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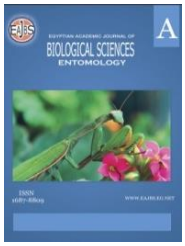

Evaluate the Effects of Entomopathogenic Fungi Isolates on Wheat Aphid, *Schizaphis graminum* (Rondani) (Hemiptera: Aphididae)

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ABSTRACT

Four native isolates of entomopathogenic fungi (EPF) *Beauveria bassiana* (B1and B2), *Metarhizium anisopliae* (M1 and M2) were isolated and evaluated against wheat aphid, *Schizaphis graminum* (Rondani). Three concentrations of spores suspension from each of the four fungal isolates (1×10^6, 1×10^7 and 1×10^8conidia) were used against adult aphid (one day old). The results showed that (B1) was the most effective according to LC_{50}. Whereas, when testing the toxicity of crude metabolites of fungal isolates in four concentrations for each isolate, it was found that the highest effective toxin was (M1) followed by (B1), (M2) and (B2). Scanning electron microscope photographs (SEM) showed the mode of action of entomopathogenic fungi and its ability to colonize and how it infects aphid.

INTRODUCTION

Cereal aphids are the serious pests attacking cereal crops, particularly wheat which caused yield losses either directly by sucking the sap of the plants or indirectly by transmitting viral and fungal diseases (Abro *et al*., 2004). Damages to wheat caused by aphids were estimated by up to 23%, particularly in Upper Egypt, where the highest infestation mostly occurs (El-Heneidy and Adly, 2012). Some of the cereal aphids are efficient vectors of different strains of Barley yellow dwarf virus (BYDV) (Emden and Harrington, 2007). The most important and economic cereal aphid species in Egypt are; bird cherry-oat aphid (*Rhopalosiphum padi* L.), green bug (*Schizaphis graminum* Rondani), *Rhopalosiphum maidis* Fitch.) and (*Sitobion avenae* F.) (El-Fatih, 2000).

Aphid is commonly controlled by pesticides. However, resistance to pesticides has guided research to find new methods intended to control Aphid. Additionally, the indiscriminate use of different synthetic insecticides to avoid serious health hazards for mammalians. Biological control agents such as entomopathogenic fungi can be used as a component of integrated pest management. Under natural conditions, fungal pathogens are frequent and often cause natural mortalities to the insect populations. Entomopathogenic fungi (EPF) such as *Metarhizium anisopliae* (Metschnikoff) and *Beauveria bassiana* (Balsamo) are well characterized with respect to pathogenicity to several insects, and they

have been used as agents for the biological control of agricultural pests worldwide (Sandhu, *et al*., 2012). Most of the published reports reveal that multiple toxic substances were derived from entomopathogenic fungi, which is an attractive approach for identifying important bioactive compounds from EPF. Based on this approach, few studies on EPF have been directed at elucidating the relevant virulence factors of insecticidal metabolites, antifeedants. EPF produces secondary metabolites that disable several immune mechanisms allowing the fungus to overcome and then kill its host. This characteristic makes *B. bassiana* and *M. anisopliae* promising models for biological control of insect pests (Zibaee *et al.*, 2011). The insecticidal activities of secondary metabolites against many pests have been described in several reports (Ríos-Moreno *et al.*, 2017 and Shin *et al.*, 2016).

Therefore, the present work was carried out as an attempt to investigate and suggest some alternative agents be incorporated into IPM control of wheat aphid; *S. graminum* by determining the toxicity of four isolates of entomopathogenic fungi *Beauveria bassiana* (Blas.) and *Metarhizium anisopliae* (Metsch.) and their metabolites. *S. graminum* adult females will be treated under laboratory conditions, with entomopathogenic fungi and their extracts to evaluate their effects and efficiency as natural insecticides.

MATERIALS AND METHODS

Rearing of Wheat Aphid*:*

Aphid colonies were maintained according to (El-Gendy,2009). The Laboratory strain of wheat aphid, *Schizaphis graminum* (Rondani) was obtained from a colony cultured at Biological control department, Plant Protection Research Institute, ARC, Ministry of Agriculture, Dokki, Giza, Egypt. These strains were reared for several generations under laboratory conditions on a wheat plant in plastic pots. Aphid colonies were prevented from external contamination by placing infested plants in cages covered with a muslin cloth.

Bioassay:

Three concentrations ($1\times10^{6,}$ 1×10^{7} and $1\times10^{8)}$ of spores suspension were prepared in 0.1% TritonX-100 (added as surfactant) for each one of entomopathogemic fungi isolates, *Beauveria bassiana* (B1and B2)& *Metarhizium anisopliae* (M1 and M2) were isolated in Bio-insecticides Production Unit, Plant Protection Research Institute, Agricultural Research Center, Giza, Egypt. Spray method was used to test the virulence of fungi. The mortality percentage was corrected according to Abbott's formula (Abbott, 1925). LC_{50}, LC_{90} and slope values were calculated according to (Finney, 1971), using "Ldp line" software by Bakr (2000).

