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MOLECULAR IDENTIFICATION OF *TRICHODERMA LONGIBRACHIATUM* CAUSING GREEN MOLD IN *PLEUROTUS ERYNGII* CULTURE MEDIA

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Abstract

The study was conducted to identify the causal agent of green mold contaminating the oyster fungus *Pleurotus eryngii* culture media–six different isolates were isolated from sacs containing *P. eryngii* culture media, showing green mold symptoms, collected from six locations in Iraq. The morphological examination of fungal isolates colony on PDA indicated that the isolates belong to *Trichoderma longibrachiatum*. The morphological identification of the fungal isolates was confirmed by PCR protocol using DNA ITS gene. The analysis of PCR products, of ITS amplification, using ITS_1 / ITS_4 primers, on 1.5% agarose gel by electrophoresis revealed a band of 660 bp for each isolate. The DNA sequencing of PCR product according to GenBank database by last searching confirmed the identity of the six isolates and that belong to *Trichoderma longibrachiatum*. Iraqi isolates of *T. longibrachiatum* shows similarity of 99% with china, Colombian, and Iranian isolates in National Center for Biotechnology Information (NCBI) GenBank database BLAST and were placed in the same center.

Keywords: Molecular, Green, Mold, *Pleurotus eryngii*, *Trichoderma Longibrachiatum.*

Introduction

Green mold is considered as the main contaminant of oyster fungi cultivation media that developed early during cultivation as well as during seed production causing high losses in the production (Mwangi *et al.,* 2017). The first observation of the disease was on Agaricus in North Ireland 1946, then in England and Scotland 1987, Holland 1994, France 1997, Spain 1998, USA and Canada 1990 causing heavy losses in mushroom fungus.

The disease was observed on oyster fungi, *Pleurotus ostreatus* in North America 1997 in commercial farms then seen in South Korea, Italy, Hungary and Spain (Colavolp *et al.,* 2014). The disease was reported to cause losses in oyster fungi in Britain's Islands during 1985-1986, and during 1990-1991 attained losses about 3-4 million sterling and in North America up to 30 million dollars (Hatvani *et al.,* 2010).

The causal agent of green mold was identified basing on morphological characteristics as *Trichoderma spp., including T. harzianum, T. aggrissivum f. agressivum, T. aggrissivum, F. curapaecum, T. pleuorotum, T. pleuroticala, T. fluvidum, T. pleurotophilum and T. longibrachiatum* in European countries causing high losses in oyster fungi (Hatvani *et al.,* 2007; Zelazowska *et al.,* 2007).

It was reported that *T. longibrachiatum* grown in oyster fungi culture media and causing losses estimated up to 8.6 % of total *P. eryngii* production as well as producing lytic enzymes that lead to decompose fungal cell walls (Choi *et al.,* 2003).

Due to variation in the morphological characteristics correlated with environmental variation leading to erroneous identification, so the efforts were oriented toward molecular identification by using ITS region of fungal DNA (Kubicek and Harman, 1998).

Green mold growth was observed in *Pleurotus eryngii* culture media at different locations, in Iraq causing some problems in fungal cultivation. The study was conducted to identify the causal agent at molecular level using polymerase chain reaction PCR.

Material and Methods

Isolation and Identification

Samples, from sacs containing *Pleurotus eryngii,* formed of wheat hay, showing green mold symptoms in six locations in Iraq, college of Al-Anbar province (College of Agriculture / University of Al-Anbar), Baghdad province/ Mohammed Al-kassim Location for fungus culturing / Agriculturist Protection Office, Diyala province (special farm), Dhi Qar province (special farm), Kirkuk province(special farm) and Salah El-din province (College of Agriculture / University of Tikrit), were collected as small pieces from the culture media in the sacs which were placed separately in test tubes contained 9ml distilled water. The tubes were agitated and a serial dilution 10^{-1}–10^{-6} were done. 1ml of 10^{-6} dilution was placed in each of petriplates containing 20ml of potato dextrose agar (PDA) before solidification with slight cycloid movement for homogenization. The plates were maintained at 25±2ºC for 3days. The growing fungi were purified and identified to genus level according to calcification key described by (Bissett, 1984). The purified fungal isolates were conserved on PDA in small tubes at 4ºC.

