

**The insecticidal activity of actinomycete metabolites, against the mosquito
Culex pipiens.**

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ABSTRACT

Twenty seven actinomycetes were isolated from desert soil of different Egyptian sites and tested for production of insecticidal agents against the 3rd instar larvae of mosquitoes *Culex pipiens*. The obtained data exhibited that the isolate metabolites have a lethal effects. Metabolites of seven isolates cause 100 % total mortality. These isolates were identified as *Streptomyces fungicidicus*, *Streptomyces griseus*, *Streptomyces albus*, *Streptomyces rochei*, *Streptomyces violaceus*, *Streptomyces alboflavus* and *Streptomyces griseofuscus*. However, some isolate metabolites exhibited its insecticidal effect on the development of larvae. In addition, some pupal deformities (pupal-adult intermediate) were recorded by isolates no. A7, A8, A13, A24 & A26.

Keywords: *Culex pipiens*, actinomycetes, mortalities, development.

INTRODUCTION

Mosquitoes are vectors of many vertebrate blood parasites. In Egypt, *Culex pipiens* (Diptera: Culicidae) is the main vector of *Rift Valley Fever Virus* (Meagan *et al.*, 1980; Darwish and Hoogstraal, 1981) *Wuchereria bancrofti* (Khalil 1930; Gad *et al.*, 1996) and *Western Nile Virus* (Pelah *et al.*, 2002).

Repeated use of synthetic insecticides for mosquito control has disrupted natural biological control systems and led to resurgences in mosquito populations. It has also resulted in the development of resistance (Brown, 1986), undesirable effects on non-target organisms and fostered environmental and human health concern (Hayes and Laws, 1991), which initiated a search for alternative control measures.

Biological control is slow but can be long lasting, inexpensive, and harmless to living organisms and the ecosystem; it neither eliminates the pathogen nor the disease, but brings them into natural balance (Ramanathan *et al.*, 2002). At present, microbial insecticides are the main component of the bio-pesticide industry (Xie 1998; Shi 2000). Most of the pesticidal micro-organisms, however, have been isolated from entomopathogens and the terrestrial environment (Zhang 1996; Leonard and Julius 2000). Several varieties of microorganisms including fungi, actinomycetes, bacteria, viruses and nematodes that are antagonistic to insects have been reported as strategies to biologically control them.

The actinomycetes are noteworthy as antibiotic and enzymatic producers, making three quarters of all known products; the *streptomyces* are especially prolific and can produce a great many antibiotic and other class of biologically active secondary metabolites. If we include secondary metabolites with biological activities other than antimicrobial, actinomycetes are still out in front, over 60%; *streptomyces* spp. accounting for 80% of these (Hopwood, *et al.*, 2000).

Actinomycetes play an important role in the biological control of insects through the production of insecticidally active compounds against the house fly *Musca*

domestica (Hussain *et al.*, 2002). Actinomycetes gave a good effect, shown as lowest pupal formation percentages of *Drosophila melanogaster* (Gadelhak *et al.*, 2005). Dhanasekaran *et al.*, (2010) found that the actinomycete isolates producing strong larvicidal activity against *Anopheles* mosquito larvae. However, actinomycetes were effectively used against *Culex quinquefasciatus* (Sundarapandian *et al.*, 2002). Many actinomycete strains caused larval mortality, of the cotton leaf worm *Spodoptera littoralis*, ranging from 10-60% (Bream *et al.*, 2001). In addition, considerable lethal effect of some actinomycetes was observed on pupae. The secondary metabolites of new strain of streptomycetes give displayed growth inhibition on the test pathogenetic insects, such as *Spodoptera exigua*, *Dendrolimus punctatus*, *Plutella xylostella*, *Aphis glycines* and *Culex pipiens* (Huamei *et al.*, 2008).

