CHAPTER II
MATERIALS
AND
METHODS
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Effects of land application of effluent on soil microflora in relation to a soil borne disease has been studied as irrigation with effluent has been a regular practice in agricultural fields around carpet dyeing and processing industries. It’s continuous use significantly changes the physical, chemical and biological composition of soil affecting the growth and yield of the crops.

Two fields situated in Khamaria, district Ravidas Nagar Bhadohi near industrial unit, one irrigated with effluent obtained from the industrial unit (hereafter called as treated field), and the other irrigated with tube well water (hereafter called as control field) were selected for the present study. The crops grown in these fields are linseed, wheat, barley, paddy and some vegetables like tomato, cauliflower and potato. Tomato was selected for the present because study this is very economically important plant and greatly suffer from wilt disease. The wilt of tomato is caused by *Fusarium oxysporum f. sp. lycopersici*.

The incidence of the disease in effluent irrigated and in control fields was recorded. The soil samples of both the fields were
collected in sterilized polythene bags while effluent and tube well water were collected in sterilized plastic bottles from industrial unit and tube well at seasonal interval. The samples were brought into laboratory for the study.

**Sampling of Effluents and Water**

Three replicates each of two liter of the effluents and tube well water were collected randomly in new plastic sampling bottles and brought into laboratory in ice boxes for the analysis of various physico-chemical properties i.e. temperature, colour, odour, pH, electrical conductivity, total solids, dissolve oxygen (DO), biological oxygen demand (BOD), chemical oxygen demand (COD), total alkalinity, carbonate, chloride, phosphate, sulphate, nitrate nitrogen, ammonium nitrogen, total nitrogen, sodium, potassium, calcium, magnesium, chromium (VI) and dye. During the collection of effluents and water, samples were analysed for the physical properties temperature, colour, odour and pH value at the time of sampling. Dissolved oxygen of effluent and tube well water was fixed immediately at the sampling site with magnous sulphate and alkalilodize acid for transportation. The collected samples were stored in cool, dark and dry place till analysis. Care was taken to see that no contamination of the collection sample took place during
transportation and storage. Necessary sampling precautions and specialized sampling equipments were used whenever need (Michael, 1984).

I. Physical Properties

The analysis of effluents and water has been done for the following physical properties.

1. Temperature –

In addition of plant growth physical, chemical and biological processes occurring in the soil are strongly influenced by the temperature. Solubility, saturation and stability of chemicals and in general reaction operations, the water temperature is very important environmental factors. Elevated temperature resulting from heated water discharges may have significant ecological impact.

A good quality of mercury filled thermometer with minimal thermal capacity and rapid equilibration, has been consistently used for the temperature measurement. The temperature of effluent and water was recorded in degree Celsius upto 0.1°C at the time of sampling.

2. Colour –

The colour of discharged water is result of the presence of metallic ions, industrial wastes, refuses of dyeing and washing etc.
The colour of woolen mill industrial effluents is predominated by the colours of dyeing with wool dust particles. The term colour means the true colour of turbidity removed water, whereas the apparent colour donates to the colour resulted due to suspended matter and the substances in solution.

The usual comparison method was applied and the colour chart has been used for the purpose (Michael, 1984).

3. Odour –

In chemical sense odour depends on actual contact of the stimulating substances with the appropriate human receptor cell. Most organic and inorganic substances contribute odour. These chemicals originate from, chemicals used in industrial purpose, their reaction on wool-yarn and water at higher temperature. Wool-yarn gives special odour when comes in contact with water. The ultimate odour testing device is the human nose.

4. Solids –

The term ‘residue’ refers to solid matter suspended or dissolved in water and waste water. It is of the following types:

a) Dissolved Solids –

The dissolved solid is defined as solids capable of passing through a glass fibre filter and dried to constant weight at 105°C.
100 ml of well mixed water sample is filtered through a standard glass fibre filter disc. The filtrate is allowed to evaporate and then dried to constant weight at 105°C cooled in desicator and weight in mg per liter (ppm) (APHA, 1985).

b) Suspended Solid –
Suspended solid is defined as those solids which are retained by a glass fibre filter and dried to constant weight at 103°C to 105°C.

100 ml of well mixed water sample is filtered through a standard glass fibre filter disc and the residue on filter disc is dried at 105°C in an oven and cooled in a desicator and weight in mg per liter (ppp) (APHA, 1985).

c) Total Solids –
The total solids refers to the sum of the suspended and dissolved material left in the vessel after evaporation of a water sample and its subsequent drying in an oven at 103°C to 105°C (APHA, 1985).

II. Chemical Properties
1. pH –
The pH of a solution means its activity of hydrogen ion and is expressed as the logarithm of the reciprocal of the hydrogen ion concentration in moles per liter at a given temperature.
It was measured with the help of B. D. H. Universal indicator at the time of sampling in the field. An accurate measurement was done electrometrically with the help of pH meter (Elico make) with cabinate electrode in the laboratory (Michael, 1984).

2. Conductivity –

Conductivity or the ability to carry electric current in water depends on the total ionic concentration of the dissolved substances in the water and the temperature at which the measurement is made. Conductivity is directly related to the mobility of each of the various dissolved ions and hence it is measured to get an idea of total concentration of soluble salts or ionized constituent in water. It is related to the sum of cations and anions as determined chemically and it is also usually correlated with the total dissolved salts.

The specific conductance of all the samples was measured with the help of conductivity meter. Temperature correlated were made and results are reported in m mhos per cm at 25°C (APHA, 1985).

Dissolved Oxygen (DO) –

Dissolved oxygen (DO) was estimated by Winkler's modified azide method. DO of sample is measured by precipitating as
manganic basic oxide which is dissolved by concentrated sulphuric acid forming manganic sulphate. It immediately reacts with potassium iodide, already present, liberating iodine which is determined by titration with sodium thiosulphate (0.025 N).

