MOLECULAR AND BIOLOGICAL IDENTIFICATION OF *METARHIZIUM ANISOPLIAE* EG016 AS POTENTIAL BIOCONTROL AGENT INFECTED *SPODOPTERA EXIGUA* AGAINST *SPODOPTERA LITTORALIS* (LEPIDOPTERA: NOCTUIDAE)

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Abstract

Entomopathogenic *Merarhizium* fungus infects and kills insect pests in the green house and is used as agent in biological control. In this study, it was isolated from naturally infected pupae of *Spodoptera exiguae* which known to infest cabbage plants. The infection resulted in color change, malformation, cell death as well as fungal mycelium growth. *Metarhizium* sp. was isolated from infected pupae on SDYA Sabouraud dextrose yeast extract agar specific medium and showed fully colonies with round shape on medium. The fungus was isolated from pure single colony on SYDA medium by hyphal tip method. The purified isolate was identified based on morphological characteristics including hyaline and branched conidiophore,formation of a sporulating layer,single phialides or in pairs, conidia produced in chains, compacted into columns and long-ovoid to cylindrical shape was observed. Pathogenicity potential was 66.8% after 12 days of treatment. The molecular identification was conducted via 18srDNA gene amplification (335 bp PCR product resulted) and sequencing recorded in gene bank *M. anisopliae* EG016 (KU965593) isolate.

Key words: Entomopathogenic fungi, *Spodoptera exigua*, Isolation, Identification, DNA, PCR, Nucelotide Sequences.

INTRODUCTION

Entomopathogenic fungi represent effective and promising biotic components in the natural regulation of arthropods (Meyling and Eilenberg, 2007). There are more than 750 entomopathogenic fungal species identified worldwide. The genus *Metarhizium* (Ascomycetes) includes multiple species such as *M. anisopliae*, *M. flavoviride* and *M. acridum* and considered as the best characterized entomopathogens (Meyling and Eilenberg, 2007). They have the potential to infect a wide range of insects compromising agronomically important pests such as locusts. Termites, beetle larvae and spittlebugs. The taxonomy of *Metarhizium*, particularly *M.*

anisopliae mophospecies has been performed through applying morphological, biochemical and molecular based characteristics (Pantou *et al.,* 2003).

Numerous biological control agents were developed using *Metarhizium* species. The Deuteromycete fungus *Metarhizium anisopliae* infects insect pests of commercial importance and has been studied extensively as a possible biological control agent as potential commercial product (Barra *et al*., 2013).The maximum germination temperature of many isolates of *M. anisopliae* is about 37°c. It is widely dispersed in nature and commonly isolated from infected insect or soil (Razinger *et al*., 2014).

In addition, Entomopathogenic fungi for instance *M. anisopliae, B. bassiana* and *Bacillus thuringiensis* were tested against the insect pests (the diamondback moth, the cabbage worm and beet armyworm) in the green house and in the field (Sabbour and Sahab, 2005).

The present work aims to isolation, of *a Metarhizium* fungus that could be a promising biological control agent from *Spodoptera exigua* and further identification using Biological and Pathogenicity characteristics as well as 18srDNA gene amplification and sequencing.

MATERIALS AND METHODS

Isolation of *Metarhizium* sp.: The naturally infected pupae of *Spodoptera exigua* showing fungal symptoms were collected from fields in which cabbage (*Brassica oleracea*) was infested in Sharkiya governorates. Pupae were surface sterilized using sodium hypochlorite (1%) for 30 second, then washed several times by sterilized distilled water and dried carefully using sterilized filter paper. Sterilized insect surface was transferred onto Sabouraud dextrose yeast extract agar (SDYA) medium; containing 1% peptone, 0.2% yeast extract, 4% dextrose and 1.5% agar in distilled water and incubated at 25°c for 14 days. The fungal growth was investigated daily to detect any growth of fungal colonies. All fungal colonies were reinoculated on SDYA medium for maintenance and purification (Hashim *et al*., 2002).

Purification of the isolated fungus.

The fungal isolate was purified using hyphal tips from the external growth by taking aloopful, followed by inoculation on appropriate SYDA agar media ,then incubated at 25°C as described by Hildebrand, (1938). The colony of each fungal isolate was re-cultured on slant medium and kept at 4°C as stock for further studies.

