M.Sc. (Previous) MICROBIOLOGY

Practical-II : MICROBIAL PHYSIOLOGY, ENVIRONMENTAL AND AGRICULTURAL MICROBIOLOGY

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1. DETERMINATION OF BACTERIAL GROWTH CURVE

Aim: To determine the bacterial growth curve by colorimetric method.

Principle: Under optimal conditions, Bacteria exhibits 'S' shaped growth curve pattern. Growth curve includes 3 phases lag phase, log phase and stationary phase. A 4^{th} phase called death phase follows the stationary phase which gives the sigmoid shape to the growth curve.

Requirements: Test tubes, conical flasks, measuring cylinder etc.

Procedure: Prepare 150 ml of nutrient broth medium by using the following composition. Beef extract: 3g; Peptone: 5 g distilled water 1000 ml. Disperse about 10 ml of the medium into each of the 12 test tubes. Sterilize the tubes by autoclaving at 121^oC temp. and 12 lbs pressure for 15 minutes. After sterilization, cool the test tubes under running tap water. Inoculate the tubes with loopful of bacterial suspension in front of a spirit lamp, in an inoculation chamber. Then incubate them at 28^oC in an incubator. At bihourly intervals take one test tube and observe the growth of bacteria by measuring the O.D. of the culture at 600 nm using a colorimeter. Tabulate the OD values at bi-hourly intervals, upto 24 hours. Plot a graph, by taking the time in hours on 'X' axis and O.D. values on Y axis.

Result:

S.No.	Hours after inoculation	Optical density
1	2	
2	4	
3	6	
4	8	
12	24	

Under optimal availability of nutrients, in a batch culture, bacteria usually exhibit ' _I¯ ' shaped growth curve. It is divided into lag phase, exponential phase, stationary phase and death phase. In lag phase, no growth was observed, because bacteria when inoculated fresh into a medium, require sometime to divide In (log phase) exponential phase cell divide rapidly as per the doubling time of individual bacteria. In stationary phase there is no net increase or decrease in number of bacterial cell number. In death phase, decrease in OD values was observed due to death of the cells by lysis.

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2. EFFECT OF TEMPERATURE ON BACTERIAL GROWTH

Aim: To study the effect of temperature on bacterial growth by colorimetry.

Principle: The optimum range of temperatures requires for better growth of bacteria differ from one organism to another. Depending on this optima, bacteria are broadly classified into psycrophiles, mesophiles and thermophylies.

Requirements: Test tubes, measuring cylinder, conical flask, colorimeter etc.

Procedure: Prepare 100 ml of nutrient broth medium by using the following composition: Beef extract 3 g peptone 5 g dis. water 1000 ml. Take 3 big test tubes and disperse about 25 ml of the medium into each test tube. Sterilize them by autoclaving. After sterilization, cool the test tubes under running tap water. Inoculate the tubes with loopful of bacterial culture in front of the flame, in inoculation chamber. Label them as 10^oC, 37^oC and 50^oC, and incubate them at temperatures according to their labelling for 24 hrs. After incubation, study the growth of bacteria by taking the optical density values at 600 nm wave length using a colorimeter. Tabulate the results and report the type of given bacterial suspension.

S.No.	Temp.	OD values
1	10^oC	0.03
2	37^oC	0.45
3	50^oC	0.30

Result: As in the given example, Maximum OD value was observed for the test tube incubated at 37^oC. Therefore, the given bacterial culture is mesophelic type.

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3. EFFECT OF pH ON BACTERIAL GROWTH

Aim: To study the effect of pH on bacterial growth.

Principle: Bacteria usually prefer neutral pH for optimal growth. However, some bacteria are acidophilic and some are alkalophilic which can grow in a acidic and alkaline pH respectively.

Requirements: Test tubes, measuring cylinder, conical flasks, cotton etc.

Procedure: Prepare 150 ml of nutrient broth medium by using the following composition: Beef extract 3 g, peptone 5 g distilled water 1000 ml. Transfer 50 ml aliquots of medium into 3 conical flasks. Adjust the pH of 1st conical flask to pH 5, 2nd flask to 7 and 3rd flask to pH 9 by adding either diluted alkali or diluted acid. Take 25 ml of medium from 1st conical flask transfer into a boiling test tube and label it as 5. Similarly from 2nd and 3rd conical flasks, transfer 25 ml aliquots in to two separate boiling test tubes and label them as 7 and 9. Sterilize the test tubes at 15/121 lb/^{o}C for 15 minutes. After sterilization, cool the test tubes under running tap water. Inoculate the tubes with loopful of bacterial suspension (*B. subtilis* culture). Incubate the tubes at room temp. for 24 h. After incubation, study the effect of pH on bacterial growth by taking the optical density of the culture tubes at 600 nm wave length, using colorimeter. Tabulate the results and comment of them.

