3. MATERIALS AND METHODS

3.1. Type and preparation of soil:

Composite soil samples of alluvial sandy loam typical of Northern Region of India were taken from a cropped field of the botanical garden of Panjab University, Chandigarh. Soil had no history of pesticide treatment. Surface soil (0-15 cm) that passed through 2 mm sieve was stored in a humid atmosphere (Relative humidity to be 80 per cent) at 20°C for a maximum of 7 days. Appropriate quantity of soil was removed as required. Drying of soil was prevented for increased carbon dioxide production which results on remoistening (Wilkinson et al., 1964). Quantities of soil mentioned in all experiments are on dry weight basis. The weight of the soil used in the experiment was corrected for the amount of moisture present in soil. For example, a 300 g soil sample on dry weight basis was equivalent to 360 g if the sample had 20% of moisture.

3.2. Physical and chemical analysis of soil:

Freshly procured samples of soil were analysed for physical and chemical characteristics. Mechanical analyses were determined by the hydrometer method (Bouyoucous, 1936); pH was determined by a pH meter (soil : water ratio, 1:2.5); and organic matter by the chromic acid titration method (Walkley and Black, 1934). Cation exchange capacity, available phosphorous, total nitrogen and nitrate and
ammonium-N were determined by the procedures outlined by Jackson (1967). Nitrite-N was determined by the method of Prince (1954). The physical and chemical characteristics of the soil used are given in Table 1.

Table 4: Characteristics of soil used

<table>
<thead>
<tr>
<th>Determination</th>
<th>Average value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water holding capacity</td>
<td>44.5 %</td>
</tr>
<tr>
<td>Cation exchange</td>
<td>24 meq 100g⁻¹</td>
</tr>
<tr>
<td>Mechanical analysis:</td>
<td></td>
</tr>
<tr>
<td>Sand</td>
<td>50 %</td>
</tr>
<tr>
<td>Silt</td>
<td>25 %</td>
</tr>
<tr>
<td>Clay</td>
<td>25 %</td>
</tr>
<tr>
<td>pH</td>
<td>7.3</td>
</tr>
<tr>
<td>Nitrogen</td>
<td></td>
</tr>
<tr>
<td>Ammonium</td>
<td>14.5 ppm</td>
</tr>
<tr>
<td>Nitrite</td>
<td>-</td>
</tr>
<tr>
<td>Nitrate</td>
<td>6.5 ppm</td>
</tr>
<tr>
<td>Kjeldahl</td>
<td>0.05 %</td>
</tr>
<tr>
<td>Organic matter</td>
<td>0.65 %</td>
</tr>
<tr>
<td>Phosphate-P</td>
<td>2 ppm</td>
</tr>
</tbody>
</table>

- = No NO₂⁻-N detected.

3.3. Organisms:

3.3.1. Rhizobia:

Cultures of rhizobia isolated from root nodules of
pea (*Pisum sativum*), berseem (*Trifolium alexandrinum*), fenugreek (*Trigonella foenum-graecum*) and **gram** (*Cicer arietinum*). *Rhizobium japonicum* D-338 from *Glycine max*. *Rhizobium* from *Vigna unguiculata* strain CP-20 and *Rhizobium* from *Phaseolus* *mungo* strain U-4 (obtained from department culture collection) were maintained on asparagine mannitol agar (*K₂HPO₄*, 0.5 g; MgSO₄·7H₂O, 0.2 g; NaCl, 0.2 g; CaCO₃, 0.1 g; mannitol 10.0 g; asparagine, 0.50 g; one liter distilled water; pH 7.0) at 4°C. Subculturing was done periodically on the same medium at an incubation temperature of 28°C.

3.3.2. **Pseudomonas aeruginosa**

TMTD degrading organism tentatively identified as *P. aeruginosa* was isolated from soil. The organism was maintained on TMTD-minimal-salt medium (*K₂HPO₄*, 7.0 g; KH₂PO₄, 3.0 g; MgSO₄·7H₂O, 0.1 g; TMTD, 300 ppm; one liter distilled water; pH 7.0) at 4°C. TMTD was the sole source of carbon and nitrogen. Subculturing was done periodically on the same medium at an incubation temperature of 28°C.

