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Studies on the Bioactivity of Diflubenzuron on *Caenorhabditis elegans*

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ABSTRACT. The present study shows that the population growth of *Caenorhabditis elegans* was significantly reduced after treatment with 5-40 µg/ml of the wettable formulations of diflubenzuron, a phenyl benzoyl urea compound ($P < 0.01$), whereas treatment with similar concentrations of the analytical grade diflubenzuron in acetone or dimethyl sulphoxide were less effective. The percentage reduction in population growth of *C. elegans* was shown to be greatest (62-99%) in treatments with wettable formulations of diflubenzuron (5-40 µg/ml). Formulation B of diflubenzuron has the lowest EC_{50} value: 0.2 µg/ml. The study also shows that diflubenzuron significantly affects egg production of *C. elegans* ($P < 0.05$) at low concentrations (20 µg/ml) while other biological activities such as development, survival, feeding and movement are not significantly affected ($P < 0.05$). About 56, 85 and 91% reduction in egg production was observed in treatments with 5, 10, 20, µg/ml diflubenzuron for 96 hr, respectively.

Diflubenzuron is a phenyl benzoyl urea compound; 1-4 chlorophenyl -3-(2,6 diflubenzoyl - urea), found to inhibit the formation of insect cuticle, which is mainly composed of chitin (Post *et al.* 1974). Diflubenzuron has also been suggested to have potential anthelmintic or nematicidal effects (Veech 1977, Ibrahim 1978). A significant reduction in population growth of the free living nematodes *Pelodera* sp. and *Panagrellus redivivus* was reported after treatment with 10 µg/ml diflubenzuron. Ibrahim (1978) similarly demonstrated a significant reduction in population growth of *Caenorhabditis elegans* when treated with 5-80 µg active ingredient (a.i)/ml of formulated diflubenzuron. However, the effect of the formulation material incorporated with the active ingredient was not investigated in either of the above studies.

Therefore, the present study, firstly, investigates the effects of analytical grade diflubenzuron on the population growth of *Caenorhabditis elegans* compared to

that of two wettable formulations of diflubenzuron and to the formulation material alone. Then, the study examines the various effects of the most active preparations of diflubenzuron on development, survival, feeding, movement and fecundity of *C. elegans*.

Experimental Work

Culture of Experimental Organism

C. elegans Bristol (strain N) was cultured with OP 50 a uracil mutant of *Escherichia coli* on a medium of NG agar (Brenner 1974).

Experimentation

I. *Effects of diflubenzuron preparations on population growth of* ***C. elegans***

I.A. *Effects of analytical grade diflubenzuron:* Stock solutions of diflubenzuron were prepared in acetone or dimethyl sulphoxide, due to its low solubility in water (0.2 µg/ml). The toxicity of these two solvents has been reported to be minimal when applied to axenic cultures of *Aphelenchoides rutgersi* at 0.1 and 0.01 (w/v), respectively (Myers 1972).

Standard solutions of 0.025, 0.05, 0.1, 0.2 and 0.4% (w/v) of analytical grade diflubenzuron (purity 95-99%, I.C.I. Plant Protection Ltd., U.K.) in acetone or dimethyl sulphoxide (2 ml) were added to 98 ml of autoclaved NG agar kept at 45° C and rapidly mixed to make 5,10,20,40 and 80 µg a.i./ml solution of diflubenzuron. Controls of pure NG agar with acetone or dimethyl sulphoxide (2% w/v) were also prepared. Diflubenzuron - agar suspensions were then poured into sterile 45 mm diameter plastic petri dishes and once solidified the agar inoculated with *E. coli* OP 50 and incubated at 25° C. After 48 hr, three second stage juveniles (J_2) of *C. elegans* collected from 3-5 day old stock cultures were transferred to each plate. The number of adult and juvenile nematodes on each plate was recorded after 4 and 5 days at 25° C. Each treatment was replicated five times.