Molecular Identification by Polymerase Chain Reaction (PCR)

DNA Extraction

The six isolates obtained of *Trichoderma Spp.* (TA, TB, TD, TK, TTH and TS) were grown on PDA by single spore technique on petriplates at 25±2ºC for 6 days. The mycelium and spores from each isolates were collected in eppendorf tubes.

The mycelium and spores of the isolates Trichoderma were lyophilized separately in 2ml screw cryo tubes with holes in the cap. 200ml of extraction buffer I (20ml of 1M

Tris, 5ml NaCl, 5ml of 0.5M EDTA, 70ml Ionic distilled water and 20ml Protinase K) were added to each tube , mixed by vortex and centrifuged at 2000 rpm for 2 minutes then 90ml of supernatant were added to 10ml of extraction buffer II (5% sodium dodecyle sylfate SDS in distilled water) in eppendorf tubes and mixed by vortex. The tubes were maintained at 65ºC for one hour with agitation each 30 minutes for mixing and then centrifuged at 3500 rpm for 10 minutes. 40ml pf the supernatant were added to 100ml of absolute ethanol and centrifuged at 2000 rpm for 10 minutes. The DNA precipitation was air dried and dissolved in 100ml ionic distilled water, let at 4ºC overnight and centrifuged at 2000 rpm for 5 minutes. The supernatant was conserved at 4ºC for PCR protocol.

PCR Protocol

The DNA was cleaned up by using ultraclean gel spin purification kit from Bioneer Laboratories. Gel-Bind was added to DNA solution, and the mixture was passed through spin filter by centrifugation at 10000 xg for 30 seconds. The filtrate was discarded and 300ml of Gel-wash buffer was added to the filter and centrifuged at 10000 xg for 30 seconds. The last step was repeated and the filter was carefully transferred to 2ml clean tube. 50ml of elution buffer was added onto the center of filter and centrifuged at 10000 xg for 30 seconds. The concentration of DNA in the filtrate was determined by spectrophotometer, No-1000 Nano drop and adjusted to 5ng/ml. PCR mixture for ITS rRNA gene consisted of 12.8ml ionic distilled water, 2.5ml of 10x buffer, 2.5ml mgcl2, 0.5ml dNTP mix (10mM each), 0.8ml forward primer (10mM), 0.8ml reverse primer ($^{5'}$ TCC TCC GCT TAT TGA TAT GC^{3}), 0.1ml Tag and 5ml DNA in ice bath. The cycling parameters were, initial denaturation at 95ºC for 1 minute, denaturation at 95ºC at 20 seconds, annealing at 53ºC for 30 seconds, extension at 72ºC for 30 seconds, final extension at 72ºC for 5 minutes and hold at 10ºC.

DNA Analysis on Agarose Gel 1.5% by Electrophoresis

The PCR product was analyzed in 1.5% agarose gel prepared in TBE buffer 10X. The mixture was heated by microwave for 3 minutes, then let to refrigerate. 5ml of ethidium bromide were added to gel at 45ºC and poured in gel container after inserting the comb at one side of the gel container. The gel was left at room temperature for 30-40 minutes for solidification and the comb was removed. 5ml of DNA ladder were deposited in the first well and 5ml of PCR product were deposited in the other wells. The gel container was covered and the electrophoresis was performed at 70ºC for 60 minutes. The DNA nucleotides sequencing was carried out by Korean Bioneer Company.