3.3.3. **Trichoderma reesei** QM-9414 (obtained from Dr. M. Mandels, U.S. Army Natick Laboratories) was maintained on potato dextrose agar at 4°C. Subculturing was done periodically on the same medium at an incubation temperature of 28°C.

3.4. Pesticides and Reagents:

Tetramethylthiuram disulphide (TMTD; Fluka AG,
Switzerland; Purity 99%), sodium dimethyldithiocarbamate (NaDDC; BDH; Purity 99%) and zinc dimethyldithiocarbamates (ZnDDC; Fluka; Purity 99%) were used. Analytical reagents obtained from BDH or E. Merck were used for most of the chemical estimations.

3.5. Mixing of TMTD with soil:

Unless stated otherwise, the desired concentration of TMTD was mixed with soil and was sieved six times to ensure proper mixing before transfer to flasks/polyethylene bags/pots. Soil was adjusted to 50% water holding capacity. Flasks were covered with polyethylene film which allowed gaseous diffusion but prevented moisture loss. The incubation temperature for the studies on soil biological processes was 28°C.

For bacterization experiment, the soil and sand were mixed in the ratio of 2:1 and then TMTD was mixed before filling the pots. The soil was also amended with 1% CaCO₃ and 0.05% K₂HPO₄.

3.6. Isolation of Thiram (TMTD) degrading organisms:

Organism active in degrading TMTD was isolated by the enrichment and adaptation technique. Two grams of soil (obtained from different places) was put into 100 ml of minimal media containing 200 ppm of TMTD. Sterile solution of TMTD in DMSO (Dimethyl sulfoxide) was added. The solution of TMTD in DMSO was sterilized by passing through
the bacterial sintered glass (G-5) filter. The mixture was incubated at 28°C on a rotary shaker at 100 rpm. After incubation for 4 to 5 days an aliquot of this enriched medium was inoculated into the fresh medium containing higher concentration of TMTD. Every transfer to the higher concentration was made when more than 200 colonies per 0.1 ml of suspension were observed. This process was repeated with medium containing higher concentration of TMTD. The maximum concentration of TMTD used was 600 ppm. The organism was isolated by plating on the minimal medium containing 300 ppm TMTD. TMTD degrading organisms formed clear zone around the colonies. The colonies with clear zone around it were picked and purified.

3.7. Identification of the isolate:

The morphological, cultural and biochemical characteristics of the isolate were studied (Conn, 1957; Skerman, 1967). The isolate was identified according to the schemes of (Breed et al., 1974; Skerman, 1967; Finegold et al., 1978).

3.8. TMTD estimation:

TMTD in the soil and in the medium was extracted in chloroform and estimated according to Rangaswamy et al. (1970). The description of the method is given in Appendix-I. DMA was detected according to Day et al. (1966).

3.9. Degradation of TMTD in soil:

300 ppm TMTD was added to soil and subjected to
various treatments. TMTD degrading organisms added were $2.5 \times 10^6$ CFU g$^{-1}$ soil. The per cent TMTD degraded and total number of bacteria (colony forming units; CFU) in soil was determined after different days of incubation. The per cent TMTD degraded was calculated on the basis of average amount of TMTD recovered from all soil samples on day 0, which were then normalized to 100%.

3.10. Functional longevity of TMTD degrading organisms in soil:

The culture of TMTD degrading organisms (P. aeruginosa) was inoculated to 10 g samples of autoclaved and non-autoclaved soil in tubes. After inoculation with P. aeruginosa (day 0), 300 ppm TMTD was added on day 0, 4, 8, 12, 16 and 20. The retention of TMTD degradative activity of the organisms in each of these groups was determined by extracting the remaining TMTD and finding out amount of TMTD degraded in triplicate samples after 0, 4 and 8 days of TMTD addition.

