DOI: https://doi.org/10.26682/ajuod.2020.23.2.13

Journal of University of Duhok., Vol. 23, No.2 (Agri. and Vet. Sciences), Pp 106-113, 2020

MOLECULAR IDENTIFICATION AND INSECTICIDAL ACTIVITY OF *Albifimbria verrucaria* ISOLATED FROM CUCURBIT PLANTS AND SOIL IN IRAQ

LAZGEEN **H**AJI **A**SSAF
General Directorate of Agriculture-Duhok, Kurdistan Region–Iraq

(Received: June 28, 2020; Accepted for Publication: August 19, 2020)

ABSTRACT

***Albifimbria* (=*Myrothecium*) *verrucaria* (Alb. & Schwein.) L. Lombard & Crous. has been involved as promising bio-pesticides for wide range of weeds and nematodes. Furthermore, has a promising insecticidal activity because of its ability to produce many enzymes as lipase, protease and kinase that have the ability to degrading the cuticles of insects. Seven isolates of *A. verrucaria* were isolated from cucurbit plants and the soil. The identification was based on molecular (ITS-rDNA) and morphological analyses. Laboratory bioassays to evaluate the insecticidal activity of *A. verrucaria* (cgh-38) against different stages of squash beetle, *Epilachna chrysomelina* (F.) (Coleoptera: Coccinellidae) were conducted. Three days post treatment, 87.5% mortality rate was obtained when the 1st instar larvae treated, compared to 31.45 for 4th instar larvae. The highest mortality percent was 25 % for adults after 9 days of treatment.**

***KEY WORDS**: Albifimbria verrucaria*, *Myrothecium*, insecticidal activity, *Epilachna chrysomelina*, soil, Iraq.

INTRODUCTION

The genus *Myrothecium* was first described in the 18 century, comprising three species (Tode, 1790). Tulloch (1972) revised the genus and described eight species, two new species and three new combinations. Recently, Lombard, et al. (2016) who performed multi-locus phylogenetic analyses, renamed *Myrothecium verrucaria* under the name of *Albifimbria verrucaria* (Alb. & Schwein.) L. Lombard & Crous. Persoonia 36: 177 (2016).

In general *Albifimbria* (=*Myrothecium*) species have a worldwide distribution as entophytic fungi that colonize various hosts - (Baute et al. 1978), as saprophytic fungi in the soil and decaying tissues of plants (Wang et al. 2005; Domsh and Anderson 2007), or as a weak pathogens on various hosts (QuezadoDuval et al. 2010).

A. verrucaria is particularly virulent against several weedy plant species and is potentially useful as a bio-herbicide (Hoagland et al. 2007) and reported to act as nematocides (Quarles, 2011, Nguyen et al. 2018). *A. verrucaria* is also has a promising insecticidal activity because of its ability to produce many enzymes as lipase, protease and kinase that have the ability to degrading the cuticles of insects (Wagenaar and Clardy 2001; Podieiablonskaia et al. 2017). Thus the *A. verrucaria* represents potential tool for many insect's biocontrol as woolly aphid *Ceratovacuna lanigera* on sugarcane (Chavan et al. 2009), Mealy bug (Vidhate et al. 2015) and Mosquitoes (Podieiablonskaia et al. 2017).

In this study we aimed to identify *Albifimbria* spp isolated from cucurbit plants and the soils based depending on both morphological and molecular analysis and then evaluate its insecticidal activity to squash beetle *Epilachna chrysomelina* (F.) one of the important insect pests of cucurbit plants in Iraq.

MATERIALS AND METHODS

Isolation

From soil

The soil samples were collected from agricultural soils cultivated with different cucurbit plants as cucumber (*Cucumis sativus* L.), squash (*Cucurbita pepo* L.), snake cucumber (*Cucumis melo* Var. *flexuoses* Naud), melon (*Cucumis melo* L.), watermelon (*Cirullus lanatus*) and pumpkin (*Cucurbita sp* L.) from the villages Qidishi, Bawan, Chalki, Hareka-shikha, Harek-agha, Mergi and Kalaka (that related to Amadia district (1122m above sea level), Duhok province, Kurdistan region, Iraq.