Extraction of Fungal Metabolites:

Czapek-Dox broth (pre-culture medium) and plate media or slant were Czapek-Dox media were prepared. The conidia spores from the slants of different strains were suspended; 10 mL of conidia suspension was poured into a flask with 100 mL culture broth and incubated for 21 days at 240 r/min and 27 ± 1°C. Each isolate was replicated 4 times. Each flask was filtered two times through a Whatman 1 filter paper to remove the spores and mycelia (Ting-yan *et al*., 2016).

Evaluation Toxicity of Fungal Metabolites:

Metabolites concentration of each fungal isolates were (100%, 75%, 50 % and 25%) crude/ml each treatment had five replicates. Sterilized distilled water used as a control to each concentration. Mortality was assessed daily.

Scanning Electron Microscopy:

Adult aphids were treated after (24h, 48h, 72h, 96h, and 120h) then fixated by gluteraldhyde 2.5% and freezing. Then the samples drying using CO_2, finally coated by gold sputter coater (SPI-Module, USA).images were taken under low vacuum scanning electron

microscope by scanning electron microscopy (Model: JSM-5500 LV; JEOL Ltd-Japan) by using high vacuum mode at the Regional Center of Mycology and Biotechnology, Cairo, Egypt.

Data Analysis:

All experiments contained. 3-5 replicates and data were analyzed by one – way analysis of variance (ANOVA) using SPSS 17.0 statistical software. When the ANOVA statistics were significant ($P < 0.05$), the means were compared by Duncan's multiple range test.

RESULTS AND DISCUSSION

Virulence of Entomopathogenic Isolates:

The susceptibility of adult *S. graminum* to entomopathogenic fungi *B. bassiana* and *M. anisopliae* isolates were conducted. The mortality percentage values after exposure to a series of concentrations of 1×10^6, 1×10^7, and 1×10^8 spores/ml were shown for seven days after treatment in Fig. (1). the mortality percentage gradually increased along with spores concentrations and time. The Lowest concentration of 1×10^6 spores/ml revealed 65.2, 58.3, 54.7 and 67.56 % for B1, B2, M1, M2 and respectively, 7 days after treatment. While the highest concentration of 1×10^8 spores/ml revealed 80.0, 73.08, 87.7and 81.01 % for B1, B2, M1, M2 and respectively, When mortality was assessed after the same consecutive days, respectively.

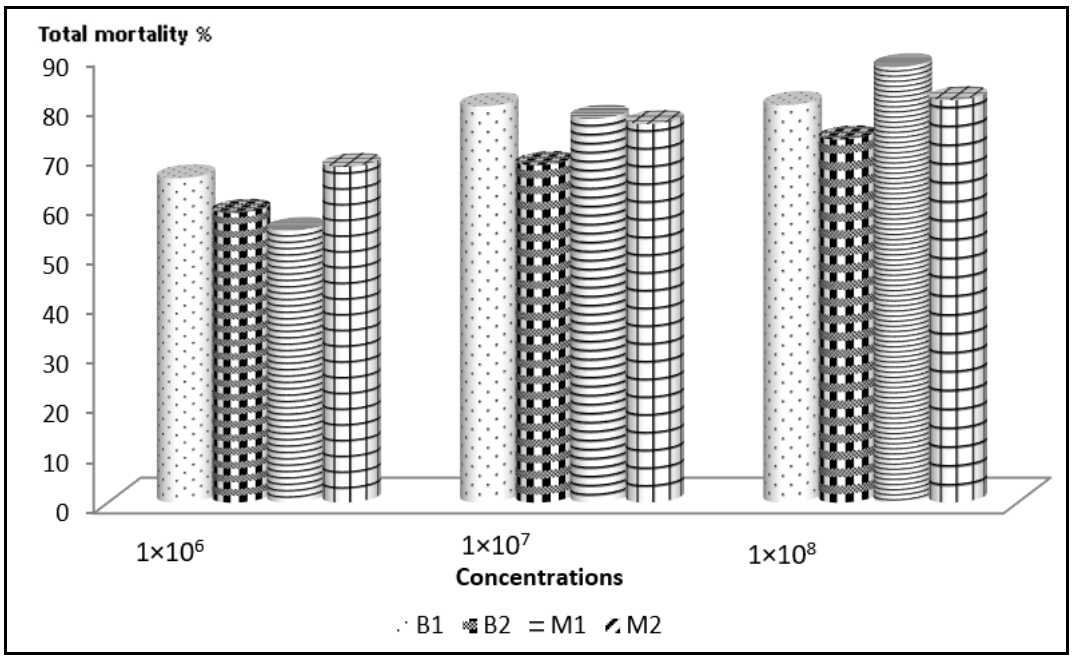

Fig. 1: Percentage total mortality of adult *S. graminum* treated with three concentrations of (B1 and B2) and (M1 and M2).

