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Fatty Acid Composition of Eight Isolates of Entomopathogenic Nematodes from Five Egyptian Governorates

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ABSTRACT

The fatty acid composition of infective juveniles (IJs) related to five isolates of *Heterorhabditis indica* (EGAZ1, EGAZ2, EGAZ3, EGAZ4, and EGAZ5) and three isolates of *Steinernema carpocapsae* (EGAZ9, EGAZ10 and SA) collected from five Egyptian Governorates was assessed. Also, fatty acid composition of IJs from two commercially relevance strains of *H. bacteriophora* (HP88) and *S. carpocapsae* (All), was examined for comparison. Newly emerged IJs of all isolates had fatty acid number and pattern similar to that of the corresponding commercial species. Of the ten fatty acids identified, oleic, was the main fatty acid in all species and isolates. Unsaturated fatty acids were dominant and total amount of saturated fatty acids of *H. indica* isolates was significantly higher than that of *S. carpocapsae* isolates. Palmitic was the second most abundant fatty acid in IJs of *H. indica* isolates, while linoleic was the second most abundant fatty acid in IJs of *S. carpocapsae* isolates. Of the tested Egyptian isolates, IJs of EGAZ3 and EGAZ5 of *H. indica* had the highest amounts of fatty acids and their contents of saturated fatty acids/gram body weight are comparable to that of the commercial strain. It is suggested that isolates EGAZ3 and EGAZ5 of *H. indica* are the candidates for developing practical Egyptian bio-control product based on nematode formulation.

INTRODUCTION

Egyptian orchards are inhabited by diverse and abundant communities of native entomopathogenic nematodes (EPNs). These nematodes, belonging to the genera *Steinernema* and *Heterorhabditis*, have qualities which make them potentially suited to become an important factor in the biological control of insects, as they actively disperse, seek out their hosts, can be mass produced and can be formulized and commercialized (Beekman *et al.*, 1994). However, the success of EPN as biological control agents of insects would be accelerated by improvements in nematode persistence after field application and by increasing the shelf life of formulations containing infective juvenile (IJ) stage of the candidate EPN (Fodor, *et al.*, 1994). Shelf life and the field activity of formulated IJs are dependent on amount of energy reserves stored and the rate of utilization during storage (Fitters *et al.*, 1999). IJs of *Steinernema* and *Heterorhabditis* store high levels of lipids to provide the non-feeding IJs with the energy necessary for host finding or to persist in the soil when hosts are unavailable (Fitters *et al.*, 1999).

The amount of energy provided by lipids depends on the amount of fatty acids and the degree of un-saturation of fatty acids since more energy is yielded by oxidation of saturated than of unsaturated fatty acids (Fitters *et al.*, 1999). There are many species and isolates of EPN that vary enormously in their fatty acid contents, and consequently vary in their ability to maintain biological activity during formulation, storage, and application. Accordingly, to choose the appropriate nematode for formulation in applicable products the most logical approach is to understand the physiological chemistry of the candidate nematode isolates, particularly, their appropriate composition of fatty acids (Georgis and Kaya, 1998).

In the present study, the fatty acid composition of eight geographically diverse Egyptian isolates of *Steinernema* and *Heterorhabditis* was determined. Also, fatty acid contents of IJs from two commercially relevance strains of *Steinernema* and *Heterorhabditis* were examined for comparison. This will provide detailed information on the degree of variation in the fatty acid composition of these nematodes. The availability of nematode isolates with a higher fatty acid contents, especially saturated fatty acids would mean remarkable progress on the road to longer shelf life of nematode bio-pesticide formulations (Perry *et al.*, 2012), which is an important requirement for Egyptian agriculture and environment.

MATERIALS AND METHODS

Nematode Populations:

Eight EPN isolates collected randomly from several locations of five Egyptian governorates, and maintained at the Pest Physiology Department, Plant Protection Research Institute, Egypt, and two commercial nematode species were analyzed for their fatty acid composition (Table 1). Species of the isolated populations were identified based on both detailed morphological and molecular characterizations according to Dolinski *et al.* (2008) (Azazy, A.M., Manal, F. and R.M. Abdelrahman, unpublished data). Fatty acid composition of *S. carpocapsae* isolates was compared with *S. carpocapsae* (All strain) extracted from the commercial product Ecomask (BioLogic, Inc., PA, USA). Due to unavailability of commercial products of *H. indica* to us, fatty acid composition of *H. indica* isolates was compared with that of the closely related species, *H. bacteriophora* (HP88 strain) extracted from the commercial product Heteromask (BioLogic, Inc., PA, USA).

