INHIBITION OF AZOTOBACTER ACTIVITY BY SOME BACTERIA STIMULATED IN SOIL TREATED WITH THE INSECTICIDE SEVIN

(N-methyl-1-naphthyl carbamate)

By

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INTRODUCTION

Azotobacter was suppressed in soil treated with the insecticide sevin (N-methyl-1-naphthyl carbamate) whereas some other bacteria were stimulated (the authors 1969, a & b). The recorded inhibitory effect of sevin on soil microorganisms might be due to the susceptibility to the insecticide and/or to the activity of highly resistant microbes on other less resistant ones. Although *Azotobacter* was suppressed in sevin treated soil, yet it was not completely inhibited, indicating therefore a certain degree of resistance (the authors, 1969 a). On the other hand, the bacteria stimulated in sevin treated soil (3 *B. megaterium* strains & 4 pseudomonads) were found highly resistant to sevin ; one of them (*B. megaterium*, 2) utilized it as only carbon and nitrogen source (the authors, 1969 b)...

The present paper is an endeavour to elucidate the role of these sevin-resistant stimulated bacteria in suppressing *Azotobacter* activity in the sevin-treated soil This would clarify an aspect of the nature of effect of sevin on soil microorganisms

MATERIAL AND METHODS

Azotobacter strain used proved to be *A. chroococcum.* It was identified according to the keys of Bergeys manual of Determinative Bacteriology ; the keys of Norris and Chapman (1968) were also consulted. Identifications of bacteria stimulated in sevin treated soil were previously given (the authors, 1969 b). These bacteria are *B. megaterium* strains 2, 9 & 11 and pseudomonad strains 6,7, 10 & 13. Variability in characteristics between these strains has been also previously given (the authors, 1969 b).

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Azotobacter inoculum : *Azotobacter* cells were transferred from the surface of 72 hours old Nitrogen free agar plates, into a bottle of 250 ml. capacity containing 100 ml. of sterile distilled water. The bottle was shaken by hand for 2 minutes and 1 ml. of the obtained homogenous suspension was used as an inoculum.

Effect of bacterial exudates : Bacteria were allowed to grow in Czapek 's liquid medium with 0.1% NaN03 for 48 hours at 30° C. The supernatents of cultures centrifuged at 4000 r.p.m. for 5 minutes, were filtered through bacteriological cintered glass filter under aseptic conditions to get cell-free filtrates. One ml of a neutral N-free liquid medium (x 10 strength) and 9 ml. of the prepared bacterial filtrate were mixed in a sterile conical flask of 25 ml capacity. The contents were inoculated with the above mentioned *Azotobacter* inoculum. Potentiality of N -fixation was determined in treatments and in the respective unconsumed liquid medium as control. Effect of bacterial exudates in the filtrates on activity of *Azotobacter* could thus be determined.

Effect of diethyl ether extract of bacterial exudates :

Cell-free filtrates of the most potent strains were extracted with diethyl ether " Merck " (1 : 1, v/v) . The residue of the etherical layer was raised to the original volume by neutral N-free liquid medium. The extract was dispensed under aseptic conditions, in conical flasks of 25 ml. capacity (10 ml. for each) and inoculated with *Azotobacter* inoculum. Control in this case was an equal amount of N-free liquid medium similarly inoculated with the *Azotobacter* inoculum.

Inoculation of Azotobacter and bacteria into soil : One ml. of actively growing 48 hours old homogenized bacterial culture in modified Czapek 's liquid medium was used as bacterial inoculum for 100 gm. of a 2 mm. sieved air dried garden soil. 100 grams of the air dried soil were weighed in sterile conical flask of 250 ml. capacity. One ml. of the prepared homogenous bacterial culture was dispensed asepatically in small drops on the surface of the soil. The soil was allowed to stand for 30 minutes, and then was moulded by sterilized spatula for homogenization. One ml of *Azotobacter* suspension was similarly dropped on the surface and after 30 minutes of allowing for percolation, homogenization was similarly made. 8 mls of sterile distilled water were then dispensed on the even surface of the soil to adjust the water content at 28% of the saturation capacity, allowed to settle for 30 minutes, and then rehomogenized by the sterile spatula. All possible precautions to get uniformity in soil were taken in consideration. The amount of water lost by evaporation during incubation was compensated for, every week. Incubation was at 30° C. for 3 weeks. Control was soil inoculated with *Azotobacter* only to be compared with soil treated with *A. chroococcum* together with a bacterial strain.

Sampling and counting of A. chroococcum : A sample of soil was transferred aseptically to heat sterilized Petri - dish and left to dry. A load of a standardized sterile spatula (16 mg. in the present case) as used by Elwan and El - Sayed (1964), was sprayed upon the surface of solid N - free agar plates. One, two, and sometimes more than 2 spatula were used as inoculum for each plate. Triplicates of each treatment were incubated in an inverted position at 30° C, counting was made after 3 days.