S.No.	pH	O.D. values
1	5	
2	7	
3	9	

Result: If the maximum O.D. values were observed at pH 7 indicating that the given bacteria suspension is a common bacterial species that requires neutral pH. If it was observed at pH^{5} it is acidophilic and at pH^{9} it is alkalophilic bacteria.

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4. EFFECT OF CARBON SOURCES ON BACTERIAL GROWTH

Aim: To study the effect of two different carbon sources – Glucose, Dextrose and Sucrose on bacterial growth.

Principle: Bacteria respond positively to the application of additional carbon source in the growth medium. Among the different carbon sources simple sugars like Glucose and Dextrose are preferred to disaccharides like sucrose.

Requirements: Test tubes, measuring cylinders, conical flask etc.

Procedure: Prepare 150 ml of 0.5% peptone water by dissolving 750 mg of peptone in 150 ml of distilled water. Disperse the peptone water in to 3 conical flasks in equal volumes of 50 ml each. Label them as control, glucose and sucrose. Take 0.5 g of D-glucose and sucrose and add to medium in the flasks labeled as Glucose, Dextrose and Sucrose. Sterilize the four flasks (including the control) by autoclaving at 121^{o}C temp. and 15 lbs pressure for 15 minutes. After sterilization cool them under running tap water. Take 3 sterilized test tubes and transfer 20 ml of medium from each of the three conical flasks separately into these test tubes. Inoculate all the three test tubes with a loopful of bacterial suspension and incubate them at room temperature for 24 hours. After incubation, study the bacterial growth by measuring the O.D. values at 600 nm wave length using a colorimeter. Tabulate the O.D. values and plot a histogram by taking different carbon sources on 'X' axis and the O.D. values on 'Y' axis.

Result: Usually maximum growth was observed in the test tube with medium amended with glucose, followed by sucrose and control.

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5. EFFECT OF SALT CONCENTRATION ON BACTERIAL GROWTH

Aim: To study the effect of NaCl concentration on growth of bacteria.

Principle: Bacteria, which tolerate the NaCl concentration of more than 3% are referred to as halophiles.

Requirements: Test tubes, conical flask, measuring cylinders etc.

Procedure: Prepare 150 ml of nutrient broth medium by using the following composition : Beef extract : 5 g Peptone 5 g; distilled water : 1000 ml.

- Disperse the medium into 4 conical flasks in equal proportions of 50 ml each.
- To the first conical flask add 250 mg of NaCl to get 0.5% concentration.
- To the second conical flask add 2.5 g of NaCl to maintain 5% concentration, and
- To the third conical flask add 5 g of NaCl to maintain 10% concentration. Then
- Transfer 10 ml of medium from each of the 4 conical flasks into one set of 4 test tubes. Label them as 0.5, 5, 10 corresponding to the NaCl concentrations from which medium was transferred. Similarly, prepare the second set of 3 test tubes with 10 ml of medium from each of the 3 conical flasks.

Sterilize all the 6 tubes by autoclaving at 121^{o}C temp. and 15 lb's pressure for 15 minutes. After sterilization, cool the test tubes under running tap water. Inoculate 1st set of 3 test tubes labeled as 0.5, 5 and 10 with a loopful of bacterial suspension and incubate them at room temp. for 2-3 days. The second set will serve as blanks.

Maintain a test tube with nutrient broth (without NaCl) medium and inoculate with bacterial culture. Observe the OD values along the above 3 test tubes this will serve as control for comparison.

After incubation, study the growth of bacteria by measuring the O.D. values of the 3 test tubes at 600 nm wavelength using colorimeter. Tabulate the O.D. values.

Results: If best growth with maximum O.D. values was observed in the first test tube, the bacteria was considered as non-halophilic. If maximum O.D. values was observed in the second and third test tubes, the bacteria wave considered as salt tolerant.

Sl. No.	Salt concentration	O.D. values
1	0.5%	
2	5%	
3	10%	
4	control (without NaCl)	

6. ISOLATION OF BACTERIA AND FUNGI FROM AIR

Aim: To isolate the bacteria and fungi present in air.

Requirements: Sterilized petriplates, conical flasks measuring cylinder, cotton etc.