Identification of the isolated fungus

Morphological characteristics of the fungal isolate was determined in the plant pathology Dept., National Research Center, Cairo (NRC) based on the cultural and morphological characteristics after growing the fungus on specific media. The morphological criteria were compared with the available literature as well as with the description given by (Barent and Hunter, 1977) for the genus *Metarhizium.*

Pathogenicity test: Healthy *Spodoptera littoralis* had been reared in the laboratory of Plant Protection Research Institute for several generations under 25±1ºC and 50-60% Relative Humidity condition. Cotton leaf worm larvae, 2nd instar larvae (*S. littoralis*) were dipped in each concentration of *Metarhizium* for 20 seconds (Er *et al.*, 2007). Control larvae were treated with sterile water only. All treated larvae were placed in cylindrical cages and fresh castor leaves were added, then covered using cheesecloth. Ten larvae for each cage and three cages as replicates for each treatment in a completely randomized design were used. Treated larvae were kept in a dark champers maintained at an average temperature of 28±2 ºC.

Malformed and dead larvae were checked daily. Percentages of malformed dead cotton leaf warm larvae were calculated. The infected larvae were daily transferred to media in dishes, kept at saturated humidity and at 28±2 ^{O}C for presence of a sporulating fungal isolate. All experiments were prolonged for12 days after inoculation. (Fargues *et. al.,* 2001). Data analysis was performed using probit analysis (Finney, 1971).

Data analysis

Daily corrected cumulative and accumulative larval mortality was reported for *Metarhizium* isolate and corrected according to Abbott, (1925). The lethal concentration ((LC_{50}) was computed for each of the fungal suspensions through probit analysis using the Propan program.

Isolation of total genome DNA

Metarhizium isolate was grown into Czapeks Dox broth media followed by 5 days incubation at 25ºC with shaking at 200 rpm. After incubation the mycelium mass was harvested using centrifugation at 6000 rpm for 10 min. The pellets were washed twice with a buffer solution containing 145Mm NaCL; 100 mM Na2HPO4; pH 7.5 and stored at 20ºC. The pellets were suspended in 500 ml PBS buffer and kepton ice three times for 20 sec. at 60 w to disrupt *Metarhizium*.Total genomic DNA was isolated

using a lysozyme/dodecyl sulfate. Lysis method was conducted according to Chang *et al.*, (1995). The mycelia were lysed by the addition of 10% SDS solution followed by incubation with 100µl RNase. 10 µl of Proteinase K solution (10 mg/ml) was added, to lysedmycelia and incubated for 1 hr at 37ºC. The DNA in the aqueous phase was precipitated with 95% ethanol followed by washing with 70% ethanol. The DNA pellets were allowed to dry then dissolved in TE buffer (10 mM Tris HCL, 0.5mM EDT, pH 8.0) and stored at 20º C for further studies.

Amplification of *18srDNA* gene

The Primer sets used for identification of Metarhizium was designed as universal 18srDNA gene for Deutromycetes fungi. The primers sequence for the primer pair was; the forward primer; 5CAGCTCGAAAACAAACCCCG-3 and the reverse primer; 5-AGAGCATCCTAGCAAAGCCG-3. The primer sets generates a 335 bp PCR product with optimal annealing temperature at 55ºC.

The **18s rDNA** gene was amplified using conventional PCR. The PCR reactions consisted of ; 12.5 µl DreamTaq Green PCR Master Mix (2X), 1 µl forward primer, 1 µL reverse primer, 2 µl Template DNA and water in a total volume of 25 µl. The PCR reaction conditions were; Initial denaturation at 95ºC for 5 min, 40 cycles of denaturation at 95ºC for 30s, annealing at 58 ºC for 30 s and extension at 72 ºC for 30 s , and final extension step at 72ºC for 10 min.

Agarose gel electrophoresis

The PCR product (50µl) was detected by 1.5% agarose gel electrophoresis running in 1X TAE buffer,stained with ethidium bromide as described by Sambrook et al., (1989). The amplified DNA fragments were visualized usingUV light transilluminator and the size of expected DNA fragments was estimated as compared to a DNA ladder of 100 to 2000 bp (Bio-rad).The run was performed at 100 V in Bio-Rad submarine and photographed with Gel Documentation 2000 systems.

Purification of PCR product

The 18sr DNAPCR products were eluted from agarose gel using the gel DNA extraction kit and purified using a QIA quick gel extraction kit (Qiagen Inc., Germany).

DNA sequencing

The nucleotide sequence of the purified DNA fragments was sequenced using automated DNA sequencing.The DNA amplicons returned as electropherogram files.

Electropherogram showed distinct peaks for each base cell as well as high values for each calls. The Primers were easily identified in either the forward or reverse direction in each sequence fragment and easily used to piece together the individual sequence. Sequences obtained for each primer for each isolate had sufficient overlap between them and used to form one continuous sequence (Contig).

