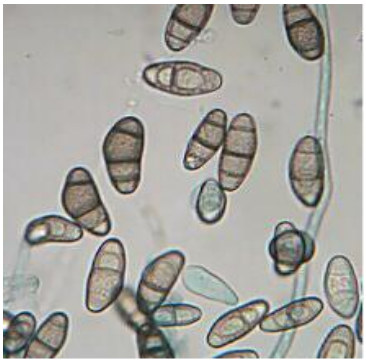


Pic. No. 1 -a -

- b –

Pic. No.3 -a -

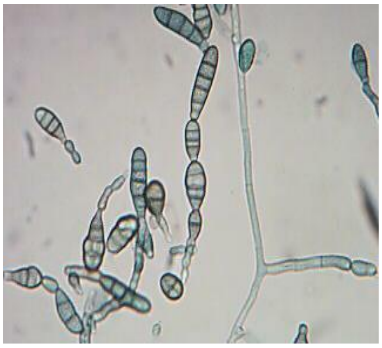
-b-



Pic. No. 5 -a-

-b-

Pic. No. 6 -a-



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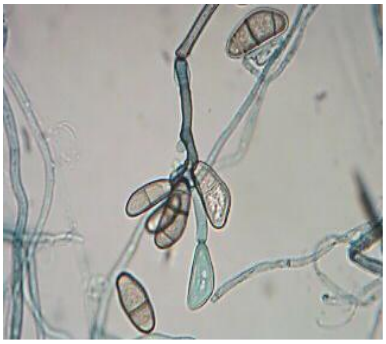

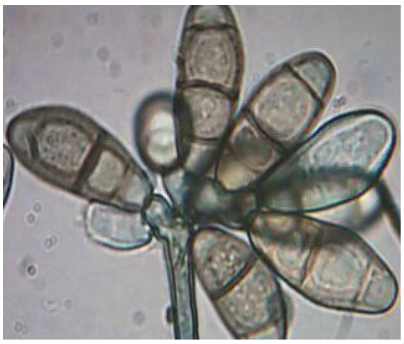

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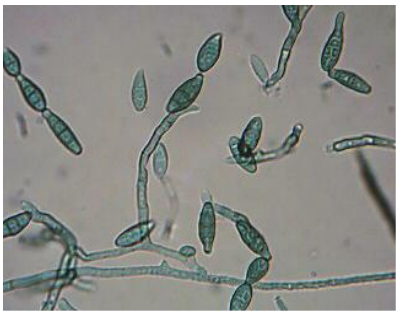

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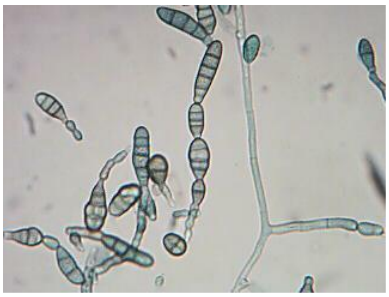

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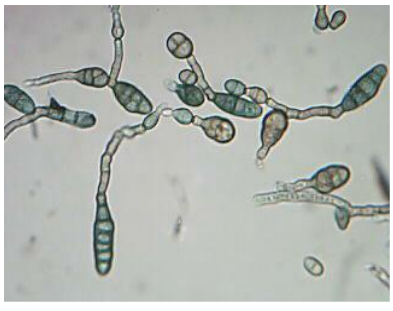

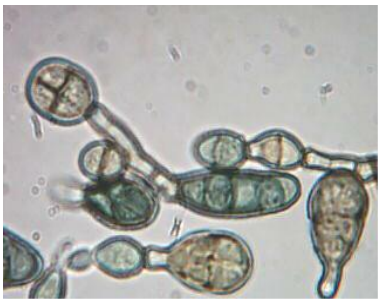

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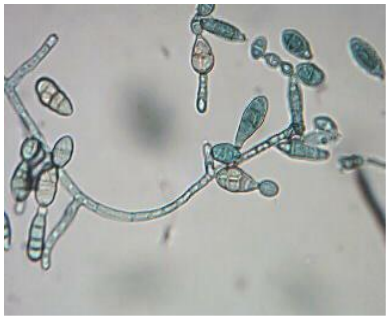

