SOME STIMULATIONS OF BACTERIA IN SOIL TREATED WITH THE INSECTICIDE SEVIN

(N-methyl-1-naphthyl carbamate)

By

S. H. ELWAN* and A. A. KHODAIR**

Botany Department, Faculty of Science,
Ain Shams University, Cairo, A.R.E.

INTRODUCTION

On studying the effect of the insecticide sevin (N-methyl. 1 - naphtyl carbamate) on soil bacteria (Elwan and Khodair, 1969a) using total viable counts as critria, no great effect was recorded. However, it was sometimes observed that certain morphologically distinct bacterial colonies particularly mucoid and non mucoid circulars, and lenticulars were evidently dominant on the counting plates of various treatments as compared with controls. Therefore, interest in this investigation was directed towards quantitative following of the development of morphologically distinct bacterial colonies on the counting plates of soil treated with sevin. Apart from the possibility of providing a quick tool for discovering some aspects of bacterial stimulations, the quantitative determination of the development of these colonies might point at the nature of colonization of bacteria in the soil, particularly when the medium used for the colony determination is soil extract.

Revealing of certain bacterial stimulations in the soil treated with sevin, on the basis of colonial morphology presented in this paper — raised a question regarding the nature of this stimulation. An answer of this question was attempted in this investigation.

MATERIAL & METHODS

Sevin was applied to soil at doses of 0.5, 2.5, 5, 10,50,100, and 500 ug/gm for 2 consecutive times ; the second was 6 weeks after the first. Agar media for counts wer supplemented soil extract and Bunt and Rovira with calcium car bonate as devised by Louw & Webley (1959) for counting acid producing bateria. Techniques and media were similar to those previously described (Elwan and

* Present Address : Botany Department, Faculty of Science, AL AZHAR UNIVERSITY Cairo, A.R.E.
** Now at Faculty of Girls, Ain Shams University, Cairo.

khodair, 1969a). Circular colonies were those more in diameter than 1 mm., lenticulars were lens-shaped colonies, and acid producers were those showing halos on calcium carbonate medium. Mucoid colonies were those showing evident mucilage.

Identification of bacteria was carried out according to the methods recommended in the "Manual of the microbiological methods" 1957, using the keys of Bergey's manual of determinative bacteriology, 1957.

Representative data of distinct stimulations only were given tabularly, other data are discussed in the text. Responses of morphologically distinct colonies on $CaCO_3$ medium at the doses of 10, 100, & 500 ug/gm soil, only were given graphically as representatives of the other medium and doses.

In attempts of investigating the nature of stimulation of isolated bacteria, the resistance degree and ability of utilization of these to sevin were tested as follows. Stimulated bacteria were streaked separately on gradient plates (Szybalski & Bryson, 1952) containing successive concentrations of the insecticide; streaking started at the point of higher concentration extending towards that of the lower one. In another attempt, the microorganisms were inoculated in sevin powder located on supplemented soil extract agar plates. This technique was made to reveal if growth occurred, the unlimited degree of resistance.

As for the ability to utilize sevin, a basal liquid medium of the following constitution was used, 0.1% KH_2PO_4, 0.05% KCl, 0.05% $Mg SO_4 . 7H_2O$, traces of $FeSO_4 . 7H_2O$. BDH chemicals were used. 0.05% sevin was supplemented as carbon and nitrogen source. Ability of utilizing sevin was tested for the highly resistant forms only (*B. megaterium*, strains 2 & 9). Nutrient free cells washed in physiological saline solution were used as inocula. Viable counts were the criteria of growth. Plate culture decimal dilution technique was used. Test for significance was made by the probable error difference (PED) determination

RESULTS

Stimulations of morphologically distinct bacterial colonies :

Percentages of circular bacterial colonies on both supplemented soil extract and calcium carbonate complete media showed that they represented in general the greatest part of the colonies of both treated and untreated soils. Their values were more than 50% in most counts. Application of two consecutive sevin doses showed stimulatory effects on circulars in some counts and inhibitory ones in others.

Following the stimulations of circular bacterial colonies on soil extract agar, it was observed that sevin dose of 500 ug/gm soil, resulted in a maximum stimulation effect (about 79% of the whole viable bacterial colonies), three weeks after the second application (Table 1). These circulars were nonmucoid, a representative of which was subcultured purified, and identified. It was suggestive of being *Bacillus megaterium*. It was given the name a strain 11.