On the other hand streptomycetes metabolites not only effective against insect but may also protect the insect themselves from other microbial pathogen and other insect as in Beewolf wasps which cultures a strain of antibiotic – producing *Streptomyces philanthi* within specialized glands on her antenna. *Streptomyces philanthi* then excrete antibiotics into the cocoons, protecting the beewolf larvae from harmful pathogen. (Kroiss *et al.*, 2010).

The present study aims at investigating the production of insecticidal activity for some actinomycetes against the mosquito, *Culex pipiens*.

MATERIALS AND METHODS

A- Origin and rearing of the mosquitoes.

Mosquitoes used in this study were *Culex pipiens*. They were collected from Abu Rawash, Giza governorate, Egypt, then they were reared for several generations, in the insectariums of medical entomology at the Department of Zoology, Faculty of Sciences, Al-Azhar University, Egypt, under controlled conditions at temperature of $27 \pm 2^\circ\text{C}$, relative humidity $70 \pm 10\%$ and 12-12 light-dark regime. Adult mosquitoes were kept in (30 × 30 × 30 cm) wooden cages and daily provided with sponge pieces soaked in 10% sucrose solution for a period of 3-4 days after emergence. After this period the females were allowed to take a blood meal from a pigeon host, which is necessary for laying eggs (anautogeny). Plastic cup oviposition (15 × 15 cm) containing dechlorinated tap water was placed in the cage. The obtained egg rafts picked up from the plastic dish and transferred into plastic pans (25 × 30 × 15 cm) containing 3 liters of tap water left for 24 h. The hatching larvae were provided daily with fish food as a diet. This diet was found to be the most preferable food for the larval development and a well female fecundity (Kasap and Demirhan, 1992).

B- Collection of actinomycetes:

Different samples were collected randomly from different desert areas in Cairo-Egypt, during 2010. The actinomycete were isolated from soil samples by dilute plating using starch nitrate agar medium (El-Nakeeb and Lechevalier, 1963) and then incubated at $30 \pm 2^\circ\text{C}$ for four days. All isolates were purified by repeated streaking on starch nitrate agar medium.

C- Extraction of extracellular metabolites from actinomycete isolates:

Actinomycete isolates were tested against the *Culex pipiens*. The selected isolates were inoculated into a 250 ml conical flask containing 100 ml of starch nitrate liquid medium and shaken at $30 \pm 2^\circ\text{C}$ and 200 rpm for seven days. The cells free culture filtrates were separated by centrifugation and screened for larvicidal activity.

D- Identification of actionmycetes:

The most potent insecticidal production actinomycetes were characterized by morphological and biochemical method. Morphological methods consist of macroscopic and microscopic methods. The microscopic characterization was done by cover slip culture method (Kawato and Sinobu, 1959). The mycelium structure, color and arrangement of conidiospore and arthospore on the mycelium of isolates were observed by smear from colonies and stained by Gram's Method as described by (Hucker and Conn 1923). The Colonies were identified on the basis of their morphology and color (Shirling and Gottlieb, 1966) and compared with Williams *et al.* (1989).

E- Insect treatment:

Ten *Culex* larvae were tested for each 50 ml of actinomycetes filtrates in plastic cup. The control tubes were maintained as tap water (50 ml) free from actinomycetes filtrate. The experiment was checked daily for recording the biological effects.

F- Criteria studied.

Toxicological activity: The larvae were observed daily until pupation and adult emergence to estimate the following parameters.

Larval mortality percent: was estimated by using the following equation: Larval mortality % = $(A - B) / A \times 100$, where A = number of tested larvae and B = number of tested pupa (Briggs, 1960).

Pupation rate: The pupation percent was estimated by using the following equation: Pupation % = $A / B \times 100$, where A = number of pupae and B = number of tested larvae.

Pupal mortality: The pupal mortality percent was estimated by using the Following equation: Pupal mortality % = $(A - B) / A \times 100$, where A = number of produced pupae and B = number of observed adults.