I.B. *Effects of diflubenzuron formulation (A) and (B):* Two samples (A and B) of a 25% wettable formulation of diflubenzuron having the number median diameter (NMD) of 1.8 µm and 2.0 µm, respectively were obtained from I.C.I. Plant Protection Ltd., U.K. Formulations A and B were mixed thoroughly with 100 ml of molten autoclaved NG agar at 45° C to give uniform suspensions of 5,10,20, and 40 µg (a.i.)/ml diflubenzuron. Agar suspensions were then prepared as described in I.A. After 48 hr, second stage juveniles of *C. elegans* were added as described above and the population size was recorded after 4 and 5 days.

In a separate experiment, control agar plates with 0,15,30,60,120 and 240 µg/ml of inert carrier for the wettable formulation (supplied by I.C.I. Plant

Protection Ltd.,) were prepared corresponding to the equivalent amount of this carrier incorporated with the formulated diflubenzuron tested above. The population growth of *C. elegans* on plates with diflubenzuron carrier was determined after 3,4, and 5 days. In both experiments, each was replicated five times.

I.C. *Effects of diflubezuron on biological activities of* ***C. elegans:*** Formulation (B) of diflubenzuron, the most active preparation of this compound (see Results, B), was used in the following experiments, C. 1-5.

C.1. *Effect of diflubenzuron on development of* ***C. elegans:***
Eggs of *C. elegans* were collected from 5 day old cultures and batches of 25-30 eggs transferred to NG agar plates containing 0,10,20,40, and 80 μg/ml of diflubenzuron. After 24, 48 or 72 hr at 25° C nematodes were collected individually using a fine pasteur pipette with a bulbous head. These nematodes were mounted on thin films of 5% (w/v) agar on microscope slides, narcotized in a drop of 0.25% (v/v) propylene phenoxetol (Ellenby and Smith 1964) and observed under a light microscope. Body and pharyngeal length and the maximum body width (at the vulval region) were recorded. Five nematodes from each treatment were examined at each time interval.

C.2. *Effects of diflubenzuron on survival of* ***C. elegans:***
J_2 of *C. elegans* were collected from 3 or 5 day old cultures and batches of 25-30 juveniles transferred onto NG agar plates containing 0,5,10,20,40 and 80 μg/ml diflubenzuron. The percentage nematodes surviving was recorded after 48 hr at 25° C. Each treatment was replicated five times.

C.3. *Effect of diflubenzuron on feeding of* ***C. elegans:***
J^2 of *C. elegans* were treated with 0-80 μg/ml diflubenzuron as described above and the rate of feeding of each nematode was determined after 72 hr at 25° C. Each nematode was transferred into a drop of water on a microscope slide which had been previously coated with a fine layer of 1% (w/v) ion agar smeared with *E. coli* OP 50. The rate of pharyngeal pulsation was then recorded over a 2 min period. Eight nematodes from each treatment were examined *in situ* on agar plates using an inverted microscope with a ×10 objective. The percentage of nematodes exhibiting a pumping motion of the pharynx in 5 sec was determined as described by Byerly *et al.* (1976).

C.4. *Effect of diflubenzuron on movement of* ***C. elegans:***
Individual adult nematodes from diflubenzuron-treated agar plates (0-40 μg/ml) were transferred onto a thin film of 1.5% (w/v) agarose (0.1 ml)

on a glass slide. After 30 min, tracks produced by the nematodes were photographed and measured using an x-y digitiser (FERRANTI CETEC) linked to a computerised board. This method has previously been found to be more accurate and less time consuming than using calibrated dividers (Turner 1980). Five nematodes were examined for each treatment.

C.5. *Effect of diflubenzuron on egg production of* ***C. elegans:***
Groups of 25-30 j_2 of *C. elegans* were inoculated onto NG agar plates containing 0,5,10,20 μg/ml diflubenzuron as described above. After 48 hr at 25° C juveniles from each treatment were divided into two groups; nematodes in one group were transferred individually to fresh plates containing the same concentration of diflubenzuron, the other group was transferred to clear agar plates. The fecundity of both groups was recorded after a further 48 hr. For each treatment 24 nematodes were examined.

