Journal of the Egyptian Society of Parasitology, Vol.50, No.2, August 2020
J. Egypt. Soc. Parasitol. (JESP), 50(2), 2020: 221 - 227

CONTROL OF HUMAN FILARIAL VECTOR, *CULEX QUINQUEFASCIATUS* (DIPTERA: CULICIDAE) THROUGH COMBINATION OF THE ENTOMOPATHOGENIC FUNGUS, *METARHIZIUM ANISOPLIAE* AND NANOPARTICLES OF ZINC OXIDE AND ALUMINUM OXIDE

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Abstract

Culex quinquefasciatus (Diptera: Culicidae) is a vector of many pathogens and parasites of humans, as well as domestic and wild animals. Its eradication or control is regarded as one of the important alternative available in preventing and controlling such diseases. Thus, there is an urgent need to check the proliferation of the population of vector mosquitoes in order to reduce vector-borne diseases by appropriate control methods. This study investigated the effect of the entomopathogenic fungus, *Metarhizium anisopliae* against *Cx. quinquefasciatus* larvae, as well as its effect when combined with zinc oxide (ZnO) and aluminum oxide (Al_2O_3) nanoparticles (NPs). Larvae were exposed to different concentrations of fungus, *M. anisopliae* (1.42×10^5, 1.42×10^6, 1.42×10^7, 1.42×10^8 spore/ml). Mortality rates of fourth instars ranged from 100% for the highest spore concentration to 80% for lowest spore concentration tested. Larvae exposed to different concentrations of ZnO & Al_2O_3 (5, 10, & 30mg/ l) combined individually with low concentration of fungus, *M. anisopliae*, showed significantly high rates of mortality reaching 100% and 96.25% for ZnO & Al_2O_3, respectively after 4 days of treatment. SEM micrographs confirmed the attachment and penetration of the fungus *M. anisopliae* into the larval cuticle in different body parts. The combination of NPs and fungus had a synergistic effect on larvae mortality. This study showed that addition of the synthesized nano-metal oxides to *M. anisopliae* have greater efficacy on control of *Cx. quinquefasciatus* larvae.

Key words: *Culex quinquefasciatus*, Entomopathogenic fungi, *Metarhizium anisopliae,* Nanoparticle metal oxides**,** combination effect.

Introduction

Culex quinquefasciatus Say 1823 is a domestic to peridomestic mosquito found in North America, South America, Australia, Asia, Africa, the Middle East, and New Zealand and belongs to the globally distributed *Cx. pipiens* species complex which contains a number of related species, ecotypes or forms and hybrids occur along geographical introgression zones on multiple continents (CDC, 2012). Generally, *Culex* mosquitoes are vector many diseases as filariasis, Rift Valley Fever, West Nile Fever, Sindbis Fever, encephalitis (Elnakib *et al*, 2018) and widespread over large areas of the tropical and subtropical regions (Benelli *et al,* 2018). Control of mosquito populations is most effective when the aquatic stage is targeted because that is the most concentrated and immobile stage (Cetin *et al*, 2010). So, effective control measures were needed to eradicate these risky mosquitoes Using of entomopathogenic fungi and their derived products were promising approach for biological mosquitos' control (Kirschbaum, 1985). The nanotechnology is one of the most promising new approaches for pest control (Bhattacharyya *et al,* 2010).

With the progress of nanotechnology, many laboratories worldwide have investigated metal oxide NPs production. Goswami *et al.* (2010) have studied the applications of different kinds of NPs, silver (SNP), aluminum oxide (ANP), zinc oxide, and titanium dioxide NPs in the insects' control. Some nanomaterials proved effective in mosquitoes co-

ntrol such as nano-silica (Deanath *et al*, 2011; Barik *et al*, 2012), silver NPs (Marimuthu *et al*, 2011; Arjunan *et al*, 2012), and aluminum NPs (Stadler *et al*, 2010; 2017).