Adult emergence: The emerged males and females adults were counted and the adult emergence percent was calculated by using the following equation:

Adult emergence % = $A / B \times 100$, where A = number of emerged adults and B = number of tested pupae.

Pupal malformation: was estimated by failure to develop to adult stage (pupaladult intermediate). All malformed pupae were counted and removed immediately. The pupal malformation percent was calculated by using the following equation: Pupal malformation % = $C / A \times 100$, where C = number of malformed pupae and A = number of tested pupae.

(G)Statistical Analysis of Data: Data obtained were analyzed by the Student's t-distribution, and refined by Bessel correction (Moroney, 1956) for the test significance of difference between means.

RESULTS

Insect treatment:

Twenty seven actionmycetes were isolated from desert soil of different Egyptian sites. These isolates were encoded from A1 to A27 and their secondary metabolites were investigated in vitro as insecticidal agent against the 3rd instar larvae of mosquitoes *Culex pipiens* (Table 1). The obtained data exhibited that the isolates have a lethal effects. Complete mortality of treated larvae with secondary metabolites of isolate no. A1, A2, A3 & A4 was recorded while the other isolates cause mortalities ranged from 66.7 to 16.7 % in comparison with 16.7 % mortalities in control. Also the lethal effect was extended to the pupal stage by the metabolites of isolates no. A5, A6 & A7 which cause 100% mortalities in the resulted pupae from the treated larvae while no mortalities were observed in the adult stage.

With regard to the development, the larval duration was significantly prolonged with some isolates (A8, A15, A19, A20 & A23) in comparison with that of the control. No significant effect was exhibited for pupal durations (Table 1).

Table 1: Screening the biological effects of actinomycetes secondary metabolites against *Culex pipiens*.

Isolates code	Larval mort. %	Larval duration	% of pupation	Pupal* mort. %	pupal adult int.	pupal duration	Adult emer. %	Total mort. %
A1	100.0	-	-	-	-	-	-	100.0
A2	100.0	-	-	-	-	-	-	100.0
A3	100.0	-	-	-	-	-	-	100.0
A4	100.0	-	-	-	-	-	-	100.0
A5	66.7	5.2± 0.93a	33.3	100.0	-	-	-	100.0
A6	50.0	5.1± 0.81a	50.0	100.0	-	-	-	100.0
A7	33.3	5.4± 0.49a	66.7	100.0	50.0	-	-	100.0
A8	16.7	6.4± 0.55c	83.3	60.0	20.0	1.0± 0.00a	40.0	66.7
A9	33.3	5.0± 0.63a	66.7	50.0	-	1.5± 0.70a	50.0	66.7
A10	66.7	5.7± 0.80a	33.3	-	-	2.0± 0.00a	100.0	66.7
A11	66.7	4.7± 0.54a	33.3	-	-	1.5± 0.70a	100.0	66.7
A12	66.7	5.3± 0.90a	33.3	-	-	1.5± 0.70a	100.0	66.7
A13	33.3	4.9± 0.96a	66.7	50.0	50.0	1.5± 0.70a	50.0	66.7
A14	66.7	5.1± 0.43a	33.3	-	-	1.5± 0.70a	100.0	66.7
A15	66.7	6.5± 0.71b	33.3	-	-	1.0± 0.00a	100.0	66.7
A16	66.7	5.8± 0.77a	33.3	-	-	2.0± 0.00a	100.0	66.7
A17	66.7	4.8± 0.85a	33.3	-	-	2.0± 0.00a	100.0	60.0
A18	50.0	5.4± 0.69a	50.0	-	-	1.3± 0.57a	100.0	50.0
A19	33.3	6.0± 0.82b	66.7	-	-	1.5± 0.57a	100.0	40.0
A20	33.3	6.5± 0.58c	66.7	-	-	1.2± 0.50a	100.0	33.3
A21	33.3	4.7± 0.53a	66.7	-	-	1.5± 0.57a	100.0	33.3
A22	33.3	5.0± 0.56a	66.7	-	-	1.5± 0.57a	100.0	33.3
A23	-	6.3± 0.52c	100.0	33.3	-	1.2± 0.50a	66.7	33.3
A24	-	5.3± 0.70a	100.0	33.3	33.3	1.5± 0.57a	66.7	33.3
A25	33.3	5.1± 0.83a	66.7	-	-	1.5± 0.57a	100.0	33.3
A26	-	4.9± 0.80a	100.0	33.3	33.3	1.2± 0.57a	66.7	33.3
A27	16.7	5.0± 0.71a	83.3	-	-	1.2± 0.45a	100.0	16.7
Control	16.7	4.7± 0.88a	83.3	-	-	1.4± 0.55a	100.0	16.7