The chemical reaction involve are:

$$2\text{Mn (OH)}_2 + \text{O}_2 = 2 \text{MnO (OH)}_2 \quad \text{(Manganic Basic Oxide)}$$

$$\text{MnO (OH)}_2 + 2\text{H}_2\text{SO}_4 = \text{Mn (SO}_4\text{)}_2 + 3\text{H}_2\text{O} \quad \text{(Maganic sulphate)}$$

$$\text{Mn (SO}_4\text{)}_2 + 2\text{KI} = \text{MnSO}_4 + \text{K}_2\text{SO}_4 + \text{I}_2$$

$$2\text{Na}_2\text{S}_2\text{O}_3 + \text{I}_2 = \text{Na}_2\text{S}_4\text{O}_6 + 2 \text{NaI}$$

The quantity of iodine liberated during these reactions is equivalent to the quantity of oxygen present in the sample. The value of DO was calculated with the help of following formula:

$$\text{Dissolved Oxygen} = \frac{V \times N \times 8 \times 1000}{\text{ml Sample}} \text{ mgL}^{-1} \text{ (ppm)}$$

where, V is volume and N is normality of the titrant. The values is expressed in ppm.

**Biochemical Oxygen Demand (BOD) —**

BOD of sample was estimated by the difference of the initial DO of the sample and DO after 5 days incubation at C in dark condition. The values of BOD was calculated as follows and expressed in ppm
\[
\text{BOD} = \frac{(D1 - D2)}{\% \text{ Dilution}} \times 100 \text{ mg L}^{-1} \text{ (ppm)}
\]

where, \( D1 \) = Initial DO and \( D2 \) = DO after 5 days

**Chemical Oxygen Demand (COD) —**

Chemical oxygen demand (COD) of sample was estimated by dichromate reflux method. COD is a measure of oxygen equivalent of those constituents in the sample which are susceptible to dichromate oxidation in acid condition. Reflux known volume of potassium dichromate and conc. Sulphuric acid was kept in reflux flask, for two hours. The whole content of the reflux flask was taken and the flask was washed with distilled water and the whole amount of \( K_2Cr_2O_7 \) was titrated with ferrous ammonium sulphate using ferroin indicator. The values of COD is expressed in ppm

\[
K_2Cr_2O_7 + 4H_2SO_4 = K_2SO_4 + Cr_2(SO_4)_3 + 4H_2O + 3O
\]

COD was calculated by the following formula :

\[
\text{COD} = \frac{(a - b) \times N \times 8000}{\text{ml sample}} \text{ mg L}^{-1} \text{ (ppm)}
\]

where, \( a \) = ml of ferrous ammonium sulphate used for blanks

\( b \) = ml of ferrous ammonium sulphate used for effluent sample

\( N \) = normality of ferrous ammonium sulphate
3. Nitrate-Nitrogen —

The water sample were treated with phenol-disulphonic acid. The intensity of yellow colour produced is directly proportional to the nitrate concentration in water. The intensity of yellow colour was measured colourimetrically at 460 nm. The amount of nitrate in water is determined with the help of standard curve and expressed in ppm (APHA, 1985).

4. Ammonium-Nitrogen —

Ammonia is largely produced by deamination of nitrogen containing organic compound proteins, peptides, nucleic acids, urea and numerous synthetic organic materials.

The sample is buffered at 9.5 pH with a borate buffer (mixed 88 ml of 0.1 N NaOH solution to 500 ml of 0.025 M sodium tetraborate solution and diluted to 1 lit) in order to decrease hydrolysis of cyanates and organic nitrogen compounds and is then distilled into a solution of boric acid. The ammonia in distillate was determined titrimetrically using mixed indicator with sulfuric acid, matching the end point against a blank containing the same volume of distilled water and boric acid solution. For the removal of chlorine the sample is pretreated with thiosulfate before distillation (APHA, 1985). The amount of ammonia nitrogen is represented in ppm.
5. Total Nitrogen –

Total nitrogen was determined by micro Kjeldahl procedure (APHA, 1985). Samples were digested with concentration H₂SO₄ and catalyst (copper sulphate + potassium sulphate + selenium powder). The extract was distilled in Markham steam distillation unit and ammonia liberated was collected in boric acid which was titrated against dilute HCl.

6. Calcium, Potassium, Sodium and Magnesium –

Potassium and sodium content of the effluent and water were determined by flame photometer. Calcium was determined by oxalate method and calcium + magnesium was determined by titration against EDTA (Ethylene diamine-tetra acetate). Magnesium content was then calculated by the substraction of calcium (obtained by oxalate method) from calcium + magnesium (determined by EDTA method). All the determination were made according to Richards procedure (1954) as described in Agricultural Hand Book No. 60 and expressed in ppm.

7. Chromium –

In woolen mill or yams the chromium is used in chrome dyes and to sharpen the colour, potassium dichromate is also used or added. Hexavalent chromium in water was determined by colourimetry, a red violet colour of unknown composition is produced
by reaction with 1, 5, diphenyl carbazide in acid solution. New bottles were used for sample collection. Samples were acidified by about 2 ml of HNO₃ per liter at the time of collection to keep the metal in solution and prevent their plating out on the container wall. The samples were digested with sulfuric nitric acid mixture to decompose the organic matter. An addition of 1.5 ml diphenylcarbazide solution (0.25 g, 1,5, diphenyl carbazide in 50 ml acetone) in 5 ml of aliquot yields the red violet product and its absorbance was measured photometrically at 450 nm. A blank titration with distilled water and other reagents was run and the amount of chromium is determined with the help of standard calibration curve, prepared with known solution of K₂Cr₂O₇ (APHA, 1985) and expressed in ppm.

8. Chloride –

Chloride in water is determined according to the procedure described in standard methods for the examination of water and waste water (APHA, 1985) and expressed in ppm.

9. Sulphate –

The sulphate in water is estimated by turbiditimetric method by the precipitation of sulphate as BaSO₄ on addition of 1 g solid barium chloride to 20 ml of aliquot, in the presence of 2 ml of 0.258 acacia solution to make the colloid very stable. pH of 4.8 is maintained by adding sodium acetate-acetic acid buffer. The
suspension was made to 25 ml volume and shaken for 1 minute. Absorbance of this turbid reaction mixture is noted at 440 nm and calculated the amount of sulfate by comparing with standard curve prepared from potassium sulfate solution (Richards, 1954) and expressed in ppm.

10. Phosphorus –

High phosphatic compounds containing detergents and soaps are extensively used for the washing of woollen yarns before dyeing and again for washing of woolen carpets. These phosphatic compounds, when washed away come out with the effluents.

