

EFFECT OF FOUR BIOACTIVE COMPOUNDS SEPARATELY AND IN COMBINATION WITH *Metarhizium anisopliae* ON THE ACTIVITY OF SOME HAEMOLYMPH ENZYMES OF *Schistocerca gregaria* (FORSKAL).



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ABSTRACT

The effect of *Metarhizium anisopliae* var. *acidum* and four bioactive compounds (Neem, L-Glutamic acid, *Schinus molle* and abamectin) in sole treatments and in combination with *M. anisopliae* var. *acidum* was studied on activity of Lactate Dehydrogenase, Trehalase, Phenol oxidase, and Acid Phosphatase in 5th nymphal instar haemolymph of desert locust. Obtained results showed that the integration of *M. anisopliae* var. *acidum* and the four bioactive compounds caused 100% mortality after six days post treatments, also caused severe reduction in activity of all studied enzymes. *M. anisopliae* var. *acidum* treatment caused significant reduction in Lactate Dehydrogenase by the second day after treatment, but all treatments caused significant reduction in Lactate Dehydrogenase by 4th day post treatment except Neem. In case of trehalase all treatments were significantly lower than control treatment except abamectin two days post treatment, but by day four all treatments were lower than control, while in the 6th day post treatment *S. molle*, L-Glutamic acid and Neem treatments caused significant increase in Trehalase activity. All treatments caused significant increase in Phenol oxidase activity two days post treatment except L-Glutamic acid and abamectin treatment, while after four days Neem and *M. var. acidum* treatment were still higher than control, but other treatments were significantly lower than control treatment, by the 6th day post treatment only L-Glutamic acid caused significant increase in Phenol oxidase activity. In the situation of Acid phosphatase activity two days post treatment abamectin, L-Glutamic acid and *M. anisopliae* var. *acidum* + L-Glutamic acid were significantly lower than control, while after four days all treatments were significantly lower than control, after six days post treatment only *M. anisopliae* var. *acidum* and L-Glutamic acid were lower than control.

Keywords: *Schistocerca gregaria*, *Metarhizium anisopliae*, plant extract, amino acid, L-Glutamic acid, *Schinus molle*, abamectin, Lactate dehydrogenase, trehalase, Phenol oxidase, and acid phosphatase.

INTRODUCTION

Control of desert locusts has traditionally relied on synthetic insecticides, preventive, integrated control strategies with early interventions will reduce the financial cost and environmental hazards associated with large-scale plague treatments, *Metarhizium* biopesticide kills 70%–90% of treated locusts within 14–20 days, with no measurable impact on non-target

organisms (Lomer *et al.*, 2001). These products act slowly and are thus inappropriate for emergency situations. However, they should have a role in an integrated control strategy alongside classic insecticides (Lomer *et al.*, 1999). Many studies were done to evaluate the ability of *M. anisopliae* var. *acidum* to integrate with its other control agents in order to increase efficacy of *M. anisopliae* var. *acidum* for desert locust control operations *i.e.* integration with some insect growth regulators (IGRs) and antifeedant (El-Gamal *et al.*, 2004), and with abamectin and D-limonene (Mohamed *et al.*, 2014). *S. molle* or pepper tree, is an interesting plant that has been used worldwide as an insecticide, however different authors have evaluated the effect of *S. molle* extracts on varied pests, both important on crops and forests (Abdel-Sattar *et al.*, 2009 and López *et al.*, 2014). L-Glutamic acid is known as excitatory transmitter at the neuromuscular junction of invertebrates (Cull-candy, 1976 and Wafford and Sattelle, 1989), while (Clements and May, 1974) showed that when *Schistocerca gregaria* nerve-muscle exposed to glutamate caused a variety of responses, some of which were shown to be abnormal and were much more severely affected.

Insect phenoloxidase is considered an important mediator in defense reaction against pathogens and parasites. It is present in the hemolymph of most insects as an inactive proenzyme, called prophenoloxidase (Da Silva 2002).

Acid phosphatase (AcP) is hydrolytic enzymes, which hydrolyse phosphomonoesters under acid conditions (Bai *et al.*, 1993). AcP may have a role in autophagy and cell turn over as well as defence, Phagocytosis is known to stimulate production of lysosomal enzymes of which AcP is a key component (Xia *et al.*, 2000).