a: non significant data, b: significant data, c: highly significant data, mort.: mortality, int.: intermediate, emer.: emergence, *:pupal mortalities include mortality of pupae and pupal adult intermediate

Some pupal deformities (pupal-adult intermediate) were observed with percents of 50.0, 20.0, 50.0, 33.3 and 33.3 by isolate no. A7, A8, A13, A24 & A26, respectively. The recorded deformations were pupal- adult intermediate and Incompletely emerged adult with legs and abdomen attached to the pupal skin (Plate 1).

On the other hand, the percentage of adult emergence was decreased in some isolates to 40, 50, 50, 66.7, 66.7 & 66.7 % at A8, A9 A13, A23, A24 & A26, respectively, vs 100 % of adult emergence in control (for more details see Table 1).

Identification of actinomycete isolates:

The isolates from no. A1 to A7 that exhibited total mortality (100 %) were identified to species level based on morphological, cultural and physiological

characteristics. Isolate A1 was *Streptomyces fungicidicus*, Isolate A2 was *Streptomyces griseus*, Isolate A3 was *Streptomyces albus*, Isolate A4 was *Streptomyces rochei*, Isolate A5 was *Streptomyces violaceus*, Isolate A6 was *Streptomyces alboflavus* and Isolate A7 was *Streptomyces griseofuscus* (see Table 2 & Plate 2).

Table 2: Morphological and physiological data of actinomycete isolates.