Nano-metal oxides such as ZnO & Al_2O_3 are promising materials due to their highly advantageous characteristics such as large surface area, small particle size and high chemical stability (Klabunde, 1996). These unique properties provide the ability of NPs to penetrate through exoskeleton and cause insect mortality (Benelli, 2016; Foldbjerg *et al*, 2015). Mostafa *et al*. (2018) showed that synthesized nano-oxides have a high mortality effect on larvae of *Cx. quinquefasciatus.*

The present study aimed to evaluate the activity and penetration impact of fungus *M. anisopliae*, combined with the synthesized nano-metals ZnO and Al_2O_3, against the larvae of *Cx. quinquefasciatus* mosquitoes.

Materials and Methods

Mosquitoes rearing: Larvae of *Cx. quinquefasciatus* were reared in the Chemistry and Biochemistry lab, Texas State University, San Marcos TX, USA (Mostafa *et al*, 2018) under laboratory conditions at 25±2^{o}C, 80± 5% relative humidity (R.H.) and photoperiod 14 L: 10 D. Adult mosquitoes were kept in cages (40cmx40cmx40cm) and fed 10% glucose solution. While, the females laid eggs two days after a blood meal, egg rafts were collected in plastic bowls containing water and kept for hatching. Larvae were reared in plastic trays (30cmx25cmx5cm) and fed on fish food.

Preparation of metal oxide nano-powder: Nano-metal oxides have been prepared by Co-precipitation method in Chemistry and Biochemistry lab, Texas State University, San Marcos TX. USA (Mostafa *et al*, 2018). To obtain ZnO and Al_2O_3 nano-powder the samples dried at 80^{o}C for 24 hours followed by annealing at 500^{o}C for 2 hours.

Fungal source and preparation of spore suspension: Entomopathogenic fungal cultures were obtained from Agriculture Research Service (ARSEF) in Ithaca New York, USA. *M. anisopliae* (Balsamo-Crivelli). The fungal isolates were cultivated and maintained on Sabouraud Dextrose Agar yeast (SDAY) medium. Spores were harvested by scraping the culture surface with a sterile loop in 10 ml distilled water. A drop of 0.01% Tween 80 was added. Spore suspension was then filtered through muslin cloth to remove mycelia, and counted by using an improved Neubauer haemocytometer.

Larvicidal effect of fungus *M. anisopliae* alone and combined with NPs (ZnO & Al_2O_3) against *Cx. quinquefasciatus* larvae: Four concentrations ($1.42x10^5$, $1.42x10^6$, $1.42x10^7$ & $1.42\ x10^8$ spore/ml) of *M. anisopliae* were prepared. Spore suspension of each concentration was applied against *Cx. quinquefasciatus* larval instars. The lowest concentration ($1.42x10^5$ spore/ml) were tested with different concentrations (5, 10 & 30 mg/l) of NPs (ZnO & Al_2O_3). For each case, larvae were divided into four replicates. Twenty larvae per replicate were transferred into 200ml sterilized water in 250-ml plastic cups covered with mosquito's netting (Scholte *et al*, 2004). A group of larvae was treated with sterile water solution as control. All cups were maintained at 25±2^{o}C, 80±5% R. H. and 14 L: 10 D photoperiod. Mortality was observed daily for 14 days.

SEM: Infected larvae were prefixed in 2.5% glutaraldehyde (Sigma) for 2hr, washed with 0.1M cacodylate buffer (pH 7.2) for about 15 min and then the specimens were post-fixed in 1% OsO4 in the same buffer for 1hr. Furthermore, specimens were washed with buffer, dehydrated in ethanol series and treated in acetone solution. Specimens were sputter coated with Iridium and examined using Scanning Electron Microscope (Model, Helios Nano-lab. 400) at 10 KV.