The aim of present study is to evaluate the effect of four bio active agents namely: Neem, *S. molle*, Abamectin (Aba.) and L-Glutamic acid also the fungus *M. anisopliae* var. *acidum* (M.) isolate (IMI330189), as well as their mixture with *M. anisopliae* var. *acidum* on activity of Lactate dehydrogenase and Trehalase of treated desert locust.

MATERIALS AND METHODS

Desert locust nymphs

Fifth nymphal instar of *S. gregaria* two days after final molting, were obtained kindly from desert locust colony maintained in Locust and Grasshoppers Research Dep., Plant Protection Research Institute, ARC, Egypt. Desert locust individuals were reared in the laboratory according to (Robert *et al.*, 2002). The colony was fortified with wild insect collect from the field each year.

Metarhizium anisopliae var. *acidum*

The entomopathogenic fungus used during the study is *M. anisopliae* var. *acidum* isolate (IMI330189) was kindly obtained from BASF Company, South Africa, under the commercial name Green Muscle®. The spores were suspended in sterilized water; trace of Tween (80) was added. The

concentration was adjusted to 5×10^8 spores/ ml, each nymph received 5 μ l of the final solution.

Neem (Azadirachtin)

Azadirachtin, under the commercial name Safe-oil 0.03 % EC, at concentration of 1ml/liter distilled water.

Schinus molle extract

50 g of fresh aerial part of *S. molle*, were air-dried at lab temperature, dried in oven at 40°C till constant weight then ground to fine powder, add to 800 ml liter of distilled water in volumetric flask for 3 days and repeated 4 times, then filtrated. Combined filtrates were evaporated under reduced pressure using rotary evaporator apparatus until a minimum amount of solvent remained which gives (3 g) at last. The extract (brownish sticky) was stored in a refrigerator at 5 °C and kept for using in different analysis. The concentration used was 1 g from extract added to 100 ml distilled water (Woo *et al.*, 1977).

Amino acid (L-Glutamic acid)

Molare solution from L-glutamic acid is prepared by addition of 147.13 gm \ litter distilled water. concentration are used is 0.1 from molar solution (Krasilnikov and Bakhrarov, 1983) each nymph received 5 μ l of the final solution.

Abamectin

5-O-demethylavermectin A_{1a} (i) mixture with 5-O-demethyl-25-de(1-methylpropyl)-25-(1-methylethyl) avermectin A_{1a} (ii). Under the commercial name, Agromic 1.8 % EC, was used at concentration of 1ml/liter distilled water, each nymph received 5 μ l of the final solution.

Mixtures

Metarhizium anisopliae var. *acidum* was used in combination with Neem, *S. molle*, L-Glutamic acid and Abamectin, mixture solution contain the same concentration for each infeluintal in alone case.

Treatments

Ten 5th instar nymphs were used in each treatment. *M. anisopliae* var. *acidum*, L-glutamic acid and Abamectin treatments were used as topical application, while Neem and *S. molle* were used as follow: 20 g of fresh clover were dipped in 100 ml of each used concentration, dried for 1 hr. in room temperature, then introduced to the nymphs. The mixtures were used as topical application, but in case of Neem and *S. molle* the nymphs were treated with *M. anisopliae* var. *acidum* first then were feed on treated clover as described previously.

Haemolymph Sample collection

The haemolymph was collected through a fine puncture in the hind leg and from beneath the dorsal pronotal shield membrane and transferred into dry centrifuge tubes (Metaweh *et al.*, 2001), at days 2, 4 and 6 post treatments.

Estimation activity of Lactate Dehydrogenase Enzyme (LDH):

Activity of Lactate Dehydrogenase was estimated according to German Society for Clinical Chemistry (DGKC, 1972). NADH is oxidized to NAD. The rate of NADH decrease is directly proportional to the LDH activity and is determined photometrical. The reaction mixture consisted of

phosphate buffer (68 m/mol/L, pH 7.5), pyruvate (0.73 m/mol/L) and NADH (1.1 m/mol/L). Haemolymph (100 µL) was mixed with the mixture (2.5 ml) that pre-incubated at 37°C, then the absorbance was measured by the spectrophotometer at 340 nm/min.