Test name	A1	A2	A3	A4	A5	A6	A7
DAP type	L-DAP	L-DAP	L-DAP	L-DAP	L-DAP	L-DAP	L-DAP
Spore chains Rectiflexibiles	-	+	-	+	+	+	-
Spore chains Spirales	+	-	+	-	+	-	+
Spore mass color	Buff	Gray	Gray	White	Gray	White	Rose
Substrate mycelial color	White	Green	Brown	White	Pink	Violet	Violet
Diffusible pigment produced	-	-	Brown	-	-	-	violet
Melanin on peptone yeast iron agar	-	-	-	-	+	-	-
Melanin on tyrosine agar	-	-	-	-	+	-	-
<i>Bacillus subtilis</i>	+	+	+	+	-	-	+
<i>Micrococcus luteus</i>	+	+	+	+	+	-	+
<i>Candida albicans</i>	-	-	-	-	-	+	-
<i>Saccharomyces cerevisiae</i>	-	-	-	-	-	+	+
<i>Streptomyces murinus</i>	-	+	+	+	+	-	+
<i>Aspergillus niger</i>	-	-	-	-	-	+	-
Lecithinase activity	+	-	-	-	+	-	-
Lipolysis	+	+	+	+	+	+	+
Pectin hydrolysis	-	+	-	-	-	-	+
Nitrate reduction	+	+	-	-	+	-	-
H ₂ S production	+	+	+	+	+	+	+
Hippurate hydrolysis	-	-	-	-	+	-	-
Elastin degradation	+	+	+	+	+	+	+
Xanthine degradation	+	+	+	+	+	+	+
Arbutin degradation	+	+	+	+	+	+	+
Neomycin (50µg/ml)	-	-	-	-	-	-	-
Rifampicin (50µg/ml)	+	+	+	+	+	+	+
Oleandomycin (100µg/ml)	-	+	+	-	-	+	-
Penicillin G (10 i. u.)	+	+	+	+	+	+	+
Growth at 45 C°	+	-	+	+	-	-	+
NaCl (7% w/v) growth	+	+	+	+	-	+	+
NaN ₃ (0.01% w/v) growth	+	-	+	+	-	+	+
Phenol (0.1% w/v) growth	+	+	-	+	+	+	+
Potassium tellurite (0.001% w/v) growth	+	+	+	+	+	+	+
Thallos acetate (0.001% w/v) growth	+	+	-	+	-	+	+
DL-α-Amino-n-butyric acid	-	-	-	-	+	+	-
L-Cysteine	-	+	-	+	-	+	-
L-Valine utilization	-	-	-	-	+	-	-
L-Phenylalanine utilization	-	+	-	+	+	+	-
L-Histidine	+	+	+	+	-	-	+
L-Hydroxproline	-	-	+	-	+	-	-
Sucrose	+	-	-	+	-	-	+
meso-Inositol	+	-	-	+	+	-	+
Mannitol	+	+	+	+	-	+	+
L-Rhamnose	+	-	+	+	-	-	+
Raffinose	+	-	-	+	+	-	+
D-Melezitose	+	+	+	+	+	+	+
Adonitol	-	+	+	-	-	+	-
D-Melibiose	+	-	+	+	+	-	+
Dextran	+	+	+	+	-	-	+
Xylitol	-	-	-	+	-	-	+

DISCUSSION

Biological control or 'biocontrol' is the use of natural enemies to manage mosquito populations. There are several types of biological control including the direct introduction of parasites, pathogen and predators to target mosquitoes (Kenneth, 1995) or by using the dead spores of varieties of the natural soil bacteria and actinomycetes which used to interfere in the digestion systems of larvae. These

spores were no longer effective after the larvae turn into pupae because they stop eating (Walker and Lynch, 2007).