Statistical analysis: Mortality data was analyzed with SPSS version 14. Using one-way analysis of variance (ANOVA) followed by pair wise comparisons based on Tukey's HSD tests. The results were expressed as means (±SE) and mortality was corrected using Abbott's formula (1925). LT_{50}, LT_{90} & their associated confidence intervals were

estimated from regression analysis.

Results

M. anisopliae against *Cx. quinquefasciatus* larvae showed significant concentration-dependent larval lethal effects i.e. as concentration increased, lethality also significantly increased. At concentrations of $1.42x10^5$, $1.42x10^6$,$1.42x10^7$, $1.42x10^8$ spore /ml, rates of the larval mortality was 80±0.98%, 89.25±0.52%, 94.75±1.62% & 100±0.0% respectively. As well, percentage of larval mortality was significantly affected by periods of exposure to the fungus, longer periods of exposure showed higher rates of mortality. At exposure periods of 1, 3, 5 & 7 days to fungal concentrations of $1.42x10^8$ spore/ml, the mean larval mortality was 78.75±0.75%, 83.25±0.65%, 98.25±1.54% & 100±0.0%, respectively. The differences among rates of mortality were statistically significant (F =5.998; df = 3; P < 0.006). The LT_{50} & LT_{90} dramatically decreased with the increase of spore concentrations and periods of exposure (Tab.1). With spore concentrations of $1.42x\ 10^5$, $1.42x\ 10^6$, $1.42x\ 10^7$, $1.42x\ 10^8$, LC_{50} was calculated as 4.06, 0.97, 0.97 & 0.54 days, and LT_{90} as 8.18, 7, 5.65 & 4.12 days respectively.

Table 1: Efficacy of different spore concentrations of *M. anisopliae* (spore/ml) on larval instars of *Cx. quinquefasciatus*

(spore/ml) conidia Concentration	Mean percentage of daily mortality (Mean ± S.E.)				LT_{50} (Days)	LT_{90} (Days)	R^2
	1	3	5	7			
1.42×10^5	31.25±1.06	45± 0.25	70± 1.47	80±0.98	4.06	8.18	0.986
1.42×10^6	57.5± 0.45	72.25± 1.76	80± 0.98	89.25±0.52	0.97	7	0.897
1.42×10^7	67.5 ± 0.75	77.75±1.05	86.25±0.62	94.75±1.62	0.97	5.65	0.789
1.42×10^8	78.75± 0.75	83.25±0.65	98.25±1.54	100±0.0	0.54	4.12	0.976

LT_{50}, LT_{90} lethal time caused 50% & 90% larval mortality of *Cx. quinquefasciatus* after exposure to spore concentration of *M. anisopliae*. R^2= Regression coefficient, S.E.: Standard Error.

In spite of the high activity of *M. anisopliae* against the *Cx. quinquefasciatus* larvae (Tab. 1), addition of NPs (ZnO & Al_2O_3) raised significantly the fungal activity by increasing larval mortality at shorter periods of exposure (Tab. 2). Combination of *M. anisopliae* (1.42×10^5 spore/ml) with different concentrations (30, 10 & 5mg/l) of ZnO, showed that the mean percentages of the larval mortality reached 100%, 86.25±1 and 80±1.08, respectively, after 4 days of treatment (Fig. 2). As well, results of combination of *M. anisopliae* (1.42×10^5 spore/ml) with Al_2O_3 of concentrations of 30, 10 & 5 mg/l showed mean larval mortality of 96.25%, 92.5±1.5% & 77.5 %, respectively after 4 days of treatment. Combination effect of *M. anisopliae* with NPs of ZnO & Al_2O_3 against *Cx. quinquefasciatus* larvae was shown (Tab. 2)

Table 2: Efficacy of *M. anisopliae* combined with NPs against the larval instars *Cx. quinquefasciatus*

