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Degradation of Parathion by *Penicillium waksmani* Zaleski Isolated from Flooded Acid Sulphate Soil

A. V. Rao and N. Sethunathan

Department of Soil Microbiology, Central Rice Research Institute, Cuttack-6, Orissa

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Abstract. By enrichment culture technique, a fungus *Penicillium waksmani* Zaleski which can degrade parathion was isolated from an acid sulphate soil under flooded condition. The fungus tolerated parathion at concentrations as high as 1000 ppm. Initially, medium containing parathion supported less growth but at later stages the growth was equal to that of control treatment. Parathion was converted to aminoparathion by the fungus. The increase in the radioactivity in the aqueous phase of the culture filtrate after solvent extraction indicated the formation of certain polar metabolites.

Key words: Acid Sulphate Soil — Flooded Soil — Parathion — Degradation — Fungus.

Among acid rice soils of India, acid sulphate *Kari* soils of Kerala are characterised by extremely high acidity, high sulphate content, high organic matter, low nitrogen and high electrical conductivity. Rice is cultivated in this unique peaty soil despite its low pH and infertility. Folidol, a commercial formulation of parathion (O,O-diethyl O-*p*-nitrophenyl phosphorothioate), is extensively used for controlling common pests of rice grown in this problem soil. Sethunathan (1973a) reported rapid loss of parathion from this soil and attributed the loss to the microbial participation. Further studies were undertaken to determine the specific microorganisms and their role in the degradation of parathion in acid sulphate soil and results are reported hereunder.

Materials and Methods

Enrichment Culture. Acid sulphate (*Kari*) soil (pH 3.15, organic matter 12.2%, total nitrogen 0.27%, electrical conductivity 4.6 M mho/cm) was dried, pulverized and passed through 100 mesh sieve. Twenty g soil samples were placed in the tubes (200 × 25 mm) and flooded with 20 ml of water. At weekly intervals, 1000 µg of parathion as Folidol was added to the soils. One week after the fifth addition, the soil solution was collected from several tubes and used as an enrichment culture. An aliquot of this soil solution was sterilized by autoclaving at 15 psi for 15 min. One ml each of autoclaved and nonautoclaved enriched soil solution was added to 20 g soil samples which were previously sterilized by autoclaving at 15 psi for 1 h

for 3 days consecutively. The soils were flooded with 20 ml of sterile distilled water and 0.1 ml of 10 g/l solution of parathion was added to the soils. After incubation of the soils at room temperature for 3 to 4 weeks, a mycelial growth developed in the standing water of the soils receiving nonautoclaved enrichment culture. The mycelial growth was subcultured in Petri dishes containing Martin's rose bengal agar medium at pH 4.0 (Martin, 1950) supplemented with streptomycin to inhibit the bacterial contamination. This fungus was further purified in plates containing Czapek's dox agar medium having pH 3.0.

The fungus was maintained in Czapek's dox agar slants (pH 3.0). For identification, the fungus was grown in plates containing the same medium at both pH levels, i.e., 3.0 and 6.5. The fungus was identified by studying the growth characters in plates and measuring the spores and other structures.

Tolerance Studies. Martin's rose bengal broth medium (pH 3.0) without rose bengal and streptomycin was prepared, dispensed in 50 ml aliquots in 250 ml Erlenmeyer flasks and sterilized at 15 psi for 20 min. A known amount of ethanolic parathion (0.2 ml) was incorporated in the flasks to get a final concentration of 5, 10, 50, 100 and 1000 mg/l. Controls received 0.2 ml of ethanol. The media were inoculated with 1 ml of mycelial suspension prepared in sterile water from 10 day old fungal culture. The inoculated cultures were incubated at 28 $\pm$ 1°C. At the end of 5 and 8 days of incubation, the contents were filtered through Whatman No. 1 filter paper under vacuum for separating the mycelium. The pH of the culture filtrate was measured in Elico's pH meter. The mycelia were dried at 70°C for 36 h for determination of the dry weight. Two replicates were maintained for each treatment.

Degradation of C^{14} Labelled Parathion by the Fungus. Czapek's broth at pH 3.0 was prepared, dispensed in 25 ml aliquots in 100 ml flasks and sterilized at 15 psi for 20 min. Ethoxy labelled C^{14} parathion (Radiochemical Centre, Amersham, England) with a specific activity of 40 mC/mM was equilibrated in aqueous phase for 24 h and passed through a Millipore filter (pore size — 0.45 μ). Five ml of the aqueous labelled parathion were incorporated. Nonlabelled ethanolic parathion was added at the rate of 1000 μg per flask. The flasks were inoculated with 0.5 ml of the mycelial suspension and incubated at 28 $\pm$ 1°C. At different intervals of incubation, two replicates were taken and analyzed for parathion and aminoparathion both in the mycelium and culture filtrate.