An interesting observation based on the morphological characters of the circular colonies was the mucoid nature. Mucoid circular colonies were dominant on soil extract counting plates of the treated soil whereas they were not so on the control plates. This happened at the time when the stimulation of the total viable bacteria and the total circulars (Table 1) was rather slight. This was recorded 4 weeks after applying 100 ug of sevin per gram soil for the first time. This mucilaginous circular was subcultured, purified and indentified as a member of Pseudomonads (Pseudomonad sp., strain 6). Pseudomonad strains were thus called since they produce gas and acid from carbohydrates, hence could not be related to the genus *Pseudomonas*. On the other hand, they could not grow in nitrogen free liquid medium, hence not related to the genus *Azotomonas*. They are or the moment considered as members of Pseodomonadales.

Table (1) : Stimulation of circular bacterial colonies in soil treated with sevin. Representatives were the nonmucoid *B. megaterium*, 11 in 500 ug/gm dose and the mucoid *Pseudomonad sp*., 6 in 100 ug/gm dose, The medium used was supplemented soil extract agar.

Dose and time of stimulation	Total viable bacteria 10^6/gm.		Total circulars (%)	
	control	treated	control	treated
500 ug/gm. soil (3 weeks after 2*nd* application).	4.77	3.40	45.0	79.3
100ug/gm. soil (4 weeks after 1*st* application).	1.748	2.935	54.1	62.4

Table 2 : Stimulation of circular acid producing colonies (representative *B. megaterium*, strain 9) in soil treated with the insecticide sevin (500 ug/gm soil), 4 weeks after application.

Medium	Total viable bacteria10^6/gm. soil		Total circulars (%)		Acid producing circulars (%)	
	control	Treated	Control	Treated	control	Treated
Bunt & Rovira with CaC03	1.244	0.835	51.7	45.2	20.5	63.2

Table 3 : Stimulation of total mucoid lenticular colonies on supplemented soil extract agar (representatives, *pseudomonad sp.*, strains 10 & 13) in soil treated with sevin, 4 days after second application.

Dose	Total viable bacteria 10^6 / gm. soil		Total lenticulars (%)	
	Control	Treated	Control	Treated
5 ug/gm. soil	2.50	3.97	5.30	14.4
500 ug/gm. soil	2.50	3.37	5.30	15.5

Table 4 : Stimulation of lenticular acid producing colonies (representative *B megaterium*, strain 2) in soil treated with sevin (2.5 ug/gm), few hours after application.

Media	Total viable bacteria 10^6 / gm soil		Total lenticulars		Lenticular acid producers (%)	
	Control	Treated	Control	Ttreated	Control	Treated
Bunt & Rovira with CaC03 :	1.06	0.89	8.1	3.4	44.1	66.7
supplemented soil extract :	1.92	2.72	10.5	6.2	—	—

Table 5 : Test for significance of growth of bacterial cells in a medium with sevin as only carbon and nitrogen sources. S = significant, NS = non significant.

Bacteria	Number of cells (x10^6) /ml culture		Difference	PED x 3	Significance.
	before incubation	after 4 weeks incubation			
B. megaterium, 2	31.25	134.060	+102.8	38.5	(S)
B. megaterium , 9	63.50	105.975	+42.5	76.1	(NS)

In the first count of soil supplied with 500 ug/gm, a negligible difference was observed between percentages of total circulars on control & treated plates, yet a particular circular (fading white colonies) has dominated the treated counting plates, while it was not observed at all on the control ones. Although time elapsing after treatment was only few hours, yet prompt bactericidal action might have abolished a probable competitive effect, thus allowing space for their development. This type of colony has been subcultured, purified and identified as a member of Pseudomonads (Pseudomonad sp., strain 7).

Thus, isolation of two stimulated strains of Pseudomonads and one *B. megaterium* strain, was the result of following the effect of various doses of sevin on circular bacterial colonies on soil extract agar.

In an attempt to combine physiological and morphlogical bases of distinction, circular acid producing colonies (colonies clearing the medium around them; also considered a morphological character) on calcium carbonate medium were counted and expressed as percentages of total circular bacterial colonies recorded on the same plates. At the time when total viable bacteria (Table 2) and total circulars (Table 2 & Fig. 1) were not greatly affected, acid producing circular colonies (Tables 2 & *Fig.* 1) were evidently stimulated in sevin treated soil 4 weeks after first appliation. A representative of these colonies was isolated, purified and identified as *B. megaterium*, strain 9.

The rhythm of response of circular acid producing colonies was in general different from that of the total circulars (Fig. 1, upper). Fluctuations were more drastic in case of total circulars than in case of acid producing ones particularly in the second application.