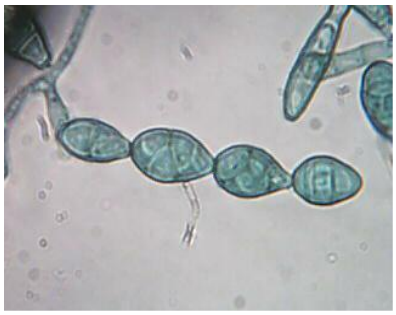

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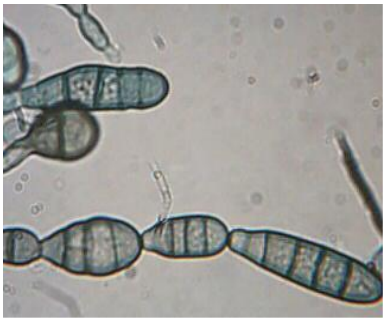

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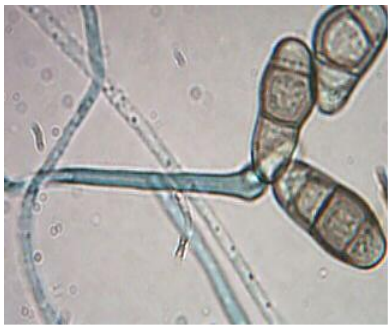

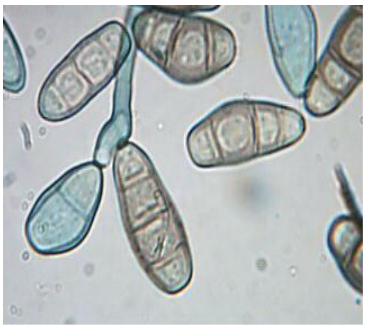

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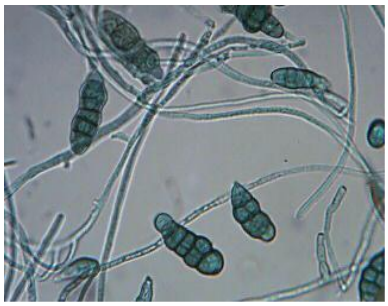
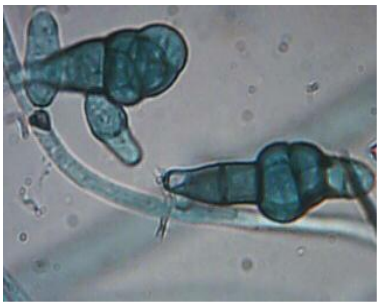
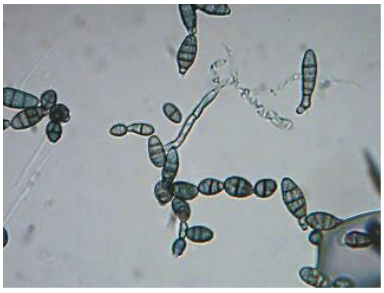
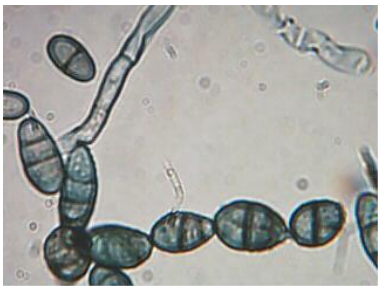




Pic. No. 20 -a- -b-

(Pic. No. 2,4,9,10,13,14,15, 16 revealed isolates that similar to *Alternaria alternata* by classical methods, a- hyphae, b- conidia)





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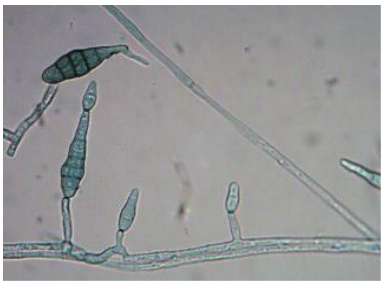