Extraction of Parathion. The contents of the flask were centrifuged at 2500 rpm for 30 min. The mycelium was suspended in distilled water and centrifuged. After the second centrifugation the mycelium was resuspended in distilled water and homogenized with a sonifier (Branson Sonic Power Company, Danbury, Connecticut, USA) for 9 min at 5 min intervals after every 3 min. The culture filtrate and the mycelial homogenate were extracted separately with chloroform: diethyl ether (1:1) as used earlier for parathion by Sethunathan (1973a). The solvent fraction was evaporated to dryness in a beaker at room temperature. The residues were dissolved in 2 ml methanol, stored in glass vials and analyzed by thin-layer chromatography.

Thin-Layer Chromatography. The method of separation of parathion and its metabolites by tlc was essentially the same as adopted earlier for parathion (Sethunathan, 1973a). The thickness of the silica gel G layers was 300 μ. After spotting the samples along with the authenticated standards of parathion and its predicted metabolite, aminoparathion, the chromatograms were developed in hexane: chloroform: methanol (7:2:1) solvent mixture for a distance of 15 cm. The silica gel of the samples exactly opposite to the authenticated standards, made visible by spraying with 0.5% palladium chloride in 2% HCl, was taken in conical

centrifuge tubes. Two ml of methanol was added for elution. After centrifugation at 2000 rpm for 15 min, 0.1 ml of the supernatant was mixed with 5 ml of NE 220 scintillation solution (Electronics Corporation of India Limited, Hyderabad, India) which consists of 7 g of PPO, 0.3 g of POPOP and 100 ml of naphthalene in 1000 ml of dioxane and their activities were determined in a liquid scintillation counter (Electronics Corporation of India Limited, Hyderabad, India).

Results and Discussion

Based on the morphological and growth characteristics, the fungus which was isolated from flooded acid sulphate soil by enrichment culture technique, was identified as *Penicillium waksmani* Zaleski (Raper *et al.*, 1949). Colonies on Czapek's agar growing rather restrictedly, attained a diameter of about 2 cm in 10 days at room temperature, strongly wrinkled with central area generally raised, consisting of closely woven basal felt of delicate hyphae, medium to light sporulating after one week in pale blue green shades and deep to dark gray in older colonies. Conidiophores arising from the basal felt are about 150 μ in length and 2.0 μ in diameter. Penicilli are monoverticillate after appearing singly, sometimes irregular clusters, sterigmata in compact clusters mostly 8 by 2 μ. Conidia are globose to subglobose mostly 2.5 μ in diameter and occur in chains.

P. waksmani was grown in media supplemented with parathion at concentrations ranging from 0 to 1000 mg/l. The fungus appeared to tolerate parathion at a concentration as high as 1000 mg/l. However, at 5 days, growth was slow in the media amended with parathion particularly at high concentrations (Table 1). The mycelial yield was indirectly proportional to the amount of parathion incorporated. But, at 8 days, good growth occurred at all concentrations of parathion. This indicated that the fungus developed enzyme systems for its adaptability to parathion after an initial lag. During 8 day incubation, the pH of the media increased. This was particularly evident at higher concentrations of parathion.

In another experiment, Martin's rose bengal agar medium supplemented with 1 mg/ml parathion was inoculated with *P. waksmani* in a

Table 1. Tolerance of *Penicillium waksmani* to parathion

Parathion added (μg/ml)	Mycelial dry weight in mg		pH	
	5 days	8 days	5 days	8 days
0	449	425	3.07	3.55
5	394	388	3.15	3.67
10	371	377	3.22	3.87
50	357	384	3.15	6.07
100	242	378	3.50	6.32
1000	155	394	3.60	5.15

Table 2. Metabolism of C^{14}-parathion by *Penicillium waksmani* isolated from flooded acid sulphate soil

Incubation (days)	Radioactivity ($\times 10^4$ cpm/flask)						Total
	culture filtrate			mycelium			
	parathion	aminoparathion	aqueous phase	parathion	aminoparathion	aqueous phase	
5	30.49	0.22	2.19	2.07	0.17	0.25	35.39
10	10.29	2.20	9.48	2.24	0.48	3.42	28.11
15	7.82	5.95	16.83	2.19	0.76	2.67	36.22

Total radioactivity recovered at the start of the experiment = 42.38×10^4 cpm.