With regard to lenticular colonies (lens shaped from top view), induction of both stimulatory and inhibitory effects was observed (Fig. 1, lower). Two enticular types of colonies (one type was smaller than the other) were producing mucilage and were dominating lenticulars stimulated on soil extract agar plates (Table 3) in the counts made 4 days after sevin second application. These two types were subcultured, one from a lower dose (5, ug/gm) andthe other from the highest dose used (500 ug/gm.). The two subcultures, after being purified and identified, proved to be pseudomonads (Pseudomonad spp. 10 & 13, respectively).

Following acid producing lenticular colonies revealed some interesting observations. At the time when stimulation of total lenticular percentages was observed one week after first application, acid producing lenticulars were rather inhibited in most cases (e.g. Fig. 1). However, few hours after applyig sevin for the first time, lenticulr acid producers were found to dominate counting plates of most of the doses (e.g. Table 4). At the time when total lenticulars were suppressed on both calcium carbonate and supplemented soil extract agar media, acid producing lenticulars were stimulated (Table 4). Purified subcultures of these stimulated lenticular acid producers proved to be *B. megaterium* strain 2.

Lenticular acid producing bacteria (representatives suggestive of being *B. megaterium*) were highly stimulated at the doses of 10, 100 & 500 ug/gm soil after the second application (Fig. 1, lower, right). One strain of these bacteria (*B. megaterium*, 2) proved to be able to utilize sevin as only carbon and nitrogen source (Table 5).

Strain variations of stimulated bacteria :

Pseudomonad strains No. 6, 7, 10, & 13 differed morphologically on counting plates. Pseudomonads 6 & 7 were circular colonies isolated from soil extract plates and calcium carbonate ones respectively. Pseudomonads 10 & 13 were lenticulars producing mucilage, the colony of the former was - relatively minute dominating the soil extract counting plates of 5 ug/gm dose, while that of the latter was relatively long and dominating soil extract plates of 500 ug/gm dose. Pseudomonad strains 7 & 13 were relatively short rods (about half the length of strains 6 & 10). Strain 10 was of relatively thicker rods. Pseudomonad strain 13 grew well in saline broth up to 5% NaCl, while the other strains grew well in saline broth only up to 3%. Strain 7 could not utilize lactose as sole carbon source. Under anaerobic conditions strain 6 was the only organism which produced gas from alkaline nitrate broth, while strain 10 was the only strain which could not produce gas from alkaline glucose broth. The above mentioned differences are in evidence of morphological, physiological, and biochemical variations of strains 6, 7, 10 & 13.

B. megaterium strains 2, 9, & 11 differed in certain characters. *B. megaterium* strains, 2 & 9 were acid producers, the former was lens shaped while the latter was circular. *B. megaterium*, 11 could not utilize lactose as sole carbon source Strain 9 was shorter in dimensions than strain 11. Both strains 9 & 11 were isolated from plates of the highest dose (500 ug/gm.), while strain 2 was isolated from a lower dose (2.5 ug/gm.).

Degree of resistance of stimulated bacteria to sevin :

On sevin gradient plates, all the stimulated bacterial strains showed growth but differed in the length of the streak. *B. megaterium* strains 2, 9 & 11 only grew at higher concentration ; strains 2 & 9 were highly resistant to sevin since they were able to grow on sevin powder located on supplemented soil extract agar. Pseudomonad strains 6,7, 10 & 13 were less resistant than *B. megaterium* ones.