Table 1: Locality, crop, and source of entomopathogenic nematode populations used in the present study.

Species	Population	Geographic location	Crop	Source*
Heterorhabditis bacteriophora	HP88	USA	Commercial product	BioLogic, Inc., USA
Heterorhabditis indica	EGAZ1	Suez, Egypt	Nktarin	Soil
Heterorhabditis indica	EGAZ2	El-kasasein, Ismailia, Egypt	Mango	Soil
Heterorhabditis indica	EGAZ3	El-kasasein, Ismailia, Egypt	Palm	Soil
Heterorhabditis indica	EGAZ4	Suez, Egypt	Plum	Soil
Heterorhabditis indica	EGAZ5	El-kasasein, Ismailia, Egypt	Alfalfa	Soil
Steinernemacarpocapase	All	USA	Commercial product	BioLogic, Inc., USA
Steinernema carpocapase	SA	Aswan, Egypt	Palm	Soil
Steinernema carpocapase	EGAZ9	Belbeis, Sharkia, Egypt	Mango	Soil
Steinernema carpocapase	EGAZ10	Qaha, Qalyubiya, Egypt	Lettuce	Soil

*Nematodes were isolated from soil using last instar larvae of *Galleria mellonella* as bait

Preparation of Nematode:

All populations were cultured in the wax moth, *Galleria mellonella* larvae at 25°C following isolation as described by Kaya and Stock (1997). Freshly harvested IJs were rinsed in de-ionized water, three times and the number of IJs in each sample was estimated. Harvested IJs were concentrated, vacuum filtered, weighed and then stored in 2 ml microcentrifuge tubes at –20 ^{o}C. Three replicates of 100,000 nematodes from each nematode isolate were prepared for fatty acid analysis.

Extraction and preparation of Fatty acids:

The fatty acids were extracted and prepared for analyzing by gas chromatography coupled with mass spectrometer (GC/MS) using the methods of Sasser (2001) as described in details by Kyung *et al.*, (2012). Briefly, fatty acids were extracted from IJs and saponified by adding 2 ml of extraction reagent (45 g sodium hydroxide, 150 ml HPLC grade methanol, and 150 ml distilled water) to 100 mg of IJs from each isolate in capped test tubes. The tubes were heated in a boiling water bath for 30 min with vortex every 10 min for 5-10 s. The cooled tubes were uncapped and 2 ml of methylation reagent (325 ml of 6.0 N hydrochloric acid and 275 ml methyl alcohol), was added to each tube. After vortexing, the tubes were heated for 10 min at 80^{o}C. Methylated fatty acids were extracted from the solution by adding 1.25 ml of hexane-methyl tert-butyl ether mixture (1:1) to each of the cooled tube and tumbling for approximately 10 min. The tubes were then uncapped and the aqueous (lower) phase was pipetted out and discarded. Each of the remaining organic phases in the tubes was washed by adding 3 ml of ca 1% sodium hydroxide in distilled water and tumbling for 5 min. The organic phase containing methylated fatty acids was then pipetted into a GC vial for GC-MS analysis.

GC-MS Analysis:

Fatty acids extracted from the nematode isolates were measured by using Shimadzu GC/MS-QP2010 Plus (Japan) Gas Chromatography/mass spectrometer system equipped with a capillary column DB-5MS (30 m length, 0.25 mm thickness, 0.25m diameter). Injections (at 250°C) were made in split mode (50:1) with Helium as carrier gas at flow rate of 1ml/min. The temperature program was isothermal at 50°C for 2 min, 7 °C/ min to 200°C and 5°C/min to 220°C and held for 20 min. An external standard of fatty acid mixtures (PUFA-3, Supelco, Bellefonte, USA) was used for confirming the identity of the different peaks in nematode samples, and for determining the quantity of each fatty acid. Fatty acids were expressed as the percentage of total fatty acid contents of each isolate, nanograms (ng) fatty acids per nematode, and also as ng fatty acids per 100 ng of IJs fresh weight.