Nitrogen determination : Digestion was made using conc. H_2SO_4 and H_2O_2. Ammonia was distilled in a semimicro kjeldahl distilling apparatus, after adding an excess amount of 40% NaOH. Ammonia was received in 2% boric acid (Hawk et al. 1947). The indicator used was that described by Sobel, Mayer & Gottfried (1944). The N - content of the samples was calculated from the volume of the standard H_2SO_4 needed for the back titration (1 ml N/70 H_2SO_4 = 0.2 mg., nitrogen). The amounts of nitrogen were calculated as mgm. per 100 ml. in case of cultural work and per 100 gm. soil in case of soil work.

Test for significance : The test was considered significant when the difference from control was more than three times the probable error difference.

RESULTS

A.—*Effect of the exudates of B. megaterium and pseudomonad strains, and their extracts on the potentiality of nitrogen fixation in culture* :

The cell - free exudates of all bacterial strains stimulated in soil treated with the insecticide sevin, had exerted significant depressive effect on potentialitty of nitrogen fixation by *A. chroococcum* (Table 1). The most potent strains were *B. megaterium* 2 & 11, and pseudomonad 13. These strains were selected to investigate the effect of the diethyl ether extracts of their cell - free exudates on the potentiality of nitrogen fixation. Significant depressive effects (Table 1) were exerted in all cases. The substances responsible for the depressive effect were therefore ether soluble.

B—*Development of A. chroococcum and its potentiality of nitrogen fixation when inoculated into the soil together with B. megaterium and pseudomonad strains*

Development of *Azotobacter* cells as determined by their counts per gram soil, was depressed by both *B. megaterium* and pseudomonad strains. (Table 2)

Nitrogen fixation in the soil was significantly depleted (Table 3).

Table 1.—Effect of exudates and exudate ether extracts of strains of *B. megaterium* and Pseudomonad sp., on the potentiality of nitrogen fixation by *A. chroococcum* (mean mg. nitrogen 100 ml culture).

	Control	B. megaterium strains :			Pseudomoad sp. strains :			
		2	9	11	6	7	10	13
Exudates*	15.2	5.12	8.12	6.48	11.24	10.28	8.41	6.92
Extracts*	12.6	4.64	—	6.92	—	—	—	6.10

* Differences from control were all significant.

Table 2 : Development of *Azotobacter chroococcum* inoculated in soil together with bacteria stimulated in "Sevin" treated soil. Control was soil inoculated with *Azotobacter* only.

Mean total number of *Azotobacter* cells per gm. soil

Weeks after inoculation	Soil inoculated with : Azotobacter only	Azotobacter and B. megaterium			Azotobacter and pseudomonad sp.			
		Strain 2	Strain 9	Strain 11	Strain 6	Strain 7	Strain 10	Strain 13
Start	16875	1041	1041	2083	1041	1875	2083	2293
1	7853	771	875	604	1000	2063	2271	729
2	4792	937	417	625	875	417	1854	729
3	4250	979	542	521	708	646	667	1104

Table 3 : Effect of *B. megaterium* and *pseudomonad* sp., strains on the potentiality of N-fixation by *A. chroococcum* (mean mg. nitrogen/100 gm. soil) when inoculated together at the same time in the soil.

Time after inoculation (weeks)	Soil inoculated with : *Azoto-bacter*	*Azotobacter* + B. *megaterium*, strains :			*Azotobacter* + *Pseudomonad* sp., strains :			
		2	9	11	6	7	10	13
Start	57.6	61.2	56.4	54.8	56.4	60.0	59.2	55.8
1	93.4	73.6	56.8	70.4	53.6	62.0	67.2	64.4
2	116.0	80.4	72.0	72.4	82.0	69.6	71.2	71.4
3	108.0	51.8	73.6	68.0	82.0	76.8	90.4	74.8

. Differences from controls-except start values were all significant.

DISCUSSION

The finding that all the bacterial strains isolated from the soil treated with sevin, had exerted depressive action on *Azotobacter* in culture and soil, is very interesting. It contributes to the understandig of the nature of effect of this insecticide on soil microorganisms. *Azotobacter* was found depleted in the soil due to application of sevin at the time when certain bacterial stimulations were recorded (the authors 1969, a & b). The recorded suppression of *Azotobacter* and its activity, might indicate that the direct effect of sevin in the soil seems unlikely to be the only effect. The stimulated bacteria being resistant to sevin might have exuded certain diethyl ether soluble substances which are able to suppress the development of *Azotobacter* and its potentiality of nitrogen fixation. The action of these stimulated bacteria on *Azotobacter* might be different from their action on other microorganisms ; it might be stimulatory.

These stimulated bacteria which are resistant to sevin (the authors 1969, b) might not only exert their inhibitory action on less resistant microbes but also on highly resistant ones as well.