The soil samples about 500 g each was taken randomly from the depth of 0-10cm (standard depth of sampling for fungi) with a trowel after removing litter or weed plants, then placed in plastic bags labeled and transferred to the

lazgeenassaf@yahoo.com

laboratory (25˚C ± 2). Before using, the samples were thoroughly mixed and passed through a fine mesh sieve to break soil lumps and separating litter remnant. *Albifimbria* species were isolated with direct soil plating method (Warcup 1960). The plates were incubated at 25ºC for 7 days, then checked for the occurrence of *Albifimbria* spp. and other opportunistic fungi from soil using Oat amended with Cetyl trimethyl ammonium bromide CTAB (0.6) as selective media. Pure cultures from the growing colonies obtained were transferred to fresh appropriate PDA (Potato Dextrose Agar) media for identification depending on their morphological characteristics and reproductive structures with the aid of several taxonomic references (Tode, 1790; Tulloch, 1972; Goettel and Inglis, 1997).

- **From plants (Endophytes)**

To isolate endophytic *Albifimbria*, the above mentioned cucurbit plants were collected. The samples were kept in plastic bags and transferred to the laboratory. For each plant, tissues including leaves, stems and roots were washed by tap water and then surface- sterilized (Arnold, 2007). The tissues then well dried on sterile paper towels and the edges were cut to remove dead tissues ensuing from the disinfection process. Five sections of each plant part (root, stem and leaves) were placed in Petri dishes containing the selective media. Four replicates were used/ plant part. The plates were incubated at 25˚C for two weeks to record the occurrence of *Albifimbria* spp and other opportunistic fungi.

Genomic DNA extraction, PCR and sequencing

The extraction was done according to a commercial animal and fungi DNA preparation kit protocol (Jena Bioscience, Germany) at Duhok Research Center labs/ College of Veterinary Medicine/ Duhok University.

The Genomic DNA was used as template for PCR amplification of ITS region using universal primers ITS5/ITS4 (White *et al.* 1990). The sequencing (15 samples) was performed at Macrogen Company/ Korea. The resulting sequences were checked and aligned using BioEdit sequence alignment editor 7.0.0 (Isis Pharmaceuticals, Inc., Carlsbad, CA, USA). The sequences were submitted to Gen Bank. The similarity of the sequence with homologous sequences deposited in GenBank was calculated using the "BLAST" tool on the National Center for Biotechnology Information (NCBI) website. Alignment of selected sequences was done with clustalW. The phylogenetic tree was constructed using the Neighbor-Joining method with Jukes-Cantor model in MEGA7. Branch support was estimated by bootstrap analysis with 1000 replicates.

Insecticidal acticity of *Albifimbria verrucaria* under laboratory conditions

The virulence of *Albifimbria verrucaria* **(**isolate cgh-38) isolated from soil cultivated with cucumber (Hareka-shikha village) (Genbank No. MT019873) was conducted against the squash beetle *Epilachna chrysomelina* eggs, larvae, pupae and adults. Under laboratory conditions, a conidial concentration of treatment was 10^7 /ml water. The squash beetles individuals sprayed directly by 3ml of spore suspension/ stage/replicate. Tween 80 at a conc. of 0.02% was added to the suspension. Four replicates were determined (10 individuals/stage/ replicate) in a small plastic container (20 x 10 x10 cm) lined with moisten filter paper, supplied with fresh and clean pieces of squash leaves and fruit when required. For control treatment the replicates were sprayed with 3ml of distilled water by a new parfan sprayer (50 ml capacity). The application was repeated twice. The mortality percentage was recorded daily for 3 days for larvae (development period of each larva instar) and 9 days after treatment for adults. Cumulative mortality counts obtained from experiments were corrected for natural mortality, using Abbott's formula (Abbott, 1925). Data of lab experiments were statistically analyzed by SAS program using a Complete Randomized Design (CRD) with 4 replicates and the means were compared, using Duncan's multiple- range tests at $P \leq 0.05$.