For the conversion of organically bound and condensed phosphate-phosphorus into soluble orthophosphate, 1 ml of 1 NH₂SO₄ was added to 50 ml water in a 150 ml Erlenmeyer flask. It was boiled gently until the final volume reduced to about 10 ml, cooled and diluted to 40 ml and filtered 40 ml of acidified ammonium molybdate reagent and 0.5 ml stannous chloride reagents were added for the developments of blue colour at constant temperature (between 20 to 30°C). The intensity of this blue colour was measured photometrically at 690 nm invariably at interval of 10 minutes in all the cases and the amount of phosphorus in water was determined with the help of calibration curve (APHA, 1985). The concentration of phosphorus is reported in ppm.
11. Carbonate and Bicarbonate –

Carbonate and bicarbonate in water were determined by titration with sulfuric acid, using phenolphthalein and methyl orange as indicator (Richards, 1954) and expressed in ppm.

12. Concentration of Dyes –

Initially at higher temperature the major portion of the dyes is absorbed by the wool-yarns and only a residual amount of the dyes comes out with the effluents. In order to remove the turbidity creating larger particles like wool waste etc., unaltered water samples were filtrated with the help of glass-wool. The absorbance at certain wavelength is determined with the help of spectro-colorimeter. The amount of dyes is determined with the help of standard curve, obtained by the absorbance of known concentration of solution of the dyes at certain pH. The amount of dyes is expressed in ppm.

**Analysis of Soil**

Soil samples in triplicate were collected seasonally from 0 – 30 cm depth on each of the three study sites. The soil samples were analysed for the following physical-chemical characters:

**I. Physical Characters**

Soil were analysed for the following physical characters.
1. Soil Colour –

Soil colour is often regarded as an index of soil fertility. The colour of soil is mainly due to presence of organic matter, salts of various colours etc.

2. Soil Texture –

In mechanical analysis the estimation of percentage of three types of soil particles was done by Robinson’s Pipette Method (Piper, 1966). With the help of which the soil texture was determined from the soil textural triangle.

The rate of water intake of plants, soil, water supplying power, the fertility, erosion, aeration and energy required to till the soil are closely related to soil texture. Soil texture refers to weight proportions of different size particles. The diameter sizes of texture groups are for coarse sand 2 – 0.2 mm fine sand 0.2 – 0.02 mm, silt 0.02 – 0.002 mm and clay below 0.002 mm.

3. Bulk Density –

Bulk density is the ratio of mass to the bulk or apparent volume including pore space of the soil i.e., the oven dry content weight per unit volume of naturally occurring soil.

A known volume of soil sample as existing in situ having field structure is removed from the fields by cylindrical metal
sampler. The soil samples are dried at 105°C for a constant weight, cooled in a desiccator and weighed. Oven dried mass of soil divided by field volume of the sample gives the bulk density, which is expressed in terms of grams per cubic centimeter (gm/cm³). It was determined by the methods as described by Mishra (1968).

\[ B. \ D. = \frac{W}{V} \]

\( B. \ D. = \text{Bulk Density} \)

Where, \( W = \text{weight of oven dried soil} \)

\( V = \text{volume of sample which is used in field} \)

4. Porosity of Soil –

Interstitial space around the soil particles is known as pore-space, i.e., the part of a given soil volume occupied by air and water. Porosity depends upon the texture composition and aggregation of soil particles. Per cent porosity of soil was determined by using the following formula:

\[
\text{Per cent Porosity (PP)} = \frac{1 - \text{Bulk Density}}{\text{Particles Density (2.65)}} \times 100
\]

5. Soil Moisture –

The effect of mineral elements in soil on plants is related to their concentration and ionic balance in the soil solution, which is fully dependent on the amount of water present therein. Therefore soil
water content measurement is of prime importance on the practical studied of edaphic condition of an environment. Soil moisture is determined as percentage of water on dry soil weight basis. It was determined by the methods which are described by Michael (1984).

**Soil moisture content by weight (MW%)**

\[
(MW\%) = \frac{\text{Loss in weight on drying}}{\text{Weight of oven dry soil}} \times 100
\]

6. **Water Holding Capacity (WHC)** –

Amount of water taken up by unit weight of dry soil. When immersed in water in a standardized condition, which is equal to the volume of the pores i.e. the amount of moisture in a soil when its total pore-space both macro and micro is completely filled with water (Mishra, 1969).

Air dried unclosed, sieved soil is transferred by spatula in small pots into weighed \((W_1)\) metallic circular brass boxes fitted with a filter paper at its perforated bottom. The box with uniformly packed and level surfaced soil is submerged in water upto 0.65 cm, so that water enters into the soil through the bottom pores only. It was placed for overnight in a tray of water and then after 30 minutes of drainage the box was weighed \((W_2)\). The saturated soil is then dried to
a constant weight in an oven at 105°C, cooled in desicator and again weighed \( W_3 \), which a correction of average weight \( W_4 \) of moisture taken by a saturated filters paper used. The method was determined by Mishra (1968).

\[
\text{Maximum Water Absorbed by the Soil} \quad \text{WHC}\% = \frac{W_2 - W_3 - W_4}{W_3 - W_1} \times 100
\]

\[
\text{or} \quad \text{WHC}\% = \frac{W_2 - W_3 - W_4}{W_3 - W_1} \times 100
\]

II. Chemical Characters

Soil samples were analysed periodically for the following chemical characters:

1. Soil pH –

Soil pH determines the capacity for the growth of plants, activity of macro and micro elements availability of nutrients, microbial activity and physical conductions of the soil. The pH is defined as the negative logarithm of hydrogen ion activity (effective concentration). The effective concentration of hydrogen ions include all sources arising by association of soluble acids, dissociates from soil particles and the relative amounts of absorbed hydrogen ion and metallic cations on the exchange complex.
The pH of soil sample was measured electrometrically (pH-meter, Elico Make, Model L-1-12, with Combined Glass Electrode) as described by Michael (1984).

2. Conductivity of Soil –
Fertile soils always have some amount of soluble salts bearing cations and anions in them. Soil solution conduct the passage of electric current through them. Higher ionic content increased conductivity of the soil or reduced the resistance for the flow of electric current. Conductivity is the reciprocal of resistance (Ohm) and is expressed in mho. Systronic conductivity meters type 301 – 1, Sr. No. 4007 was used and measurement was done in m mhos/cm.