Statistical Analysis:

Percentage data were normalized using arcsine (square root) transformation and subjected to analysis of variance (ANOVA) using CoStat software, release 5.5. Duncan's multiple range test was used to compare means at $p<0.05$. Results were recorded as mean ± standard deviation (SD).

RESULTS

Tables (2&3) and Fig. (1) show the relative percentage and pattern of fatty acids found in all studies EPN isolates. Although there were high variation in the percentage of each fatty acid among the studied EPN species and isolate, IJs of all isolates had the same ten fatty acids.Also, IJs of all isolates had fatty acid number and pattern similar to that of the corresponding commercial species.

In *Heterorhabditis* isolates; oleic acid (C18:1) was the most abundant as it representing50.4-60.8% of the total fatty acids (Table 2), followed by palmitic acid (C16:0) (12.5-21.5%), then linoleic acid (C18:2) (3.7-14.0%). Oleic acid was also the most abundant among *Steinernema* isolates (Table 3) representing 49.8-59.8% of the total fatty acids but followed by linoleic acid (16.2-24.5%) then palmitic acid (5.83-9.96%). Unsaturated fatty acids were

predominated in all the tested nematode species, as it accounted for more than 75% of total fatty acids of each isolate. The percentage of saturated fatty acids differed among nematode isolates. In general, *Heterorhabditis* isolates contained significantly higher ($P < 0.05$) proportions of saturated fatty acids than *Steinernema* isolates (Fig. 1). Notable differences were the significantly higher ($P< 0.05$) proportions of the palmetic acid and significantly lower proportions ($P< 0.05$) of linoleic acid in *Heterorhabditis* than *Steinernema* isolates (Fig. 1).

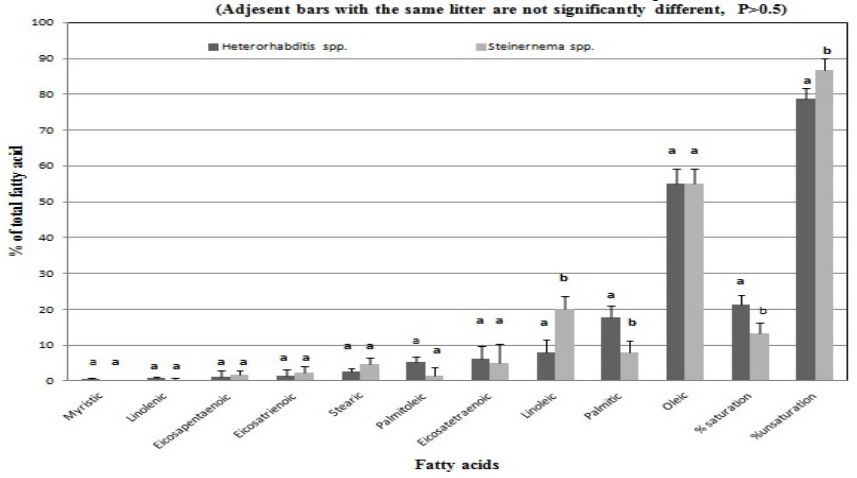
Table 2: Fatty acid composition (% of total FA) of freshly harvested infective juveniles of *Heterorhabditis bacteriophora* HP88 (commercial) and *Heterorhabditis indica* isolates:

Fatty acid		Nematode Species and Isolates					
		H.bacteriophora	*H.indica*				
Common Name	Lipid Numbers	HP88	EGAZ1	EGAZ2	EGAZ3	EGAZ4	EGAZ5
Myristic	C14:0	0.59	0.66	0.8	0.88	0.91	0.57
Palmitic	C16:0	12.5	18.9	17.9	21.5	19.7	15.9
Palmitoleic	C16:1	7.93	5.25	5.91	4.74	4.62	4.11
Stearic	C18:0	3.45	2.68	1.71	2.49	1.97	4.11
Oleic	C18:1	50.4	59.2	53.2	54.0	52.0	60.8
Linoleic	C18:2	14.0	7.68	8.2	3.74	7.33	7.78
Linolenic	C18:3	1.37	0.84	0.8	0.82	0.01	0.78
Eicosatrienoic	C20:3	1.39	0.01	0.01	4.75	2.51	0.01
Arachidonic	C20:4	5.56	4.23	10.8	2.08	10.1	4.24
Eicosapentaenoic	C20:5	2.48	0.01	0.01	4.25	0.02	0.95

Table 3: Fatty acid composition (% of total FA) of freshly harvested infective juveniles of *Steinernema carpocapase* isolates.