RESULTS AND DISCUSSION

Morphological observation

By using Oat amended with CTAB (0.6) as selective media, a total of 48 isolates of different endophytic and soil fungi were isolated from cucurbit plants and the soil based collected from agricultural fields in Amadia district, Duhok province, Kurdistan region, Iraq. The most occurrence percentage of fungi was recorded with the *Fusarium*, *Aspergillus*, *Penicillium*, *Alternaria*, *Albifimbria*, *Chaetomium* and *Trichoderma*. Based on microscopic observation, 15 samples isolated from plants and soil displays the typical morphological characteristics found in species of *Albifimbria* that described elsewhere (Tode, 1790; Tulloch 1972) (fig.1).

lazgeenassaf@yahoo.com

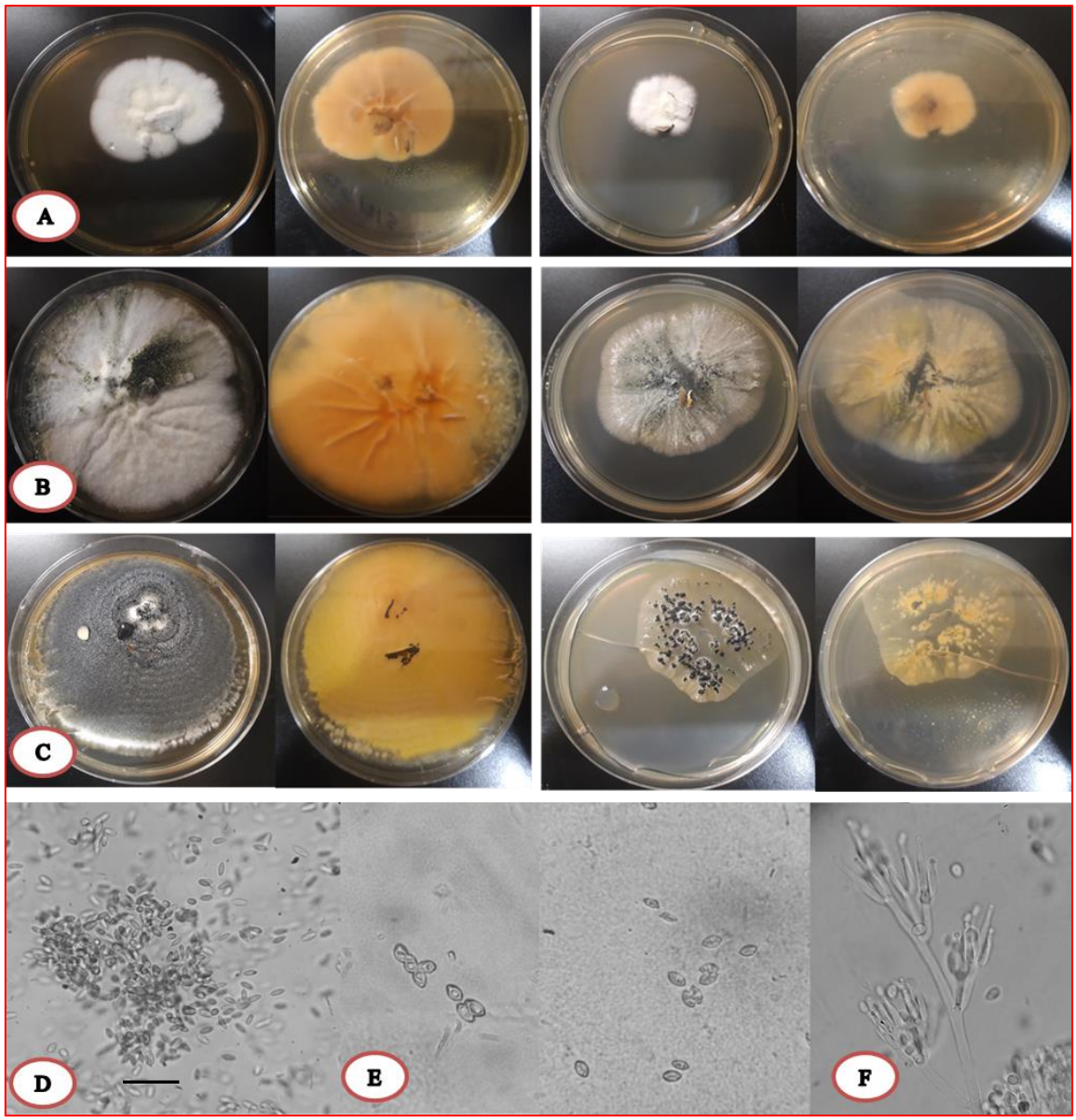

Fig. (1): Morphological characters of *Albifimbria verrucaria* three isolates grown for 10 days on MEA (left, obverse and reverse) and PDA (right, obverse and reverse) at 25C˚. A- MT019869 isolate, B- MT019872 isolate, C- MT019874 isolate, D- *A. verrucaria* conidia cluster scale bar= 5µm (apply to E and F) E- blastospores F- *A. verrucaria* conidia and phialides.

