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Effectiveness of *Pseudomonas aeruginosa* for Detoxification of Tetramethylthiuram Disulfide (TMTD) from Contaminated Soil

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Much of the work that has been done on the use of microorganism for the decontamination of pollutants in the environment has concerned insecticides and herbicides, presumably because of much greater usages of these agrochemicals. However, there is a growing interest in the fate of fungicides (Woodcock 1978). McRae and Alexander (1965) attempted to protect alfalfa seeds from the herbicides 4-(2,4-dichlorophenoxy) butyric acid by inoculation with a heavy suspension of 4-2,4-dichlorophenoxy) butyric acid utilizing Flavobacterium sp. prior to planting. Phytotoxicity of isopropyl-N-phenylcarbamate (IPC) fortified soils was decreased by inoculation with IPC-utilizing bacteria (Clark and Wright 1970). Perhaps the most thorough and successful experiments were those of McClure (1972). Several phenyl carbamate herbicides were added to non-sterilized soil flats under green house conditions, at the rates up to 15 kg/ha. When a mixed bacteria culture capable of growth on IPC as the sole carbon source was applied to the soils, final plant yields were increased over uninoculated control.

High concentrations of tetramethylthiuram disulfide (TMTD) in seed bed soils have been shown to be quite persistent (Duffield and Eide 1962), and may affect the Rhizobium-legume symbiosis. We have previously reported the effectiveness of Pseudomonas aeruginosa towards the degradation of high concentrations of TMTD in autoclaved soils (Shirkot and Gupta 1985). Our results, reported here, concern the use of biological assay (plant growth) to measure the ability of P. aeruginosa to detoxify TMTD in soil contaminated with 100 to 2500 ppm TMTD.

MATERIALS AND METHODS

Pseudomonas aeruginosa active in degrading TMTD was isolated by enrichment and adaptation technique using TMTD as sole carbon source. The organisms were grown and enumerated in minimal medium (Shirkot 1983). A strain of Rhizobium leguminosarum was isolated from root nodules of Pisum sativum and were maintained and enumerated on yeast extract mannitol (YEM) agar (Vincent 1970). The organisms were grown at 30°C in 250 ml medium in 500 ml

Erlenmeyer flasks on a rotary shaker operating at 180 rpm. After 36 h, the cells were collected by centrifugation at 10,000 x g for 10 min at $4^{o}C$, and suspended in 250 ml sterile distilled water. These cells were used to inoculate the soil.

Composite soil samples of alluvial sandy loam typical of the Northern Region of India were obtained from the two to six inch surface layer and seived through a 2 mm wire mesh. The physical and chemical analyses of the soil were made by standard procedures and are given in Table 1.

Table 1. Properties of soil used in this study

Property	Average value
Moisture holding capacity	44.5 %
Cation exchange	24 m eq 100 g^{-1}
Mechanical analysis	
Sand	55 %
Silt	24 %
Clay	20 %
pH	7.3
Nitrogen	
Ammonium	14.5 ppm
Nitrite	0 ppm
Nitrate	6.5 ppm
Total nitrogen	0.05 %
Phosphate -P	2 ppm
Organic matter	0.65 %

The soil and sand in the ratio of 2:1 were mixed to ensure uniformity. The mixture was amended with 1% $CaCO_3$ and 0.05 % K_2HPO_4. Fortification with a proper amount of technical tetramethylthiuram disulfide (TMTD, Fluka AG, Switzerland, Purity 99%) was done with neat material. The TMTD was then evenly dispersed and an aliquot of 4 kg soil was placed into pots. Other treatments included inoculation with the culture of P. aeruginosa and R. leguminosarum (at most 2.5 x 10^6 CFU/g soil), bringing to 40% saturation with water and finally mixing to ensure homogeneity. Three replicate pots per treatment with six plants in each pot were placed in a glass house in a completely randomized block design. After 8 weeks of growth the above ground portion of plants and root system were washed free of soil particles and number of nodules were counted. The plants were dried at $30^{o}C$ for two weeks for weight determination. The plant material were also analysed for total nitrogen by the conventional kjeldahl method (Jackson 1967). The data were subjected to statistical analysis by following the randomized block design (Panse and Sukhatme 1978).