Petri dish. The agar medium was cloudy in appearance. After 10 days of incubation at 28 ± 1°C, a clearing zone appeared around the growing fungal colony indicating the dissolution of parathion.

To determine whether parathion was degraded by *P. waksmani*, the fungus was grown in Czapek's broth containing an aqueous solution of nonlabelled parathion. After incubation for 14 days, the insecticide concentration in the culture filtrate declined from 2305 to 425 µg whereas in uninoculated medium no significant loss of the insecticide was noticed. This decline in insecticide level in the culture filtrate following fungal growth can be attributed to its accumulation by the mycelium and/or to the degradation. Fungi are known to accumulate insecticides from culture solutions (Chacko and Lockwood, 1967; Ko and Lockwood, 1968; Anderson *et al.*, 1970; Zuckerman *et al.*, 1970). Degradation of parathion by microorganisms isolated from nonflooded soil via nitro-group reduction has been demonstrated earlier (Lichtenstein and Schulz, 1964; Matsumura and Boush, 1968).

In order to determine which of the two factors are operating, the fungus was grown in the media supplemented with labelled parathion. As in the earlier experiment, the concentration of parathion in the culture filtrate decreased from 42.38×10^4 to 7.82×10^4 cpm during 15 day incubation period (Table 2).

Analyses of solvent extracts of culture filtrate and mycelial homogenate showed that aminoparathion which was formed by nitro group reduction of parathion increased with incubation in both culture filtrate and mycelial homogenate (Table 2). Radioautograph of solvent extracts of culture filtrate and mycelial homogenate also showed that parathion concentration decreased after 15 days of incubation accompanied by the formation of aminoparathion and two unidentified metabolites with R_f values of 0.00 and 0.05 particularly in the culture filtrate and could not be extracted by the method employed. The total radioactivity recovered. In 15 days, about 61% of parathion was decomposed with no appreciable

increase in the radioactivity of the mycelial homogenate. Perhaps, parathion was used as a co-metabolite and not as a carbon source. The total radioactivity recovered in the mycelium as well as in the culture filtrate on the 10th day was 28.11×10^4 cpm whereas it increased to 36.22×10^4 cpm in 15 days. This indicated that part of the radioactivity which was not extracted from the mycelium on 10th day was released into the culture filtrate subsequently evidently as a water soluble substance. This resulted in a increase in the counts in the aqueous phase remaining after solvent extraction of the culture filtrate. No attempt was made, however, to determine the radioactivity which was bound in the mycelium and could not be extracted by the extraction method employed in our study. According to Ahmed and Casida (1958) organophosphorus insecticides are rapidly absorbed by *Torulopsis* and *Chlorella* and then released slowly from the living and dead cells into the culture medium. Recently, the cells of another alga, *Chlorella pyrenoidosa* proteose (Chick van Niel's strain) were shown to absorb parathion rapidly and then form aminoparathion which was subsequently released into the culture medium (Zuckerman *et al.*, 1970). But, in our studies, the fungus does not seem to accumulate parathion prior to its conversion to aminoparathion. Moreover, the clearing zone around the growing colony of the fungus indicates that parathion is metabolized by *P. waksmani*. The radioactivity bound in the mycelium at 10 days, must have been due to the presence of a compound other than parathion and aminoparathion, which could have been extracted from the mycelial homogenate by the method employed.

In flooded soils which become predominantly anaerobic within few days after submergence, anaerobic bacteria, facultative or strict, seem to play more important role in the transformation of organic pesticides. A strict anaerobe, *Clostridium* sp., and a facultative anaerobe, *Flavobacterium* sp., both isolated from flooded soil readily decomposed certain related chlorinated hydrocarbon and organophosphorus insecticides respectively (Sethunathan, 1973b). But, in a problem soil such as acid sulphate soil, fungi also appear to play a role in the transformations of pesticides under flooded conditions. Fungi are known for their ability to tolerate extreme conditions, such as extremely low pH. Perhaps, oxidized conditions in the thin layer of soil on the surface and in standing water (Sethunathan, 1973b) favour the activity of *P. waksmani*.

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Dr. N. Sethunathan
Department of Soil Microbiology
Central Rice Research Institute
Cuttack-6, Orissa, India