Samples NPs + Fungus	NPs (mg/l) Concentrations	Mean percentage of mortality (Mean ± S.E) 1	2	3	4	LT_{50} (days)	LT_{90} (days)
ZnO+*M. anisopliae*	5	45±0.50	55±1.04	67.5±0.70	80±1.08	1.86	5.08
	10	46.25±0.41	71.25±0.35	78.75±0.28	86.25±1.40	1.65	4.11
	30	82.5±0.65	88.75±1.05	95±0.75	100±0.0	.31	2.07
Al_2O_3+*M. anisopliae*	5	38.75±1.05	60±1.22	75±1.28	77.5±1.53	1.88	5.60
	10	53.75±1.40	70±0.72	85±0.77	92.5±1.54	1.01	3.94
	30	66.25±1.47	80±0.75	91.25±0.25	96.25±0.85	0.89	3.02

LT_{50}, LT_{90} lethal time caused 50% & 90% larval mortality after exposure to nanoparticle concentrations (mg/l) combined with fungus *M. anisopliae*, P<0.05, Mean value of five replicates, (SE) stander error. Control (distilled water), nil mortality

LT_{50} & LT_{90} decreased with NPs addition and with the concentrations increase. Adding ZnO NPs at concentrations of 30, 10 & 5 mg/l, LT_{50} decreased to 1.86, 1.65 & 0.31 days & LT_{90} to 5.08, 4.11 & 2.07 days, respectively. Adding Al_2O_3, LT_{50} decreased to 1.88, 1.01 & 0.89 days & LT_{90} to 5.60, 3.94 & 3.02 days, respectively.

SEM for individual fungus and combined with NPs: Germination of *M. anisopliae* conidia was observed on dead larvae surface (Fig. 3); conidial germination was observed in a high density indicated by mycelium growth on the larval cuticle (Fig. 3a, b, & c). Growth of mycelium and spor-es on respiratory siphons; blocked respiratory system and

led to suffocation (Fig. 3d, e, & f).

The ZnO NPs were detected on different body parts of larvae combined with the fungus, *M. anisopliae* that confirmed NPs have no antifungal effect. High density of ZnO nano-particles on insect abdominal cuticle with fungus, showed infected siphon openings & gills with mycelium (Fig. 4, a, b, c, d, & e). So, fungus mycelium and spores with NPs destructed the respiratory system leading to suffocation and larval death.

After exposure of *Cx. quinquefasciatus* larvae to *M. anisopliae* with Al_2O_3 NPs, SEM showed high density of Al_2O_3 NPs in abdominal region and germination of mycelium, and conidia in cuticle, confirmed the infection of siphons and gills with fungus & NPs of Al_2O_3 (Fig 5a, b, c, d, e & f).

Discussion

In the present study, the insecticidal effect of *M. anisopilae* on *Cx. quinquefasciatus* larvae caused significantly high percentage of larval mortality in short periods of time. These results agreed with Mnyon *et al*. (2010) who reported that *B. bassiana* and *M. anisopliae* fungal species were the commonest bio-control agents for African mosquito vectors. Furthermore, the results revealed that the entomopathogenic fungi attacked and invaded bodies' mosquito larvae that agreed with Amóra *et al.* (2010) and Benserradj and Mihoubi (2014) who found that some strains of *M. anis-opliae* were virulent against mosquito larvae.

In the present study, combination of ZnO & Al_2O_3 NPs with entomopathogenic fungus, *M. anisopilae* had higher insecticidal effect at its lower concentration. *M. anisopilae* caused 100% & 91.25% mortality in shorter period when combined with ZnO & Al_2O_3 NPs, respectively. None showed advantages of synergistic interaction between metal oxide NPs and entomo-pathogenic fungi when applied simultaneously, but many studies fungi used in nanotechnology for producing NPs and showed larvicidal activity against mosquito larvae (Soni and Prakash, 2013; 2016). ZnO & Al_2O_3 NPs alone caused 96% & 74% of larvae mortality, respectively in 14 days (Mostafa *et al,* 2018). Metal NPs proved effective against plant pathogens & insect-pests. NPs were used in new formulations such as insecticides, and repellants (Barik *et al,* 2008; Owolade and Ogunleti, 2008; Gajbhiye *et al,* 2009; Goswami *et al,* 2010).