RESULTS AND DISCUSSION

Since our TMTD-utilizing bacteria could degrade high concentrations of TMTD in soil under laboratory conditions (Shirkot and Gupta 1985), we first determined whether this organism could remove the toxic effect of TMTD on rhizobia in soil. The effectiveness was initially assessed by determining nodulation and plant yields.

Table 2 shows the results from uninoculated soil, soil inoculated with Rhizobium leguminosarum only and soil inoculated with R. leguminosarum plus P. aeruginosa. These results indicate that nodules were present in uninoculated pots and this may be because of the presence of autochthonous rhizobia in the soil. However, uninoculated plants still had less nodules than the inoculated plants and the difference was found statistically significant. Average number of nodules decreased with the increase in TMTD concentration and nodulation was completely inhibited at concentration above 200 ppm TMTD in soil. This may be attributed to the toxicity of TMTD to rhizobia. While studying the toxicity of fungicides in rhizobia strains, TMTD was included among the most toxic group (Staphorst and Strijdom 1976). Addition of P. aeruginosa significantly increased the average number of nodules to 5.08 as compared to 2.97 in plants grown in soil inoculated with R. leguminosarum alone. This is not because of the direct effect of P. aeruginosa as shown by the number of nodules in control (0 ppm TMTD). The data clearly show that at 10 and 100 ppm TMTD the number of nodules were more in the presence of P. aeruginosa, an effect that was statistically significant. This is because of the accelerated degradation of TMTD by P. aeruginosa to the level where it is not toxic to rhizobia. We have already reported that this strain of P. aeruginosa could degrade more than 95 % of 100 ppm TMTD in 24 days of which about 80 % is degraded in first 8 days of incubation (Shirkot and Gupta 1985). To our knowledge, this is the first report of removing the toxic effect of pesticide on rhizobia by inoculating the soil with acclimated bacterial culture.

As the organism was found highly effective in reducing the toxic effect of 100 ppm TMTD on rhizobia, and nodulation does not always result in increased plant growth (Vincent 1980), its effect on plant growth was also determined. The results in Table 3 indicate that average dry weight increased significantly with increase in TMTD concentration upto 250 ppm. Further increase in TMTD concentration had phytotoxic effect on the growth, and average dry weight was reduced to minimum (1.51 g) at 2500 ppm TMTD concentration. While studying the interaction between TMTD and three plant groups important observation made was that dry weight was significantly more in plants grown in soil having TMTD degrading bacterial culture when compared to the uninoculated soils. These results indicate that a selective organism like P. aeruginosa in sufficient number is required for detoxification of TMTD in soil. Also, during the accelerated degradation of TMTD in soil certain metabolites having the growth promoting effects on plants may be produced. Increase in amino acid contents of soil at low TMTD concentration has

Table 2. Effect of TMTD on nodulation of Pisum sativum in soil inoculated with TMTD-utilizing bacteria

TMTD concentration (ppm)	Number of nodules*/plant			
	Uninoculated soil	R. leguminosarum inoculated soil	P. aeruginosa plus R. leguminosarum inoculated soil	Mean
		(+0.106)		(+0.064)
0	7.11	11.55	11.62	10.09
10	3.26	4.23	10.77	6.09
100	1.00	1.00	9.18	3.73
250	1.00	1.00	1.00	1.00
500	1.00	1.00	1.00	1.00
1500	1.00	1.00	1.00	1.00
2500	1.00	1.00	1.00	1.00
Mean(+0.042)	2.00	2.97	5.08	

*Average of three replicates

Standard Error (S.E.) of TMTD concentrations means = 0.064

Critical difference between any two means of TMTD concentrations ($C.D._{0.01}$) = 0.24

S.E. (Uninoculated soil or R. leguminosarum inoculated soil or P. aeruginosa plus R. leguminosarum inoculated soil) means = 0.042

$C.D._{0.01}$ (uninoculated soil or R. leguminosarum inoculated soil or P. aeruginosa plus R. leguminosarum inoculated soil) means = 0.16