3. Soil Organic Matter –
Method suggested by Walklay (1947) based upon the oxidation of carbon by nascent oxygen, liberated from K₂Cr₂O₇ in presence of sulphuric acid was applied for the determination of soil organic matter (Jackson, 1962). Percentage of carbon present was determined by using diphenyl amine as indicator and titrating with ferrous ammonium sulphate solution in burette

\[
\text{Percentage of Carbon (C\%) = } \frac{V_1 - V_2}{W} \times 0.603 \times 100
\]

where, \( V_1 = \text{ml of } 1 \text{ N } K_2Cr2O_7 \text{ used} \).
\[ V_2 = \text{ml of 1 N Ferrous Ammonium Sulfate Used} \]

\[ W = \text{Weight of Soil Sample Used.} \]

Percentage Organic Matter = Percentage Organic Carbon \( \times 1.724 \)

Organic matter in soil improves the soil fertility especially nitrogen, which are continuously released by microbial activity. Humus prevents compaction, increases infiltration, aeration and moisture retaining capacity of soil.

4. Exchangeable Cations —

When the soil is placed in a solution of salt, such as ammonium acetate, ammonium ions are adsorbed by the soil and equivalent amount of cations is displaced from the soil into the solution. This reaction is termed “cation exchange” and the cations displaced from the soil are referred to as exchangeable cations.

Most interesting exchangeable cations in soils are Na\(^+\), K\(^+\), Ca\(^{++}\) and Mg\(^{++}\) which are readily extracted and determined in 1N ammonium acetic (NH\(_4\)OAC) extracts of soil. The water soluble salts are removed by leaching the soil with 40% ethanol until the leachate is free from Cl\(^-\) and SO\(_4^{--}\) prior to the NH\(_4\)OAC extraction (Jackson, 1962).
a. Sodium, Potassium and Calcium —

The ammonium acetate leachate is analysed for $\text{Na}^+$, $\text{K}^+$ and $\text{Ca}^{++}$ directly by Flamephotometer and their amounts, determined by standard curve method (Jackson, 1962). The results obtained are expressed in me/100 g dry soil.

b. Magnesium —

Calcium is precipitated and removed by Hydrochloric acid and Oxalic acid method from 20 ml of NH$_4$OAC leachate. Magnesium in leachate is determined by Ammonium Vandate and Ammonium Molybdate method and transmittance was noted at 460 nm. The amount of Mg$^{++}$ was determined from a standard calibration curve (Richard, 1954). Results obtained are expressed in me/100 g dry soil.

5. Chromium in Soil —

1.5 ml of diphenyl carbazide solution is added to 5 ml of aliquote in conical flask. Pink colouration indicate the presence of chromium. The intensity of pink colur in solution (reaction mixture) was determined by taking the optical density (O. D.) at 540 nm in a spectrocolourimeter and the amount of chromium in the solution was determined with the help of standard curve prepared from standard solution of K$_2$Cr$_2$O$_7$ by the same method. The results obtained are expressed in (ppm) dry soil.
6. Anion in Soil -
   a. Nitrogen —
      i. Nitrate — Nitrogen —

      Three ml of toluene was added to one kg of each soil sample and nitrate content was determined by the phenoldisulfonic acid method. Rapid addition of phenoldisulfonic acid is followed by addition of 6NH₄OH slowly till the development of yellow colour in alkaline medium. The transmission percentage of the yellow coloured reaction mixture of nitrate is read in a spectrocolourimeter at 420 nm. Nitrate concentration in test sample is determined by company with standard calibration curve plotted from standard nitrate solution (Jackson, 1962). The result obtained are expressed in (ppm) dry soil.

   ii. Ammonium-Nitrogen —

      Ammonium-nitrogen of soil was estimated by the distillation of soil extract in 2N KCl solution with MgO following the method described by Hesse (1970) and results obtained are expressed in (ppm) of dry soil.

   iii. The Nitrogen —

      Two grams soil sample was digested with 10 g catalyst mixture of mercuric oxide, sodium sulphate and selenium power and 20 ml sulphuric acid in 300 ml digestion flask till the greenish white
residue was obtained. Water was added to cooled residue and filtered and maintained the volume in 100 ml volumetric flask. 10 ml of aliquot and 10 ml of 1N NaOH were distilled by microkjeldahl into boric acid solution. The nitrogen in the form of ammonia in distillate was titrated against N/28 H₂SO₄ by using Conway mixed indicator with faint pink end point. The blank digestion distillation and titration was also done with the same reagents in the same manner (Jackson, 1962). The results obtained are expressed in (ppm) dry soil.

b. Phosphorus —

Phosphorus in soil was determined by sulfomolybdic acid and chlorostanous acid blue colour developed method suggested by Jackson (1962). The intensity of blue colour is directly proportional to phosphorus content, which was determined spectrocolourimetrically at 660 nm. The amount of phosphorus in the aliquot was calculated with the help of the standard curve. The results obtained are expressed in (ppm) dry soil.

**Extraction of Salts from Soil**

Soil extracts were prepared from 1.5 soil water mixture by one hour shaking on horizontal shaker and filtering through Whatman No. 42 filter paper (Jackson, 1962).
i. Chloride in Soil –

Chloride content of soil were determined from alkaline (8.2) extract, by titrating with silver chloride using chromate indicator with red colouration of end point. Titrate value in ml (after substraction of blank) for 50 ml of aliquot is equal to the ppm of chloride in the solution. Thus the amount of chloride in mg from the soil is determined (Richards, 1954). The results obtained are reported in (ppm) dry soil.

ii. Sulphate –

Sulphate in soil extract is determined gravimetriclly by precipitating as BaSO$_4$ which is dried, ignited and cooled in a crucible and weighed (Richards, 1954). The sulphate content is reported in (ppm) dry soil.

iii. Carbonate –

The soil carbonate was determined by titrating soil mixture (1 : 2) with 1 N H$_2$SO$_4$ using phenolphalein indicator. The appearance of pink colour after the addition of phenolphalein in soil extract indicator the presence of carbonate which disappears at the end point (Richards, 1954). The results obtained are expressed in (ppm) dry soil.
**Total Alkalinity** –