In the present study, SEM showed that the *M. anisopliae* spores were attached, invaded and germinated to *Cx. quinquefasciatus* different parts of larval cuticle. Fungal growth was observed in different body parts after 48-72hr. High conidial germination density was observed on abdominal inter-segmental regions, on abdominal tracheae and on gills and respiratory siphons. These data were reported on mosquito larvae, fungal germination was seen on *Cx. quinquefasciatus* larvae in contact with aqueous solution of *Aspergillus clavatus* spores (Seye *et al*, 2009). Silva *et al*. (2004) showed that mosquito larvae treated with *M. anisopliae* had high amounts of conidial adhesion to colloid chitin with at least 90% germination after 24hrs incubation. Other possible routes of invasion were via respiratory siphon or alimentary canal (Lacey, 1988). ZnO & Al_2O_3 NPs high density appeared in larval different body parts.

NPs may provide an alternative strategy to traditional broad-spectrum insecticides, to manage pests which have become resistant to conventional pesticides in integrated pest management programs (Korunic *et al*, 1999). SEM observations may support that larval death might be related to the effect of NPs on the cuticle wax layer, and the biotoxicity of NPs against mosquito larval instars may be related to the ability of NPs to penetrate through the exoskeleton (Benelli, 2016 and Foldbjerg *et al*, 2015). Larval death occurred via desiccation of insect cuticle by physicosorption of lipid, and damage in the cell membrane resulting in cell lysis and death of the insects. Also, Rouhani *et al.* (2012) proved the toxic effects of metal NPs (such as silver, zinc, aluminum, and titanium

oxide) on plants, crustaceans, bacteria, fungi, pathogens and pests.

Conclusion

The current study revealed that combination of the insecticidal *M. anisopliae* fungi and nano-metal oxides ZnO & Al_2O_3 gave a great larvicidal effect against *Cx. quinque-fasciatus* larvae through penetration of the exoskeleton leading eventually to insect death. Further study is needed to understand the mechanisms of how nanoparticles and entomopathogenic fungi interact and work together in controlling mosquito larvae.

The synthesized nano-metal oxides raised and accelerated entomopathogenic fungus, *M. anisopliae* activity against *Cx. quinquefasciatus* larvae. Combination of synthesized nano-metal oxides with entomopathogenic *M. anisopliae* was recommended as an effective bio-control agent not only for *Cx. quinquefasciatus* larvae but also for all mosquitos' vectors.

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Explanation of figures

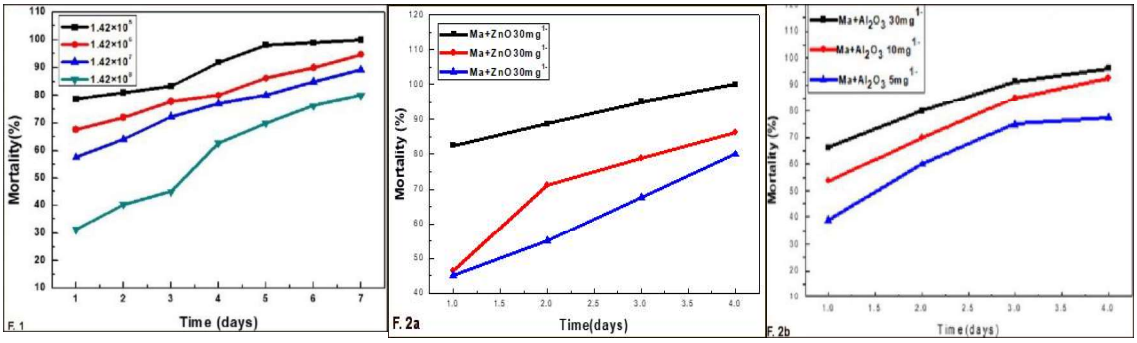
Fig. 1: Mean percentage of accumulative daily mortality of larval instars of *Cx. quinquefasciatus* after exposed to different *M. anisopliae* concentrations.