S.E. (Interaction between TMTD and nodulation) = 0.106

$C.D._{0.01}$ (Interaction between TMTD and nodulation) = 0.41

Table 3. Effect of soil treatment with TMTD and Pseudomonas aeruginosa on the growth of Pisum sativum

TMTD concentration (ppm)	Dry weight*(g) Uninoculated soil	R. leguminosarum inoculated soil	P. aeruginosa plus R. leguminosarum inoculated soil	Mean
		(+0.064)		(+0.035)
0	2.12	2.53	2.69	2.44
10	2.55	2.68	2.78	2.67
100	2.41	2.48	3.30	2.71
250	2.41	2.45	2.87	2.58
500	1.77	1.88	2.03	1.89
1500	1.37	1.51	1.85	1.57
2500	1.26	1.42	1.86	1.51
Mean (+0.021)	1.98	2.13	2.48	

*Average of three replicates

Standard Error (S.E.) of TMTD concentrations means = 0.035

Critical difference between any two means of TMTD concentrations ($C.D._{0.01}$) = 0.14

S.E. (Uninoculated soil or R. leguminosarum inoculated soil or P. aeruginosa plus R. leguminosarum inoculated soil) means = 0.021

$C.D._{0.01}$ (uninoculated soil or R. leguminosarum inoculated soil or P. aeruginosa plus R. leguminosarum inoculated soil) means = 0.08

S.E. (Interaction between TMTD and Dry weight) = 0.064

$C.D._{0.01}$ (Interaction between TMTD and Dry weight) = 0.24

Table 4. Effect of TMTD and Pseudonomas aeruginosa in soil on the total nitrogen uptake by Pisum sativum

TMTD concentration (ppm)	Total nitrogen*(mg)			
	Uninoculated soil	R. leguminosarum inoculated soil	P. aeruginosa plus R. leguminosarum inoculated soil	Mean
		(+1.195)		(+0.686)
0	64.09	95.95	97.50	85.85
10	83.12	93.03	94.13	90.09
100	93.93	95.56	137.60	109.03
250	79.80	82.46	105.04	89.33
500	71.13	72.03	86.72	76.09
1500	55.67	58.00	76.68	63.45
2500	46.60	48.13	71.70	55.48
Mean (+0.446)	70.62	77.80	95.68	

*Average of three replicates

Standard Error (S.E.) of TMTD concentrations means = 0.686

Critical difference between any two means of TMTD concentrations ($C.D._{0.01}$) = 2.62

S.. (Uninoculated soil or R. leguminosarum inoculated soil or P. aeruginosa plus R. leguminosarum inoculated soil) means = 0.446

$C.D._{0.01}$ (Uninoculated soil or R. leguminosarum inoculated soil or P. aeruginosa plus R. leguminosarum inoculated soil) means = 1.70

S.E. (Interaction between TMTD and total nitrogen) = 1.195

$C.D._{0.01}$ (Interaction between TMTD and total nitrogen) = 4.57

been reported earlier (Wainwright and Pough 1975).

The effectiveness of P. aeruginosa in reducing the harmful effect of TMTD on Rhizobium-legume symbiosis is clearly revealed by the chemical analysis of plants. The results in Table 4 show the combined effect of TMTD and P. aeruginosa on total nitrogen contents. Total nitrogen of plants was significantly more in P. aeruginosa inoculated soil at all the TMTD concentrations used as compared to plants grown in uninoculated and R. leguminosarum inoculated soil among which the difference from total nitrogen was statistically non-significant. The rate of decrease of total nitrogen at higher TMTD concentration was more rapid when soils were not inoculated with TMTD utilizing bacteria.

The phytotoxicity of TMTD contaminated soils was decreased by inoculation with TMTD utilizing bacteria. The toxic effect of 100 ppm TMTD on nodulation was completely removed by P. aeruginosa. Combined use of TMTD and P. aeruginosa significantly increased the final plant yield and total nitrogen over uninoculated control. Previous studies on the decomposition of parathion by acclimated bacteria under field conditions have demonstrated the usefulness of such cultures in removing this compound from the contaminated areas (Barles et al. 1979). Our studies reconfirm the effectiveness of specially designed bacterial strains for the removal of toxic chemicals from the environment.

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