Procedure:

(a) for isolation of fungi:

Prepare 100 ml of Czapeck-Dox medium by using the following composition:

Sucrose	: 30.0 g
Sodium Nitrate	: 2.0 g
Dipotassium Phosphate	: 1.0 g
Potassium Chloride	: 0.5 g
Ferrous sulphate	: 0.01 g
Agar Agar	: 20.0 g
Distilled water	: 1000 ml
pH	: 4.5

Sterilize the medium by autoclaving at 15 lb pressure and 121^{o}C temp. for 15 min. After sterilization, when the medium was about to solidity, add 5 mg/l of streptomycin sulphate and mix the contents thoroughly. Then disperse about 27 ml of the medium into each of the sterilized petriplates, in front of the flame, in an inoculation chamber. The plates were allowed to solidity.

Expose the media plates to air by placing them in an inclined position i.e., 45^{o} against the direction of the wind for 10 minutes. Repeat the exposures at different heights (Ground level, 5 m, 10 m and 15 m). Incubate the plates at room temperature for 3-4 days. After incubation, count the number of colonies per plate and tabulate the results and discuss on them.

(b) for isolation of bacteria:

Prepare 100 ml of Nutrient Agar medium using the following composition:

Beef extract	: 3 g
Peptone	: 5 g
Agar Agar	: 20 g
distilled water	: 1000 ml
pH	: 6.8

Sterilize the medium by autoclaving at 15 lb pressure and 121^{o}C temp. for 15 min. After sterilization, cool the medium to 40^{o}C, disperse about 27 ml into each sterilized petriplate in front

of the flame in an inoculation chamber. Allow the petriplates to solidify. Expose the plates to air by placing them in an inclined position (at 45^{o}) against the wind direction for 10 minutes. Repeat the exposures at different heights (ground level, 5 m, 10 m, 15 m). Incubate the plates at room temp. for 24 hrs. After incubation count the number of colonies per plate, tabulate the results and discuss.

Result:

S.No.	Place of exposure	No. of fungal colonies/plate	No. of bacterial colonies / plate
1	Ground level	15	65
2	5 m	10	45
3	10 m	5	25
4	15 m	4	10

As given in the example, the number of colonies decrease with increase in height.

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7. OBSERVATION ON AIR SPORA

Aim: To study the structure of different air spora present in air.

Principle: Air spora includes the spores of fungi, actinomycetes and angiosperm pollen grains etc. Air spora can be trapped by different methods. The spores can by photographed from spore trap or from any standard reference books, slides and studied for the structure of spores/pollen.

Requirements: Microphotographs taken from burkard spore trap slides.

Procedure: Draw the neat diagrams from the microphotographs. Draw the diagrams of fungal spore types, including conidial types, basidiospore types and ascospore type and pollen grains.

Write a few morphological characters of each spore types.

Result: Characteristic features of different air spora types.

(a) Conidial types:

(i) Cladosporium: Conidia are produced in chains. It is an aggregate spore types. Each conidia is pale olivaceous, oval, single celled with prominent scars on both sides.

(ii) Aspergillus: Spores are very small; round to oval thin walled, hyaline, with smooth or attenuated cell wall. Spores produced in chains.

(iii) Periconia: Conidia are round, reddish brown, thick walled, either smooth or attenuated of varying sizes.

(iv) Nigrospora: Conidia are round, dark black in colour, thick walled of varying sizes.

(v) Alternaria: Conidia are dictyospore type with horizontal and vertical septa, 6-10 celled, thick walled, reddish brown with an elongated beak at the base and prominent scar at the apex. Conidia produced singly or in chains.

(vi) Corynespora: Conidia are elongated, multicellular with 3-11 cells, very thick walled hyaline to pale olivaceous in colour with a prominent scar at the apex.

(vii) Trichoconis: Conidia are multicellular with 3-6 cells. Cells are broader at the center, thin walled, reddish brown with a beak at the base and a scar at the apex.

(viii) Phaeotrichoconins: Conidia are multicellular with 3-8 celled, dark reddish brown, very thick walled, heavy spore with a long beak and a prominent crescent shaped scar at the apex.

(ix) Curvularia: 3-4 celled structure, 3rd cell enlarged, dark brown in colour, with a prominent scar at the apex.

(x) Dreschlera: 3-9 celled, thick walled, brown, multicellular structure with a prominent scar at the apex.

(b) Basidiospore types:

(i) Bunt spore: Round, blackish spore with wrinkled margins and big in size.

(ii) Ganoderma: Oval shaped, small, single celled, with two cell walls, outer wall in smooth and inner wall is attenuated.

(iii) Uredeniospores: Rust spores are round or sub spherical, thick walled, reddish brown spores with smooth are attenuated cell walls.