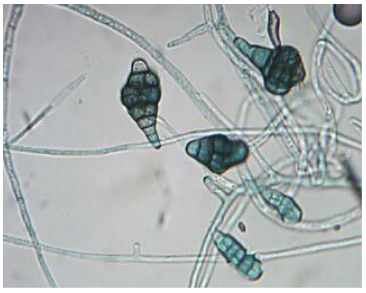

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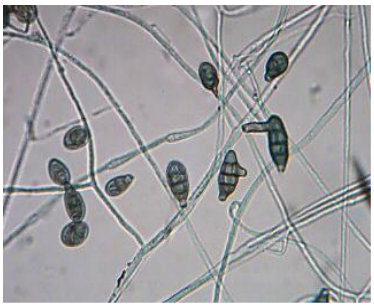

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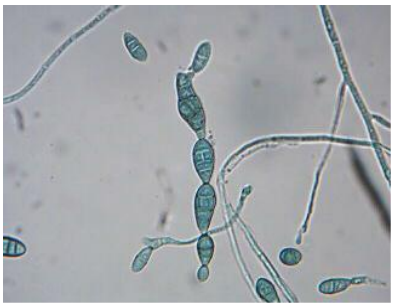
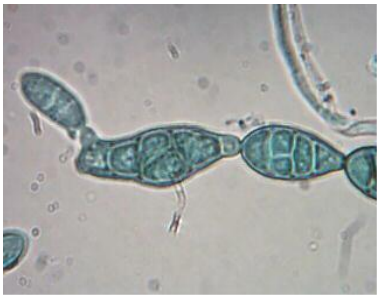
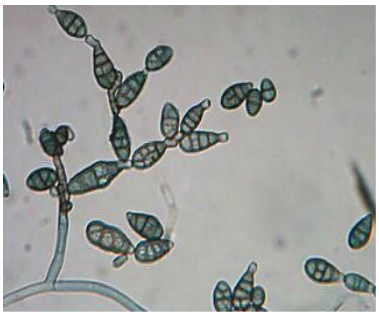
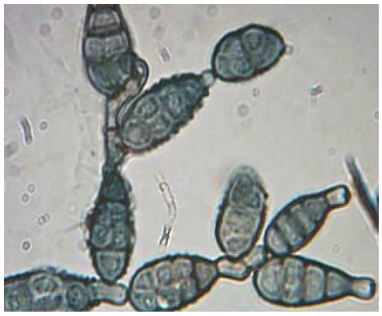
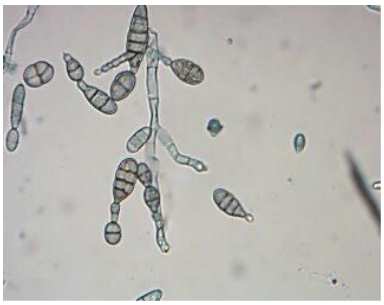
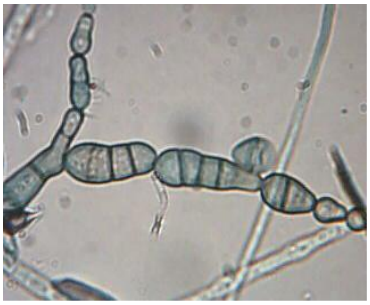




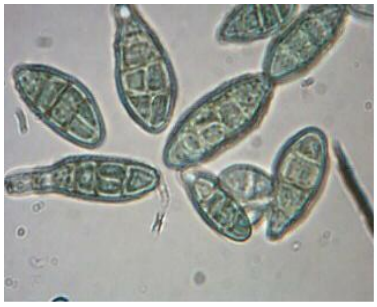
Pic. No. 10 -a- -b-

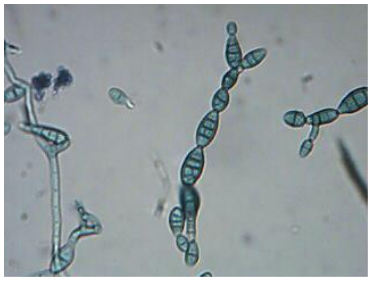
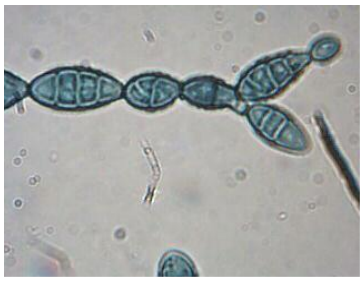
Pic. No.13 -a- -b-