The filamentous actinomycetes are gram-positive bacteria with high G+C content and are well known as prolific producers of biologically active secondary metabolites of economic significance to the chemical, pharmaceutical and agricultural industries. Among them, streptomyces is by far the most prolific genus, and has provided about 10000 known antibiotics, 45-55% are produced by streptomyces. Most of the antibiotics are extracellular metabolites which are normally secreted in culture media and have been used as herbicides, anticancer agents, drugs, immunoregulators and antiparasitic drugs (Demain, 1999; Lazzarini *et al.*, 2000 and Charoensopharat *et al.*, 2008).

In the present study the metabolites of 27 actinomycete isolates from soil were tested as insecticidal agent against the 3rd instar larvae of mosquitoes *Culex pipiens*. Isolates no. A1, A2, A3 & A4 caused complete mortality on the treated larvae. Also the lethal effect was extended to the pupal stage where the isolates no. A5, A6 & A7 caused 100% mortalities in the resulted pupae from the treated larvae.

The present results are, however, in accordance with several results performed with actinomycetes and other insect species. Dhanasekaran *et al.* (2010) found that the actinomycete isolates producing strong larvicidal activity against *Anopheles* mosquito larvae. Only 4 isolates had the potentiality inhibits (100%) the growth of *Anopheles* mosquito larvae. In *D. melanogaster*. The application of chitinase producing actinomycetes to the rearing medium of the fruit fly, had a significant effect on their mortality. The actinomycete isolates were all considerably effective compared to their controls. Both *A. philippinensis* and *A. missouriensis* have significantly reduced insect pupal formation when applied to the medium individually (Gadelhak *et al.*, 2005). Many actinomycete strains caused larval mortality, of the cotton leaf worm *Spodoptera littoralis*, ranging from 10-60% (Bream *et al.*, 2001). Nair *et al.*, (1989) observed that a poly polyene macrolide lacton antibiotic (Faeriefungin) was isolated from *Streptomyces griseus* caused 100% mortality of mosquito larvae (*Aedes aegypti*, Rockefeller strain) at concentration of 100ppm. In addition, considerable lethal effect of some actinomycetes was observed on pupae. The secondary metabolites of new strain of streptomyces give displayed growth inhibition on the test pathogenetic insects, such as *Spodoptera exigua*, *Dendrolimus punctatus*, *Plutella xylostella*, *Aphis glycines* and *Culex pipiens* (Huamei *et al.*, 2008).