Alkalinity of sample were determined by potentiometric titration method. Titrant sulphuric acid (0.025 N) was used to lower down the pH of the samples (100 ml) at 8.3 (phenolphthalein alkalinity) and to the pH 3.7 (methyl orange alkalinity). Alkalinity of the samples were calculated by followed formula and expressed in ppm.

\[
\text{Total Alkalinity} = \frac{V \times N \times 50,000}{\text{Volume of Sample (ml)}} \text{ mgL}^{-1} \text{ (ppm) CaCO}_3
\]

V and N are volume and normality of the titrant respectively

Carbonate alkalinity = 2 x phenolphthalein alkalinity

Bicarbonate alkalinity = Total alkalinity – Carbonate alkalinity

**Composition of Culture Media used during the Present Study**

Different culture media with their constituents are given below which were used during the course of the present study for various experiments:

- Peptone-Dextrose – Rose Bengal
  Agar (Martin, 1950)
- Dextrose 10.0 g
Peptone  5.0 g
KH₂PO₄  1.0 g
MgSO₄ 7H₂O  0.5 g
Rose Bengal  0.006 g
Agar  18.0 g
Distilled Water  1000 ml
pH  5.0

Czapek Dox + Yeast Extract Medium

(Raper and Thom, 1949)

MgSO₄ 7H₂O  0.5 g
KCl  1.0 g
NaNO₃  2.0 g
KH₂PO₄  1.0 g
FeSO₄  0.066 g
Sucrose  30.0 g
Yeast Extract  0.5 g
Agar – Agar  18.0 g
Distilled Water  1000 ml
*pH  5.6
*Streptomycin
(added after autoclaving)

(Liquid medium was prepared without the addition of agar – agar)
Potato-Dextrose Agar (PDA Medium)
(Riker and Riker, 1936)

Potato (Peeled and Sliced) 200 g
Dextrose 20 g
Agar – Agar 18 g
Distilled Water 1000 ml
Streptomycin 30 µg/ml
(added after autoclaving)

Thornton’s Agar Medium
(Thornton, 1922)

K₂HPO₄ 1.0 g
MgSO₄ . 7H₂O 0.2 g
CaCl₂ 0.1 g
NaCl 0.1 g
FeCl₃ 0.002 g
KNO₃ 0.5 g
Asparagine 0.5 g
Mannitol 1.0 g
Agar – Agar 15.0 g
Distilled Water 1000 ml
pH 7.4
Mycostatin 25 µg/ml
(added after autoclaving)
**Penta Chloro Nitro Benzene (PCNB)**

**Agar Medium**

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**Starch Casein Agar Medium**

(Adapted from Kuster and Williams, 1964)

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</tr>
<tr>
<td>pH</td>
<td>7.2</td>
</tr>
<tr>
<td>Mycostatin</td>
<td>25.0 µg/ml</td>
</tr>
</tbody>
</table>

(Added After Autoclaving)
### Synthetic Medium for the Growth of Actinomycetes and Fungi Both

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>KH₂PO₄</td>
<td>1.5 g</td>
</tr>
<tr>
<td>KCl</td>
<td>1.0 g</td>
</tr>
<tr>
<td>NaNO₃</td>
<td>2.0 g</td>
</tr>
<tr>
<td>MgSO₄·7H₂O</td>
<td>0.5 g</td>
</tr>
<tr>
<td>FeSO₄</td>
<td>0.06 g</td>
</tr>
<tr>
<td>Yeast Extract</td>
<td>1.0 g</td>
</tr>
<tr>
<td>Sucrose</td>
<td>20.0 g</td>
</tr>
</tbody>
</table>

Casein (Dissolved in 10 ml 0.5 g of 0.1 N NaOH)

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Agar – Agar</td>
<td>12.0 g</td>
</tr>
<tr>
<td>Distilled Water</td>
<td>1000 ml</td>
</tr>
<tr>
<td>pH</td>
<td>6.0</td>
</tr>
</tbody>
</table>

### Synthetic Medium for the Growth of Bacteria and Fungi

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>K₂HPO₄</td>
<td>1.0 g</td>
</tr>
<tr>
<td>MgSO₄·7H₂O</td>
<td>0.2 g</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>0.1 g</td>
</tr>
<tr>
<td>NaCl</td>
<td>0.1 g</td>
</tr>
<tr>
<td>FeCl₃</td>
<td>0.002 g</td>
</tr>
<tr>
<td>KNO₃</td>
<td>0.5 g</td>
</tr>
<tr>
<td>Asparagine</td>
<td>0.5 g</td>
</tr>
<tr>
<td>Mannitol</td>
<td>2.0 g</td>
</tr>
<tr>
<td>Sucrose</td>
<td>20.0 g</td>
</tr>
<tr>
<td>Agar – Agar</td>
<td>12.0 g</td>
</tr>
<tr>
<td>Distilled Water</td>
<td>1000 ml</td>
</tr>
<tr>
<td>pH</td>
<td>7.0</td>
</tr>
</tbody>
</table>
Isolation of Soil Micro-flora

Fungi, bacteria and actinomycetes were isolated from treated soil (irrigated with effluent) and control soil by dilution plate technique at monthly interval (from July 2003 to June 2004). Soil samples were collected separately in sterilized polythene bags from both the fields control as well as the treated one i.e. field irrigated with effluent. Ten gram well mixed soil, from each sample, was taken separately into 250 ml conical flask containing 100 ml sterilized distilled water. The flasks were shaken on electric shaker (120) throws min⁻¹ and 1.5 cm replacement to get a homogenous suspension. Ten ml suspension was immediately taken out with the help of sterilized pipette and was transferred into 250 ml Erlenmeyer flask containing 90 ml sterilized distilled water. Ten ml suspension was again transferred successively in sterilized distilled water until the desired final dilution (10⁷) was obtained. The suspension was not allowed to remain in any dilution for more than 10 min. The preceding method for making soil dilution yielded dilutions of soil in water as 10¹, 10², 10³, 10⁴, 10⁵, 10⁶ and 10⁷.

One ml of 10³ and 10⁴ dilutions for fungi, 10⁶ and 10⁷ for bacteria and 10⁵ and 10⁶ for actinomycetes were inoculated separately into each of 3 replicate sterilized Petri dishes (90 mm diameter).
Twenty ml sterilized and cooled modified Martin’s, Jensen’s and Thornton’s media were poured separately into each inoculated Petri dish for the isolation of fungi, actinomycetes and bacteria, respectively.