Fatty acid		Nematode isolate			
Common Name	Lipid Numbers	All	EGAZ9	EGAZ10	SA
Myristic	C14:0	0.15	0.39	0.32	0.3
Palmitic	C16:0	6.95	5.83	9.64	9.96
Palmitoleic	C16:1	0.37	0.48	0.56	5.07
Stearic	C18:0	3.59	3.76	6.59	4.96
Oleic	C18:1	56.3	49.8	54.1	59.8
Linoleic	C18:2	24.5	20.3	19.9	16.2
Linolenic	C18:3	0.39	0.01	0.78	0.57
Eicosatrienoic	C20:3	2.88	4.55	1.95	0.57
Arachidonic	C20:4	1.33	12.8	4.42	2.0
Eicosapentaenoic	C20:5	3.42	1.76	1.49	0.32

Figure 1. Varuations of different fatty acids contents (Mean ± SD) between *Heterorhabditis and Stinernema* infective juveniles (Adjesent bars with the same litter are not significantly different, P>0.5)



Quantitative analysis of total fatty acids content (Table 4) revealed that infective juveniles of the two commercial nematode species, *H. bacteriophora* (HP88) and *S. carpocapsae* (All) had approximately equal amounts of fatty acids (40.4 ± 3.5 and 36.0 ± 4.2 ng/IJ, respectively). However, such amount of total fatty acids is significantly much higher ($P < 0.05$) than the amount of total fatty acids detected in any of the studies Egyptian isolates that ranged from 16.6±2.2ng/IJ for isolate EGAZ3 of *H. indica* to 7.1±1 ng/IJ for isolate EGAZ9 of *S. carpocapsae* (Table 4). Also, the amount of saturated fatty acid per IJ was significantly higher ($P < 0.05$) in the two commercial nematode species than the amount of saturated fatty acids detected in each of the other eight Egyptian isolates. The amount of fatty acid contents in relation to nematode weight mirrored in general their amount per IJ despite the differences in IJ weight between species and isolates (Table 4).

Table 4: Fresh weight and total fatty acid contents of freshly harvested infective juveniles of the tested *Heterorhabditis* and *Steinernema* isolates.

Nematode species	Nematode isolate	Fresh Weight (ng/IJ)	Fatty acid contents per IJ (ng ± SD		Nanogram fatty acid/100 ng of IJ weight	
			Total FA	Saturated FA	Total FA	Saturated FA
H. bacteriophora	HP88	188±10a	40.4±3.5a	6.7±0.4a	21.6±1.9a	3.6±0.21a
H. indica	EGAZ1	123± 8b	11.3±1.9b	2.5± 0.22b	9.2±0.7b	2.1±0.17b
	EGAZ2	101± 5b	13.7±1.7b	2.8± 0.21b	13.6±0.69c	2.8±0.21b
	EGAZ3	133± 6b	16.6±2.2b	4.2± 0.35c	12.4±1.7c	3.1±0.26a
	EGAZ4	136± 6b	7.1±1.8c	1.6±0.19b	5.2±1.3d	1.2±0.06c
	EGAZ5	100± 4b	16.8±3.3b	3.5±0.33c	16.8±1.3c	3.5±0.33a
S. carpocapsae	All	171± 11a	36.0± 4.2a	3.9±0.38c	21.1±2.4a	2.3±0.22b
	EGAZ9	204± 13a	7.4±1.3c	0.8±0.4d	3.6±0.6d	0.4±0.1d
	EGAZ10	169±9a	9.6±1.7c	1.6±0.1b	5.7±1.0d	1.0±0.06c
	SA	229± 15c	13.0±1.9b	0.6±0.5d	5.7±0.8d	0.2±0.20d

Means within the same column followed by the same letter are not significantly different (Duncan's multiple range test, $P > 0.05$).