Resulted DNA sequences of *Metarhizium* isolates were aligned using MEGA 5.1 and further analysis was conducted using BioEdit software version 7 (www. Mbio-NCUs. Edu/bio. Edit). The nucleotide sequence of 18srDNA gene was checked on GenBank for highly similar sequences with known accession numbers. Furthermore, the *Metarhizium anisopliae* EG016 18s rDNA nucleotide sequence was submitted using NCBI BankIt submission tool and an accession number (KU965593) was assigned for this isolate. The dendrogram showing relationships between the *Metarhizium* isolate isolated in this study and the other close isolated was constructed using Neighbor joining method.

RESULTS AND DISCUSSION

Identification of *Metarhizium* associated with *Spodoptera exigua*.

M. anisopliae EG016was isolated from naturally infected pupae of *S.exiguae* infested cabbage onto SDYA specific medium. The external symptoms such as dead impact, malformation, colour change and mycelium growth was revealed (**Fig.1a).** *Metarhizium*was purified by hyphal tip assay on SDYA medium and was identified as *Metarhizium anisopliae* based on the morphological characteristics which werehyaline and branched Conidiophores, formation of asporulating layer; phialides either as single or in pairs, or in whorls; conidia (phialospores) produced in basipetal chains, compacted into columns with long-ovoid to cylindrical and1-celled, shape)as shown in Fig.1b. These results were in agreement with Pik-kheng *et al.*, (2009) and Razinger *et al.*, (2014) who reported that *M.anisopliae* produced yellow mycelia mat with circular rings of green conidia, fluffy colonies and gave round shape colony on SDYA or Potato dextrose agar (PDA) media.

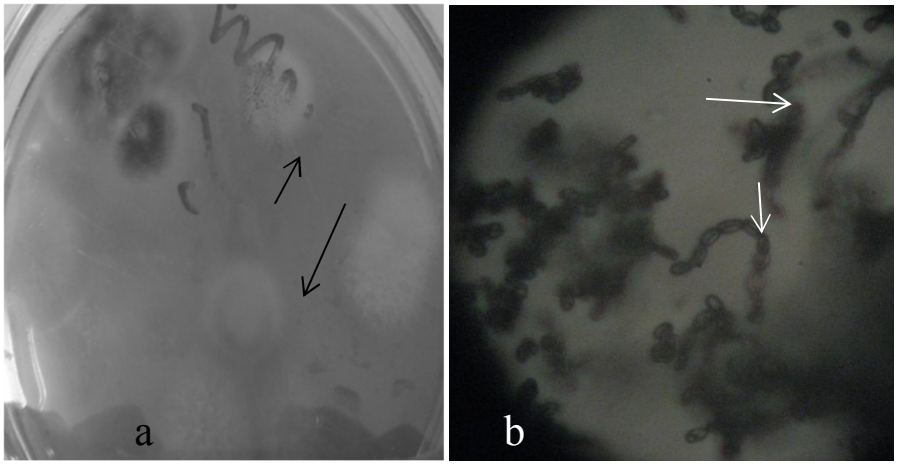

Fig. 1**. (a)***Metarhizium* growth after isolation from the naturally infested cabbage leaves with pupae of *S.exiguae* **(b***) Metarhizium* conidiophores and magnification at400x**.**

Entomopathogenicity tests

The entomopathogenicity tests were performed against 2nd instar larvae of *S.littoralis*. Results indicated that mortality percentage after 2 days of treatment was14.6% for all concentrations,while after 4 days the mortality percentage was 27.1%, then there was a gradual increase in mortality percentage appeared with increasing time elapsed post treatment. The mortality percentage increased to 66.8% after 12 days as shown by Lotfy *et al.,* (2010). Kumar and Chowdhry, (2004) reported that *M.anisopliae* gave mortality percentage ranged between 50-92.5%. The larvae showed progressive symptoms of fungal infection; beginning with a stop to eat that was associated with sluggishness. As infection progressed, the larvae became immobilized, moribund and darker in color. These symptoms were similar to those observed by Blanford and Thomas, (2001). Daily corrected cumulative and accumulative larval mortality for *M.anisopliae* isolate was recorded according to Abbott (1925) as shown (Table 1). In addition, Thompson and Brandenburg, (2005) reported that death caused by the fungi usually was more than 48 hs post infection after attachment of conidia to the insect cuticle.

Table 1. Cumulative corrected mortality percentage of *S.littoralis* 2nd instar larvae after dipping on *M.anisopliae* isolated from *S.exigua*.