Estimation activity of Trehalase Enzyme:

Trehalase activity were determined according to the method described by (Ishaaya and Swirski, 1976), using trehalose, as substrates for trehalase. The enzyme activity was expressed as µg glucose released /min/g fresh weight.

Estimation activity of Phenoloxidase Enzyme:

Phenoloxidase activity was monitored spectrophotometrically as formation of quinines according to Ishaaya, 1972 with some modifications, 200 µl haemolymph, 2ml phosphate buffer (0.2 M, pH 7) and 0.5 µl 2 % Catechol were mixed gently, incubated for 5 min at 25°C, The activity was then recorded at absorbency 470 nm, the optical density was recorded every 1 min for 10 min. The specific activity of phenoloxidase was expressed as units of activity per mg of protein. One unit of activity was defined as the amount of enzyme that increases the absorbance by 0.001 units per min.

Estimation activity of Acid Phosphatase Enzyme:

For determination of acid phosphates in haemolymph, Powell and Smith (1954) method with slight modification were used, where the hydrolysis of disodium phenylphosphate by acid phosphates releases phenol, which reacts with 4-aminoantipyrine, and by the addition of potassium ferric cyanide a brown color is produced. The produced color was measured, immediately, by spectrophotometer at 510 nm. The optical density was converted to enzymatic activity units which expressed as mg phenol released/ ml haemolymph.

Statistical analyses

Data were subjected to Analyses of Variance using costat 6.4 software from CoHort Software, 2 way completely randomized procedure was utilized.

RESULTS

Data illustrated in Table (1) showed that, the effect of different treatments on the activity of lactate dehydrogenase in muscle of desert locust, it's clear that the treatment of *S. molle*, M + Neem, M + L- Glutamic acid, L- Glutamic acid and M+ *S. molle* caused significant increase in the activity of lactate dehydrogenase comparing with control treatment (there values were 971.00, 882.67, 857.67, 721.00 and 645.33 respectively), while *S. molle* was significantly higher than M + Neem and M + L- Glutamic acid which were significantly higher than L- Glutamic acid which were higher than M+ *S. molle* and Neem treatments. There were no significant differences between control treatment and both Abamectin and M+ Abamectin (there values were 548.33 and 556.00 respectively), but the control treatment were significantly higher than M treatment. By day four post treatment the application of Neem caused significant increase in compeer with control.

While other treatments were significantly lower than control, also M treatment showed the lowest value. By the 6th day post treatment, the treated insects in the mixtures treatments were died, while abamectin showed significant increase in the activity of lactate dehydrogenase than control treatment. There were no significant differences between control and *S. molle* treatments. While other treatments were significantly lower than control.

The obtained result in Table (2) showed the effect of same previous treatments on trehalase activity. The results showed significant increase in trehalase activity than control after 2 days post treatment with abamectin, while such activity in control treatment were significantly higher than other treatments except M+ *S. molle*. In case of day 4 post treatment the enzyme activity in control treatment was significantly higher than other treatments. By day 6 post treatment all nymphs treated with mixtures were died. While the enzyme activity in *S. molle*, L- Glutamic acid and Neem treatments were higher than control treatment (there values were 342.00, 322.67 and 306.67 respectively), but such activity in control treatment were higher than abamectin treatment followed by M treatment.

The obtained result in Table (3) illustrated the effect of the for mentioned bioactive compounds on the activity of Phenoloxidase, 2 days post treatment both Abamectin and L- Glutamic acid caused significant decrease of Phenoloxidase activity than control treatment (there values were 3592.67 and 3569.33 respectively), while other treatments were significantly higher than control. By the 4th day post treatment only Neem treatment was significantly higher than control, while M + Neem, M + L- Glutamic acid, treatments did not differ significantly than control (there values were 5480.00 and 5406.33 respectively), but the treatments of M alone, M+ *S. molle*, *S. molle*, and L- Glutamic acid were significantly lower than control (there values were 2549.67, 2500.00, 1390.00 and 1060.66 respectively). L- Glutamic acid treatment caused significant increase in Phenoloxidase than control treatment but other treatments were significantly lower than control.