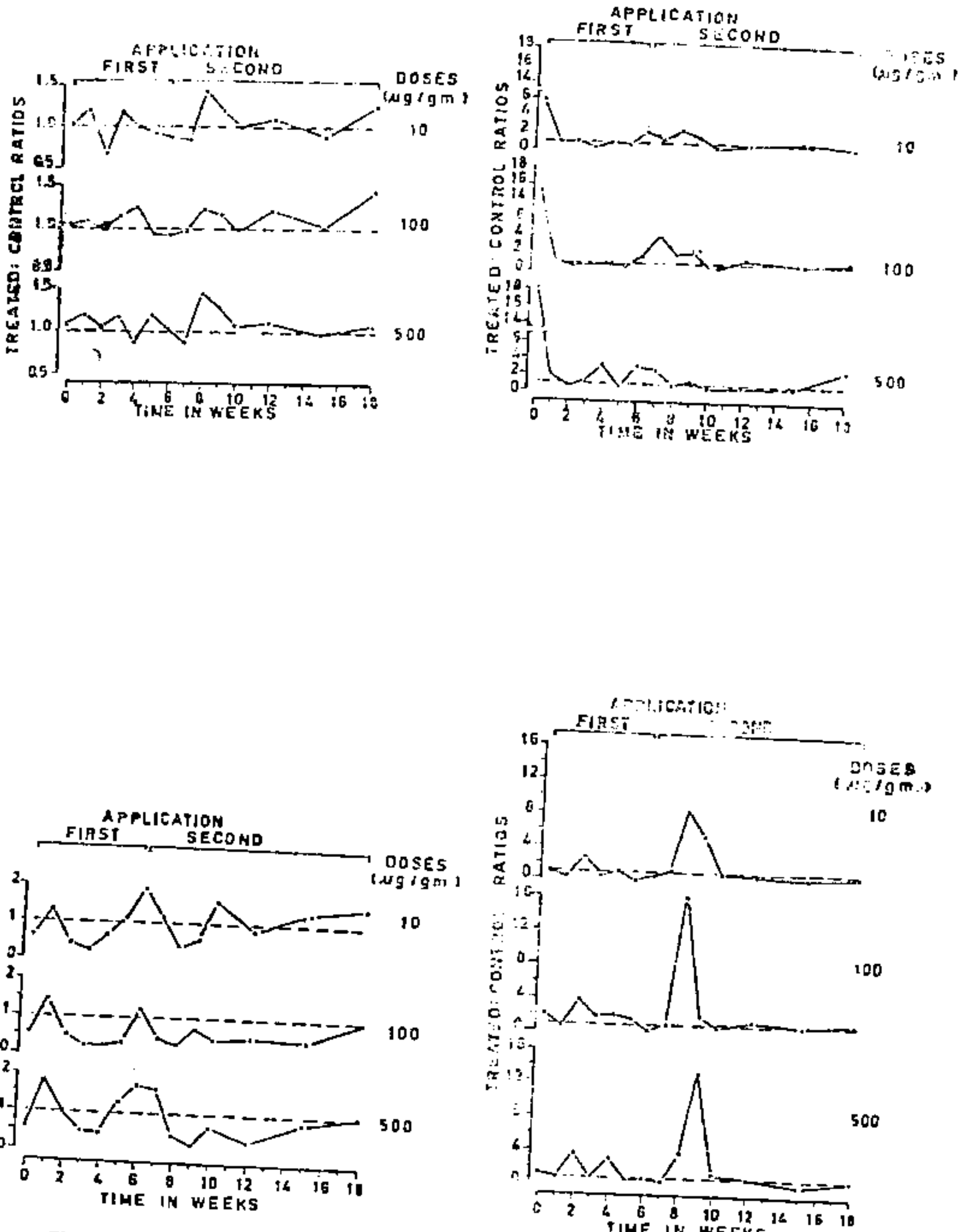

Fig. 1 — The rhythm of responses to sevin, of total circular bacetrial colonies (upper, left), circular acid producing colonies (upper, right). total lenticular colonies (lower, left) and lenticular acid producing colonies (lower, right). The medium in all cases was Ca CO_3 complete agar.

Ability of sevin utilization :

Highly resistant forms (*B. megaterium* 2 & 9) were chosen to test their ability of utilizing sevin as only carbon and nitrogen source. Definite growth was observed visually in both strains.Counts showed in strain 2 about 4—5 times increase. This increase proved to be significant (Table 5). However, the observed growth and the increase in cell counts of strain 9 proved to be not significant.

DISCUSSION

The use of the morphological character of the bacterial colonies as criterion for following the effect of sevin on soil microorganisms enabled to observe certain stimulations of bacteria. Although the shape of the colony may change with the change of the medium and probably on the same medium because of other factors, yet if conditions were uniform the shape of the singl well isolated colony would be almost the same. As early as 1923, Lohnis and Fred stated that under practically uniform conditions the pictures presented by growing colonies are remarkably constant, characteristic, and of considerable diagnostic value. Familiarity with morphological characteristics becomes very helpful as a guide for the recognition of major groups of bacteria (Pelczar & Reid 1958). In this investigation, representatives of stimulated acid producing bacterial colonies were *B. megaterium* strains either circular or lenticular in shape. These colonies were not mucoid as compared with the mucoid stimulated circulars and lenticulars which proved to *Pseudomonad* strains, in most cases. In other words, it might be claimed that non-acid producing mucoid circulars or lenticulars on the media used in this investigation were most probably members of the *pseudomonads* whereas acid producing non-mucoid circulars or lenticulars were strains of *B. megaterium*. In an investigation on the effect of sulpha compounds on soil microorganisms, using the same media employed here, representatives of stimulated non mucoid circular and lenticular acid producing bacterial colonies and mucoid circulars and lenticulars were, *B. megaterium* & Pseudomonad strains, respectively (Elwan & Khodair, 1969b) However, these authors isolated also plumose *B. megaterium* colonies and star-shaped Pseudomonads among the representatives of the stimulatad bacteria in the soil treated with sulpha compounds.