Results

A. *Effects of Analytical Grade Diflubenzuron on Population Growth of* ***C. elegans***

C. elegans populations treated with 0-80 μg/ml diflubenzuron (in acetone or dimethyl sulphoxide) were compared using Duncan's multiple range test. The data obtained is summarised in Fig. 1. There was no significant difference between the mean number of nematodes in the plain agar, or acetone and dimethyl sulphoxide controls ($P < 0.05$) after 4 or 5 days treatment (Figs. 1 and 2). The population growth of *C. elegans* was however, significantly retarded ($P < 0.01$) in plates containing 20-80 μg/ml diflubenzuron acetone or dimethyl sulphoxide after 4 days treatment. But, after 5 days treatment, a significant reduction in population size was only observed with 40 and 80 μg/ml diflubenzuron acetone or dimethyl sulphoxide ($P < 0.01$; Figs. 1 and 2).

B. *Effects of Diflubenzuron Formulations A and B on Population Growth of* ***C. elegans***

Population growth of *C. elegans* was not significantly affected compared to control by treatment with 15-240 μg/ml diflubenzuron carrier ($P < 0.05$). But, population was significantly reduced in all diflubenzuron treatments ($P < 0.01$; Fig. 3) although there was no significant differences between the effects of equivalent concentrations of formulations A and B ($P < 0.05$; Fig. 3). Table 1 summarizes the effects of various treatments of diflubenzuron upon population growth of *C. elegans*. The percentage reduction in population growth appeared to be greatest (62-99%) in treatments with wettable formulations of diflubenzuron (5-40 μg/ml).

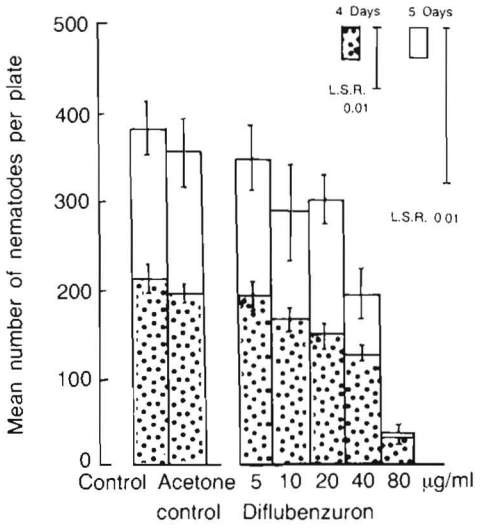

Fig. 1. The effect of analytical grade diflubenzuron in acetone on population growth of *C. elegans* after 4 and 5 days treatment.
Bar Lines = ± standard error.
$L.S.R_{(0.01)}$ = least significant range, P = 0.01.

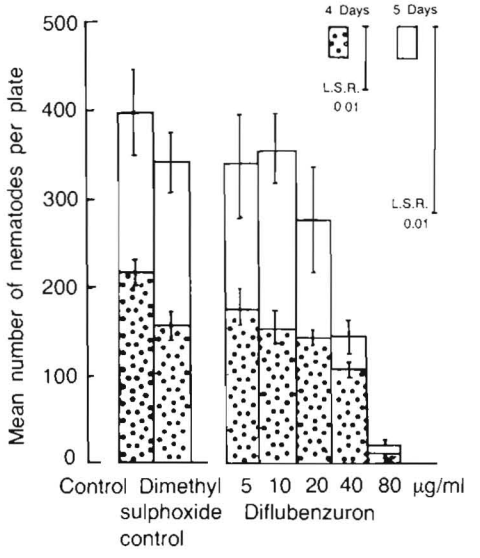

Fig. 2. The effect of analytical grade diflubenzuron in dimethyl sulphoxide on population growth of *C. elegans* after 4 and 5 days treatment.
Bar Lines = ± standard error.
$L.S.R_{(0.01)}$ = least significant range, P = 0.01.

The EC_{50} values for the analytical grade diflubenzuron in acetone or dimethyl sulphoxide were estimated to be 44.6 and 46.0 μg/ml, respectively whereas EC_{50} values of formulation A and B of diflubenzuron were found to be 1.1 and 0.2 μg/ml, respectively.