The Petri dishes were rotated clockwise and anticlockwise to get a homogeneous distribution of the suspensions into the medium. The Petri dishes were incubated at 25 ± 2°C for fungi, 35 ± 2°C for bacteria and 30 ± 2°C for actinomycetes. The qualitative and quantitative estimation of fungal flora was observed after 7 days of incubation whereas the colonies of bacteria and actinomycetes were counted after two and ten days of incubation, respectively.

Isolation of the Test Pathogens and Performance of their Pathogenicity Test

The wilted plants of tomato was collected from the selected fields during November 2003 to March 2004. The test pathogen *Fusarium oxysporum* f. sp. *lycopersici* was isolated from the root region of the wilted plant by the method of Harley and Waid (1957). The roots were cut into small pieces (2 cm length) and were thoroughly washed in sterilized distilled water and then were surface sterilized with 0.1% mercuric chloride for 1 min. The treated root bits
were washed thoroughly (8 - 10 times) with sterilized distilled water and soaked in between the folds of sterilized blotting paper to remove the excess water content and then placed on czapek dox + yeast extract and potato dextrose agar medium in Petri dishes. Each plate was inoculated with five root bits and incubated at 25 ± 2°C in continuous light and darkness of 12 hr each for 5 days. The pathogen growing on root bits were picked up and transferred on slants in culture tubes as well as in Petri dishes containing PCNB, Czapek dox agar and Martin’s media. The pathogen was subcultured at monthly interval on Czapek dox + yeast extract medium on slants in culture tubes and Petri dishes.

The pathogenicity test of the isolated test fungus was performed according to Koch’s postulate in pot culture experiment as well as in selected fields. The test was performed in pure sand inoculums of the test pathogen mixed with soil sample irrigated with effluent and control soil samples in pots in ratio of 1 : 9. The test pathogens were prepared in 100 g of acid washed sand + 3 g of maize meal and 13% of distilled water.

The inoculums soil mixture was filled in earthenware pots (15 cm × 12 cm). Twenty surface sterilized seeds of the
susceptible and resistant varieties of tomato were sown in different 
pots containing soil sample from treated and control field. Tomato 
was sown in pots which were filled with soil, mixed with inoculums 
of *F. oxysporum* f. sp. *lycopersici*. Seeds were also sown in control 
pots containing only sterilized soil. Five replicate pots were taken for 
each treatment. Sterilized water was added to the pots at suitable 
intervals to maintain the moisture of the soil at 15 – 20% level. The 
pots were kept in garden and the wilting was recorded from the pots 
as well as from the fields. The wilted plants were taken out and the 
pathogen was reisolated to follow the further steps of Koch’s postulate 
for confirming pathogenicity.

**Isolation of Non-Rhizosphere, Rhizosphere and Rhizoplane 
Microflora of Healthy and Wilted Plant of Tomato**

The non-rhizosphere soil samples from tomato field, 
from both the sites, (Irrigated with effluent and control fields) were 
collected separately in sterilized polythene bags from nearly regions, 
which was away from the influence of root system. Ten gram well 
mixed soil, from each sample, was taken separately into 250 ml 
Erlenmeyer flasks containing 100 ml sterilized and cooled distilled 
water. the flasks were shaken well on an electric shaker (120 throws 
min⁻¹ and 1.5 cm displacement per throw) for 30 min to get a uniform
soil suspension and thereafter, soil dilution series of $10^3$, $10^4$ (fungi) and $10^5$, $10^6$ and $10^7$ (for actinomycetes and bacteria) were prepared in sterilized distilled water. Three replicate petri dishes for each sample and dilution were inoculated with one ml aliquot of soil suspension separately for fungi, actinomycetes and bacteria. Separate sterilized pipettes were used for each dilution. The inoculated Petri dishes were poured separately with melted and cooled 20 ml modified Martin’s, Jensen’s and Thornton’s media (at 40°C) for fungi, actinomycetes and bacteria respectively. The plates were incubated separately at 25 ± 2, 30 ± 2 and 35 ± 2°C for fungi, actinomycetes and bacteria, respectively. The fungal colonies appeared in plates were examined and identified, whereas the total number of colonies of bacteria and actinomycetes were counted and recorded.

For the isolation of rhizosphere microflora, the roots of healthy and wilted tomato plants were collected separately in sterilized polythene bags from the selected fields in November 2003, December 2003, January 2004, February 2004 and March 2004. The roots were carefully dug out with the help of sterilized spatula and were gently tapped to remove the loosely adhering soil articles. They were cut into small bits (2 cm length) of different diameters and transferred into a 250 ml Erlenmeyer flask containing 100 ml
sterilized distilled water. The individual flasks for each sample were
shaken vigorously on a horizontal electric shaker so as to get a
homogeneous soil suspension. The stock solution was further diluted
to ten times. Five replicate Petri dishes each for fungi \((10^3)\),
actinomycetes \((10^5)\) and bacteria \((10^6)\) were inoculated with one ml
suspension of stock as well as diluted suspensions separately and were
poured with 20 ml melted and cooled (at 40°C) modified Martin's,
Jensen's and Thornton's media separately for isolation of fungi,
actinomycetes and bacteria, respectively. The Petri dishes were
incubated separately at 25 ± 2, 30 ± 2 and 35 ± 2°C for fungi,
actinomycetes and bacteria, respectively. The total number of fungal
colonies and the number of colonies of individual fungus appearing in
each plate were recorded to determine frequency and occurrence on
the 7th day of incubation while only the colony number were noted on
the 10th and 3rd day of incubation in case of actinomycetes and
bacteria, respectively. The method of Timonin (1940) was followed to
estimate the number of micro-organisms per gram of soil in the
rhizosphere.

The rhizoplane microflora was isolated by the method
described by Harley and Waid (1955). The root bits used for the
isolation of rhizosphere microflora were removed and taken out from
the flasks and were washed thoroughly with sterilized distilled water and were soaked between the folds of sterilized blotting paper. There replicate Petri dishes each containing 20 ml Martin’s, Jensen’s and Thornton’s agar medium in each plate were incubated with five root bits. The plates were incubated at 25 ± 2, 30 ± 2 and 35 ± 2°C for isolation of fungi, actinomycetes and bacteria, respectively.