Data demonstrated in table (4) revealed, activity of acid phosphatase was significantly higher than control 2 days post treatment in M+ Aba., M + Neem, M, M+ *S. molle*, Neem and *S. molle* treatments (there values were 24.70, 25.70, 26.70, 29.00, 30.70 and 31.70 respectively), while such activity was significantly lower than control, in Aba., M + L- Glutamic acid and L- Glutamic acid (there values were 17.33, 19.00 and 22.50 respectively). At day 4 post treatment the activity of acid phosphatase in all treatments were significantly lower than control treatment. By the 6th day post treatment *S. molle*, Abamectin and Neem caused significant increase in acid phosphatase activity than control (there values were 52.33, 52.40 and 48.11 respectively), but both L- Glutamic acid, and M caused significant decrease than control (there values were 31.50 and 24.90 respectively).

DISCUSSION

Lactate dehydrogenase:

Lactate dehydrogenase catalyzes the inter-conversion of pyruvate and lactate with concomitant inter-conversion of NADH and NAD⁺ in glycolysis cycle (Kaplan and Pesce, 1996). In this study the activity of LDH are decreased than control after 2,4 and 6 days when insect treated with *M. anisoplaiae* this result are in parallel to (Mohamed, 2009) which found decreased in activity of LDH rather than control after 1,2 and 3 days when treated *S. gregaria* with *M. anisoplaiae*. Sewify and Hashem (2001) reported that, the effect of *M. anisoplaiae* on oxygen uptake of the wax moth *Galleria mellonella* L. and suggest the decrease in LDH activity may be due to the increase the oxygen uptake.

In all other treatment the activity of LDH are increased than control this result are in parallel to (Hamadah *et al.*, 2010) in case of plant extract which treated *S. gregaria* with plant extract from *Fagonia bruguieri* and found the activity of LDH increased than control.

In Since LDH is important enzyme in the carbohydrate metabolism and related to energy production in the living cell, the induced activity level in the haemolymph of nymphs of *S. gregaria*, in the present study, indicates generally an active energy metabolism in this important tissue. It, also, may indicate an effective stimulation of the portion of the Cori cycle responsible for the overall recycling lactate, since this would result in concomitant enhanced production of pyruvate and glucose via gluconeogenesis (Harper *et al.*, 1984).

Trehalase:

Trehalase could be used as parameter for assessing the availability of nutrients (Ishaaya and Swirski, 1976) in this study the activity of trehalase after 2 days from inoculation in all treatments except abamectin and mixture from *M. anisoplaiae* and plant extract from *S. molle* and in all treatments after 4 days is lower than control, this decrease due to The decreasing in trehalase activity may be due to toxicity stress of the treatments as indicating by many authors (Fahmy and Dahi, 2009, Elbanna *et al.*, 2013 and (Rashwan, 2013). Increase in activity of trehalase after 2 days from inoculation in abamectin are in parallel to (Dahi, *et al.*, 2009) which found increase in activity of trehalase when treated Egyptian cotton leaf worm *Spodoptera littoralis* with avermectin While the activity is increased after 6 days from infection in case Neem, plant extract from *S. molle* and L- glutamic acid this result are in parallel to (Mohamed, 2014) which treated *S. gregaria* with Neem and found the activity of trehalase in insect which treated with Neem are higher than control. While in case of insect which treated with abamectin after 6 days the activity of trehalase is lower than control this result are in parallel to (Abo El-Ghar *et al.* 1995) which found that when feeding *Spodoptera littoralis* larvae 5 ppm abamectin caused remarkable decrease in trehalase activities.

Phenoloxidase:

The innate immune system in insects is composed of a large variety of specific and non-specific responses that are activated in response to the presence of foreign agents. One important element in such responses is the

enzyme phenoloxidase (PO) (Isaac and Alex., 2012) The activity of phenoloxidase are increased when insect infected with *Metarhizium anisoplaiae* after 2 days from inoculation while after 4 and 6 days the activity of phenoloxidase is decreased which match with (Gabarty *et al.*, 2013). When conidia land on the cuticle of a suitable host, they attach and germinate, initiating cascades of recognition and enzyme activation reactions by both the host and the fungal parasite. The defensive responses to the fungi infection lead to activation of the phenoloxidase and other enzymes of cascade increased levels of phenoloxidase may help to suppress microbial infection during the time interval. (Gillespie *et al.*, 2000b) illustrated that. When insect infect with *Metarhizium* the hemolymph phenoloxidase are decline over the course of the infection until the death of *Schistocerca gregaria*.