3.11. Effectiveness of TMTD degrading organisms towards degradation of high concentrations of TMTD:

Five TMTD concentrations in the soil were tested i.e., 100, 250, 500, 1500 and 2500 ppm. Each of these treatments were performed in groups of 18; one set of triplicates was extracted on day 0 and other groups of triplicates were extracted on 4, 8, 12, 16 and 24 days of incubation. Per cent TMTD degraded and total number of bacteria were determined for each treatment.
3.12. Degradation of TMTD in liquid medium:

50 ml of liquid minimal salt medium was transferred to 250 ml conical flasks. Filter sterilized TMTD solution in DMSO was added to give the final concentration of 300 ppm. The flasks were then inoculated with 0.1 ml of the culture in sterilized distilled water (0.5 O.D. at 600 nm; obtained after repeated washing with sterilized distilled water). Left over TMTD was estimated and growth was determined by pour plate method (in triplicate samples) after incubation at 28°C on rotary shaker (100 rpm) at the time intervals of 0, 12, 24, 48, 72 and 96 h.

To show that degradation of TMTD by the isolate might not be due to the process of cometabolism, degradation was studied using TMTD as sole source of carbon, nitrogen and sulphur in minimal salt medium. Also the degradation was studied by supplementing the medium with glucose and yeast extract.

3.13. Effect of possible TMTD-degradation products on growth of TMTD degrading organisms:

Different concentrations of possible degradation products of thiram (dimethylamine, formaldehyde, \(-keto\)-glutaric acid and methionine) were added to the minimal salt medium and then inoculated with 24 h old culture of TMTD degrading organisms as described earlier. The tubes were incubated at 28°C and growth was studied by taking O.D. at 600 nm after every 6 h.
3.14. Effect of possible TMTD-degradation products on TMTD degradation in liquid medium:

To 50 ml of minimal-salt-medium containing yeast extract and 300 ppm TMTD; dimethylamine (50 ppm); formaldehyde (50 ppm); methionine (50 ppm) and α-ketoglutaric acid (150 ppm) were added aseptically. The flasks were then inoculated with 0.1 ml of the 24h old culture of *P. aeruginosa*. Left over TMTD and growth was determined at different time intervals.

3.15. Crude enzyme preparation and TMTD degradation by the enzyme:

Minimal salt medium containing 300 ppm TMTD and 0.05 % yeast extract was used. 50 ml of the medium was inoculated with 0.1 ml of the culture (0.5 O.D. at 600 nm) and incubated at 28°C on rotary shaker (100 rpm). Flasks in triplicates were removed every 24 h and centrifuged at 14,000 g for 10 min at 4°C. The supernatent obtained was used as crude enzyme preparation. Protein was estimated by the method of Lowery *et al.* (1951) and TMTD degradation was studied as a function of protein.

3.16. Effect of TMTD on carbon dioxide production:

Carbon dioxide production by microorganisms in soil was measured with biometeric flasks (Bartha and Pramer, 1965). An equivalent of 50 g of dry soil was supplemented with 6 mg of ammonium hydrogen phosphate. TMTD was used at 10, 100, 250, 500, 1500 and 2500 ppm and the experiments were carried out in triplicates. Appropriate controls were included.
Carbon dioxide production was measured volumetrically (Bartha and Pramer, 1965 and Jackson, 1967) after 48 h for 45 days.

3.17. Effect of TMTD on Nitrification:

The substrate used for nitrification was 100 ppm of diammonium hydrogen phosphate in soil. Appropriate quantities of TMTD were mixed with soil and transferred to polyethylene bags and incubated at 28°C. Each study included appropriate control and triplicate treatments. Nitrification in soil was determined at 1, 2, 3 and 4 weeks intervals by measuring amino-nitrogen, nitrate and nitrite nitrogen production. Nitrate and nitrite-N were extracted by the methods of Jackson (1967). Nitrite-N and nitrate-N were estimated by the methods of Onken and Sunderman (1977).

3.18. Effect of TMTD on Ammonification:

Three hundred grams of soil was mixed with sufficient peptone to give a nitrogen content of 500 ppm. TMTD was added as described earlier and incubated at 28°C. Appropriate soil samples were removed at 1, 2, 3 and 4 weeks intervals and ammonium, nitrite, and nitrate-N were extracted (Jackson, 1967). Ammonium, nitrite and nitrate nitrogen were estimated by the methods of Onken and Sunderman (1977).