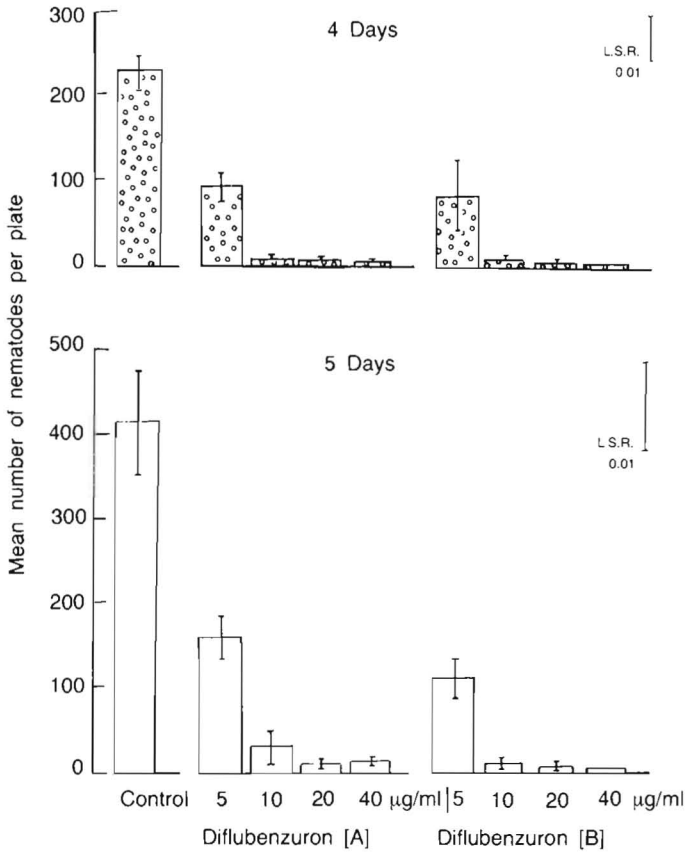

Fig. 3. The effect of diflubenzuron formulations A and B on population growth of *C. elegans* after 4 and 5 days treatment.
Bar Lines = ± standard error.
$L.S.R_{(0.01)}$ = least significant range, P = 0.01.

C. *Effects of diflubenzuron formulation B on biological activities of* ***C. elegans***

C.1. *Development*

Measurements of *C. elegans* treated with diflubenzuron while at different stages of post embryonic development are given in Fig. 4. In the first 24 hr of treatment, *C. elegans* were able to develop normally in all concentrations of

diflubenzuron, but after 48 and 72 hr treatment nematode development was significantly reduced ($P < 0.01$) in 80 μg/ml diflubenzuron. Measurements of the total body length, pharynx length and body width of diflubenzuron treated (80 μg/ml) *C. elegans* after 48 hr represented about 33%, 60% and 43% of the corresponding measurements in control nematodes.

Table 1. Population growth of *C. elegans* expressed as population percentage,* after 5 days treatment with different preparations of diflubenzuron.

Treatment	Diflubenzuron			
	in acetone	in dimethyl sulhpoxide	formulation	
			A	B
5 μg/ml	96.0	98.8	37.7	34.8
10 μg/ml	83.7	102.0	1.1	0
20 μg/ml	79.8	79.5	0	0
40 μg/ml	52.0	40.6	0	0
80 μg/ml	7.6	3.8	–	–

* Percentage changes in population, calculated according to Myers (1972).

Population percentage $\frac{T-N}{C} \times 100$

T = total number of living and treated nematodes.
N = number of nematodes originally inoculated.
C = net increase of nematode numbers in control treatment.

C.2. *Survival*

Concentrations of diflubenzuron up to 80 μg/ml had no singificant effect on nematode survival ($P > 0.05$; Table 2).

C.3. *Feeding*

Treatment with 10 or 20 μg/ml diflubenzuron did not significantly affect feeding activity of *C. elegans* ($P > 0.05$), whereas 40 and 80 μg/ml diflubenzuron significantly reduced feeding activity ($P < 0.05$ and < 0.01, respectively; Table 3). However, the percentage of nematodes feeding was not significantly affected by any treatment ($P > 0.05$; Table 3).