Sequencing of ITS and phylogenetic analysis

Molecular identification of 15 fungal isolates that morphologically described as *Albifimbria* spp was performed based on rDNA-ITS sequence. The results showed 580-590 bp of special DNA fragments sequenced. The sequencing results of 15 isolates showed that the obtained sequences confirmed that the taxon of seven isolates belong to *Albifimbria verrucaria* (Table1).

Using blast search, to compare the resulting sequences with sequences of rDNA accessed in Genbank, phylogentic analysis showed that the obtained sequences shares 99% homology to *Albifimbria verrucaria* isolates: USA isolates (MH855118, KM215639, MH864009, MN227479 and MH855118), Chinese isolates (FJ235085and MG988393) and Netherlands isolates (KU845892 and KU845885) (Fig. 2).

lazgeenassaf@yahoo.com

Table (1): Isolate code, Genbank accession numbers, locality, hosts, colony color and conidia measurements of *A. verrucaria* isolated from cucurbit plants and soil based.

Isolate	Locality	Host/ Substrate	Genbank No.	Conidia (µm)	Colony color
sq-34	Qidishi	Snake cucumber based soil	MT019869	2-2.8 x 2.2-2.8 Oval in shape	White
sq-35	Qidishi	Snake cucumber based soil	MT019870	2-2.9 x 2.1-3 Oval in shape	White
cg-36	Chalki	Cucumber stem	MT019871	2.5-3 x 4.8-8.2 oval –rounded	Green
cg-37	Chalki	Cucumber leaf	MT019872	2.1-3.1 x 5.2-8.2 Rounded in shape	White-green
cgh-38	Hareka-shikha	Cucumber based soil	MT019873	2.5-3 x 6.8-7.7 Rounded in shape	Green
Gs-44	Hareka-shikha	Pumpkin based soil	MT019874	2.2- 3 x 3-8.1 Oval-rounded	Green
psz-45	Kalaka	Melon based soil	MT019875	3-3.4 x 8.5-8.8 Rounded	Green

