The Effect of Several Organophosphorus Insecticides upon the Acetylene-reduction Activity of *Azotobacter vinelandii*

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In recent years, the reduction of acetylene to ethylene and its detection by gas-liquid chromatography (DILWORTH 1966; SCHOLLHORN and BURRIS 1967) has become a widely used method of assaying nitrogen fixation. The *Azotobacteriaceae* form an important group of free-living, nitrogen-fixing bacteria in nature, and it is of value to know whether applied pesticides may affect the normal behaviour of these organisms.

It has been reported that two insecticides, DDT and lindane, and two herbicides, Dalapon-Na and 2,4,5-T, had no significant influence upon growth or acetylene-reduction by *Azotobacter vinelandii* when added to pure cultures at normal and fifty-times normal field application rates (MacKENZIE and MacRAE 1972). The use of chlorinated hydrocarbon insecticides has been reduced in favour of the less persistent organophosphorus pesticides. The present study was aimed at determining what effects several of these latter compounds would have upon acetylene reduction by *Azotobacter vinelandii*.

MATERIALS AND METHODS

Organism: *Azotobacter vinelandii*, Lipmann, 1903 was obtained from the culture collection, Department of Microbiology, University of Queensland.

Chemicals: The following organophosphorus insecticides or their products were obtained as gifts from The Dow Chemical Company, J.R. Geigy S.A. or Farbenfabriken Bayer A.G.: chlorpyrifos, 0,0-diethyl 0-(3,5,6-trichloro-2-pyridyl) phosphorothioate, the active ingredient of Dursban insecticide; DOWCO 214, 0,0-dimethyl 0-(3,5,6-trichloro-2-pyridyl) phosphorothioate; DOWCO 217, 3,5,6-trichloro-2-pyridyl dimethyl phosphate; 2-hydroxy-3,5,6-trichloropyridine, the "leaving group" liberated from the above insecticides; methidathion, 0,0-dimethyl S-(2-methoxy-5-oxo-Δ^2-1,3,4-thiadiazolin-4-yl) methyl phosphorodithioate, the active ingredient of Supracide insecticide; diazinon, 0,0-diethyl 0-(2-isopropyl-4-methyl-pyrimidin-6-yl) phosphorothioate; 2-isopropyl-4-methyl-pyrimidin-6-ol, the "leaving group" of diazinon; Dasanit, 0,0-diethyl 0-(p-(methylsulphinyl) phenyl) phosphorothioate; and Nemacur, ethyl 4-(methylthio)-m-tolyl isopropyl phosphoramidate.

Growth medium and cultural conditions: The culture was grown and maintained on a mannitol nitrogen-free (MNF) medium (VOETS 1963). To prevent precipitation of salts after steriliz-

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ation at 110 C for 25 minutes, the medium was removed from the autoclave immediately upon loss of pressure and cooled in a water-bath shaker. The test cultures were grown in 50 ml aliquots of this medium within 250 ml Erlenmeyer side-arm flasks that were incubated in the water-bath shaker oscillating at a rate of 78 cycles per minute and with a water temperature of 30 C.

The compounds to be tested were prepared as suitably concentrated solutions in acetone, sterilized by filtration through Millipore Solvinert filters, 0.25 µm (Millipore Corporation, U.S.A.), and 100 µl aliquots added aseptically to the culture medium. To aid solubility and dispersion, a level of 0.3% Tween 80 was incorporated into the medium.

In the initial batch of experiments, the ability of cultures to reduce acetylene when grown in the presence of insecticide was compared with that of controls which contained similar levels of Tween 80 and acetone. Four replicate cultures for each treatment were established and for each growth flask three replicate assay vials were employed. Vials containing sterile medium, medium plus killed cells, and water, were included. The concentrations of pesticides to which the bacterium was exposed were 5 µg ml^{-1} (a common level for field application) and 500 µg ml^{-1}. If the latter of these levels caused interference with acetylene reduction, other intermediary levels were tested. In any one experiment no more than four control and eight test cultures could be handled.

In the second group of experiments, the flask cultures without pesticide were incubated until the exponential phase of growth was reached at which point samples were removed for testing acetylene reduction. 100 µl of the insecticide in acetone were added to the remaining cultures to give a final level of 100 µg ml^{-1} within the growth medium. The cultures were further incubated for an hour and then retested.