C.4. *Movement*

Locomotion of *C. elegans* was not affected by 10-40 μg/ml diflubenzuron but was significantly retarded by 80 μg/ml ($P < 0.05$; Table 4). Control nematodes and others treated with 10-40 μg/ml diflubenzuron typically produced sinusoidal tracks in the agarose. In contrast, traces made by

nematodes treated with 80 μg/ml diflubenzuron had a flatter shape with fewer sinusoidal waves.

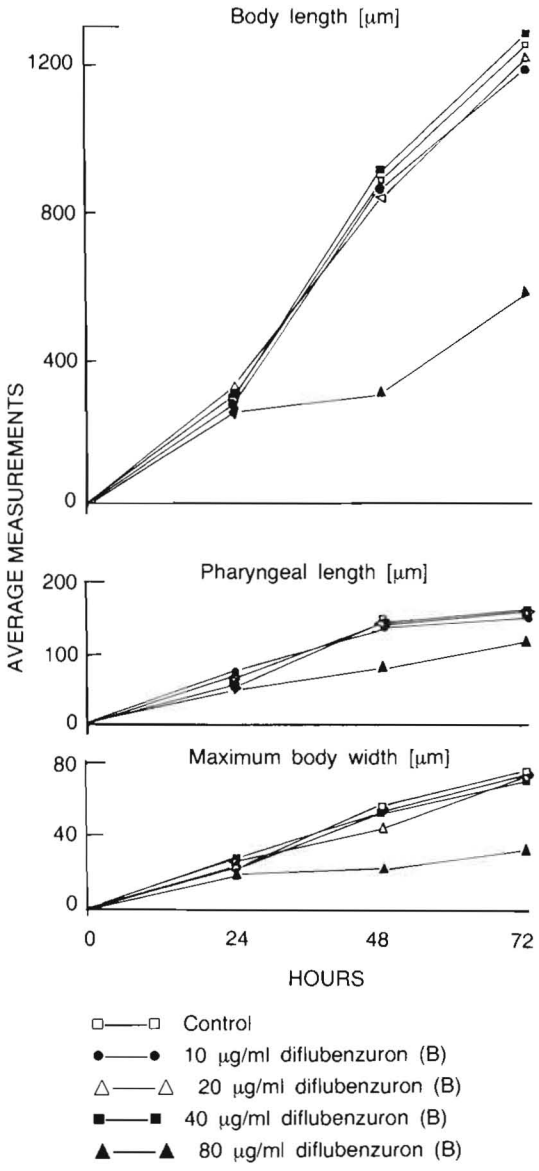

Fig. 4. The effect of (0-80 μg/ml) diflubenzuron (formulation B) on the development of *C. elegans* after 24,48 and 72 hr. Each point represents the mean of 5 replicates.

Table 2. Effect of diflubenzuron on survival of *C. elegans*

Treatment	Mean percentage of survival (n = 5)	Range	Means of transformed values ± S.E.*
Control	91.5	85-100	1.3 ± 0.07
5 µg/ml diflubenzuron	86.0	76-100	1.3 ± 0.07
10 µg/ml diflubenzuron	83.3	69-93	1.3 ± 0.09
20 µg/ml diflubenzuron	79.6	76-90	1.1 ± 0.05
40 µg/ml diflubenzuron	82.9	64-92	1.2 ± 0.03
80 µg/ml diflubenzuron	82.5	72-89	1.2 ± 0.03

* Percentages were transformed to angular values (arcsine $\sqrt{p}$).

Table 3. Effect of diflubenzuron on feeding of *C. elegans*

Treatment	Control	Diflubenzuron (µg/ml)			
		10	20	40	80
A. Feeding rate* ± S.E.	263±9	254±13	259±13.6	200±19	174±17.8 ± S.E. Range
Range	193-302	148-290	170-290	113-276	110-260
B. Mean percentage of nematodes feeding**	95	95	90	81	71
Range	88-100	77-100	84-100	74-100	64-76
Mean of transformed value ± S.E.	1.4±0.09	1.3±0.09	1.3±0.07	1.2±014	1.0±0.29

* Mean number of the nematode pharyngeal pulsations/2 min.