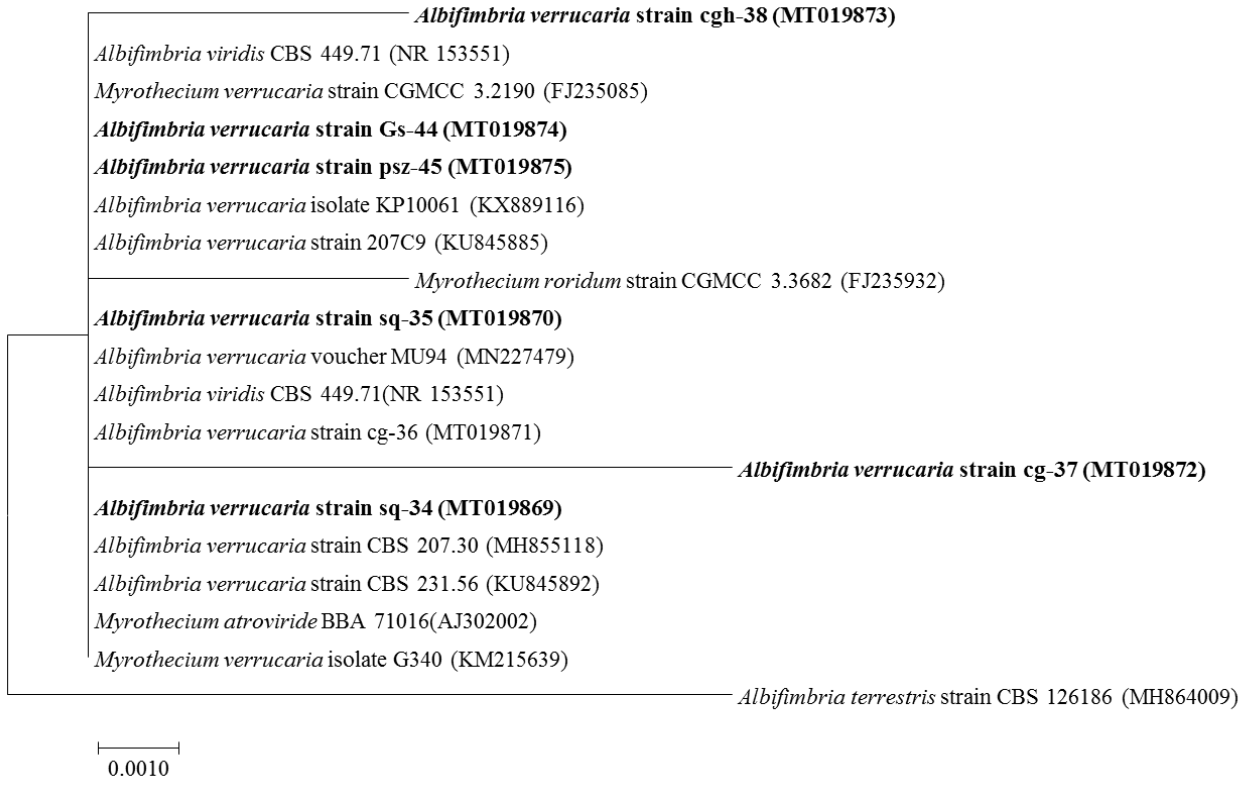

Fig. (2): Phylogenetic tree of *A. verrucaria* based on Neighbor-Joining analysis with 1000 bootstrap replicates of ITS-rDNA sequences of the new isolates from Iraq (in bold) and related *Albifimbria* species from GenBank. GenBank accession numbers provided next to species names.

lazgeenassaf@yahoo.com

Recently, several Chinese mycologists have identified *Myrothecium* species by combining morphological and analysis of ITS sequence data (Li et al. 2009; Zhao et al. 2009; Liu et al. 2014; Liu et al. 2015). Wang et al. (2015), Chen et al. (2016) reported that ITS sequence data can be considered as the primary barcode and most used for *Myrothecium* at the species level. Lombard, et al. (2016) performed multi-locus phylogenetic analyses and renamed *Myrothecium verrucaria* under the name of *Albifimbria verrucaria* (Alb. & Schwein.) L. Lombard & Crous.

Insecticidal activity of *Albifimbria verrucaria* under laboratory conditions

A. verrucaria ($1x10^7$ conidia/ ml) showed a significant effect on *E. chrysomelina* eggs hatching that a percent of 46.62 hatching was recorded after 7 days of treatment compared to 86.49% in control treatment. The data (fig.3) showed that *A. verrucaria* have an insecticidal activity to all instars of *E. chrysomelina* larvae under laboratory conditions and the mortality percentage decreased as larvae developed in age. The mortality percentages for the 1st instar larvae was 87.5% compared to 66.67, 43.67, 31.45% for 2nd, 3rd and 4th instar larvae, respectivily after three days of treatment. For pupae, *A. verrucaria* ($1x10^7$ conidia/ ml) scored a high mortality percent reaching 55.56% after 7 days treatment. A percent of (3.33) malformation was occurred with adults emerged from pupae treated with *A. verrucaria* compared to zero percent in control. Corrected mortality percentages of *E. chrysomelina* adults treated with *A. verrucaria* (10^7 conidia/ ml) as a cumulative after 9 days treatment reached to 25% compared to (0%) in the control treatment (fig.3).