Acetylene reduction method: 1 ml aliquots of the test culture were added to pre-gassed assay vials sealed with "Suba-seals". The gas phase within the vials consisted of 10% acetylene in air. After incubation of the vials for an hour in the water-bath shaker, the reaction was stopped by the addition of 1 ml of 50% trichloroacetic acid. 200 µl of the vial atmosphere were sampled with a gas-tight syringe and injected into a gas chromatograph. The instrument used was a Shimadzu gas chromatograph, Model GC4APTF, fitted with dual flame ionization detectors and matched stainless steel columns (2 m x 4 mm) of Porapak R. Operating conditions were: nitrogen carrier gas, 60 ml min^{-1}; hydrogen, 45 ml min^{-1}; air, 500 ml min^{-1}; column temperature, 60 C; detector temperature, 100 C; and injection port temperature, 75 C. Pure ethylene purchased from Union Carbide Corp., Linde Division, U.S.A., was used for the preparation of standard curves.

RESULTS

Since levels of 0.2% acetone and 0.3% Tween 80 were incorporated into the culture medium to assist solubility and dis-

persion of the pesticides, these levels were reconfirmed not to hinder acetylene reduction by *A. vinelandii* (MacKENZIE and MacRAE 1972). In the first group of experiments, the seven insecticides and two "leaving group" products were added to the culture flasks before inoculation. The effects that this had upon acetylene reduction are summarised in Table 1.

Statistical analysis of these results to obtain critical F values showed that no significant effect (5% level) could be detected for either level of application of chlorpyrifos, DOWCO 214, methidathion, diazinon, Nemacur, Dasanit or 2-isopropyl-4-methyl-pyrimidin-6-ol. The higher levels of DOWCO 217 (250-500 $\mu g\ ml^{-1}$) caused a highly significant reduction in ethylene production by this organism (0.1% level), as also did similar levels (100-500 $\mu g\ ml^{-1}$) of 2-hydroxy-3,5,6-trichloropyridine (0.1% level).

When the pesticides were added to the cultures as they entered log phase of growth, similar results were obtained (Table 2). Although DOWCO 217 at this level of application (100 $\mu g\ ml^{-1}$) did not cause a significant change in ethylene production, all levels of 2-hydroxy-3,5,6-trichloropyridine did (50-500 $\mu g\ ml^{-1}$). In addition, in contrast to the previous method, Nemacur (100 $\mu g\ ml^{-1}$) was also inhibitory.

DISCUSSION

During these experiments which extended over a six month period, there was a gradual fall in the quantity of ethylene produced by the culture of *A. vinelandii* e.g. during the testing of chlorpyrifos at the 5 $\mu g\ ml^{-1}$ level, the control cultures produced an average of 7.03 µmole of ethylene mg dry $wt^{-1}\ hr^{-1}$, but during the later testing of DOWCO 217 at a level of 50 $\mu g\ ml^{-1}$ an average of only 3.57 was recorded. It is not known whether weekly sub-culturing or the gradual uptake of nitrogenous compounds from the laboratory atmosphere by stock solutions of mineral salts were responsible. A further variation in control levels of fixed acetylene was due to the restriction of being able to use twelve flasks only during any one experiment. This meant that a large number of experiments were performed for each of which a new batch of cells were grown, and although conditions were standardized as much as possible, fluctuations in values occurred.

The reason for adding the pesticides to the culture medium before inoculation and at entry to log phase of growth was two-fold. Statistical analysis of the first method where the flasks were inoculated from the same starter culture showed that often there was a significant difference in ethylene production within a quadruplicate grouping, whether it be test or control. To obtain a closer correlation between ethylene produced in the absence of pesticide and that generated in its presence, the growing culture without pesticide was tested for acetylene reduction upon entering log phase of growth. The required level of pesticide was added, the culture incubated for a further hour, and then another sample withdrawn for ethylene production determination. This meant that each flask culture could act

TABLE 1

Effect of the presence in the growth medium of seven organophosphorus insecticides and two "leaving group" products upon acetylene reduction by *A. vinelandii*