Results that (B1) was more effective against adult compared with all other isolates according to LC_{50} value. The LC_{50} values of B1 were 3.11×10^6 spores/ml while M1, M2, and B2 revealed greater LC_{50} value, 6.09×10^6, 2.32×10^7 and 1.15×10^8spores/ml, respectively (Table 1).

One-way ANOVA statistical analysis indicated that nonsignificant levels of effect ($F_{3,16}=1.28$; $P\leq0.05$) at 1×10^8spores/ml between isolates B1, B2, M1 and M2 on adult aphid are presented. Means were 80.66, 70.41, 87.75and % 81.3 for B1, B2, M1 and M2, respectively. The LT_{50} value of the four isolates of entomopathogenic fungi (B1 and B2) and

(M1 and M2) were tabulated with their corresponding slopes at seven days after aphid treatment (Table 1).

Table 1: Comparative between four isolates of entomopathogenic fungi *B. bassiana* and *M. anisopliae* adult stage according to LC_{50}, Means ± Standard error and LT_{50}.

Isolate	LC_{50}	Lower limit	Upper limit	Slope	LC_{90}	Index	Mean±SE	LT_{50}(Day)
B1	3.11×10^{6}	2.07×10^{5}	9.95×10^{6}	0.33	2.68×10^{10}	100	80.66±6.39a	3.04
B2	1.15×10^{8}	3.67×10^{7}	1.9×10^{9}	0.30	2.34×10^{12}	2.71	70.41±4.48a	3.70
M1	6.09×10^{6}	3.82×10^{6}	9.49×10^{6}	0.79	2.39×10^{8}	51.04	87.75±3.79a	3.95
M2	2.32×10^{7}	2.28×10^{6}	6.95×10^{7}	0.30	4.03×10^{11}	13.41	81.3±9.21a	3.37

Index compared with B1
the same latter within the rows means rows do not have significantly different ($P\leq0.05$).

The data showed that isolate B1 caused high mortality in the shortest time, LT_{50} value was 3.04 days. While for the other isolates M2, B2, and M1 the LT_{50} values were 3.37, 3.70 and 3.95 days, respectively. Virulence of entomopathogenic isolates against aphid in the light of the results obtained during the present experimental work, it was clear that entomopathogenic fungi; *B. bassiana* (B1, B2) and *M. anisopliae* (M1, M2) were pathogenic to the adult aphid. The pathogenicity of fungus increased with the increase of concentration and time. Death results due to severe damage in the tissues., toxicosis, cell dehydration, loss of nutrient intake, and finally the hyphae emerge from the insect body sporulates and starts a new infection cycle (Perez *et al.* ,2014). The results compatible with Various researchers in different parts of the world like Shah *et al*. (2004) Compared isolates of *P. neoaphidis*against seven UK pest aphid species; *A. pisum*, *A.s fabae*, *B. brassicae*, *M. persicae*, *Metopolophiumdirhodum*, *Sitobionavenae*, and *R. padi*. The LC_{50} values indicated that *B. brassicae*and *R. padi*were less susceptible to infection than four other aphid species. The overall ranking of aphids to infection by *P. neoaphidis* was: *A. pisum*, *A. fabae*, *M. dirhodum*, *M. persicae*, *S. avenae*, *B. brassicae*, *R. padi*. These findings in agreement withSaranya *et al*. (2010) who examined the pathogenicity of five entomopathogenic fungi; *B. bassiana, M. anisopliae*, *Verticilliumlecanii*, *Hirsutella thompsonii* and *Cladosporium oxysporum* against the adults of *A. craccivora* in Laboratory. Six different concentrations were used. In the highest concentration (10^8 spores/ ml) 100% mortality was obtained with *V. lecanii* and *H. thompsonii* followed by *B. bassiana*, *M. anisopliae* and *C. oxysporum*. Mortality declined with the decrease in concentrations. The lowest LC_{50} value of $2.5x10^4$ spores/ ml was recorded by *V. lecanii* and *H. thompsonii* isolates, which showed higher virulence compared to other isolates. The LC_{50} values of *B. bassiana*, *M. anisopliae* and *C. oxysporum* were $4.5x10^4$, $8.9x10^5$ and $7.4x10^5$ spores/ml respectively. At the highest concentration of 10^8 spores/ ml, the LT_{50} values for *B. bassiana, H. thompsonii, V. lecanii, C. oxysporum* and *M. anisopliae* were 3.63, 3.64, 3.90, 5.24 and 5.54 days, respectively. Also, Maketon *et al*. (2013) studied the use of *B. bassiana* CKB-048 against the cowpea aphid (*A. craccivora*). LC_{50} was 6.69×10^7 conidia/ml for the nymphs and 8.25×10^7 conidia/ml for the adults. In the same line, Aker and Abacı (2016) Proved that the use of *M. anisopliae*can provide protection on hazelnuts against aphids and it can be effective as biocontrol agent on *M. coryli.* These results are consistent with laboratory bioassay studies carried out using four different concentrations ($1x10^6$, $1x10^7$, $1x10^8$, $1x10^9$ spores/ml) for each of *Beauveriabassiana, Metarhiziu manisopliae, Paecilomyces lilacinus* and *Lecanicilliumantillanum* against the adults of cowpea aphid, *Aphis craccivora* (Sahar *et al*,2016). Tang *et al*.,2019 suggest that the fungus *M. anisopliae*CQMa421 is a good