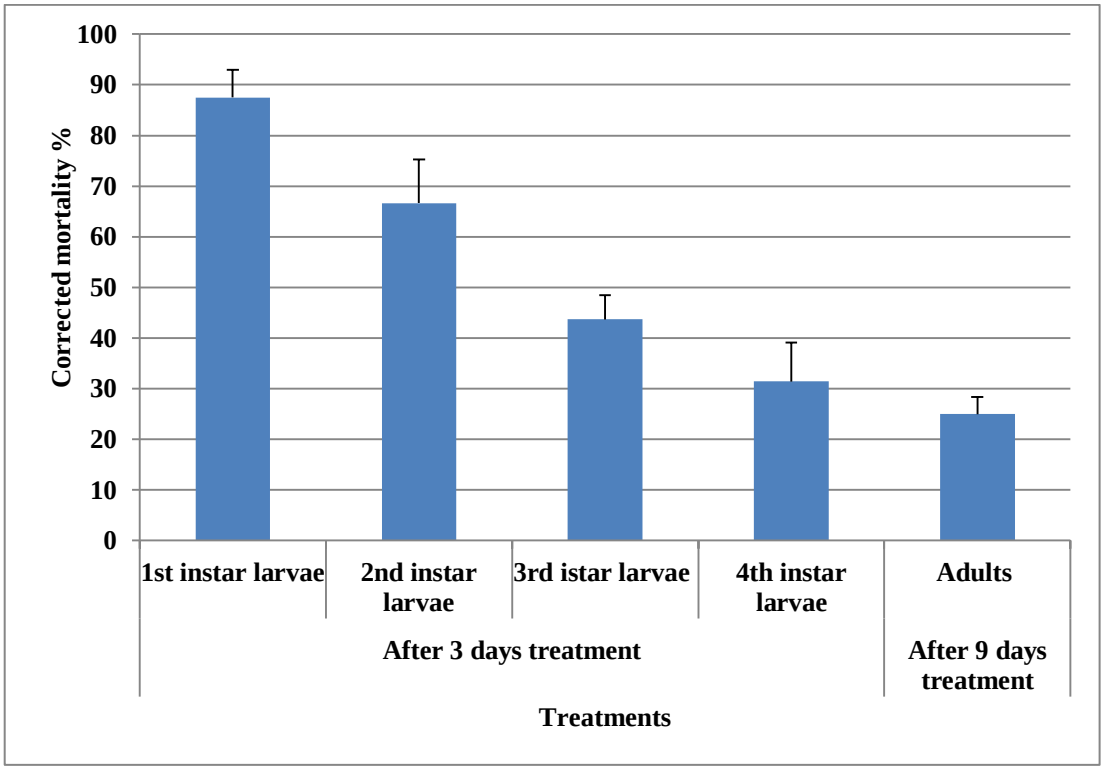

Fig. (3): Pathogenicity of *Albifimbria verrucaria* to squash beetle *Epilachna chrysomelina* larval instars (3 days after treatment) and adults (9 days after treatment).

The study of *Albifimbria verrucaria* as an entomopathogenic fungus is less developed with few works being published. *A. verrucaria* has high activity and produces chitinases, proteinases and lipases (Kobayashi et al. 1996). Vidhate et al. (2015) who used the cuticle degrading enzymes of *M. verrucaria* and the conidia of *Metarhizium anisopliae*, singly and sequentially to control mealy bug *Maconellicoccus hirsutus* Green, reported that sequential application of bioagents for mealy bug control was significantly affect the insect population, that enzymic degradation of insect cuticle possibly enhanced the entry of

lazgeenassaf@yahoo.com

germinating conidia of *M. anisopliae*. Mohamed (2016) mentioned that the fungal isolates effect on insects affected by the insect species, experiment conditions, the material used in bioassays and the pathogenicity of isolates. Li et al (2020) mentioned that *A. verrucaria* produced a total chitinase activity of 0.4 U/ml after incubation for 10 days in Czapek's liquid medium and indicated that the culture extracts of *A. verrucaria* significantly reduced disease severity caused by *Botrytis cinerea* on grape leaves.

CONCLUSION

Morphological identification of *Albifimbria* species are presently difficult to resolve because of few distinct morphological characters, therefore, it is necessary to do the molecular identification by ITS and other sequence data. *Albifimbria verrucaria* fungus has various applications in large scale production of enzymes, antibiotics and bioherbicides. *A. verrucaria* also used as a biocontrol agent for insects because of its ability to produce many enzymes as lipase, protease and kinase that have the ability to degrading the cuticles of insects but few reports are available and most of them concentrate on using the enzymes after extraction for control. This paper reports the molecular identification of *A. verrucaria* for Iraqi isolates and the success of using its spore suspension to control different stages of squash beetle *Epilachna chrysomelina* (F.). The results are very promising to use this fungus for other insects' control. Although is a weak phytopathogen, this fungus utilize as entomopathogenic fungus requires a unique risk management strategy to avoid damage to plants.

REFERENCES

Arnold AE (2007). Understanding the diversity of foliar endophytic fungi: progress, challenges, and frontiers. Fungal Biology Reviews 21: 51–66.

