Bull. Environm. Contam. Toxicol. 25, 482-486 (1980)

Effect of 2-Aminobenzimidazole on Nitrogen Fixers from Flooded Soil and Their Nitrogenase Activity

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Soil is the eventual sink for most pesticides used in agriculture or public health. The extensive use of pesticides in agriculture necessitates intensive studies on the effect of these toxic chemicals and their degradation products on non-target soil microorganisms of importance to soil fertility. Extensive studies were made on the influence of several pesticides on nitrogen fixation in pure culture and soil systems (VENKATARAMAN & RAJYALAKSHMI 1972, MACKENZIE & MACRAE 1972, WOOD & MACRAE 1974, CHARYULU & RAO 1978, NAYAK & RAO 1980). The extensive application of benzimidazole compounds like benomyl and MBC in controlling the diseases often leads to the accumulation of certain degradation products in soil-plant systems. FUCHS & DE VRIES (1978a) reported the accumulation of 2-aminobenzimidazole as one of the major degradation products of benomyl decomposition. Although the importance of Azospirillum and other nitrogen-fixers in the nitrogen economy has been stressed (NEYRA & DÖBEREINER 1977), the influence of agricultural chemicals received less attention. Studies indicated that benomyl amendment to soil greatly stimulated the Azospirillum population and nitrogen fixation (CHARYULU & RAO 1978). However, no information is available on the influence of 2-aminobenzimidazole, 2-AB, a hydrolysis product of benomyl on nitrogen fixation. This study deals with the influence of 2-AB, a degradation product of MBC on the nitrogen-fixing populations of Azospirillum and symbiotrophic associations from a submerged paddy soil.

MATERIALS AND METHODS

Soil, collected from the rice growing tract of Kerala, South India (pH 5.0; organic matter 4.3%; total nitrogen 0.24%; electrical conductivity 6.3 mmhos/cm) was air-dried, screened (<2 mm) and placed in 200 x 25 mm test tubes in 10-g portions and maintained under submerged conditions throughout the experiment. Technical grade 2-AB was applied at 0, 10, 20 and 100 ppm levels with respect to soil before submergence. At periodic intervals on 10, 20 and 30 days, samples were drawn for the isolation of Azospirillum and symbiotrophic nitrogen-fixing organisms from both 2-AB amended and unamended soils. All the treatments were replicated thrice.

Azospirillum was cultured from soils by transferring a loopful of soil to semi-solid malate medium of DÖBEREINER et al. (1976). Within 24 h at 30°C, a typical white, dense, fine pellicle developed a few mm below the surface of the medium. Microscopic observation of

0007-4861/80/0025-0482 $01.00

48-h-old cultures revealed characteristic rods with fat droplets and very active spiral movements. Nitrogen fixation in 72-h-old cultures obtained on 10, 20 and 30 day incubation of the soil was determined by acetylene reduction assay. The population of Azospirillum sp. in the soil was estimated by the most probable number (m.p.n.) technique and numbers calculated by using the probability tables (ALEXANDER 1965). Semi-solid malate medium in 5-replicate tubes per dilution was inoculated with 10-fold dilution of the soil samples. Tubes in which typical white pellicle had formed a few mm below the surface of the malate medium within 24 h at 30°C were scored positive for Azospirillum sp.

Symbiotrophic nitrogen-fixing organisms were grown from soil incubated for 10, 20 and 30 days under different treatments. For enumerating the number of facultative symbiotrophic organisms 1-mL of serially diluted (10^{-2} to10^{-6}) soil sample was inoculated to 50-mL sterile N-free medium in 100-mL Erlenmeyer flasks (CHARYULU et al. 1978) and incubated at 28°C for 30 days. The medium, initially neutral, became acidic following glucose utilization and the pH of the medium during the 30-day incubation was maintained at 7.0 by the addition of sterile dilute NaOH (KALININSKAYA 1967). Samples of 3 mL were drawn aseptically from the flasks on 5, 15 and 30-days for acetylene reduction analysis.

The B-D 75 x 13 mm vacutainer brand tubes with 3 mL cultures were stoppered and the gas phase was replaced with high pure acetylene (10% by volume) through a gas tight hypodermic syringe. Tubes were incubated at 28-30°C in the dark for 24 h. A 0.5 mL sample of the gas phase from each tube was analyzed for ethylene production on a gas chromatograph fitted with a hydrogen flame ionisation detector and 1.5 m x 3 mm column of 100-120 mesh Porapak R at the column temperature of 60°C; nitrogen at a flow rate of 30 mL/min was used. Nitrogenase activity for symbiotrophic nitrogen-fixing associations was expressed as µmoles C_2H_4 formed / flask/day and for Azospirillum as nmoles C_2H_4 formed/culture/day.