3.19. Effect of TMTD on Phosphorous solubilization:

Phosphorous solubilization in soil was studied by mixing 300 g of soil with tricalcium phosphate to provide
200 ppm of phosphorous. Soil contained 10,100, 250, 500, 1500 and 2500 ppm of TMTD and incubated at 28°C. Soil samples were removed at 1, 2, 3 and 4 weeks intervals. Available phosphorous was extracted with alkaline sodium bicarbonate solution and estimated colorimetrically (Jackson, 1967).

3.20. Effect of TMTD on Symbiotic Nitrogen Fixation:

The effect of 10,100, 250, 500, 1500 and 2500 ppm TMTD was studied on nodulation, growth and nitrogen fixation of pea (Pisum sativum); gram (Cicer arietinum); fenugreek (Trigonella foenum-graecum) and berseem (Trifolium alexandrinum) inoculated with the respective Rhizobium spp. (isolated from the root nodules of the plants) under pot house condition.

Pot culture experiments were conducted in 4 Kg soil. Experiment in sterilized and unsterilized soil was performed in case of Gram (Cicer arietinum) only. Soil was sterilized in an autoclave at 15 lb. pressure at 121°C for 1 h for three consecutive days and TMTD was mixed after sterilization. Rest of the crops were grown in non-autoclaved soil. Experiments were done in triplicates with six plants in each pot. Controls for pesticides and rhizobia were included in each study. Plants were raised under constant moisture condition. After 3 weeks, the plants were removed along with their roots by washing the soil in running water. The roots were blot dried on a filter paper. The number of nodules and dry weight of each plant was recorded. Total nitrogen of plant was determined by the conventional Kjeldahl's
method (Jackson, 1967).

3.21. Effect of TMTD on soil enzymes:

Appropriate quantity of soil was removed from flasks/pots and the enzyme activities were determined according to the methods described below.


To 2 g of soil (air dried), 0.3 ml of toluene was added, the mixture was shaken and allowed to stand for 15 min before the addition of buffers and substrates. Amylase assay used 0.1 M sodium acetate buffer (5 ml, pH 5.0) containing 50 mg soluble starch. After 24 h incubation, at 28°C, 10 ml of distilled water was added and the soil suspension was centrifuged at 12,350 x g for 10 minutes. Reducing-sugar analysis of the supernatant were done according to Nelson (1944).

3.21.2. Invertase (Hofmann and Hoffmann, 1966; Cole, 1977):

Invertase was assayed in a manner identical to the amylase, except that 0.1 M sodium acetate buffer, (pH 5.5) containing 18 mM sucrose was used, and the incubation period was 3 h.


5 g soil were placed in a 50 ml glass stoppered flask and 0.5 ml of toluene was added. The contents were
mixed thoroughly and after 15 min, 10 ml of acetate buffer (pH 5.9) were added followed by 10 ml of 1.0 % carboxymethyl cellulose (Hercules Incorpor., Willington, Dellware, USA). The flask was then incubated for 24 h at 30°C. At the end of this incubation period approximately 50 ml of distilled water were added, the suspension filtered and distilled water added to make the volume of the filtrate to 100 ml. Reducing-sugar analysis on the filtrate was done according to Nelson (1944).

3.21.4. Dehydrogenase (Casida et al., 1964; Kiss and Boaru, 1965):

3 g soil taken in a screw cap tube was saturated with 0.5 ml of 3% (w/v) solution of triphenyl tetrazolium chloride (TTC) and 1.25 to 1.75 ml of distilled water. The contents were mixed thoroughly and incubated at 37°C for 24 h. Following incubation, the triphenyl formazon formed was extracted with ethanol and estimated by spectrophotometrically.