The identification of fungi, was done with the help of literature. In case of bacteria and actinomycetes their colonies were counted without identifying the genera and species.

Population Dynamics of the Pathogens in Selected Fields

Population dynamics of the pathogen \(F. \text{oxysporum f. sp. lycopersici}\) was calculated with the help of per cent occurrence and percent frequency mycoflora of non-rhizosphere, rhizosphere and rhizoplane of tomato plant in the selected field. Population dynamics of pathogen was recorded in terms of percent frequency and percent occurrence.

Studies on Microbial Interactions Between the Pathogenic and Non-Pathogenic Microflora

The following microorganisms were tested against the test pathogen \(F. \text{oxysporum f. sp. lycopersici}\) which were isolated from the selected fields during the course of present investigation:
(a) Fungi –


(b) Bacteria –

Two unidentified bacteria (colourless and yellow colour colony).

(c) Actinomycetes – *Streptomyces rimosus* and S. rochi strain SR₁

The colony interaction between pathogenic and non-pathogenic microflora were studied in dual-culture by placing 5 mm block of the test microbes and the test pathogen over solid potato dextrose agar medium (interaction between fungi and the test pathogen) and on the synthetic media (interaction between pathogen and actinomycetes and bacteria) approximately 3 cm apart in paired combination in replicate Petri dishes. The inoculated Petri dishes were incubated at 25 ± 2°C for fungi and 30 ± 2°C for actinomycetes and bacteria. In general, observation were made after 5 days for fungi. Interactions were assessed with the help of colony interaction models of skidmore and Dickinson (1976). The control was single and dual inoculated cultures of the same pathogen. The colony growth of both the types of microorganisms was measured at both sides i.e., towards and opposing each other from their central loci. The parameters used
for the assessment of colony interaction were the breadth of inhibition zone, intermingled zone and percent inhibition of radial growth i.e. 
100 \times (r_1 - r_2)/r_1 \text{ (Fokkema, 1976)}; where r_1 denotes the radial growth of pathogenic fungus towards to opposite side and r_2 denotes the radius of pathogenic form towards the test microorganisms. The same was applied for the inhibition of the test microorganisms also.

Effect of Volatile Metabolites of Some Dominant Microflora on Radial Colony Growth of the Test Pathogens

The following 14 dominant microorganisms were selected for this study:

\textit{Alternaria alternata, Aspergillus flavus, A. luchuensis, A. niger, A. terreus, Cladosporium cladosporoides, Penicillium citrinum, P. frequentans, Trichoderma harzianum, T. viride colourless bacteria, yellow colour bacteria, Streptomyces rimosus and S. rochi (SR1).}

The method described by Dennis and Webster (1971 b) was followed for this study. The test microflora were grown in 90 mm Petri dishes on their respective media i.e., fungi on Czapek-dox agar medium for 5 days, actinomycetes on Jensen’s medium and bacteria on Thornton’s medium for 10 days. After the incubation at 25 ± 2, 30 ± 2 and 35 ± 2°C for fungi, actinomycetes and bacteria, respectively,
the lid of each Petri dish was replaced by the same size of bottom Petri dish containing 20 ml potato dextrose agar medium, centrally inoculated with 5 mm dia agar block of the test pathogen separately in each Petri dish. Both the plates were taped together nicely with cellotape. The lid of the control plate which was not inoculated with any microorganism was also replaced in the same way but with the pathogens in place of the test microorganisms. All the Petri dishes were incubated at 25 ± 2°C and the colony diameter of *F. oxysporum* f. sp. *lycopersici* was measured at an interval of 48, 72, 96, 120 and 144 hr. The percent inhibition or stimulation in the colony diameter of the test pathogens were calculated.

**Effect of Culture Filterates (Non-Volatile Metabolites) of Some Dominant Micro-Flora on Growth (Hyphal Weight) of the Test Pathogens**

The dominant microorganisms selected for the present study were the same as in the previous experiment. The method described by Upadhyay and Rai (1987) was followed for the study. 250 ml Erlenmeyer flasks containing 100 ml potato dextrose broth medium was inoculated with three agar blocks (5 mm) of each test fungus, cut from the actively growing margins of 5 day old cultures, in replicate flasks. The flasks were incubated for 10 days at 25 ± 2°C.
The static cultures were filtered firstly through Whatman filter paper No. 44 and finally through seitz filter by vacuum filter to obtain cell free culture filtrates. The metabolites of actinomycetes and bacteria were obtained the above process by inoculating spore/bacterial cell in liquid Jensen’s and Thornton’s media respectively.

Twenty ml culture filtrate of each test microbe was added in 80 ml sterilized liquid Czapek dox medium separately. This concentration was found to be most suitable for such studies by Singh (1978). The conical flask containing medium and culture filtrate was well shaken and was inoculated with three agar blocks (5 mm), cut from the actively growing edge of the colonies of *F. oxysporum* f. sp. *lycopersici*. The experiment was set in 3 replicate flasks for each type of culture filtrate. For control set same quantity of autoclaved and cooled distilled water was added in 80 ml of potato dextrose broth medium. The growth of the test pathogens were made after 10 days incubation at 25 ± 2°C when it had achieved an equilibrium. The hyphal mats were filtered through preweighed Whatman filter paper No. 42 and dried in an oven at 80°C for 48 hr and reweighed. Mean value of hyphal mat of 5 replicates were recorded. The percent
inhibition of growth of *F. oxysporum* f. sp. *lycopersici* was calculated by using the following formula:

\[
\text{Percent Growth Inhibition} = \frac{C - T}{C} \times 100
\]

where, \( C \) = Growth of Control and \( T \) = Growth of Treatment

**Effect of Culture Filterates (Non Volatile Metabolites) of Some Dominant Micro-Flora on the Mean Radial Growth of the Test Pathogen**