Baute MA, Deffieux G, Baute R, Neveu A (1978). New antibiotics from the fungus *Epicoccum nigrum. I.* Fermentation, isolation and antibacterial properties. Journal of Antibiotics 31(11): 1099-1101.

Chavan S, Kolomiets E, Kuptsov V, Mandrik M, Kulkarni M. and Deshpande MV (2009). Significance of cuticle degrading enzymes with special reference to lipase in biocontrol of sugarcane woolly aphids. *J. Mycol. Plant.Pathol.*, **39**, 118–123.

Chen Y, Ran SF, Dai DQ,Wang Y, Hyde KD,Wu YM and JiangYL (2016). Mycosphere Essays 2. *Myrothecium.* Mycosphere 7(1): 64–80.

Hoagland RE, Weaver MA and Boyette CD (2007). *Myrothecium verrucaria* fungus; A

Li BJ and Zhao YJ (2009). The identification of paint spot pathogen on eggplant(in Chinese). China Vegetable 15, 20–21.

Li Z, Chang P, Gao L and Wang X (2020). The Endophytic Fungus *Albifimbria verrucaria* from Wild Grape as an Antagonist of *Botrytis cinerea* and Other Grape Pathogens. Phytopathology 110(4): 843–850.

Liu JK, Hyde KD, Jones EBG, Ariyawansa HA, Bhat D J, Boonmee S, et al. (2015). Fungal diversity notes 1–110: taxonomic and phylogenetic contributions to fungal species. Fungal Diversity 72 (1), 1–197.

Liu WZ, Tan H, Pan QL, Li HH, Yan HJ, Tan YZ, Zhang WM (2014). Chemical constituents of the mycelia of an endophytic *Myrothecium roridum*from *Pogostemon cablin*(Blanco) Benth(in Chinese). Journal of Guangdong Pharmaceutical University 30(4), 427–429.

Lombard L, Houbraken J, Decock C, Samson R A, Meijer M, Réblová M, Groenewald J Z, Crous P W (2016). Generic hyper-diversity in *Stachybotriaceae*. Persoonia 36, 2016: 156–246.

Mohamed, GS (2016). Pathogenicity of entomopathogenic fungus *Beauveria bassiana* and bacterium *Bacillus thuringiensis* var. *kurstaki* against the lesser grain borer *Rhyzopertha dominica* F. (Coleoptera: Bostrichidae) under laboratory conditions. J. Basic and Applied Mycology (Egypt) 7: 39-44.

Nguyen LTT, Jang JY, Kim TY, Yu NH, Park AR, Lee S, Bae Ch, Yeo JH, Hur JS, Park HW and Kim JCh (2018). Nematicidal activity of verrucarin A and roridin A isolated from *Myrothecium verrucaria* against *Meloidogyne incognita*. Pesticide Biochemistry and Physiology 148: 133-143.

lazgeenassaf@yahoo.com

Podieiablonskaia EV, Kolomytseva M P, Myasoedova NM, Baskunov BP, Chernykh AM, Classen T., Pietruszka J. And Golovleva LA (2017). *Myrothecium verrucaria* F-3851, a producer of laccases transforming phenolic compounds at neutral and alkaline conditions. *Microbiology* volume 86, pages370–376.

Quarles W (2011). New biopesticides for IPM and organic production. IPM Practitioner. 33, 1-9.

Quezado Duval AM, Henz, GP, Paz-Lima ML, Medeiros AR, Miranda BEC, Pfenning LH, Reis A (2010). New hosts of M*yrothecium* spp. in Brazil and a preliminary *in vitro* assay of fungicides. Brazilian Journal of Microbiology 41: 246-252.

Tode HJ 1790 – Fungi Mecklenburgenses Selecti. Fasc. 1. Nova Fungorum Genera Complectens. i-viii, 1–50, plates 1–7.

Tulloch M (1972). The genus *Myrothecium*Tode ex Fr. MycologicalPapers 130, 1-42.

Vidhate R, Singh J, Ghormade V, Chavan SB, Patil A and Deshpande MV (2015). Use of hydrolytic enzymes of *Myrothecium verrucaria* and conidia of *Metarhizium anisopliae*, singly and sequentially to control pest and pathogens in grapes and their compatibility with pesticides used in the field. Biopestic. Int. 11(1): 48–60 (2015).

Wagenaar MM and Clardy J (2001). Two new roridins isolated from *Myrothecium* sp. Journal of Antibiotics54, 517.