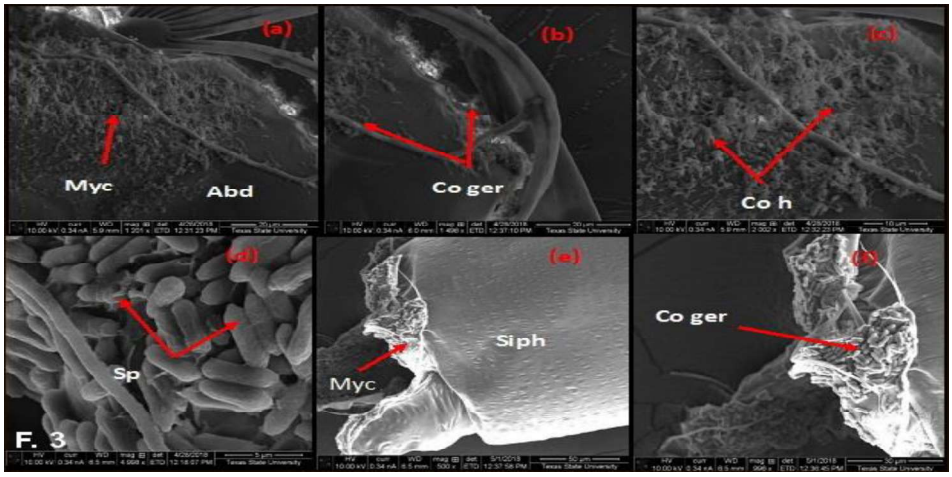
Fig. 2: Mean percentage of accumulative daily mortality of larval instars of *Cx. quinquefasciatus* after exposure to different metal oxide NPs concentrations combined with *M. anisopliae*. (a) ZnO+*M. anisopliae* (b) Al2O3+*M. anisopliae*.

Fig. 3: SEM of body surfaces (abdomen and respiratory siphon) of 2nd larval instar of *Cx. quinquefasciatus* infected with *M. anisopliae*. (a, b & c) Infected abdominal and thoracic regions with the fungus, M. anisopliae (d, e and f) Infected respiratory siphons. (Abd: Abdomen, Co h: conidia head, Co ger: conidia germination, Myc.: Mycelium, Siph: siphon, Sp: spores).

Fig. 4: SEM of body surfaces (abdomen and respiratory siphon) of 2nd larval instar of *Cx. quinquefasciatus* infected with *M. anisopliae* and ZnO NPs. (a & b) Infected abdominal and thoracic regions with combination of ZnO and *M. anisopliae* (c) Infected respiratory siphons. (d)Infected gills (Abd: Abdomen, Co h: conidia head, Co ger: conidia germination, Gill: gills, Myc.: Mycelium, Siph: siphon).

Fig. 5: SEM of body surfaces (abdomen and respiratory siphon) of 2nd larval instar of *Cx. quinquefasciatus* infected with *M. anisopliae* and Al2O3 NPs. (a, b, c & d) Infected abdominal and thoracic regions with combination of Al2O3 + M. anisopliae (e) Infected respiratory siphons. (f)Infected gills (Abd cu: Abdomen cuticle, Co ger: conidia germination, Gill: gills, Myc.: Mycelium, Siph: siphon, Sp: spores).