In all other treatment the activity of phenoloxidase are increased than control after 2 days which indicated that, the insect increase immune response and attack these foreign particles and this result are in parallel to (Isaac and Alex., 2012) which said in invertebrate immunology have documented a complex array of host defenses. These defenses include phagocytosis.

Acid phosphatase:

Untreated control locusts had constitutive levels of AcP which may have a role in autophagy and cell turn over as well as defense, Phagocytosis is known to stimulate production of lysosomal enzymes of which AcP is a key component (Xia *et al.*, 2000).

In this study after 2 days from infection the activity of acid phosphatase in majority of influential is higher than control this result is in parallel to (Eissa and. Zidan, 2009) which found elevated the activity of acid phosphatase (ACP) when treated Male Albino Rats with abamectin, result which obtained from *M. anisoplaiae* alone and when mixed with Neem, plant extract from *S. molle* and abamectin are parallel to(Xia *et al.*, 2001) which found The AcP activity in haemolymph of mycosed insects increased significantly over controls, this may be due to secretion of ACP from *M. anisoplaiae*. After 4 days from infection the activity of acid phosphatase is lower than control this result is in parallel to (Kandil *et al.*, 2009) which found inhibition in activity of ACP after three days when infected Land snails *with abamectin*.

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تأثير اربع مركبات ذات نشاط حيوي منفردة او مخلوطه مع فطر الميتاريزيم انيسوبلى على نشاط بعض انزيمات الدم للجراد الصحراوي
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تم دراسة تأثير فطر الميتاريزيم انيسوبلى وكذلك ٤ مركبات نشطه حيويًا (النيم و الحمض الأمين جلوتاميك ومستخلص نبات الشينيس مولى و الأباكتين) منفردة او مخلوطه مع الفطر على نشاط بعض الأنزيمات (اللاكتيت ديهيدروجينيز و التريهاليز و الفينول اوكسيديز و الأسيدي فوسفاتيز) فى دم حوريات العمر الخامس للجراد الصحراوي.

اظهرت النتائج المتحصل عليها ان الفعل التكاملى لفطر الميتاريزيم مع المركبات المستخدمه ادى الى نسبة موت وصلت الى ١٠٠% بحلول اليوم السادس بعد المعاملة. وفى حالة التأثير على انزيم اللاكتيت ديهيدروجينيز وجد ان بعد يومين من المعاملة ان بالفطر مع ادت الى تقليص فى نشاط الأنزيم بينما كل المعاملات المستخدمة ادت الى زيادة فى نشاط انزيم اللاكتيت ديهيدروجينيز بحلول اليوم الرابع ما عدا النيم وبعد ٦ ايام من المعامله زاد نشاط الأنزيم فى المعاملات بالنيم و مستخلص نبات الشينيس و الحمض الأميني مقارنة بالكنترول. فى انزيم التريهاليز بعد يومين من اجراء التجربه فان الابامكتين ومخلوط الفطر مع المستخلص النباتى سببا زياده فى محتوى الانزيم عن الكونترول مقارنة بباقي المعاملات اما بعد ٤ ايام فان نشاط الانزيم فى جميع المعاملات كان اقل من الكونترول اما بعد ٦ ايام فان نشاط الانزيم ازداد فى معاملات النيم والمستخلص النباتى و الحمض الامينى عنه فى الكونترول. و فى حالة انزيم الفينول اوكسيديز بعد يومين من اجراء التجربه فان كل المعاملات تسبب زياده فى انزيم الفينول اوكسيديز الا معاملات الحمض الامينيو الابامكتين اما بعد ٤ ايام جميع المعاملات نسبتها اقل من الكونترول الا فى معاملات النيم ومخلوط الفطر مع النيم ومخلوط الفطر مع الحمض الامينى اما بعد ٦ ايام فان الحمض الامينى فقط هو الذى يسبب ارتفاع فى نسبة انزيم الفينول اوكسيديز عن الكونترول. فى انزيم الاسيدي فوسفاتيز بعد يومين من اجراء التجربه فان الابامكتين و الحمض الامينى ومخلوط الحمض الامينى مع الفطر نسبتها تكون اقل من الكونترول اما باقى المعاملات فان محتوى الانزيم يكون اكبر من الكونترول وبعد ٤ ايام فان محتوى الانزيم فى جميع المعاملات يكون اقل من الكونترول اما بع ٦ ايام معاملات الفطر منفردا و الحمض الامينى هما الذان يكون محتوى الانزيم اقل من الكونترول.