It should be stressed that it is not the aim to claim that a certain mor phological character on a certain medium should be of particular species, but it is to claim that certain single or combined morphological identities might be a useful tool of revealing some ma ked effects in soil treated with additives.

In point of fact, if total viable bacteria were found stimulated due to a certain treatment, isolation of representatives of such stimulation effects, would be confronted with difficulties. Morphologically distinct colonies on counting plates — if found responding in similar to their totals — would be helpful in isolating more correct representatives as compared with random isolation of representatives of the stimulated total viable bacteria. Therefore, following up of morphologically distinct colonies is important ; its importance increases when the response of these colonies appears different from the response of their mother groups (bacterial or fungal totals). *B. megaterium*, 2, a lenticular acid producer, represented more. than 66% of the total lenticulars on the counting plates of CaC03 complete medium of soil treated with sevin as compared with 44% value of the respective control At this time, total lenticulars represented about 3%&8% of the total viable bacteria appearing on the counting plates of the treated and the control soils, respectively. Without following the halo appearance (indicative of acid production) on the counting plates, *B. megaterium*, 2 could not be isolated, and the conclusion would have been an inhibition of total lenticulars and total viable bacteria. *B. megaterium*, 2 however proved to be able to utilize sevin as only carbon and nitrogen source, which would interpret its stimulation which was masked by exerting some inhibitory effect on its mother total colonies. The finer the morphological character is the greater becomes its reliability for isolating correct representatives of effects in soils treated with additives.

Not all the isolated respresentatives of the recorded stimulations were able to utilize sevin as energy or nitrogen source although all of them were resistant to this insecticide Therefore, the ability of utilization of sevin as a cause of the recorded stimulation effects seems unlikely. Resistance might allow for selection of the stimulated strains but if this is the major factor which interprets the recorded stimulatory effect, the latter would have continued which was not the case in the present investigation, where fluctuations occurred. The nature of the microbial balance in the soil treated with sevin was not stationary (Elwan and Khodair, 1969a). The ever changing microbiological activity in a dynamic microbial balance, might therefore account for the nature of the recorded bacterial stimulations, together with resistance to and utilizability of sevin. The microbial utilization of various substances added to the soil has been recorded by many authors (e.g. Evans, 1947 ; Walker, 1954 ; Fernley and Evans, 1958 ; Gottlieb and Mandel, 1959, Rogoff and Wender, 1959 ; Kaneda and Roxburgh, 1959 ; Rogoff, 1960 ; Macrae et al, 1962 ; Lersalimskii and Skryabin, 1965; Altam and Lawlor, 1966).

Exudates of certain bacteria stimulated in soil treated with sulpha compounds decreased the development of *Azotobacter* in soil (Elwan and Khodair, 1969 c) which might point at the importance of the microbial activity in a microbial equilibrium of dynamic nature.

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SUMMARY

A garden soil was treated with various doses of the insecticide sevin. Count of total viable bacteria were made on the agar media of soil extract supplemented with sucrose and phosphate, and on Bunt & Rovira (with CaC03) using the decimal dilution technique. The latter medium was used to count acid producing bacterial forming halos). Certain stimulations of bacteria have been quantitatively revealed on the basis of colonial morpology. Identification of representatives of recorded stimulations showed that non-mucoid circular or lenticular acid producing colonies were suggestive of being *B. megaterium* strains, whereas mucoid circular or lenticular non-acid producing colonies were suggestive of being members of Pseudomonads. In some cases stimulations on basis of colonial morphology, were recorded at the time of slight inhibitions of total viable bacteria.

The stimulated bacteria were found resistant to sevin. *B. megaterium*, 2 showed growth in sevin powder located on the used supplemented soil extract It was also able to utilize sevin as only carbon and nitrogen source as indicated by the statistically significant increase of its viable counts after 1 weeks of incubation as compared with those before incubation.

The nature of the recorded stimulations was suggested to be due to one or more of the fellowing:resistance and then selection of microbes, utilization of sevin, and microbial activity of selectively stimulated microbes.

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