(c) Ascospore types:

(i) Single celled ascospore: Single celled oval dark coloured, thin walled without any scar of attachment.

(ii) Bi-celled ascospore: Bi-celled, oval dark coloured, thin walled, without any scar of attachment.

(iii) Sporormia: Ascospore type, 4 celled structure produced as set of 2 celled units, dark reddish brown, thin walled.

(iv)

(d) Pollen types:

Cyperaceae: Grass pollen are round, big, thick walled, without any visible pores.

Asteraceae: Pollen are small, round, with spinous exine.

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8. OBSERVATION OF PHYTOPLANKTON

Aim: To collect and identify the different phytoplankters in the water body.

Theory: Phytoplankton are the microscopic plant groups which are free floating in water bodies. They are abundant in surface waters. Mostly they belongs to cyanophyceae, chlorophyceae, euglenophyceae and Bacillariophyceae.

Requirements: Plankton net, microscopic slides, beakers etc.

Procedure: Collect the surface waters by towing the plankton net till sufficient quantity of plankton was accumulated in the collecting tube. Transfer the phytoplankton collected into glass voils and add few drops of 4% formalin for preservation. For direct observation, brought the sample to the laboratory without addition of formalin. Observe the collected phytoplankton by placing a drop of example from collecting tubes on clean glass microscopic slide, under compound microscope. Identify the genera by referring to standard figures.

Result: The following phytoplankton were observed and identified.

(i) Microcystis: Cyanophycean members, forms algal blooms, many small round or spherical cells irregularly distributed through out the gelatenous matrix or in a series of rows in 3 pairs perpendicular to each other.

(ii) Spirulina: Flamentous cyanophycean member. The trichome in unicellular, elongated, more or less permanently spirally coiled.

(iii) Nostoc: Filamentous cyanophycean members, cells are globular, filaments are embedded in a gelatinous tegnment. Heterocystous form.

(iv) Anabaena: Filamentous, heterocystous, cyanophycean members. Filament comprises of bead or barrel shaped cells without any gelatinous tegument.

(v) Oscillatoria: Filamentous, uniseriately multicellular, unbranched, broad cells. Mucillagenous sheath absent.

(vi) Cosmarium: (Diatom) Bacillariophycean member, cells oval or spherical, entire margin.

(vii) Pandorina: The coenobia are sub spherical to ellipsoidal and possess 4, 8, 16 or 32 flagellate cells embedded within a homogenous gelatinous matrix. Each cell is chlamydomonas type.

(viii) Eudorina: The coenobia are spherical or broadly elliptical. Each colony possesses 16, 32, 64 or 128 cells. Each cell is chlamydononas type. The common number is 32.

(ix) Volvox: The coenobia are multicellular ball like structures with many cells ranging from 500-50,000 cells. Each cell is chalmydomonas like and are distributed at the periphery margin of the ball like coenobia.

(x) Pediastrum: Chlorococcalean member. The coenobia are made up of 4, 8, 16, 32, 64 or 128 celled. Each cell is polygonal in shape. The cells are arranged in rows in the form of stellate plate. The cells are arranged in concentric rings. The peripheral cells of the colony shows processes.

(xi) Scenedesmus: Coccoid forms. 4 to 8 fusiform cells united side by side in one row, sometimes in two alternating rows.

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9. TEST FOR COLIFORMS – MULTIPLE TUBE FERMENTATION METHOD

Aim: To test the water sample for the presence of coliforms by multiple tube fermentation test.

Principle: Coliforms are facultative anaerobic organisms that ferment lactose and produce gas.

Requirements: Test tubes, Durham's tubes, pipettes, conical flasks, measuring cylinder.

Procedure: Prepare 100 ml of single strength and 50 ml of double strength lactose broth medium by using the following composition.

Lactose – 5 g
Peptone – 5 g
Beef extract – 3 g
pH – 6 g
distilled water – 1000 ml

Double the ingredients to prepare the double strength medium

Disperse 10 ml of single strength lactose broth (SSLB) in to each of the 10 test tubes.
Disperse 10 ml of double strength lactose broth (DSLB) into each of the 5 test tubes.
Introduce the durham's tube in an inverted position in all the test tubes. Sterilize the tubes with medium by placing a cotton plug on each test tube.
After sterilisation, lable 5 DSLB tubes as 10 ml and the next 5 SSLB tubes as 1 ml and the remaining 5 SSLB tubes as 0.1 ml.
Inoculate the tubes with water sample in all the 15 test tubes as per the labelling.
Incubate the tubes at 37^{o}C and examine for the gas formation in Durham's tubes after 24 h.
Record the test tubes with more than 50% gas production as positive ('+'ve) tubes.
Compare the number of '+'ve values in all dilutions with the standard MPN table values and report your results as MPN index per 100 ml.