Wang Y, Hyde KD, McKenzie EHC, Jiang YL, Li DW, Zhao DG (2015). Overview of *Stachybotrys* (*Memnoniella*) and current species status. Fungal Diversity71(1), 17–83.

Wang YU, Guo LD, Hyde KD (2005). Taxonomic placement of sterile morphotypes of endophytic fungi from *Pinus tabulaeformis* (Pinaceae) in northeast China based on rDNA sequences. Fungal Diversity 20: 235- 260.

Warpcup JH (1960). Methods for isolation and estimation of activity of fungi in soil- In Parkinson, D.P. andWaid, J.W. (eds.). The ecology of soil fungi; p3-21. Liverpool University press.

White t, Bruns T, Lee S and Taylor J (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. Inn; Innis,M.,Gelfond,d.,Shinsky,I and White,T.(eds.): PCR protocols: A guide to methods and applications;p.315-322.Academic Press,New York.

Zhao YJ, Li BJ, ShiYX and Xie XW (2009). Identification of the pathogen of tomato *Myrothecium*leaf spot(in Chinese).Acta Phytopathological ICA Sinica 39(5), 528–531.

lazgeenassaf@yahoo.com

پشکنینا گەردیلەییی و شیانییێن کەرویێ **Albifimbria verrucaria** هاتیە جوداکرن ژ رووەکێن مالباتا قرعیاتا و ئاخی ل عیراقێ

پوختە

کەرویێ Albifimbria verrucaria (Alb. & Schwein.) L. Lombard & Crous. ئیکە ژ کەرویێن هاتینە بکارئینان وەک قرکەرێن بایولوجی دژی گەلەک جورێن دەغەلا و نیماتودا. هەروەسا تیتە هژمارتن ئیک ژ کەرویێن باش بو بنبرکرنا میش و موران ژبەر شیانییین وی بو جیکرنا کەلەک انزیماتا وەکی لیبیز، بروتێز و کاینیز یین بکاردهین بو حەلاندنا کیوتکلی میش و موران. حەفت ئایزولیتین کەرویی Albifimbria verrucaria هاتنە جوداکرن ژ سامپلێن ئاخی و رووەکێن مالباتا قەرعی. شیانییێن توش بونا قوناغییێن کیزا Epilachna chrysomelina (F.) ب کەرویی(Albifimbria verrucaria (cgh-38 دبن کاودانیین لابوری دا هاتنە ئەنجامدان. ریژا مرنی کەهشتە 87.5% بشتی سی روژان ژ رشاندنا کرموکێن ژیی ئێکی بەرامبر 31.45 % بو کرموکێن ژیی جاری. بلنترین ریژا مرنێ د ناف کێزێن گەهشتی دا (بالغات) گەهشتە 25% بشتی 9 روژان ژ توشبونێ.

التصنيف الجزيئي وفعالية الفطر *Albifimbria verrucaria* المعزول من النباتات القرعية و التربة في العراق

الخلاصة

يعتبر .*Albifimbria verrucaria* (Alb. & Schwein.) L. Lombard & Crous من الفطريات التي استخدمت كمبيدات حيوية لمجموعة واسعة من الأدغال والديدان الخيطية .و ايضا يعتبر كمبيد حيوي حشري واعد بسبب قدرته على إنتاج العديد من الإنزيمات مثل الليبيز والبروتيبز والكينيز التي لديها القدرة على تحليل الكيوتكل . تم عزل سبع عزلات من الفطر *Albifimbria verrucaria* من نباتات العائلة القرعية والتربة المزروعة بها وتم تشخيصها على أساس التحليل الجزيئي (ITS-rDNA) والمورفولوجي. أجريت التجارب المختبرية لتقييم كفاءة عزلة الفطر (cgh-38) *Albifimbria verrucaria* (المعزولة من التربة المزروعة بنبات الخيار) على المراحل الحياتية لحشرة خنفساء القثاء (F.) *Epilachna chrysomelina*. سجلت نسبة الموت 87.5٪ بعد ثلاثة أيام من معاملة يرقات العمر الأول مقارنة بـ 31.45% ليرقات العمر الرابع. أعلى نسبة موت للبالغات وصلت 25% بعد 9 ايام من المعاملة.

lazgeenassaf@yahoo.com