Days	% Corrected mortality	LC_{50} (95% confidence limits)	Slope ± SE
2	14.60	$4.13566x10^{9}$	0.5489±0.3250
4	27.10	$3.56421\ x10^{9}$	0.3288±0.2506
6	48.10	$6.59060\ x10^{7}$	0.3827±0.2883
8	58.60	$1.21529\ x10^{7}$	0.3576±0.2427
10	64.10	$4.62284\ x10^{6}$	0.3510±0.0953
12	66.80	$1.99296\ x10^{6}$	0.3108±0.1350

Molecular characters of 18s rDNA gene

Total genomic DNA

Total DNA was isolated from *Metarhizium* and subjected to PCR amplification and sequencing.The integrity and quantity of purified DNA were confirmed by agarose electrophores (Fig. 2) and the UV spectrophotometer at absorption ratio A 260 / 280 was 1.7 indicating DNA purity. The concentration of DNA was 75 µg /5 gm mycelium. The results indicated that, *Metarhizium* mycelium yields high concentration and pure isolated DNA.

18s rDNA gene amplification

18srDNA gene of *Metarhizium* isolate was amplified from using conventional PCR with specific primers.The gel electrophoresis of the PCR products showed single intact and specific bands (Fig. 2). The size of the PCR products was found to be 335 bp as compared to the standard DNA ladder as shown in Figure 2.

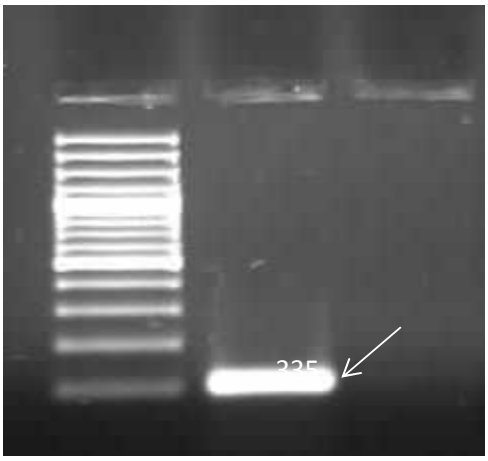

Fig 2. Agarose gelelectrophoresis (1.5%) showing DNA fragments of 18srDNA gene amplified by PCR using genomic DNA of *Metarhizium* M denotes DNA leader (100-2000 bp), IL denotes Infected larvae of *S.exiguae*;HL denotes Healthy larvae of *S.exiguae*.

The partial nucleotide sequences of the 18srDNA gene of *M. anisopliae* EG016included 571bp withas shown in Figure 3. The nucleotide sequence was aligned by using MEGA 5.1 program with other highly similar four *Metarhizium* strains published on Gen Bank (Fig.4). The nucleotide blast confirmed the identification of *Metarhizium* through sequence similarity with other known published isolates on Gen Bank. The multiple sequence alignment of *Metarhizium* 18s rDNA nucleotide sequences revealed the variation between this isolate and the other four identified *Metarhizium* in terms nucleotides addition, substitution and deletion. (Fig. 5).

CAGGCAACTACCTGATCGAGGTCACCTGGATAAAATTTGGGTTGATCGGCAAGCGCCGGCC
GGGCCTACAGAGCGGGTGACAAAGCCCCATACGCTCGAGGACCGGACGCGGTGCCGCCGCT
GCCTTTCGGGCCCGTCCCCCGGGATCGGAGGACGGGGCCCAACACACAAGCCGTGCTTGAG
GGCAGCAATGACGCTCGGACAGGCATGCCCCCCGGAATACCAGGGGGCGCAATGTGCGTTC
AAAGACTCGATGATTCACTGAATTTGCAATTCACATTACGTATCGCATTTCGCTGCGTTCTTC
ATCGATGCCGGAACCAAGAGATCCGTTGTTGAAAGTTTTAAATAATTTATATTTTCACTCAGA
CTACAATCTTCAGACAGAGTTCGAGGGTGTCTTCGGCGGGCGCGGGCCCGGGGGCGTAAGC
CCCCCGGCGGCCAGTTAAGGCGGGCCCGCCGAAGCAACAAGGTAAAATAAACACGGGTGGG
AGGTTGGACCCAGAGGGCCCTCACTCGGTAATGATCCTTCCGCAGGTTCACCTACGGAAACC
TTGTTACGACTTTTACTTCCA

Fig. 3. The partial nucleotide sequence of 18s rDNA gene of *M. anisopliae* EG016 (KU965593) isolate.