3.21.5. Phosphatase (Tabatabai and Bremner, 1969):

1 g soil taken in a test tube was incubated with 1 ml of 5 mM buffered sodium-p-nitrophenyl phosphate (Sigma Chemical Co., USA) in acetate buffer (pH 5.2) and 0.3 ml toluene at 37°C for 1 h. Determination of p-nitrophenol involved the colorimetric analysis of the extract obtained by treating the incubated soil sample with 4 ml water, 10 ml of 0.5 M CaCl$_2$ and 4 ml of 0.5 M sodium hydroxide and by filtering (Whatman No. 42) the suspension obtained by shaking the mixture for 1 min.
3.21.6. β-glucosidase (Hofmann and Hoffmann, 1966; Tabatabai and Bremner, 1969; Cole, 1977):

2 g soil taken in test tube was incubated with 5 ml of 3.3 mM p-nitrophenyl-β-D-glucoside (PNPG, Sigma Chemical Co., USA) in 0.1 M citrate buffer for 24 hours. Also 0.3 ml toluene was added 15 min before the addition of the substrate and buffer. p-Nitrophenol liberated was extracted in 4 ml water, 10 ml CaCl₂ (0.5 M) and 4 ml of NaOH (0.5 M).

3.21.7. Phenol-o-hydroxylase (Wainwright, 1979):

1 g soil was incubated with 5 ml citrate-phosphate buffer (0.5 M, pH 4.0) in screw cap bottles at 37°C for 15 min. The reaction was started by adding 2 ml phenol (10 mM), mixed thoroughly and incubated at 37°C for 1 h and ended by adding trichloroacetic acid (10 ml). The slurry was filtered through Whatman filter paper No. 42 and phenol content of the filtrate was determined.

3.21.7.1. Phenol determination:

Filtrate (5 ml) was diluted to 100 ml with distilled water and 2 ml NH₄Cl (5% w/v) and 1 ml NH₄OH (35% v/v) were added (or sufficient NH₄OH to bring the pH to 10 ± 0.2). The diluted sample was divided into 2 x 50 ml portions; to one was added 1 ml potassium ferricyanide (8% w/v) and 1 ml of 4-amino-phenazone (2% w/v). The magenta colour was developed for 15 min at room temperature and its absorbance determined at 510 nm. An aliquot of the remaining 50 ml was also measured at 510 nm to determine any soil colour extracted.
A control tube containing reaction mixture minus soil was also included to determine the initial phenol concentration. The amount of phenol lost per hour was calculated and expressed on oven dry wt. basis.

3.22. Reversal of TMTD effect on soil biological processes and soil enzymes:

Different concentrations of TMTD were mixed in the soil as described earlier along with the respective substrate for each process. In addition, the TMTD degrading organisms were added to the soil (2.5 x 10^6 CFU g^-1 soil) and estimations were done as described earlier under each soil biological process. Soil enzymes were also estimated accordingly. In case of symbiotic nitrogen fixation, each treatment was subdivided into three groups of triplicates. One sub-group contained rhizobia, the other was inoculated with rhizobia and TMTD degrading organism, while the third sub-group was not inoculated with either of the two organisms. The estimations done were same as described earlier.

3.23. Effect of TMTD and related compounds in production and activity of cellulases:

3.23.1. Enzyme production:

The basal medium used for the production of cellulase(s) was the same as used by Reese and Mandels (1963). The medium contained KH_2PO_4, 2.0 g; (NH_4)_2SO_4, 1.4 g; Urea, 0.3 g; MgSO_4.7H_2O, 0.3 g; CaCl_2, 0.3 g; FeSO_4.7H_2O, 5.0 mg;
MnSO$_4$$\cdot$H$_2$O, 1.6 mg; ZnCl$_2$, 1.7 mg; CoCl$_2$, 2.0 mg; Peptone, 1.0 g; one litre distilled water; pH 5.3. Fifty ml of liquid medium was dispensed into each of 250 ml conical flasks containing 0.5 g Avicel RC-581 (FMC Corp., American Viscose, PA, USA) and autoclaved at 15 PSI for 20 min.

Stock solution of Tetramethylthiuram disulphide (TMTD), sodium dimethyl dithiocarbamate (NaDDC) and zinc dimethyl dithiocarbamate (ZnDDC) were prepared in DMSO and filter sterilized. Different concentrations (0.0, 0.1, 0.2, 0.4 and 1.0 ppm) of TMTD, NaDDC and ZnDDC were added aseptically to the flasks containing the basal medium. They were then inoculated with 0.5 ml spore suspension which was prepared by suspending fresh spores of T. reesei (spores obtained from four day old slants) in sterilized distilled water containing 0.1 % Tween 80 v/v).