** Percentages were transformed to angular values (arcsine $\sqrt{P}$).

Table 4. Effect of diflubenzuron on locomotion of *C. elegans*

Treatment	Distance moved (mm) ± S.E.	Range
Control	8.4 ± 0.9	5.7-10.1
10 µg/ml diflubenzuron	9.1 ± 1.9	5.4-16.1
20 µg/ml diflubenzuron	9.4 ± 1.7	5.7-14.1
40 µg/ml diflubenzuron	6.7 ± 1.0	3.9- 9.6
80 µg/ml diflubenzuron	2.4 ± 0.3*	1.5- 3.4

* Significantly retarded ($P < 0.05$); $L.S.R_{(0.05)} = 5.5$

C.5. *Fecundity*

After 48 hr treatment with 20 μg/ml diflubenzuron, the fecundity of *C. elegans* hermaphrodites was found to be significantly reduced ($P < 0.01$; Fig. 5). Treatment with 5 or 10 μg/ml diflubenzuron had no significant effect on fecundity ($P > 0.05$). The fecundity of *C. elegans* was significantly affected by all concentrations of diflubenzuron (5-20 μg/ml) when the nematodes were treated for 96 hr ($P < 0.01$; Fig. 5); the number of progeny produced by nematodes on plates containing 5,10 and 20 μg/ml diflubenzuron being 44, 15 and 9%, respectively of the number produced by untreated nematodes (Fig. 5). A total inhibition of egg production was observed in some individuals treated with 20 μg/ml diflubenzuron for 48 and 96 hr.

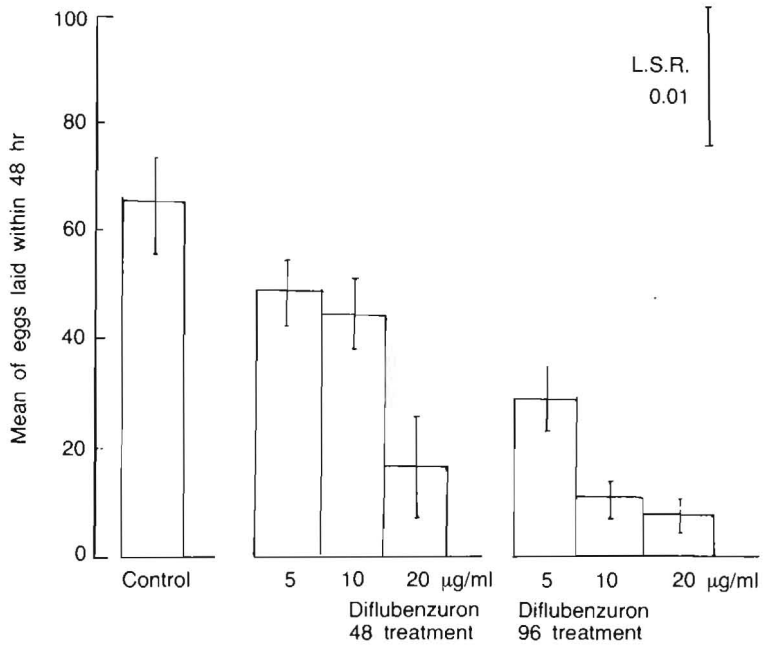

Fig. 5. Effect of diflubenzuron (formulation B) on fecundity of *C. elegans*. Each column represents the mean of 24 replicates.
Bar Lines = ± standard error.

Discussion

The present study has shown that treatment with 10-40 μg/ml diflubenzuron wettable formulation reduces population growth of *C. elegans* by more than 90% (Table 1) while treatment with similar concentrations of analytical grade diflubenzuron in acetone or dimethyl sulphoxide were less effective (Figs. 1 and 2).

The carrier material incorporated with formulated diflubenzuron proved not to have a detrimental effect on the population growth of *C. elegans*. These results thus confirm the observations of Ibrahim (1978) and Veech (1978a) on the activity of diflubenzuron on *C. elegans* and some other free living nematodes, respectively. A comparison of the population growth of *C. elegans* in various treatments of diflubenzuron showed that the lowest EC_{50} was obtained with the wettable powder formulation B (0.2 μg/ml) whereas the EC_{50} of analytical grade diflubenzuron being more than 40 times the value for the wettable formulation.