Nucleotide sequence analysis

The phylogenetic relations between the nucleotide sequence of 18s rDNA gene of *M. anisopliae* EG016 (KU965593) isolate and the closely related known isolates was constructed using neighbor-joining method. The phylogenetic tree of all *Metarhizium* isolates revealed three clusters of which the *M. anisopliae* under the current study was separated into a single cluster with close relatedness with *M. anisopliae* (KF056846.1) that existed as a separate cluster as well as *M. anisopliae* (JQ284382.1), *M. anisopliae* (JQ425479.1) and *M. anisopliae* (JQ284381.1) which constituted a single cluster.

Fig. 4. The Multiple sequence alignment of nucleotide sequences of 18s rDNA gene of *M. anisopliae* EG016 showing sequence similarity with multiple sequences of four other *Meterhizium* sp published on Gen Bank.

The molecular identification of *Metarhizium* species has been performed and contributed to the revision of the taxonomy of *Metarhizium*. Bischoff *et al.*, (2009) presented a taxonomic revision of *M. anisopliae* cryptic species complex based on a multilocus phylogenetic analysis that discriminated between nine species. A

phylogenetic tree based on multiple sequence alignment with published *Metarhizium* isolates on GenBank was carried out in this study as shown in Figure 5 to support the close relatedness of our isolates to those distributed worldwide.

The present work resulted in an efficient method for detection and identification of *M. anisopliae* that could be applied in further studies as well the bioactivity of the isolated *Metarhizium* against *Spodoptera exigua* suggests its potential as a biological control agent that might be promising for future studies to be used commercially.

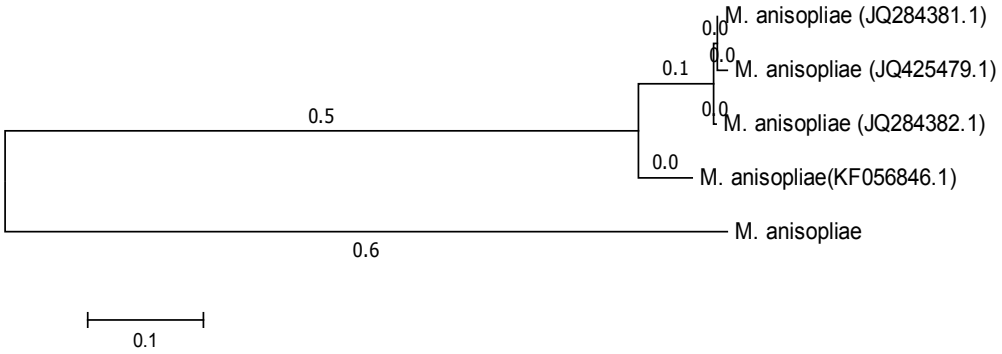

Fig 5. The Phylogenic tree showing the relatedness between all *Metarhizium* sp based on 18s rDNA nucleotide sequence similarity. The accession numbers for the four published *Metarhizium* sp obtained from GenBank is shown on the right of each isolate.

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التعريف البيولوجى والجزيئى لفطر *Metarhizium anisopliae* EG016 المعزول من حشرة دودة القطن الصغري كعنصر للمكافحة الحيوية لدودة ورق القطن

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فطر *Metarhizium anisopliae* ممرض للحشرات يصيب ويقتل الافات الحشرية ويستخدم كعامل في المكافحة البيولوجية. في هذه الدراسة، تم عزل الفطر من عذارى حشرة دودة ورق القطن الصغرى المصابة طبيعيا من على نبات الكرنب. واظهرت العذاري المصابة تغيير فى اللون، وتشوه، فضلا عن نمو الميسليوم الفطرية وموت الخلايا.

SDYA معملية متخصصة. تم عزل الفطر من مستعمرة فردية نقية على بيئة

تم تعريف الفطر المعزول بناء على الخصائص المورفولوجي للفظر بما في ذلك الهيفا وتفرع الحوامل الكونيدية ، وتشكيل طبقة من الجراثيم الكونيدية والجراثيم مفرده ا وفى ازواج او فى سلاسل فى أعمدة متراصة على شكل حصيرة ولوحظ انها بيضوية طويلة مغزليا بشكل أسطواني . والفطر له القدرة الإمراضية تصل الى ٦٦,٨% بعد ١٢ من المعاملة يوما علاج . أجرى التعرف الجزيئي عن طريق التضخيم الجيني ٣٣٥ زوج من النكلونيدات ذات وزن جزئى ١٠٠ – ٢٠٠ bp وثم تتبعها ومطابقته باستخدام PCR للتتابعات الجينية المسجلة فى بنك الجينات وتم تسجلها فى بنك الجينات تحت رقم.

EG016 (KU965593) isolate.

الكلمات المفتاحية: الفطريات اللممرضة للحشرات، دودة القطن الصغري، عزل، تعريف،

DNA, PCR, Nucelotide Sequenc