biocontrol agent for *Nilaparvatalugens* (Stål) and *Sogatella furcifera* and that the combined use of *M. anisopliae*with insecticides is an alternative strategy for pest control.

Toxicity of Crude Extract:

The comparison percentage mortality values after exposure to series concentrations of four curds extract at 100%, 75%, 50% and 25% crude/ml were shown in table (2) and figure (2). Results obtained revealed that the four tested metabolic crudes were toxic to *S. graminum.* The highest effective toxin was of (M1) followed by (B1), (M2) and then (B2). The mortality percentages on the sixth day of concentration 100% crude/ml were 91.91, 73.00, 63.83 and 62.62 % for M1, B1, M2 and B2, respectively. One-way ANOVA statistical analysis indicated that significant levels of effect between different fungal crudes *M. anisopliae* (M1, M2) and *B. bassiana* (B1 and B2) on adult *S. graminum*were presented in Table (3). The obtained results showed that there was a highly significant effect ($F_{3,16}$=5.08; P≤0.05) between M1 and other treatments on *S. graminum*after six days post-treatment at 100% crude/ml but no significant between B1, M2 or B2 were means of mortalities were 92.04, 73, 64.8, and 63.38 for M1, B1, M2, and B2, respectively. Generally, the highest values of mortality were in the M1 treatment.

Table 2: Percentage mortality of adult *S. graminum* treated with series concentrations of B1, B2, M1, and M2crudes after six days of treatments.

Concentrations	Mortality%			
	B1	B2	M1	M2
25%	48.95	48.71	69.89	38.25
50%	58.33	51.5	80.68	54.76
75%	61.00	61.00	84.26	59.03
100%	73.00	62.62	91.91	63.83

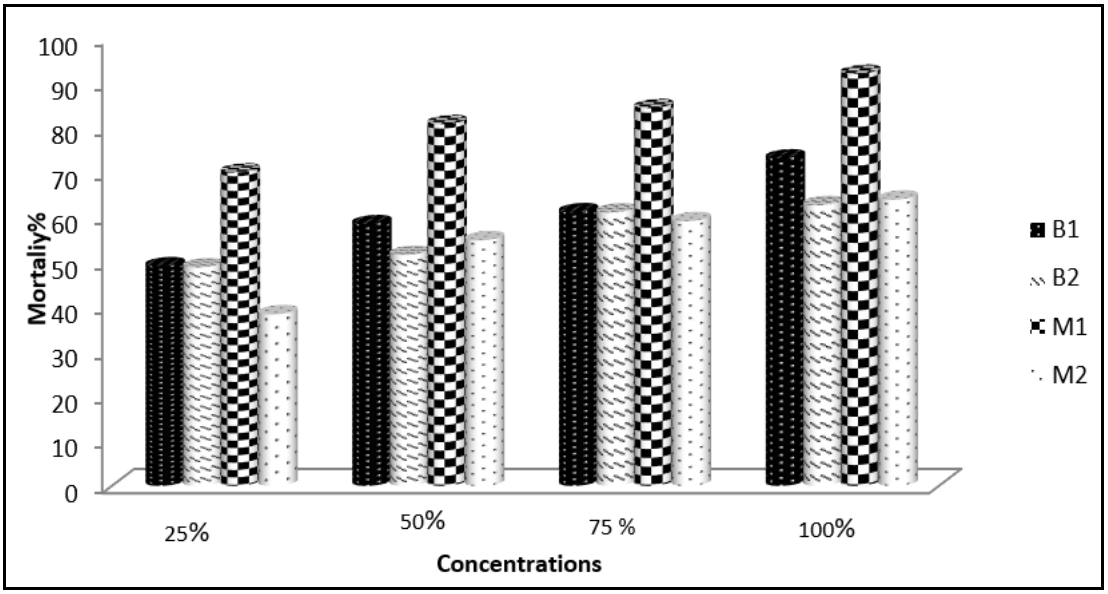

Fig. 2: Total mortality% of adult *S. graminum* treated with series concentrations of B1, B2, M1 and M2 crudes after six days of treatments**.**