After inoculation the flasks were incubated at 28°C on a rotary shaker (100 rpm) for eight days and 12 days. At the end of the incubation period, the mycelial mass was estimated according to the method of Romanelli et al. (1975). The filtrate obtained was centrifuged (15,000 g at 4°C) and the supernatant obtained was used as the crude cellulase(s) preparation.

3.23.2. Enzyme assays:

3.23.2.1. Carboxymethyl cellulase (CMCase, Cx):

Carboxymethyl cellulase assay was done according to the method of Reese and Mandels (1963). The procedure
essentially consisted of estimating reducing-sugars formed by the action of cellulase on CMC. Reducing-sugars formed were estimated by a colorimetric method of Sumner and Somers (1944) as modified by Miller (1959). The modified sugar reagent contained DNSA, 10 g; phenol, 2 g; sodium sulphite 0.5 g; NaOH, 10.0 g; sodium potassium tartrate, 200 g; one liter distilled water.

The reaction mixture contained 4.5 ml of 1.1% CMC-7 MT in citrate buffer (0.055 M, pH 5.0) and 0.1 to 0.5 ml of diluted enzyme. It was made to the final volume of 5.0 ml with citrate buffer and incubated at 50°C for one hour. After the incubation an aliquot (volume upto one ml) was withdrawn and added to 3.0 ml of sugar reagent taken in test tube which also stopped the enzymic reaction. The standard curve was prepared by taking 0.1 to 1.0 mg of glucose (in a total volume of 1.0 ml) in test tubes containing 3.0 ml of sugar reagent. The tubes were immersed in boiling water and removed after 15 minutes when the color development was completed and cooled to room temperature. The contents were transferred to 25 ml volumetric flasks and the volume made up with distilled water. The optical density was read at 550 nm in a spectronic-20.

One unit of CMCase was expressed as that amount of enzyme which produced 4 mg of reducing-sugars from CMC-7 MT as substrate in one h at 50°C.

3.23.2.2. Avicelase activity:

The method employed by Berghem and Petersson(1973)
was used. The reaction mixture contained 2.0 ml of 1 \% suspension of Avicel RC-58 in 0.05 M sodium acetate buffer (pH 5.0) and 200 \( \mu l \) of enzyme solution. After incubation at 30°C for 2 h, the mixture was filtered (Whatman filter paper No.1) and analysed for reducing-sugars by the method of Miller (1959).

One unit of enzyme activity was defined as the amount of enzyme needed to liberate reducing-sugars equivalent to 5 \( \mu g \) glucose under the assay conditions described above.

3.23.2.3.\( \beta \)-glucosidase activity (Berghem and Petersson,1973):

The assay mixture contained 1.0 ml of 1 mM p-nitrophenyl-\( \beta \)-D-glucoside in 0.05 M sodium acetate buffer, pH 5.0 and 100 \( \mu l \) enzyme solution. After incubation at 40°C for 10 minutes, 2.0 ml Na\(_2\)CO\(_3\) (1 M) was added to the mixture to stop the reaction. The mixture was diluted with 10 ml distilled water and the p-nitrophenol liberated was determined from the absorbance at 400 nm.

The unit of enzyme activity was defined as the amount of enzyme which liberated 5 \( \mu g \) p-nitrophenol under the assay condition.

The measurement of protein and pyruvic acid was done according to Lowry et al. (1951) and Friedmann and Haugen (1943), respectively. The pH of the culture medium was determined by pH meter.
3.23.3. Effect of TMTD, NaDDC and ZnDDC on cellulase(s) activity in vitro:

Cellulase(s) was prepared by incubating _Trichoderma reesei_ as described earlier. To this preparation TMTD, NaDDC and ZnDDC at 0.1, 0.2, 0.4 and 1 ppm concentrations were added. The mixture was incubated at 28°C for 15 and 30 min. After each of these incubation periods the activities of avicelase, CMCase, and B-glucosidase were determined according to methods described earlier. The solvent DMSO was also tested for its effects in vitro on enzyme activity and estimation of reducing-sugars.