The mortality of insect in this study may be due to secretion of bioactive materials which stimulate the gamma amino butyric acid (GABA) system (Willoughby *et al.*, 1987; Moar and Trumble 1987) or disruption of nicotinic acetylcholin receptors (Herbert, 2010).

With regard to the development, the larval duration was significantly prolonged with some isolates in comparison with that of the control. No significant effect was exhibited for pupal durations. Some pupal deformities (pupal-adult intermediate) were observed at some isolates. Similar effects were observed for actinomycetes on lepidopteran *Spodoptera littoralis* (Bream *et al.*, 2001).

Since the cuticle of insect species consists largely of chitin, it was postulated that chitinase produced by these isolates could be involved in insect control. Therefore, the production of chitinases was used as the criteria for the selection of potential biocontrol agents of insects. Microbial chitinolytic enzymes have been considered important in the biological control of many insects because of their ability to interfere with chitin deposition (Tripathi *et al.*, 2002).

Actinomycete metabolites exhibited its effect against mosquito *Culex pipiens*. So it can be used as, an alternative insecticides because they are free from harmful effects on the environment. Further studies needed for identification the active compounds that can be used in broad spectrum for controlling insects and also determination the mode of action of these compounds.

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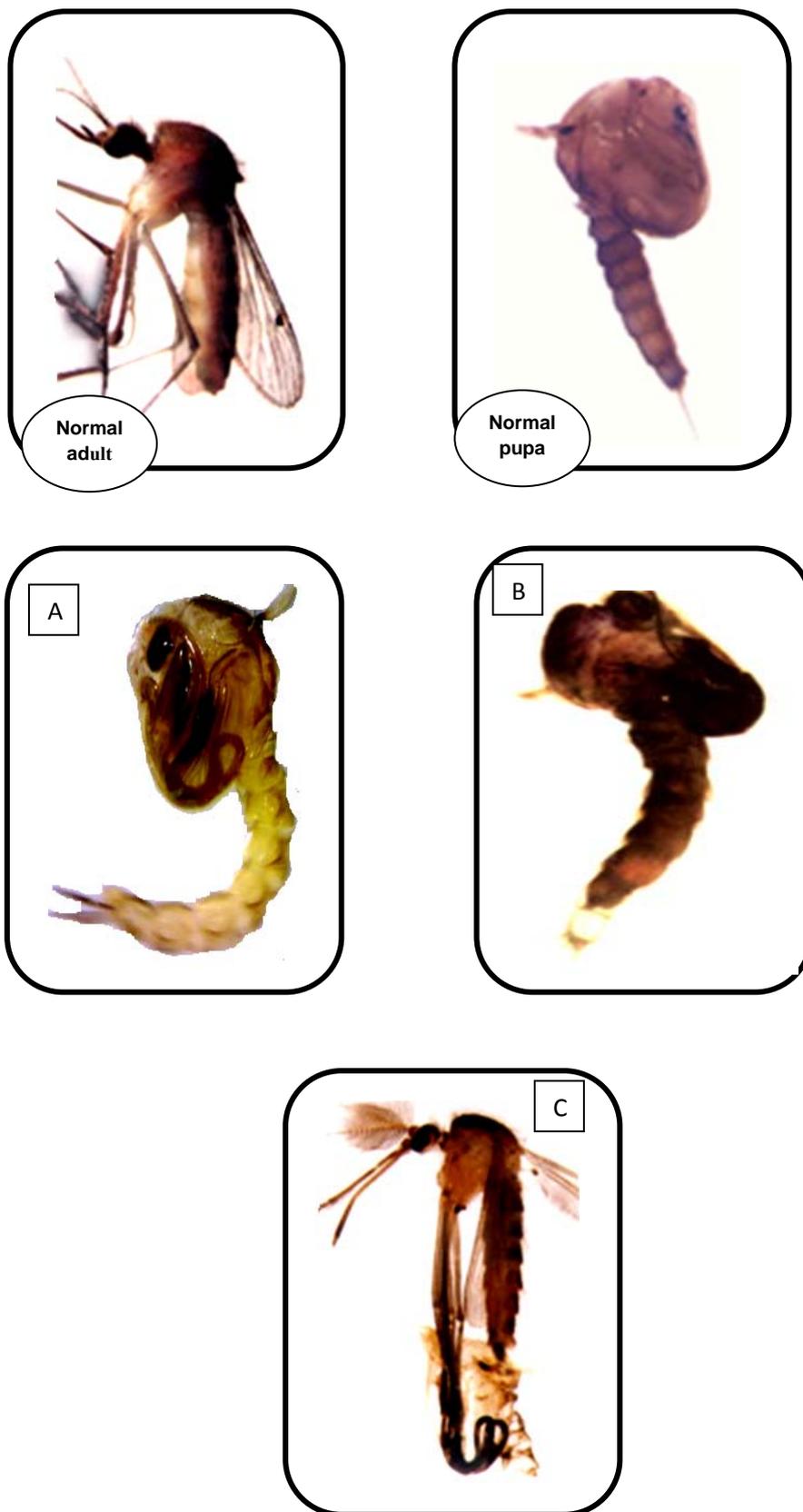