The one obvious difference was in the amount of saturated fatty acids, as the two Egyptian isolates of *H. indica*, EGAZ3 and EGAZ5 showed an amount of saturated fatty acids comparable to that of the commercial species HP88 (3.1±0.26 and 3.5±0.33 compared with 3.6±0.21 ng/100ng nematode weight, respectively). The differences in the amount of saturated fatty acids between EGAZ3, EGAZ5 and HP88 were insignificant ($P > 0.05$). Fig. (2) shows that GC-MS peaks of the main fatty acids of HP88, EGAZ3 and EGAZ5 were comparable.

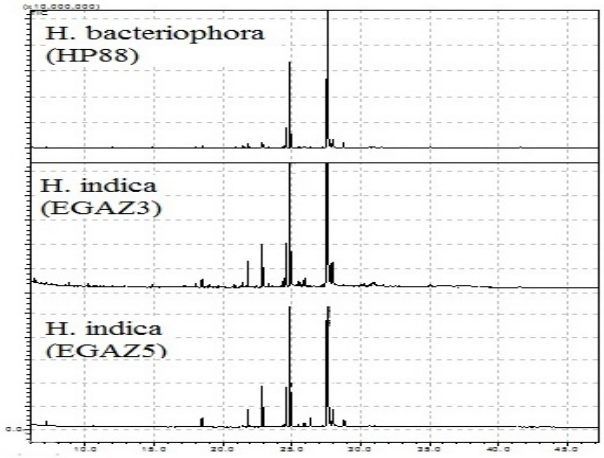


Fig. 2: GC-MS total ion chromatograms of HP88, EGAZ3 and EGAZ5 nematode isolates (Relative intensity vs time per minutes).

DISCUSSION

When aiming for nematode strains with superior stress tolerant improved traits for formulation in applicable products, first it is preferable to look for physiologically pre-adapted wild types as such genotypes are likely to be more stable and more tolerate to environmental and climatic conditions in the geographic regions from where the nematode population was isolated (Somasekhar *et al.*, 2002 and Dolinski *et al.*, 2008). It is well established that fatty acid contents and profile are important in several physiological processes, such as desiccation and osmotic tolerance of IJs (Patel and Wright, 1997 and Qui *et al.*, 2000) which in turn affect the quality and shelf life of nematode formulations (selvan *et al.*, 1993a&b and Wright *et al.*, 1999). Therefore, we analyzed the fatty acid contents of eight isolates of *S.* carpocapase and *H. indica* collected from different Egyptian geographical regions. We also included two relevant nematode species extracted from imported commercial formulations for comparison.

Our results demonstrated that IJs of all isolates tested in this study, had fatty acid number and pattern similar to that of the corresponding commercial species. Oleic, was the main fatty acids in all species and isolates, accounting for 50–60% of total fatty acid contents in newly emerged IJs. Unsaturated fatty acids dominated as their proportion was at least three-fold greater than the proportion of saturated fatty acids. These results are in agreement with the general consensus that oleic, as well as unsaturated fatty acids, predominate in EPNs (Fitters *et al.*, 1999, Abu Hatab and Gaugler, 2001, and Andalo *et al.*, 2011).

Results of this work have shown that total amount of saturated fatty acids of all the studied *Heterorhabditis* isolates was significantly higher than that of *Steinernema* isolates. This difference was due mainly to the higher amount of the saturated palmitic (C16:0) in IJs of *Heterorhabditis* isolates, as it was the second most abundant fatty acids while in IJs of *Steinernema* isolates the unsaturated linoleic fatty acid was the second most abundant fatty acids. Patel and Wright (1997), and El-Badawy*et al.*, (2011) also found the same fatty acid patterns in IJs of *Heterorhabditis* and *Steinernema* species they studied. IJs of *H. bacteriophora* and *H. indica* had higher motility nature than the *S. carpocapsae* (Campbell and Gaugler, 1992), thus *Heterorhabditis* species, in general, need more energy reserves. IJs rely on fatty acids as their sole energy source and saturated fatty acids provide more energy than unsaturated fatty acids (Selvan *et al.*, 1993a, and Andalo *et al.*, 2011). This explains our finding that amount of saturated fatty acids of *Heterorhabditis* isolates was significantly higher than that of *Steinernema* isolates as an increase in saturated fatty acid content in IJs will increase their energy reserve.