3.24. Development of resistance to TMTD:

The resistance to TMTD in strains of _Rhizobium_ was developed by the serial passage using a low concentration of the pesticide (5 ppm) and then increasing the concentration. The details of the procedure is given below:

A 3-day old broth culture (O.D., 0.3 at 620 nm) was inoculated into medium containing 5 ppm TMTD and incubated at 28°C for 3-4 days. 0.1 ml of the culture was plated on agar containing the same concentration of pesticide. A colony was picked and inoculated into medium containing 5 ppm TMTD and incubated at 28°C. After good growth was obtained, 0.1 ml of this culture was inoculated into 10 ml of medium containing 7 ppm TMTD, 2-3 transfers were made in medium having the same concentration of pesticide. Every transfer to the higher concentration of pesticide was made when more than 300 colonies per 0.1 ml of
the culture were obtained. In the same way serial transfers were made to the medium containing higher concentrations i.e., 10, 15, 20, 22, 25 and 30 ppm TMTD.

Resistant strains were picked which grew on an agar medium containing the required concentration of the TMTD. Cultures were again plated on the same concentration of the pesticide to confirm that the cultures were resistant. The resistant cultures were maintained and subcultured monthly on the asparagine mannitol agar containing TMTD.

3.24.1. Growth curves of TMTD sensitive and resistant strains of *Rhizobium* spp.:

The growth pattern of both the sensitive and resistant strains of *Rhizobium* spp., was compared by growing them in TMTD less and TMTD asparagine mannitol broth. 0.1 ml of three day old culture adjusted to O.D., 0.3 was inoculated in 10 ml of broth and incubated at 28°C. O.D., readings at 620 nm were taken at different times. The results expressed are the mean values of duplicate trials.

3.24.2. Reversion of adapted strains:

The reversion of TMTD resistant strains to TMTD sensitive ones was studied by the following procedure:

A strain which was found to grow at 30 ppm TMTD was grown in TMTD free asparagine mannitol broth for 24 h, then 0.1 ml was transferred to 5.0 ml of medium containing different concentrations of TMTD (5, 10, 20, 25 and 30 ppm),
and growth at each concentration was observed after 24 and 48 h. From the non-pesticides medium, the strain was again subcultured in non-pesticide medium and tested for resistance to different concentration of the TMTD. This procedure was repeated 20 times and reversion of resistant to sensitive strain determined on the basis of growth.

The dehydrogenase activity (Dorosinskii et al., 1966), total lipids and their fraction (Folch et al., 1957; White and Frerman, 1967) of both pesticide sensitive and their resistant counterparts were determined. The quantitative determination of phospholipids was made using thin layer chromatography. The chromatographic plates were prepared according to Mangold and Malins (1960), and activated at 110 ± 5°C for 1 h (Randerath, 1964; Marinetti, 1967). Individual phospholipids were identified on the basis of Rf values after exposure to iodine vapours (Whitehouse, 1958). The quantitative estimation of individual phospholipids was made by estimating total phosphorous by the modified method of Fiske and Subbarow (Bartlett, 1959) in the spot representing a particular phospholipid which had been removed from the chromatographic plate.

The effects of compounds like glycerol (3 %), sodium oleate (20 ppm) and sodium acetate (0.5 %) known to stimulate lipid production (Hugo and Stretton, 1966) were studied on the lipids of Rhizobium strains D-338, CP-29 and U-4 resistant or sensitive to TMTD (30 ppm).

The effects of flavin mononucleotide (10 m) which
has been shown to decrease total lipids of gram-positive bacteria (Hugo and Stretton, 1966) was studied on the sensitivity of TMTD resistant strains of *Rhizobium* to TMTD.

3.25. Statistical analysis:

The data pertaining to nitrification, ammonification, symbiotic nitrogen fixation and soil enzymes under these biological processes and reversal of the effect on these aspects were subjected to statistical analysis by following the Randomized Block Design (Panse and Sukhatme, 1978). In many cases the combination of time with the TMTD concentrations were regarded as treatments. These treatments were further decomposed into orthogonal components/contrasts, depending on the type of the information, needed in the particular experiment under study.