Table 3: Comparative between four crudes of B1, B2, M1 and M2 against *S. graminum* adult according to LC_{50} and Means ± stander error

Isolate	LC_{50}	Lower limit	Upper limit	Slope	LC_{90}	Index	Mean±SE
B1	80.45	65.10	113.66	1.42	647.07	29.19	73±6.81b
B2	133.5	90.36	450.77	1.01	2505.98	17.59	63.38±6.94
M1	23.48	14.03	30.78	1.59	149.89	100	92.04±2.51
M2	105.39	88.90	138.10	2.30	379.81	22.28	64.8±6b

Index compared with M1

a,b Means within the same row having different superscripts significantly different (P≤0.05)

Entomopathogenic fungi secrete secondary metabolites which toxic to insects. Destruxins are insecticidal metabolites of a fungus, *M. anisopliae*. More than 35 different destruxins have been characterized by a wide range of insecticidal activities (Liu *et al.*, 2004). In the present investigation, the aim was to provide data for the enhancement of entomofungal virulence and the production of a toxic macromolecular insecticidal substance from native entomopathogenic isolates. Our study showed that four Isolates *B. bassiana* and *M. anisopliae* (B1 and B2 M1 and M2) and its metabolites had toxic effects on aphidadult, M1crude is more effective may be due to variations of the chemical composition of crude.
These agree with previous studies like Yi *et al.,* 2012 pointed that destruxins has synergistic action with mixed with three botanical insecticides, rotenone, azadirachtin and paeonolum against the cotton aphid, *Aphis gossypii.* Also, Moussa et *al*, 2014 reported that the chitinase enzyme was isolated and purified from the entomopathogenic fungus *B. bassiana* has bioactivity as a bio-control factor against two different aphid species; *A. craccivora* (Koch) and *Rh. padi* (Linn.). The extracted and partially purified chitinase enzyme had a significant potency against the two tested aphids. In the our line is Khan *et al.* (2012) Screened Six entomopathogenic fungal isolates, three each of *B. bassiana* and *Verticillium lecanii*, were for pathogenicity test against the *Myzus persicae*to select high virulent isolate .Two treatments that is, conidial shower (190±23 conidia/mm2) and filtrate (3 ml filtrate per treatment from six days liquid broth culture of $1.0x10^8$ conidia ml-1). The percent mortality rates of filtrate at each day, after inoculation was found higher as compared to percent mortality of conidial showering. *V. lecanii*3 showed highest virulence or toxicity against the target pest treated either with conidial (80.70%) or filtrate (88.36%) application while *B. bassiana*70 and *B. bassiana*76 showed high toxicity (77.14 and 80.86%, respectively) in filtrate application at 6th day of incubation.

In comparison toxicity of crudes with the conidial infection, it was found that the fungal metabolites were more effective in killing the aphid than infection with the conidia. That may be due to conidial infection required more time to complete the series of infection processes, including a conidial attachment on the host cuticle, germination, hyphal penetration through the integument, and infection of the internal tissues and organs, whereas the secondary metabolite was a more direct attack. Hence, fungal secondary metabolites should be considered for pest management programs in the future. These results in line with Fan *et al.* ,2013. Zibaee *et al.* 2011, they suggested that the secondary metabolites produced by *B. bassiana*caused problem in several defense mechanisms of *Eurygasterintegriceps*, thus helping the fungus to destroy its host. Sahar and El-sayed (2015) Showed that the percentage mortality of toxins crude extraction due to isolating of *B. bassiana* investigated against 3rd inster larvae of *Spodoptera. Littoralis* after 4 days, were 51.00%, 57.50%,79.00% and 96.50% in the concentrations 25, 50, 75 and 100%, respectively.

Scanning Electron Microscopy of Entomopathogenic:

This study tested that, entomopathogenic fungi, *B. bassiana* (B1) and *M. anisopliae* (M1) on adult *S. graminum* were virulent under laboratory bioassay and Scanning electron

microscope (SEM) observation. The findings here support the potential use of entomopathogenic fungi to control aphid.

Scanning Electron Microscopy of Entomopathogenic:

Scanning electron microscopy are convenient tools to observe the mode of action of entomopathogenic fungi and to study how entomopathogenic fungus is able to colonize and infect aphid

B. bassiana:

Scanning electron microscopy (SEM) of adult aphid treated with the 1×10^8 of the fungus, *B. bassiana*, (Fig.3, A) revealed adhesion spores and penetration structures germ tube and appressorium in the infected aphid occurred between 24 and 48 hrs (Fig.3, B), while it was clearly shown that no such structures were found on untreated aphid (Fig, 4). The phase of host colonization occurred between 48 and 72 hours (Fig.39, C and D). SEM illustrated the degradation cuticle and old spores of fungus were observed fungus between 96 and 120 hours, (Fig.39, E, F and G).