Both stimulations and inhibitions of microorganisms were recorded in soil treated with sevin along 4 months (the authors 1969, a). The present findings could elucidate the significat role of the microorganisms in causig these fluctuations. The effect of sevin on soil microbes is therefore not for the whole directly exerted. The activity of the microbes which were stimulated in treated soil oftenly

due to their resistance could be considered an evidence of the also exerted indirect effect of the insecticide on soil microorganisms. If the effect was only direct the stimulated resistant bacterial strains would have caused the existence of a linear relation of bacterial development in response to sevin application to soil. This was by no means the case (the authors 1969 a).

Azotobacter, however, has been reported to be susceptible to the antagonistic action of other soil microorganisms (e.g. Nickell & Burkholder, 1947 ; Iuzhina, 1958 ; Kuznetsov, 1961 ; Rudulovic, 1962 ; Tchan & Jackson, 1966 ; Tribe and Williams, 1967 ; Elwan and El - Naggar, 1969). Decrease in *Azotobacter* counts noted in the soil inoculated with it only, might be due to competition exerted by soil microorganisms (Tchan & Jackson, 1966) or to soil noncultivation (Rakhno and Ryys, 1963). However, the actual cause needs further elucidation.

It was intersting to record parallelism between cell counts and nitrogen fixation being all suppressed. In another investigation definite gains of nitrogen were obtained when definite decreases in counts were recorded (Elwan and El - Naggar 1969). This would be discussed elsewhere.

Results have indicated that sevin application to soil might affect its fertility leivel through the microbial exudation of certain substances which deplete the process of nitrogen fixation in the soil.

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REFERENCES

Bergey (1957).

Manual of determinative Bacteriology. ed. 7 by R.S. Breed, E.G.D. Murray & N.R. Smith, Baltimore.

Elwan, S.H. & El - Naggar, M.R. (1969).

Effect of streptomyces sp. on the development of *Azotobacter* and its potentiality of nitrogen fixation in culture and soil. 6th Arab Sci. Conf., Damascus.

Elwan, S.H. and El - Sayed, M.A. (1964).

Ecology of *Azotobacter* in Egyptian soils. Ain Shams Science Bulletin No. 10, 215 - 255.

Elwan, S.H. and Khodair, A.A. (1969 a).

Effect of the insecticide sevin (N - methyl 1 - Naphthyl carbamate) on soil microorganisms Ann - Rev., Univ. College for Girls, Ain Shams Univ. (in press)

— — — AND — — — (1969 b).

Revealing and nature of some stimulations of bacteria in soil treated with the insecticide sevin (N - methyl 1 - naphthyl carbamate) on the basis of colonial morphology Ibid.

HAWK, F.P.; L. OSER AND W.H. SUMMERSON (1947).

Practical physiological chemistry, 12th edition, pp. 1323. J. and A. Churchill Ltd., London.

IUSHINA, Z.I. (1958).

Relationship between toxic properties of soil of Kola peninsula and number of bacterial antagonists of *Azotobacter*. Mikrobiologiia (Transl.) 27 (4): 452 - 456.

KUZNETSOV, V.D. (1961).

Actinomycetes which stimulate and supress the growth of certain bacteria in soils under single permanent crop. Agrobiologiia 1. 131 - 136. 1959: Referat. Zhur., Biol., 1961, No. 8B 292.

NICKELL, L.G. AND P.R. BURKHOLDER (1947).

Inhibition of *Azotobacter* by soil actinomycetes Jour. Amer. Soc. Agron. 39: 771 - 779.

NORRIS, J.R. & H.M. CHAPMAN (1968).

Classification of *Azotobacters* in identification methods for microbiologists: ed. by Gibbs & Shapton Part B, 19 - 27. Acad. Press.

RAKHNO, P.KH., AND O.O. RYYS. (1963).

The application of *Azotobacter* preparations. Mikrobiologiya 32 (3): 558 - 561

RUDULOVIC, V. (1962).

Interrelations of rhizosphere microorganisms and *Azotobacter chroococcum* Beijerinck. VIII th international Congress for Microbiology, Abstracts, Montreal, Quebic, Canada. No. B 13.3, P. 53.

SOHEL, A.E.,A.M. MAYER, AND S.P. GOTTFRIED (1944).

A convenient titremetric ultramiero - method for the estimation of urea and Kjeldahl nitrogen. J. Biol. Chem., 156, 355.

TCHAN, Y.T., AND D.L. JACKSON (1966).

Studies of nitrogen - fixing bacteria : IX. Study of inoculation of wheat with *Azotobacter* in laboratory and field experiments. Proc. Linn. Soc. New S. Wales 90 (3): 290 - 298. 1965 (reed. 1966).

TRIBE, H. AND P.A. WILLIAMS (1967).

Investigations into the basis of microbial ecology in soil, illustrated with reference to growth of Soil Diphtheroids and *Azotobacter* in a model system. Cand. J. Microbiol. Vol. 13 No. 5 P. 467.