Plate 1: Morphological abnormalities among pupae and adults resulted from larvae treated with some actinomycete metabolites. A. and B. Pupal-adult intermediate. C. Incompletely emerged adult with legs and abdomen attached to the pupal skin.

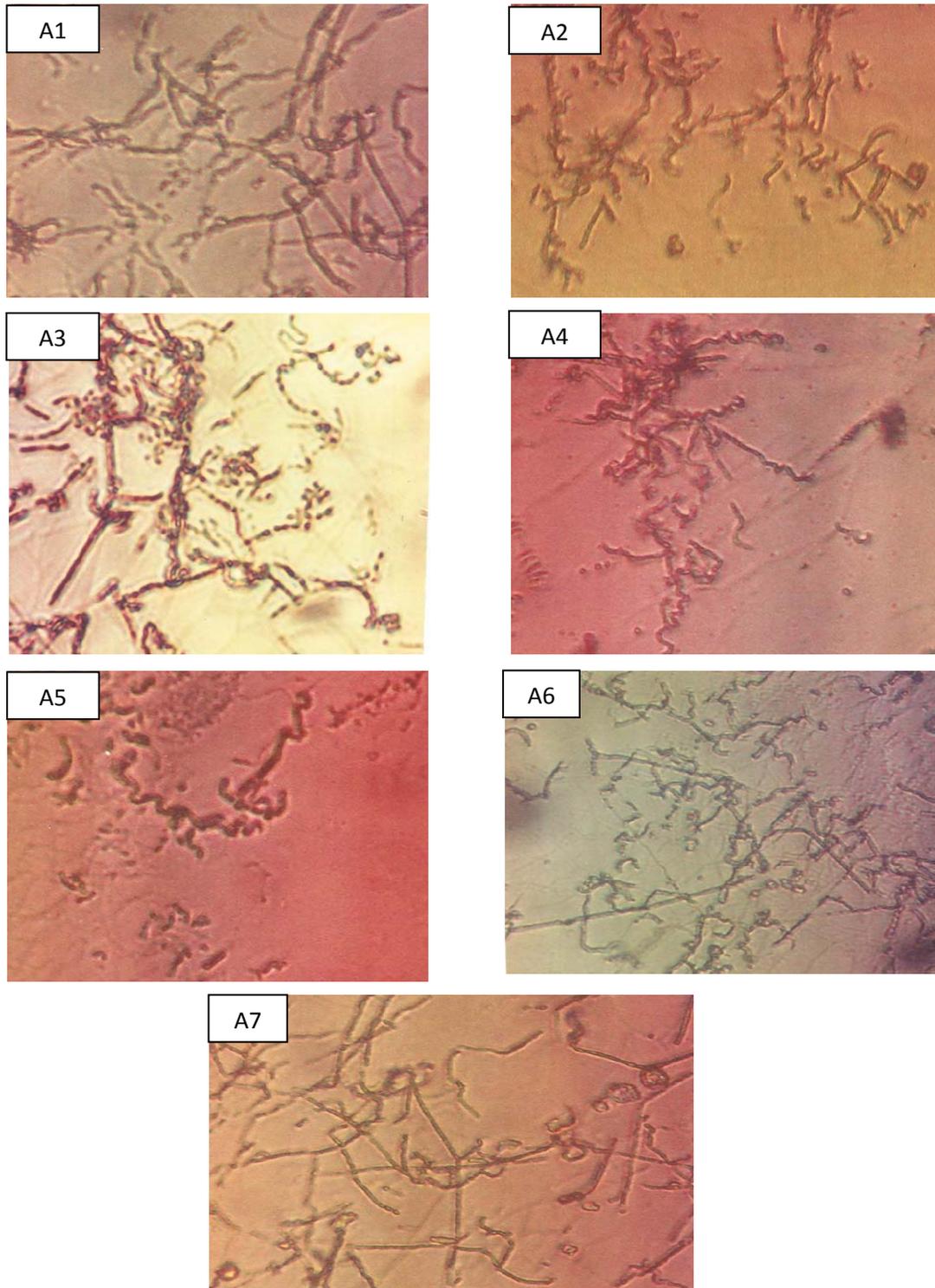


Plate 2: Morphological examination of actinomycete isolates. Where: Isolate A1 (*Streptomyces fungicidicus*), Isolate A2 (*Streptomyces griseus*), Isolate A3 (*Streptomyces albus*), Isolate A4 (*Streptomyces rochei*), Isolate A5 (*Streptomyces violaceus*), Isolate A6 (*Streptomyces alboflavus*) and Isolate A7 (*Streptomyces griseofuscus*).

ARABIC SUMMARY

النشاط الإبادي لمواد أيض الأكتينوميستيس، ضد بعوضة كيوليكس بيبينس

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تم عزل ٢٧ اكتينوميستيس من التربة الصحراوية لاماكن مختلفة من مصر وتم إختبارها كراشح ضد العمر البرقي الثالث لبعوضة كيوليكس بيبينس. النتائج المتحصل عليها أظهرت ان هذه العزلات لها تأثيرات مميتة. سبع عزلات سببت ١٠٠% وفيات كلية. هذه العزلات تم تعريفها كالاتي *استريبتوميستيس فنجيسيس*، *استريبتوميستيس جريسيس*، *استريبتوميستيس البس*، *استريبتوميستيس روتشي*، *استريبتوميستيس فيولاسيس*، *استريبتوميستيس البوقلفوس* و *استريبتوميستيس جريسيفوكس*. علاوة على ذلك، بعض العزلات أظهرت تأثيرها على انماء اليرقات. بالإضافة إلى ذلك، تم تسجيل بعض تشوهات العذارى في العزلات أ٧، أ٨، أ١٣، أ٢٤ و أ٢٦.