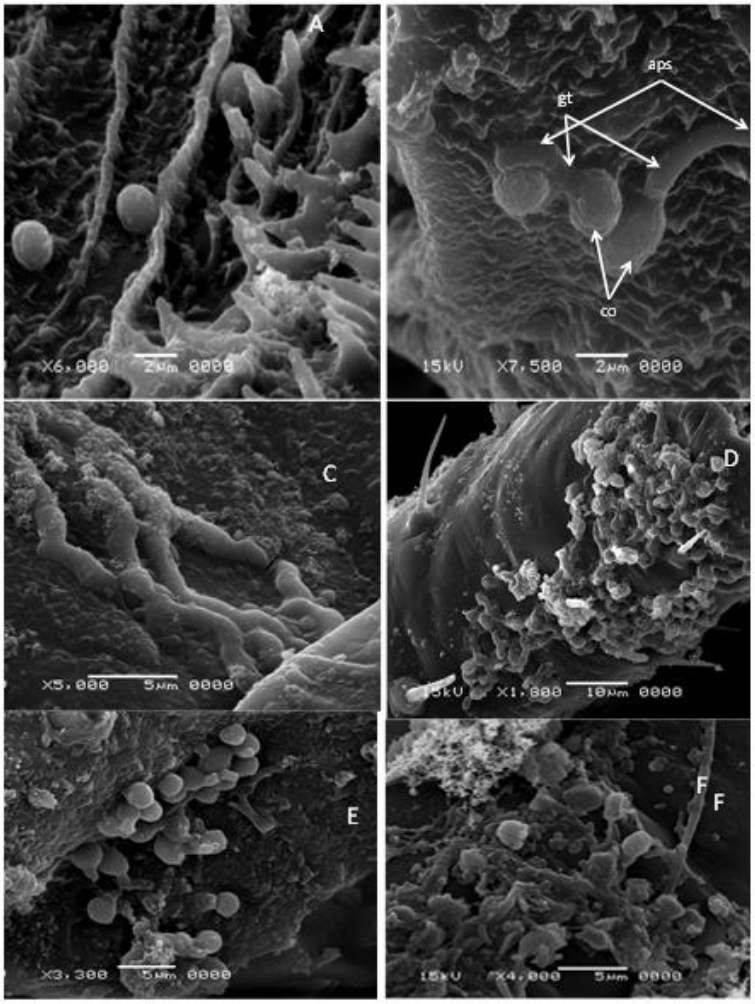

Fig. 3: Scanning electron microscopy showing *B. bassiana* infected *S. graminum*,
(A) Coindiospores (co) adhesion cuticle after 12hrs after inoculation (6,000X), (B) conidiospores germinate on the surface of the cuticle. Presence of conidia appressorium (aps) and germ tube (gt) on cuticular surface of aphid and the germinated hyphal tubes penetrate the insect's integument directly 24hrs after inoculation (7,500X,), (C) Scanning EM study of aphid post 72 hrs of inoculation Presence of conidia on surface of aphid treatment; cuticle penetration by elongated hyphae (hy) , Dislocation of the chitin plates (5,000X) (d) Leg insect covered with *B. bassiana* old conidia 96 hrs after inoculation (1,800X), (E and F) Dead insect showed degraded cuticle and old conidia (3,300and 4000X).