Pic. No. 14 -a- -b-

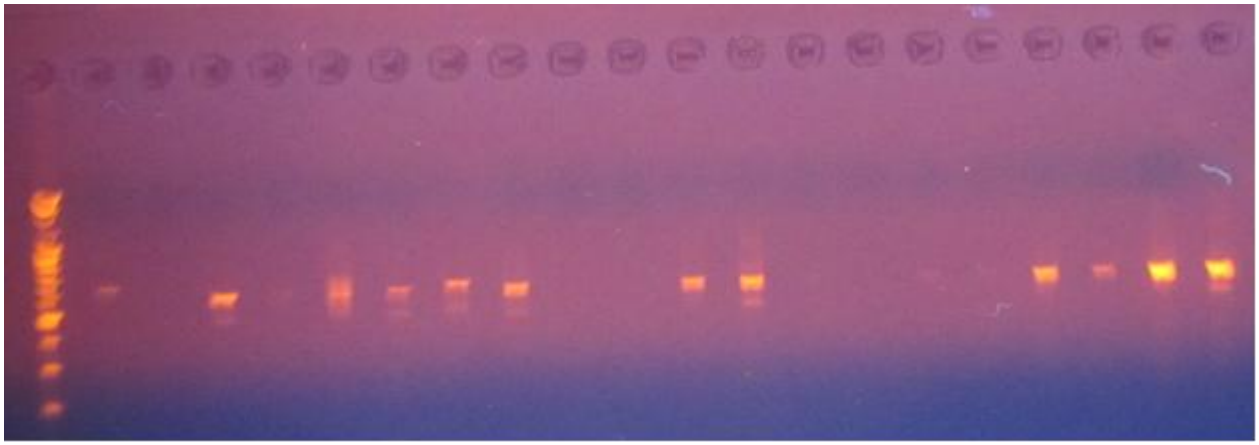
Pic. No. 15 -a- -b-

Pic. No. 16 -a- -b-

For more certainity we used DNA methods by extracting DNA and amplifying it by specific primer for *Alternaria alternata* which gave result different from classical methods, (pic. 21).

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20



Pic. 21 Electrophoresis gel visualized under U.V. light for 20 isolates.

After DNA methods revealed isolates (1,3,5,6,7,8,11,12,17,18,19,20) band at 200 bp by using specific primer AAF2,AAR3 while other isolates did not give this band.

DISCUSSION

Detection phytopathogenic fungi in plant and seeds can be difficult. A suitable diagnosis assay needs to be specific to avoid problems such as detection organism under study as another one, it is very important to monitor and quantify the occurrence of plant pathogen for efficient management of plant diseases.

So *Alternaria* as plant pathogens can cause serious problems in agriculture by reducing crop yields in the field and cause spoilage in storage mainly *Alternaria alternata* species that produce mycotoxins and allergins which cause problems in food, industry, agriculture and health services (Andersen et. Al. 2001,2002) classical tests used to detect *Alternaria alternata* in plant and seeds which involve morphological and microscopical characterization however such methods can be difficult and result may be not correct hence genetic methods as it difficult but offer alternative approaches for detection of microorganisms.so the 20 isolates in this study in classical methods revealed that they are belong to *Alternaria alternata* but by using specific primer (AAF2/AAR3) for *Alternaria alternata* that give result of 12 isolates positive specifically amplified a single 340 bp fragment from isolates which similar to result of (Konstantinova et al,2002) when used the same primer. while other isolates did not reveal amplified on there gene, so it may be another species. so we achieved that we cannot depend on classical method in detection organism with the development of biotechnology.

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