Results and Discussion

Isolation and Identification of *Trichoderma Spp.*

Morphological Identification

Six different isolates were isolated from the oyster fungi, type *Pleurotus eryngii,* culture media contaminated with green mold, these isolated were collected from different location in Iraq. The morphology of the isolates colony on PDA indicated that all the isolates were belong to *Trichoderma longibrachiatum* and assigned as, TA, TB, TD, TTH, TK and TS. The isolates growth on PDA was characterized by white color in the early stage, converted gradually to deep green associated with slight yellowing within three days, after formation of conidia spores (Figure 1). It was reported that *T. longibrachiatum* can grow at the wide range of temperature reached to 40ºC and with PH between 2-9 and able to use amino acids like, ornithine, glutamic acids, glutamine, aspartic acid, L-asparagine as carbon and nitrogen sources (Antal *et al.,* 2005). The fungus was reported to form branched conidiophores, straight or curved with short lateral branches (Ashfaqu *et al.,* 2015; Samules *et al.,* 2012; Ranga rani *et al.*, 2017 and Prameeladevi *et al.,* 2018)

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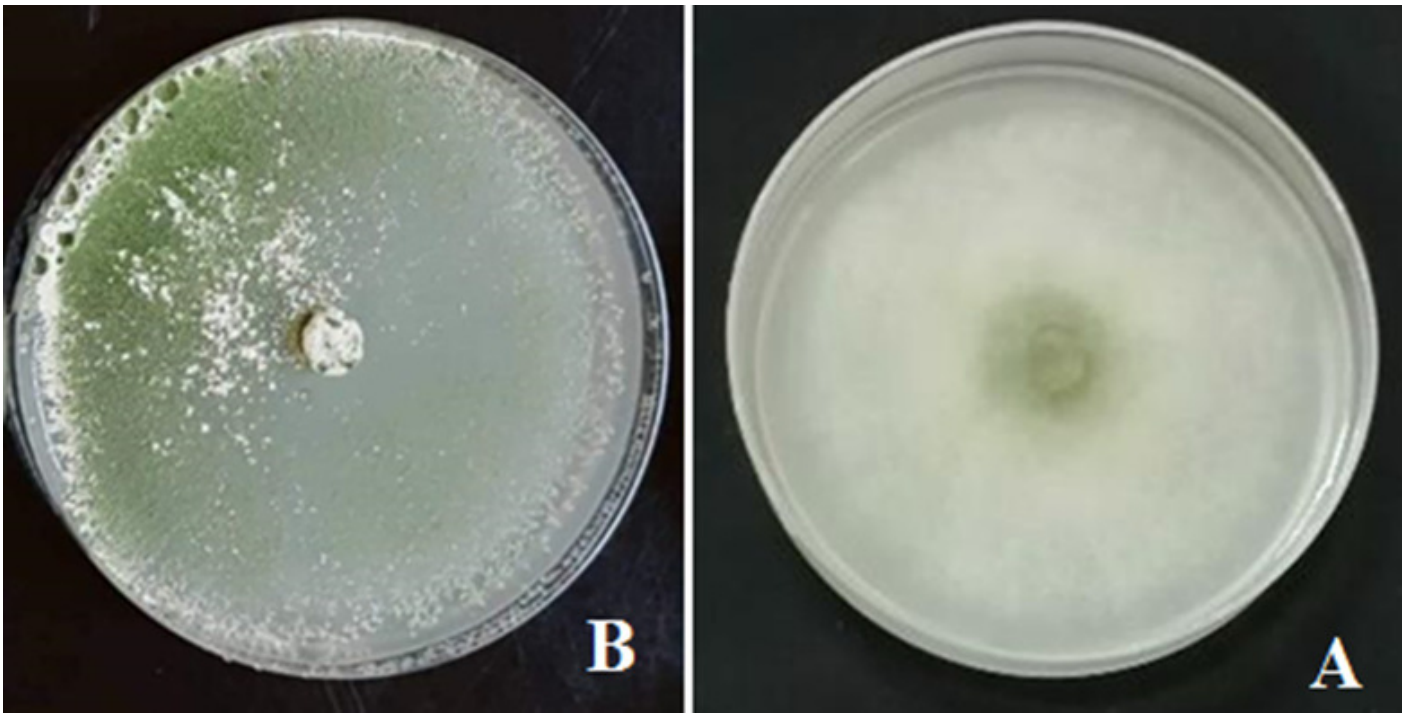