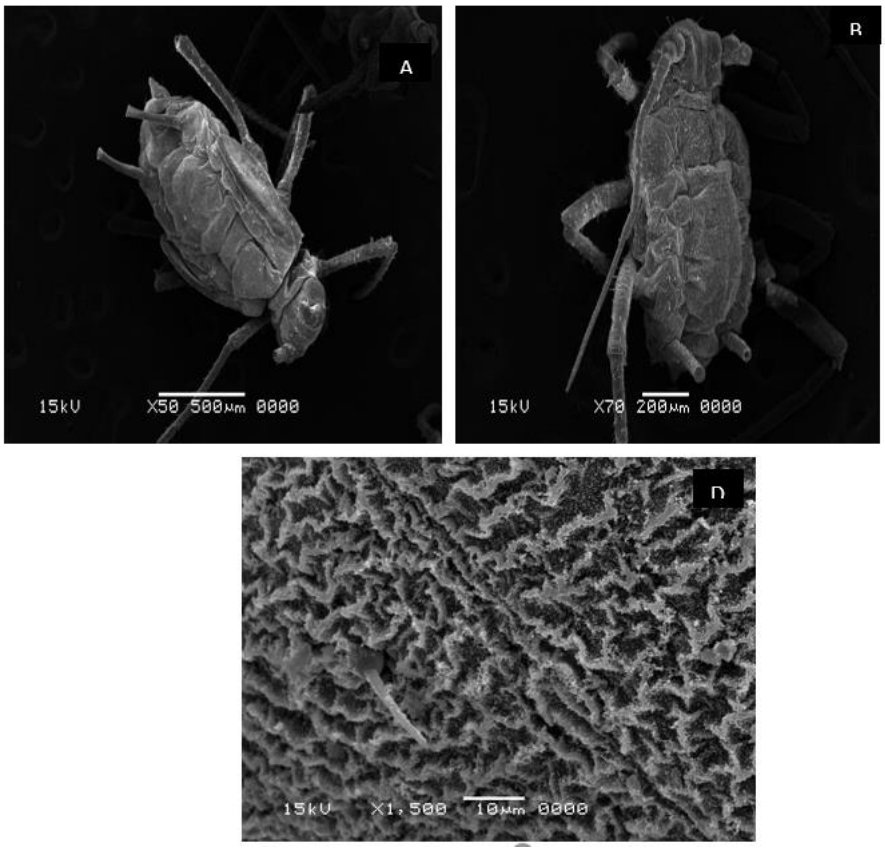

Fig.4: untreated aphid A, ventral view (50X), B, dorsal view (70X) and c, showing cuticle of aphid (1500X)

M. anisopliae

Scanning electron microscopy (SEM) of adult aphid treated with the 1×10^8 of the fungus, *M. anisopliae*, (Fig. 5, A) showed elongate spores attachment after 12 hrs. Growing germ tubes and appressorium 24-48 hrs after inoculation (Fig.5, B) promote germination and growth of the fungus across the surface of the host, and subsequent penetration of cuticular layers in the infected aphid occurred between 24 and 72 hrs. While it was clearly shown that no such structures were found on untreated aphid (Fig, 4). The phase of host colonization occurred between 48 and 72 hours (Fig.5C), Fig.5 (D) showed bilateral germination. SEM illustrated complete cuticle degradation between 72-120 hrs. (Fig.5 E).

Fungi have a unique model of infection (1) Conidia adhesion and germination in the epicuticle of the insect and germination is activated by carbohydrates present in the cuticle. Entomopathogenic fungi develop an appressorium with the purpose of beginning the penetration stage through the germ tube formation (Narayanan, 2004). (2) The cuticle penetration is the result of combined action of mechanical force and the enzymatic (3) Insect's propagation and death. Death results by severe damage in the tissues, toxicosis, cell of the insect body dehydration and loss of nutrient intake, finally the hyphae emerge from sporulates and starts a new infection cycle. proliferation inside the insect (Narayanan, 2004, and Perez *et al.*, 2014).

Our results are in the line with Nada, 2006 examined Scanning electron microscopy showing *Neozygites fresenii* infected *Aphis craccivora* aphid Germinated primary conidia with germ tubes of *N. fresenii* (arrow) penetrated *A. craccivora.* Also Scanning electron microscopy showing *Pandora neoaphidis* infected *S. graminum* aphid. *S. graminum* infected by *P. neoaphidis* showing hyphal bodies emerged through the host cuticle. Also, Sharma *et al.* (2017) Tested three isolates of entomopathogenic fungi *Beauveria bassiana* infected

nymph of *Lipaphis erysimi* Kalt. using scanning electron microscopy (SEM) to record any variation. The SEM revealed adhesion of spores of *B. bassiana* followed by penetration of *L. erysimi* nymph surface. It was observed that all *Beauveria* isolates showed little variation with respect to penetration and adhesion at different time intervals.