Table (1) Effect of some bioactive compounds on lactate dehydrogenase activity in haemolymph of *Schistocerca gregaria*

Treatments	Control	M	Neem	S. molle	L- Glutamic acid	Aba.	M + Neem	M + L- Glutamic acid	M+ S. molle	M+ Aba.
Days										
2 days	520.33 cE	251.33 bF	679.67 bD	971.00 aA	721.00 aC	548.33 bE	882.67 aB	857.67 aB	645.33 aD	556.00 aE
4 days	699.00 aB	399.00 aF	754.33 aA	653.67 bC	506.00 bD	451.00 cE	218.00 bH	472.33 bDE	624.00 aC	258.00 bG
6 days	587.67 bB	380.00 aD	440.67 cC	625.00 bB	409.00 c CD	684.33 aA	--	--	--	--

Means in same column followed by same small letter are not significantly different, while means in the same row followed by same capital letter are not significantly different. M = *Metarhizium anisopliae* and Aba.= abamectin

Table (2) Effect of some bioactive compounds on Trehalase activity in haemolymph of *Schistocerca gregaria*

Treatments	Control	M	Neem	S. molle	L- Glutamic acid	Aba.	M + Neem	M + L- Glutamic acid	M+ S. molle	M+ Aba.
Days										
2 days	318.67 c BC	270.67 a E	315.00 bc CD	299.00 b D	273.00 b E	351.67 aA	300.00 a D	271.33 a E	334.67 a AB	255.67 a E
4 days	350.00 aA	195.00 b C	330.67 ab B	315.33 b B	313.67 a B	321.33 b B	144.00 b D	148.67 b D	105.67 b E	121.33 b E
6 days	301.00 b C	37.00 c E	306.67 c BC	342.00 aA	322.67 a B	166.67 c D	--	--	--	--

Means in same column followed by same small letter are not significantly different, while means in the same row followed by same capital letter are not significantly different. M = *Metarhizium anisopliae* and Aba.= abamectin

Table (3) Effect of some bioactive compounds on Phenoloxidase activity in haemolymph of *Schistocerca gregaria*

Treatments	Control	M	Neem	<i>S. molle</i>	L-Glutamic acid	Aba.	M+Neem	M+L-Glutamic acid	M+ <i>S. molle</i>	M+ Aba.
Days	4106.33 c EF	4487.67 a E	5285.00 b D	4467.00 b E	3569.33 b F	3592.67 ab F	7029.33 a B	6446.33 a C	10967.33 aA	5684.00 a D
2 days	5380.33 b B	2549.67 b D	15053.33 aA	1390.00 c E	1060.66 c E	3360.00 b C	5480.00 b B	5406.33 b B	2500.00 b D	3633.33 b C
4 days	6030.00 a B	4480.00 a D	1393.67 c E	5353.33 a C	8866.67 aA	4109.67 a D	--	--	--	--
6 days										

Means in same column followed by same small letter are not significantly different, while means in the same row followed by same capital letter not significantly different. M = *Metarhizium anisopliae* and Aba.= abamectin

Table (4)Effect of some bioactive compounds on Acid phosphatase activity in haemolymph of *Schistocerca gregaria*

Treatments	Control	M	Neem	<i>S. molle</i>	L-Glutamic acid	Aba.	M+Neem	M+L-Glutamic acid	M+ <i>S. molle</i>	M+ Aba.
Days	24.00 c DE	26.70 b BCD	30.70 c AB	31.70 b A	22.50 b EF	17.33 c G	25.70 a CDE	19.90 a FG	29.00 a ABC	24.70 a DE
2 days	47.3 aA	43.40 a AB	39.67 b B	34.70 b C	34.20 a C	33.90 b C	20.70 b EF	17.20 a F	18.20 b F	22.70 a DE
4 days	37.90 b C	24.90 b E	48.11 a B	52.33 aA	31.50 a D	52.40 aA	--	--	--	--
6 days										

Means in same column followed by same small letter are not significantly different, while means in the same row followed by same capital letter not significantly different. M = *Metarhizium anisopliae* and Aba.= abamectin