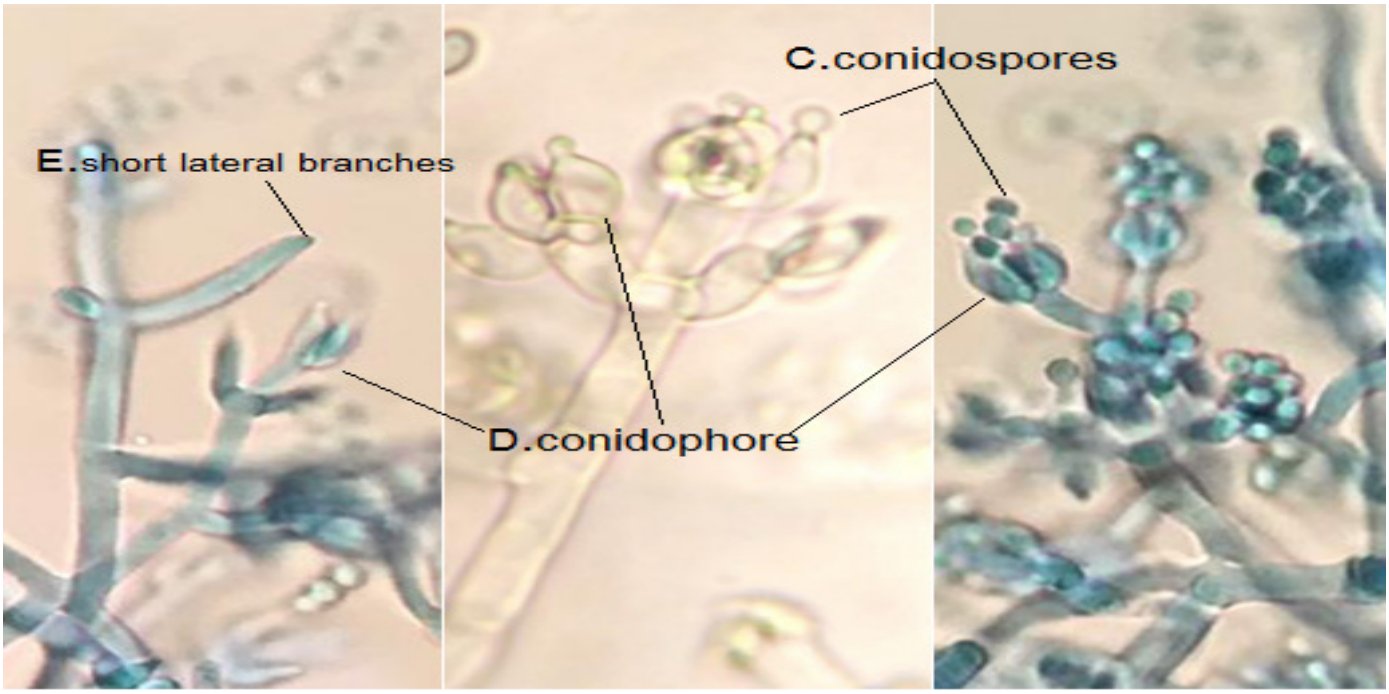

Fig. 1 : *Trichoderma longobrachiatum* colonies on PDA A: Early stage B: After sporulation C: conidospores D:conidophore E: short lateral branches

Molecular Identification

The morphological identification of *T. longibrachiatum* was confirmed by PCR protocol, using ITS gene of DNA. The analysis of PCR products of ITS amplification using the primers ITS_1/ITS_4, by electrophoresis on 1.5% agarose gel showed α band of 660bp for each isolates (Figure 2). Similar results were obtained by shahid *et al.* (2013), using the same DNA gene of *T. longibrachiatum* for amplification.