The main finding of our study is that IJs of the two commercial strains contained more than three-fold greater total fatty acid/IJ than the local Egyptian isolates. Also, total amount of saturated fatty acids was significantly higher in commercial strains than Egyptian isolates. Selvan *et al.* (1993a) suggested that increase quantity of fatty acid composition and proportion of their saturation among infective juveniles in commercial products likely to improve their shelf life. This could be achieved by feeding nematode with certain oils such as olive oil in combination with a fat such as beef fat (rich in palmitic 16:0 and stearic 18:0 acids) (Abu Hatab and Gaugler, 2001). Rouse *et al.* (1992) increased both fatty acids content (5-fold) and double the percentage of unsaturated FAs in the free-living soil nematode *Panagrellus redivivus* by adding fish oil to their diet. Accordingly, the high fatty acid contents of commercial IJs in this study could be attributed to the fact that certain techniques, mainly selection, and manipulation of dietary lipid content has been used to increase lipids and fatty acid contents of such IJs and in consequence improve longevity of commercial formulations (Abu Hatab and Gaugler, 2001

and Perry *et al.*, 2012). However, as natural populations are preferable for preparing effective formulation as mentioned above, screening among natural populations for higher fatty acid contents is the logical first step in formulation of EPN for practical use. Our results shows that of the tested Egyptian isolates, IJs of EGAZ3 and EGAZ5 of *H. indica* had the highest amounts of fatty acids. Also, the amount of saturated fatty acids/gram IJs body weight of EGAZ3 and EGAZ5 was comparable to that of the imported commercial strain. Accordingly, it is suggested that isolates EGAZ3 and EGAZ5 of *H. indica* are the candidate for further improvements through selection and dietary manipulation for developing practical Egyptian bio-control product based on entomopathogenic nematode.

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RABIC SUMMARY

محتوى الأحماض الدهنية في ثماني عزلات من النيماتودا الممرضة للحشرات من خمس محافظات مصرية

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تم تقدير تركيبة الأحماض الذهنية لخمسة عزلات من النيماتودا الممرضة للحشرات (*Heterorhabditis indica*) أطلق عليها الأسماء الرمزية EGAZ1 و EGAZ2 و EGAZ3 و EGAZ4 و EGAZ5 و ثلاثة عزلات من نيماتودا *Steinernema carpocapsae* أطلق عليها الأسماء الرمزية EGAZ9 و EGAZ10 و SA تم جمعها من خمس محافظات مصرية. أيضا، تم فحص تركيبة الأحماض الدهنية لدى سلالتين تجاريتين من نيماتودا *S. carpocapsae,* و *H.bacteriophora* للمقارنة. وقد ظهر تشابه عدد ونمط الأحماض الدهنية في جميع العزلات المصرية مع عدد ونمط الأحماض الذهنية في الأنواع التجارية المقابلة ومن بين الأحماض الدهنية العشرة التي تم تحديدها، كان حمض الأوليك هو الحمض الدهنى الرئيسي في جميع أنواع وعزلات النيماتودا. وقد تبين أن الأحماض الدهنية غير المشبعة هي السائدة وكانت الكمية الكلية من الأحماض الذهنية المشبعة في عزلات نيماتودا H. indica أعلى بكثير من عزلات نيماتودا *S. carpocapsae*. وكان حمض البالميتيك هو ثاني أكثر الأحماض الدهنية وفرة في عزلات *H. indica* في حين كان حمض اللين أوليك هو ثاني أكثر الأحماض الدهنية وفرة في عزلات *S. carpocapsae* . ومن بين كل العزلات المصرية التي تم اختبارها، احتوت عزلتي EGAZ3 و EGAZ5 على أعلى كميات من الأحماض الدهنية عامة كما أن محتواهما من الأحماض الدهنية المشبعة لكل جرام من وزن النيماتودا كان مماثلا لتلك التي تحتويها السلالة التجارية، مما يظهر أن هاتين العزلتين من نيماتودا *H. indica* هما المرشحتان لمزيد من الدراسات لتطوير منتج عملي من النيماتودا الممرضة للحشرات كوسيلة مكافحة حيوية فعالة لبعض الآفات الحشرية التى تصيب النباتات في مصر.