The fact that analytical grade diflubenzuron has less effect on *C. elegans*, whether used in acetone or dimethyl sulphoxide, suggest a possible relationship between the bioactivity and the physical properties of diflubenzuron particularly the size and the distribution of particles of this chemical in each treatment. In fact, the biological activity of a wide range of pharmaceuticals and pesticides has been reported to be dependent on the particle size of the active compound (Valkerburg 1973, Kelly *et al.* 1975, Mass 1978). In the present study, the bioactivity of diflubenzuron formulation B (NMD=2.0 μ) was, in fact, slightly greater than that of formulation A although the difference was not significant (Table 1).

The decrease in nematode population size by treatment with active chemicals could be the result of increased generation time and lowered fecundity (Samoiloff 1980) or an increased mortality of the juveniles and adult stage.

In the present work, fecundity experiments showed reduction in the number of eggs produced by *C. elegans* treated with diflubenzuron for 96 hr; about 56, 85 and 91% reduction in egg production being observed in treatments with 5, 10, 20 μg/ml diflubenzuron, respectively (Fig. 5). This effect of diflubenzuron on *C. elegans* appeared to be less pronounced when the nematodes treated for 48 hr only. Because fecundity, development and feeding activities of nematodes are known to be closely associated (Evans 1973, Cook 1977, Samoiloff 1980), the effects of anthelmintics on reproduction and development can often be associated to inhibition of feeding. But, in the case of diflubenzuron-treated hermaphrodites of *C. elegans* both development and percentage of feeding hermaphrodites were found to be normal (Fig. 4 and Table 3).

Nevertheless, the mobility of *C. elegans* hermaphrodites appeared to be normal after treatment with 10-40 μg/ml diflubenzuron (Table 4). Hence, the significant reduction of *C. elegans* population growth by diflubenzuron can be mainly attributed to retardation of *C. elegans* fecundity. In fact, diflubenzuron is believed to affect the process of chitin synthesis in the nematode eggs (Veech 1978b). The present study therefore, indicates that reduction in the population size of *C. elegans* is due to the possible action of diflubenzuron upon the formation of *C. elegans* eggs.

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دراسات على نشاط مركب الداي فلوبنزيورون البيولوجي على السينورابدايتز القانز

عادل محجوب إبراهيم

جامعة الجزيرة ـ ص . ب (٢٠) ـ واد مدني ـ السودان

أوضحت هذه الدراسة أن مركب الداي فلوبنزيورون ـ وهو أحد مركبات مجموعة الفينايل بنزوايل يوريا ـ قد قلل بصورة جلية تزايد عدد النماتودا سينورابدايتز القانز وذلك بعد معاملتها بتركيزات تتراوح بين ٥ ـ ٤٠ ميكروجرام/ملم من بودرة الداي فلوبنزيورون المخلوطة، ($P<0.01$)، بينما كانت معاملة النماتودا بتركيزات مشابهة من عينة خالصة لمركب الداي فلوبنزيورون (أذيبت في مذيبات عضوية، كالأسيتون والداي ميثايل سلفوكساييد) أقل تأثيراً. وقد تراوحت نسبة النقصان في عددية النماتودا عند معاملتها بتركيزات ٥ ـ ٤٠ ميكروجرام/ملم من خلطة الداي فلوبنزيورون بين ٦٢ ـ ٩٩٪. كما سجلت الخلطة الثانية من هذا المركب أقل جرعة مؤثرة وتعادل ٠,٢ ميكروجرام/ملم فقط. واتضح أيضاً أن مركب الداي فلوبنزيورون قد أثر بصورة فعالة على إنتاج بيض السينورابدايتز القانز عند معاملتها بتركيزات أقل من ٢٠ ميكروجرام/ملم ($P<0.05$) بينما لم تتأثر مقدرة النماتودا البيولوجية في النمو والبقاء أو على الحركة والغذاء ($P>0.05$) إلا أن مقدرتها على صنع البيض قد انخفضت بنسبة ٥٦، ٥٨، ٩١٪ عند معاملة السينورابدايتز القانز بالتركيزات ٥، ١٠، ٢٠ ميكروجرام/ملم على التوالي.