DNA Sequencing

According to the search in GenBank database BLAST, DNA nucleotides sequencing of PCR products of *T. longibranchiatum* isolates were found to confirm the identity of all isolates belong to *T. longibrachiatum* and showed similarity of 99% with the fungal isolates in the National Center for Biotechnology Information (NCBI) and saved in the GenBank and deposited in the GenBank under Accession Number (Figure 3) .

Figure 3- Iraqi isolates Accession Number

Accession Number	place of collection	Isolates
MK933755	Al-Anbar	TA
MK933743	Diyala	TD
MK933745	Baghdad	TB
MK933747	Dhi Qar	TTH
MK933749	Kirkuk	TK
MK933753	Salah El-din	TS

It was found that the nucleotides sequence of a region designated by megax program in neighbor – joining trees and compared with some isolates of *T. longibrachiatum* in NCBI GenBank, that Iraqi isolates of *T. longibrachiatum* showed 99% similarity with Chinese, Colombian and Iranian isolates of *T. longibrachiatum* and placed in one group (Fig. 4).

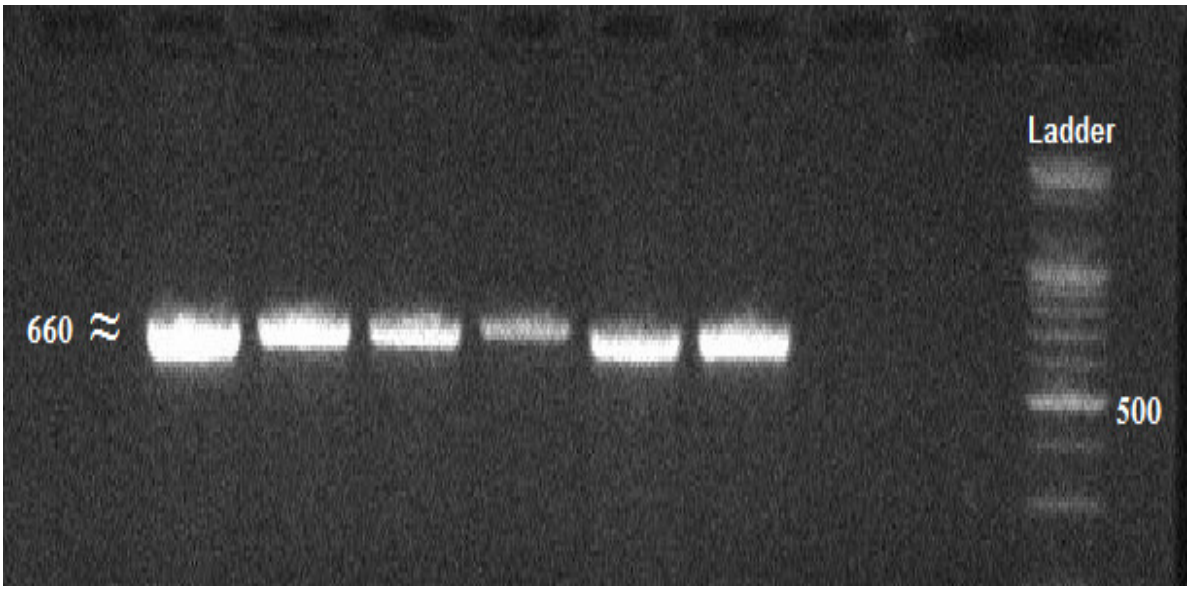

Fig. 2 : Agarose gel (1.5%) electrophoresis showing PCR amplified products of ITS rDNA gene of *Trichoderma longibrachiatum* isolates