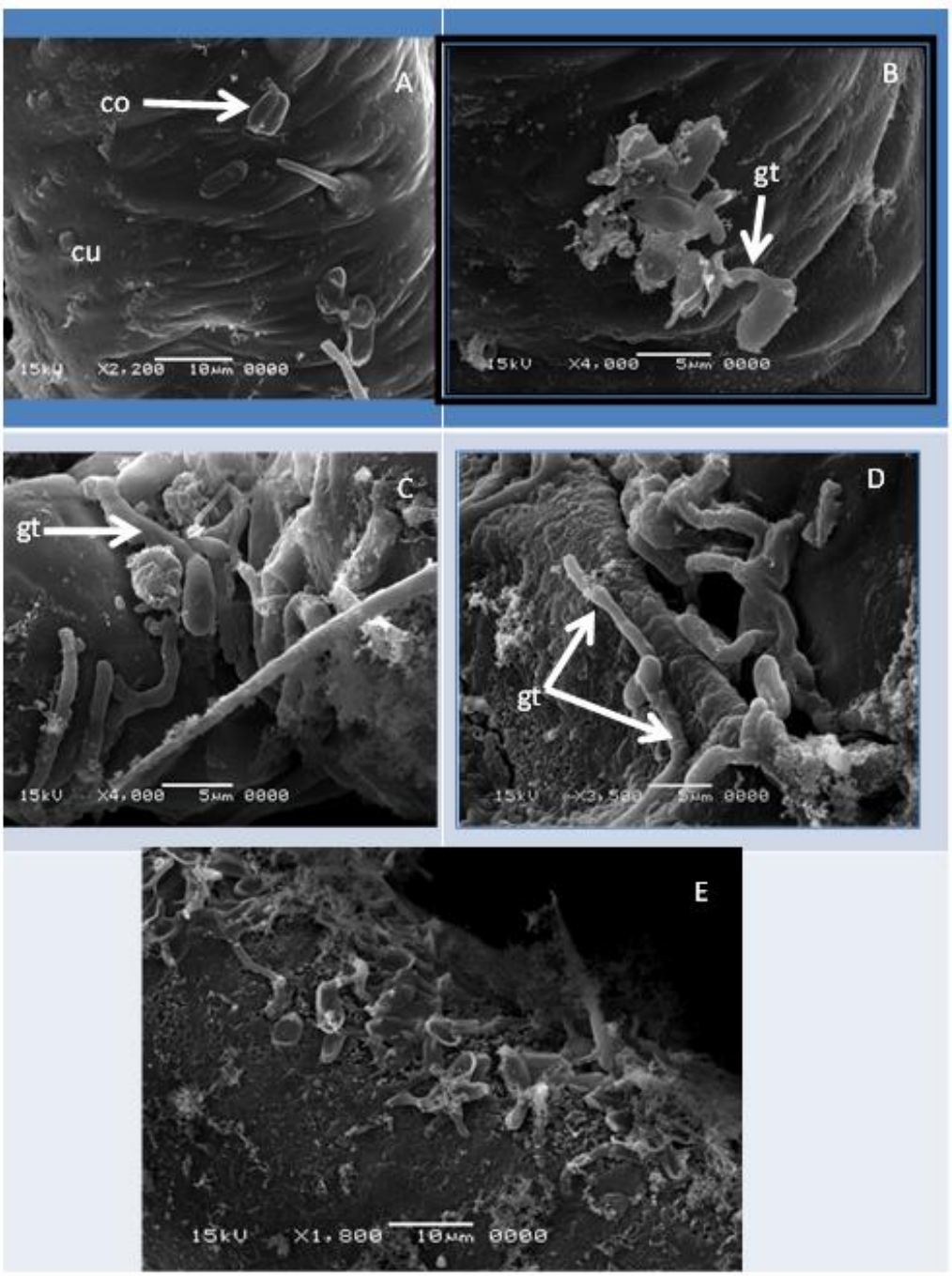

Fig.5: Scanning electron microscopy showing *M. anisopliae* infected *S.graminum*,
(A) Coindiospores (co) adhesion cuticle after 12hrs after inoculation (2200X), (B) The conidiospores germinate on the surface of the cuticle Presence of germ tube (gt) on cuticular surface of aphid and the germinated hyphal tubes penetrate the insect's integument directly between 24-48hrs after inoculation (4000X), (C) Scanning EM showed (gt) germination on cuticle (4000X),|(D) Scanning EM illustrated bilateral germination on surface of aphid treatment(3500X), (E) Dislocation of the chitin plates 120 hrs after inoculation(1800X).

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ARABIC SUMMARY

تقييم تأثير عزلات فطرية ممرضة للحشرات علي منّ القمح *Schizaphis graminum*

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تم تقييم فعالية أربع عزلات فطرية محلية ممرضة للحشرات عزلتين من فطر *Beauveria bassiana* (B1 و B2) وعزلتين من فطر Metarhizium *anisopliae* (M1 و M2) علي منّ القمح *Schizaphis graminum* تحت ظروف معملية. تم تجريب ثلاثة تركيزات (١×١٠^٦، ١×١٠^٧و١×١٠^٨) لكل عزلة علي عمر يوم واحد متجانس من الطور البالغ لمنّ القمح وأوضحت النتائج أن العزلة B1 كانت أكثر فاعلية علي الطور البالغ بالمقارنة بالعزلات الأخري طبقا للتركيز النصف مميت (LC_{50}). بينما عند دراسة سمية أربعة تركيزات للنواتج الأيضية لكل عزلة كانت العزلة (M1) اكثر سمية يليها (B1)، (M2) ثم (B2) . تم فحص العينات المصابة بواسطة الميكروسكوب الالكتروني لملاحظة ميكانيكية عمل الفطريات الممرضة وقدرتها علي اختراق وإصابة المنّ.