The Eh of the soil samples after amendment with pesticides and incubation was measured in triplicate with a portable redox meter model RM-IF (TOA Electronics Ltd., Tokyo) fitted with a compound platinum and calomel electrode type GC-211. After Eh measurements, the pH of the soil samples was determined.

RESULTS AND DISCUSSION

2-AB exerted differential influence on the nitrogen-fixing populations of a submerged soil over a period of 30-day incubation (Table 1). The population of Azospirillum sp. was stimulated at all the concentrations of 2-AB with the exception of 20-day samples amended with 20 and 100 ppm of 2-AB. In contrast to the stimulation of Azospirillum population, a drastic inhibition of the population of symbiotrophic nitrogen-fixers was evident at 2-AB concentrations beyond 20 ppm throughout the incubation period. Since the symbiotrophic nitrogen-fixing associations are mixed culture of several groups of bacteria belonging to Pseudomonas sp., Mycobacterium sp. Arthrobacter sp., and Bacillus sp. (KALININSKAYA 1967, CHARYULU et

TABLE 1. Population of nitrogen-fixing microorganisms as influenced by 2-AB in a submerged soil.

2-AB (ppm)	Population x 10^5/g dry soil					
	Soil incubation (days)					
	10		20		30	
	a	b	a	b	a	b
0	120	5.4	1600	1.1	350	0.14
10	220	5.4	16000	0.7	2000	0.14
20	320	0.7	1600	0.12	1600	0.068
100	540	0.21	1600	0.093	920	0.045

a: Azospirillum b: Symbiotrophic organisms

TABLE 2. Acetylene reduction by symbiotrophic nitrogen-fixing associations from 2-AB amended flooded paddy soil.

Soil incubation (days)	2-AB (ppm)	*Nitrogenase activity of the associations (µmoles C_2H_4 formed/flask/day)		
		Culture incubation (days)		
		5	15	30
10	0	12.4 ± 0.6	2.0 ± 0.1	0.41 ± 0.06
	10	11.7 ± 1.4	1.5 ± 0.1	0.48 ± 0.09
	20	14.7 ± 0.4	2.3 ± 0.2	0.80 ± 0.09
	100	14.4 ± 0.6	2.9 ± 0.3	0.71 ± 0.09
20	0	3.9 ± 0.4	1.4 ± 0.2	0.08 ± 0.01
	10	12.1 ± 0.3	2.1 ± 0.1	0.03 ± 0.01
	20	11.1 ± 1.2	2.7 ± 0.2	0.06 ± 0.02
	100	4.8 ± 0.2	1.0 ± 0.5	0.71 ± 0.02
30	0	0.6 ± 0.2	0.3 ± 0.04	0
	10	0.7 ± 0.3	0.4 ± 0.05	0
	20	1.0 ± 0.1	1.6 ± 0.06	0
	100	1.0 ± 0.3	0.2 ± 0.05	0

*Each value represents the mean of 9 flasks ± standard deviation.

al. 1978), the net influence of 2-AB on these nitrogen-fixing associations was inhibitory. The growth of Pseudomonas was inhibited by 2-AB and other benzimidazole compounds (FUCHS & DE VRIES 1978). 2-AB has been reported to exert inhibitory action by interfering with the normal functioning of DNA (FUCHS & DE VRIES 1978a).

The nitrogen-fixing activity of the symbiotrophic nitrogen-fixing associations isolated on 10, 20 and 30-day incubation of the soil was determined by acetylene reduction assay (Table 2). The nitrogenase of the associations was measured on 5, 15 and 30-days of incubation of the cultures in liquid medium. A gradual decrease in the nitrogenase activity with incubation was noticed irrespective of the treatment. Interestingly the associations isolated from 2-AB amended soils showed greater nitrogenase activity than the associations from unamended soil. Nitrogenase activity was high in cultures isolated from 10-day incubated soil samples, while the activity decreased in cultures from 20 and 30-day samplings. Although the population of symbiotrophic nitrogen-fixers was inhibited by 2-AB amendment, nitrogenase activity of these associations increased considerably. This suggests that conclusions on the effect of agrochemicals based on the population counts alone can often be misleading as the potential activity in certain instances as reported here is rather unaffected.