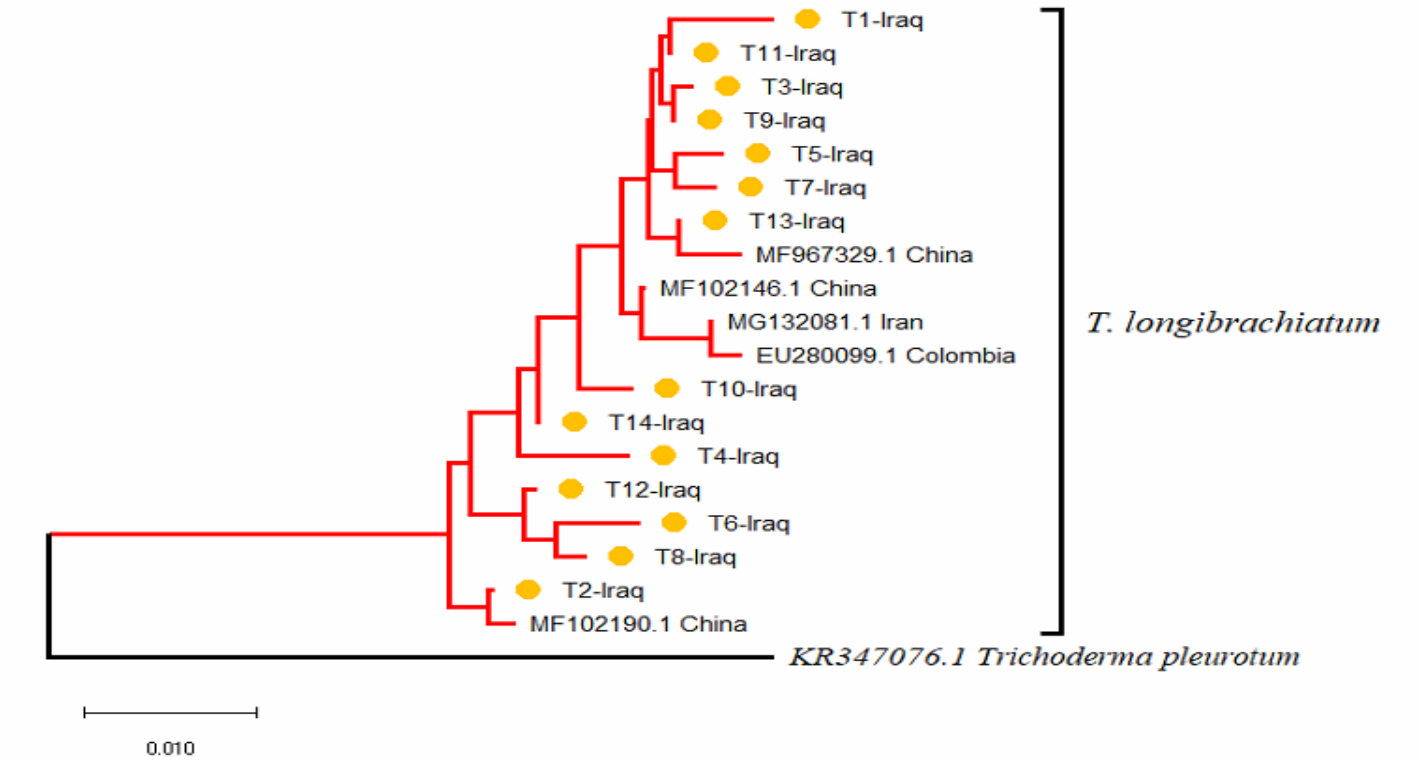

Fig. 4 : Phylogenetic relationship of *Trichoderma longibrachiatum*

Conclusion

The results of the study showed, that the oyster fungus type *P. eryngii* culture media which were subjected to contamination with green mold, caused a heavy losses in production. The result of the morphological examination together with DNA sequencing confirmed the identity of all isolates was belonging to *T. longibrachiatum*.

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