Pesticide	Concentration $\mu g\ ml^{-1}$	Ethylene production[a] $\mu mole\ mg\ dry\ wt^{-1}\ hr^{-1}$	
		With pesticide	Without pesticide
chlorpyrifos	5	6.96	7.03
	500	5.71	6.12
DOWCO 214	5	6.33	7.03
	500	6.23	6.12
DOWCO 217	5	6.02	5.89
	50	3.58	3.57
	250	1.93	3.79
	500	0.80	3.79
methidathion	5	5.75	5.89
	500	4.44	4.28
diazinon	5	5.13	5.15
	500	5.98	5.76
Nemacur	5	4.94	4.97
	500	3.77	3.79
Dasanit	5	4.90	4.97
	500	3.92	3.79
I.P.M.P.[b]	500	4.15	4.02
T.C.P.[c]	50	4.21	4.25
	100	3.17	3.68
	250	1.38	4.25
	500	0.00	4.02

a Values are averages of four replicate cultures tested in triplicate.
b I.P.M.P., 2-isopropyl-4-methyl-pyrimidin-6-ol.
c T.C.P., 2-hydroxy-3,5,6-trichloropyridine.

TABLE 2

Effect of the presence of seven organophosphorus insecticides and one "leaving group" product upon acetylene reduction by *A. vinelandii* when added at log phase of growth

Pesticide	Concentration $\mu g\ ml^{-1}$	Ethylene production[a] $\mu mole\ mg\ dry\ wt^{-1}\ hr^{-1}$	
		With pesticide	Without pesticide
chlorpyrifos	100	2.66	2.64
DOWCO 214	100	3.53	3.63
DOWCO 217	100	3.00	3.16
methidathion	100	4.27	4.19
diazinon	100	3.46	3.40
Nemacur	100	3.05	3.78
Dasanit	100	3.71	3.93
T.C.P.[b]	50	2.24	2.75
	100	1.16	4.28
	150	0.64	2.85
	500	0.00	3.76

a Values are averages of four replicate cultures tested in triplicate.
b T.C.P., 2-hydroxy-3,5,6-trichloropyridine.

both as control and test.

A common reaction of the soil microflora to the application of many pesticides has been an initial adverse effect followed by a complete recovery. The results presented support this view. When Nemacur was added prior to inoculation, the organism was able to tolerate levels as high as 500 $\mu g\ ml^{-1}$ without showing any adverse effect, but when a level of 100 $\mu g\ ml^{-1}$ was established in the flask at early log phase, there was a significant drop in ethylene production. Similarly, adverse conditions within the growth medium were generated at lower concentrations when 2-hydroxy-3,5,6-trichloropyridine was added at early log phase rather than before inoculation. Hence, *A. vinelandii* was able to develop with time a tolerance to levels of complex molecules which

at lower concentrations were inhibitory on immediate exposure.

The lack of inhibition of acetylene reduction by *A. vinelandii* by methidathion, diazinon and Dasanit at concentrations up to 100 times those normally used for pest control indicates that these pesticides are unlikely to affect this bacterium in soil. Similarly, it is suggested that should Nemacur exert an initial adverse influence when applied as a soil nematocide, this would be transient.

Chlorpyrifos and its related family of compounds presented an interesting result. Within this group, chlorpyrifos and DOWCO 214 are by far the most important commercially. The oxygen analog of chlorpyrifos, DOWCO 180, is so unstable that quantities were not available for testing, and although DOWCO 217 is relatively more stable, it is expected to have very limited use in the field. In this study, both chlorpyrifos and DOWCO 214 exerted no adverse effects when present at concentrations 100 times those normally applied in the field. The results further indicated that the inhibition of acetylene reduction by DOWCO 217 was due to its unstable nature and the resultant release of 2-hydroxy-3,5,6-trichloropyridine. There is little doubt that should this hydrolysis product accumulate in the soil, it could exert an inhibitory effect upon this particular organism. Like most other organophosphorus insecticides, chlorpyrifos and DOWCO 214 would be subjected to chemical and biological breakdown, particularly in the soil. Other compounds similar in structure to this complex "leaving group" have been found to be highly recalcitrant and phytotoxic (NAIK et al. 1972). Should any of this group of insecticides be applied frequently to a field, it would appear advisable to check not only the residue level of the parent insecticide but also that of released 2-hydroxy-3,5,6-trichloropyridine.

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