The nitrogenase activity of Azospirillum cultures isolated from 10, 20 and 30-day incubated soil samples was also measured (Table 3). Interestingly, Azospirillum cultures isolated from 2-AB amended soils exhibited low nitrogenase activity than the cultures from unamended soils. The nitrogenase activity increased with the incubation time and cultures isolated from 20 and 30 day incubated samples exhibited greater nitrogenase activity irrespective of the treatment. Although 2-AB amendment greatly stimulated the population of Azospirillum, the nitrogenase activity of the cultures was inhibited. Again, no correlation seems to exist between the population of a particular group of nitrogen-fixers and the nitrogenase activity. Thus the nitrogenase activity depends on the effectiveness of the strains rather than the number of organisms.

TABLE 3. Acetylene reduction by Azospirillum cultures isolated from 2-AB amended submerged paddy soil.

2-AB (ppm)	*Nitrogenase activity of Azospirillum cultures (nmoles C_2H_4 formed/culture/day) Soil incubation (days)		
	10	20	30
0	373 ± 12	658 ± 13	1535 ± 8
10	44 ± 2	82 ± 7	476 ± 21
20	58 ± 4	423 ± 21	372 ± 46
100	47 ± 4	160 ± 7	96 ± 5

*Each value represents the mean of 3 cultures ± standard deviation.

Like 2-AB, benomyl also stimulated the population of Azospirillum. Since benomyl is known to be unstable (FUCHS & DE VRIES 1978a), it is not clear whether the stimulation of Azospirillum population by benomyl is due to the parent compound or its degradation products (CHARYULU & RAO 1978). Interestingly addition of benomyl, the

TABLE 4. Changes in pH and Eh of the soil amended with 2-AB.

2-AB (ppm)	Incubation (days)					
	10		20		30	
	pH	Eh	pH	Eh	pH	Eh
0	7.15	-40	7.20	-180	7.40	-200
10	7.15	-35	7.20	- 75	7.40	-130
20	7.20	-35	7.25	- 50	7.40	-115
100	7.20	-25	7.30	- 35	7.50	- 95

parent compound of 2-AB, to soil systems retarded the drop in redox potential (PAL et al. 1979). Likewise, 2-AB retarded the drop in redox potential (Eh) of the soil especially at higher concentrations while no marked effect on pH was observed (Table 4). This drop in redox potential would have favored the population build up of Azospirillum since they are reported to be effected by changes in pH and Eh (DÖBEREINER et al. 1976, CHARYULU & RAO 1980). In situations where the metabolic breakdown of benonml to 2-AB occurs these results are of significance, since its presence effects the microorganisms of importance to soil fertility.

Acknowledgments. We thank H. K. Pande for his interest and encouragement. Study was supported by International Atomic Energy Agency, Vienna, Austria and Department of Atomic Energy, Government of India.

REFERENCES

ALEXANDER, M. : In "Methods of Soil Analysis". Part 2. C. A. Black (ed.), p. 1467, American Society of Agronomy, Madison, Wisconsin, U.S.A. (1965).
CHARYULU, P. B. B. N. and V. R. RAO : Curr. Sci. 47, 822 (1978).
CHARYULU, P. B. B. N., C. RAMAKRISHNA, V. R. RAO : Proc. Indian Acad. Sci. B. 87, 243 (1978).
CHARYULU, P. B. B. N. and V. R. RAO : Soil Biol. Biochem. 12, in press (1980).
DÖBEREINER, J., I. E. MARRIEL, M. NERY : Can. J. Microbiol. 22, 1464 (1976).
FUCHS, A. and F. W. DE VRIES : Antonie van Leeuwenhoek 44, 283 (1978).
FUCHS, A. and F. W. DE VRIES : Antonie van Leeuwenhoek 44, 293 (1978a).
KALININSKAYA, T. A. : Mikrobiologiya 36, 526 (1967).
MACKENZIE, K. A. and I. C. MACRAE : Antonie van Leeuwenhoek 38, 529 (1972).
NAYAK, D. N. and V. R. RAO : Soil Biol. Biochem. 12, 1 (1980).
NEYRA, C. A. and J. DÖBEREINER : Adv. Agron. 29, 1 (1977).
PAL, S. S., S. BARIK, N. SETHUNATHAN : J. Soil Sci. 30, 155 (1979).
VENKATARAMAN, G. S. and B. RAJYALAKSHMI : Indian J. Agric. Sci. 42, 119 (1972).
WOOD, P. A. and I. C. MACRAE : Bull. Environ. Contam. Toxicol. 12, 26 (1974).