The dominant microorganisms selected for the present study were the same as in the previous experiment. The method described by Upadhyay and Rai (1987) was followed for the study. 250 ml Erlenmeyer flasks containing 100 ml potato dextrose broth medium was inoculated with three agar blocks (5 ml) of each test fungus, cut from the actively growing margins of 5 day old cultures, in replicate flasks. The flasks were incubated for 10 days at 25 ± 2°C. The static cultures were filtered firstly through Whatman filter paper no. 44 and finally through seitz filter by vacuum filter to obtain cell free culture filtrates. The metabolites of actinomycetes and bacteria were obtained by the above process by inoculating spore/bacterial cell in liquid Jensen’s and Thornton’s media respectively.
5, 10, 15 and 20 ml of the filtered culture filtrate of each microbe was added to 95, 90, 85 and 80 ml Czapek dox year extract medium (sterilized and cooled) so as to give the final concentrate as 5, 10, 15 and 20% respectively. Medium without culture filtrate serves as control. The medium was poured in three replicates. Plates were inoculated with 8 mm agar block of the test pathogen. Radial colony growth was measured after 4 day of incubation at 25 ± 1° C when pathogen achieved full growth per cent inhibition of the test pathogen was calculated by the formula:

\[
\text{RGI} = \frac{C - T}{C} \times 100
\]

where, \( C \) = Growth of Control and \( T \) = Growth of Treatment.

**Efficacy of Pesticides Against Growth Behaviour of the Test Pathogen**

Nine fungicides viz., bavistin, benlate, blue copper, brassicol, dithane M-45, folfat, mancozeb, methoxy ethyl mercury chloride (MEMC), thiram and four insecticides viz., benzene hexa chloride (BHC), ekalux, monocil and thiodone were evaluated in laboratory against the growth behavior of *Fusarium oxysporum* f. sp.
lycopersici by poisoned food technique (Flack, 1970). Different concentrations of each pesticide were prepared in double strength on active ingredient basis in sterilized distilled water under aseptic condition. Double strength of CDA (czepex dox agar) medium was also prepared separately and was autoclaved at 15 lb pressure for 20 min. Ten ml of each concentration was added to 10 ml of the CDA medium in a sterilized Petri plate and was mixed thoroughly and was allowed to solidify. Thus, the desired concentration of the pesticides, to be tested, was obtained. Each Petri plate containing 20 ml treated solid medium was inoculated centrally with a 5 mm agar block cut from the leading edge of the pathogen with a sterilized cork borer. All the plates were incubated at 25 ± 2°C. For the control 10 ml of sterilized distilled water was added with 10 ml of melted CDA medium instead of Pesticide solution. Three replicates for each concentration were maintained. The radial growth of the colony of 

_Fusarium oxysporum_ f. sp. _lycopersici_ was measured after 7 days of incubation. The percent growth inhibition was calculated with the formula as mentioned earlier.
Effect of Effluent on Growth of Some Dominant Micro-Flora and the Test Pathogen

5, 10, 15 and 20 ml of effluent was added into 95, 90, 85 and 80 ml CZapek dox agar, Jensen's medium and Thornton's medium for fungi, actinomycetes and bacteria respectively, to give the final concentration as 5, 10, 15 and 20% for effluent and thereafter media were autoclaved. Twenty ml of supplemented medium was poured in each of sterilized 9 cm dia. Petri dishes in three replicate for each treatment and was inoculated at the centre of the Petridish with a 5 mm agar block, cut from the margins of actively growing 5 days old culture fungi and bacteria and 10 days old cultures of actinomycetes. The media without effluent were also inoculated and treated as control. All the Petridishes were incubated at 25 ± 2, 30 ± 2 and 35 ± 2°C for fungi, actinomycetes and bacteria, respectively and the radial growth of the pathogens and microorganisms were measured after 7 days of incubation. The percent inhibition of growth of pathogens and other microorganisms was calculated by comparing the results of the treated plate with that of control. Percent inhibition or stimulation of microorganisms were calculated by the following formula:

\[
\frac{C - T}{C} \times 100
\]

where, \( C \) = Growth of Control and \( T \) = Growth of Treatment
Effect of Some Heavy Metals on Growth of Some Dominant Microflora and the Test Pathogens

Five heavy metals viz., cadmium (Cd), chromium (Cr), lead (Pb), Nickel (Ni) and Zinc (Zn) in the form of their salts i.e. CdCl₂, H₂O, CrCl₂, Pb (NO₃)₂, NiCl₂ · 6H₂O and ZnSO₄ · 7H₂O were selected and their effects against some dominant microflora (as mentioned earlier) and test pathogen (F. oxysporum f. sp. lycopersici) was evaluated by poisoned food technique.

Dominant microflora was calculated with the help of percent frequency and percent occurrence from isolated microflora of soil sample irrigated with effluent and control soil samples.

250 ml Erlenmeyer flasks containing 100 ml sterilized and cooled (40°C) Czapek dox agar medium, Jensens’s medium and Thornton’s medium for fungi, actinomycetes and bacteria respectively, were amended with calculated quantity of each heavy metal to make the final concentrations as 25, 50, 100 and 200 ppm for Cd, Cr, Pb and Ni whereas 200, 400, 600 and 800 ppm for Zn. Twenty ml of supplemented medium was poured in each of sterilized 9 cm dia. Petri dishes in three replicate for each treatment and was inoculated at the centre of the Petri dish with a 5 mm agar block, cut
from the margins of actively growing 5 days old cultures of fungi. The media without heavy metals were also inoculated and treated as control.

All the Petri dishes were incubated at 25 ± 2°C for fungi, actinomycetes and bacteria, respectively and the radial growth of the pathogens and microorganisms were measured after 7 days of incubation. The percent inhibition of growth of pathogen and other microorganisms was calculated by comparing the results of the treated plate with that of control. Percent inhibition or stimulation of microorganisms were calculated by the following formula:

\[
\frac{C - T}{C} \times 100
\]

where, \( C \) = growth of control

and \( T \) = growth of treatment.

**Per Cent Incidence of Wilt Disease of Tomato from the Selected Fields**

During three successive cropping years of tomato i.e., 2003 – 04, 2004 – 05 and 2005 – 06 the wilted plants along with the healthy ones were counted in experimental fields every year from November to March. Twelve random samplings were done in each
month with the help of Quadrat (100 x 100 cm²). The per cent incidence of wilting was calculated by the following formula:

\[
\text{% Wilting} = \frac{\text{Number of wilted plant}}{\text{Total number of plants}} \times 100
\]

Statistical Analysis

The data obtained during the course of studies were subjected to statistical analyses by applying analysis of variance.

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