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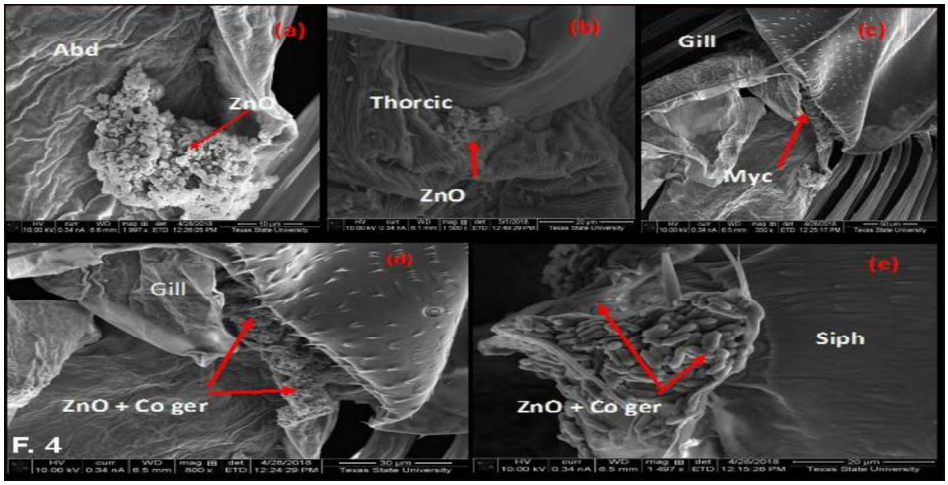
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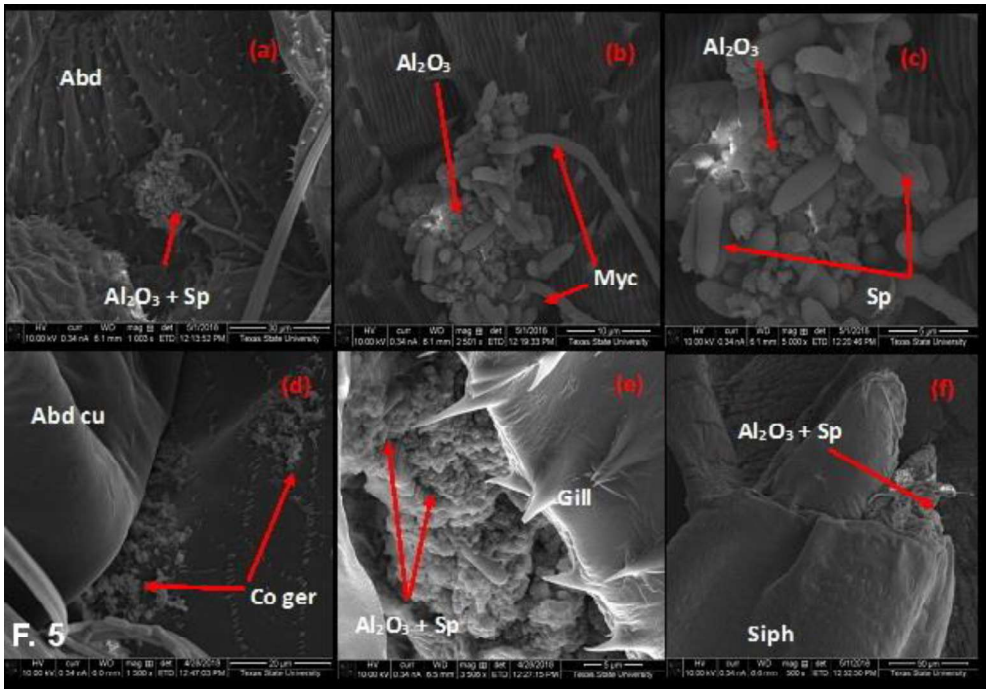
ZnO + Co ger
F. 4


Abd
(a)
Al2O3 + Sp
Al2O3
(b)
Myc
Al2O3
(c)
Sp
Abd cu
(d)
Co ger
(e)
Gill
Al2O3 + Sp
(f)
Al2O3 + Sp
Siph
F. 5