Result: 3-1-0

If your test result is 3-1-0 (3 '+' 10 ml tubes, 1 in 1 ml tubes and 0 in 0.1 ml tubes), then the corresponding MPN table value is 11. This means MPN index per 100 ml = 11.
This means MPN index per 100 ml = 11
At 95% confidence level the lowest number of coliform is 4 and upper limit is 29.

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10. TEST FOR WATER QUALITY – MEMBRANE FILTRE METHOD

Aim: To determine the quality of water sample, by detecting the coliforms, using membrane filtres.

Principles: Coliforms, especially *E. coli,* are the indicators of water pollution. By using differential media, coliforms and non-coliforms can be easily be detected. On Eudo agar *E. coli* colonies produce characteristic green metallic sheen.

Requirements: Millipore filter apparatus, filter papers; suction pump, endo agar media plates, sterile distilled water, spirit lamp, foreceps, measuring cylinders etc.

Procedure: Prepare 5 Endo agar media plates using Hi-media dehydrated medium. Allow the plates to solidify.

Assembly of filter apparatus:

- Aseptically assemble the filter apparatus and insert the membrane filter in it by the method described below:

1) Place the filter holder aseptically in the buchner flask.
2) Attach the flask to the vacuum line through rubber testing.
3) Using flamed forceps place a sterile membrane filter disc, on the porous glass support of the filter holder.
4) Set the funnel on the filter holder and fasten in place.

Filtering: Shake the water sample and pour 100 ml into the funnel, and filter under vacuum. After the sample in completed, wash the inner walls of the funnel with 100 ml of sterile water. Then carefully remove the filter from the filter holder using sterile forceps. Transfer the filter paper on to Endo media plates and incubate at 37^{o}C for 24 h. After incubation coliforms appear pink to red in colour and *E. coli* colonies appear with green metallic sheen. Non-coliforms appear colourless. Count the number of colonies in each plate and tabulate the results and calculate the coliforms by using the following formula:

$$\text{No. of coliforms / 100 ml} = \frac{\text{Colony count/plate}}{\text{Volume of sample}} \times 100$$

Result and Discussion:

The recommended number of coliforms to be present in a water to consider it as potable water is 0/100 ml according to WHO & USEPA. If coliforms are observed the water is not potable.

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11. ESTIMATION OF DISSOLVED OXYGEN

Aim: To estimate the amount of dissolved oxygen present in the water sample by Winkler's method.

Principle: A white precipitate of $Mn(OH)_2$ is generated in the sample which absorbs oxygen to form a brown Manganic oxide of uncertain composition. After acidification, Mn^{+4} reacts with iodide to liberate Iodine in an amount equivalent to that of the original oxygen. The iodine is determined by titration with thiosulphate.

Reagents Preparations:

i) **$MnSO_4$ solution (Winkler's 'A'):** Dissolve 91.0 g of $MnSO_4$ in 250 ml of distilled water.

ii) **Alkaline iodide (Winkler's 'B'):** Dissolve 15 g of NaOH and 33.75 g of Sodium Iodide (NaI) in distilled water and dilute it to 250 ml.

iii) **Sodium thiosulphate (0.0025N):** Dissolve 0.620 g of $Na_2S_2O_3$. $5H_2O$ in freshly prepared distilled water and dilute it to 1 litre.

iv) **Starch solution:** 1 gm of starch is boiled in 100 ml of distilled water.

v) **Sulphuric acid:** Mix 50 ml of conc. H_2SO_4 with 50 ml of distilled water.

Procedure: Carefully fill the 500 ml stopper bottle with the water sample. Place the stopper firmly (neglect the amount of spilled water from bottle). Care should be taken to avoid entry of air bubbles. After few minutes, introduce 2 ml of $MnSO_4$ (Winkler's A) and 2 ml of Alkaline iodide (Winkler's B) into the bottle with pipettes and replace the stopper. Entry of air bubble should be avoided. A white precipitate of $Mn(OH)_2$ is formed.

Allow the precipitate to settle, if slow, shaking may be helpful. Introduce 4 ml of H_2SO_4 into the bottle and replace the stopper immediately. Shake the contents well. The ppt. dissolves, resulting in dark brown solution. Take 50 ml of this solution into a conical flask, titrate it against hypo after adding 1 ml of starch solution. The titration should be done quickly to minimize the loss of Iodine by volatilization. First disappearance of blue colour is taken as the end point. Tabulate the results and calculate the amount of dissolved oxygen present in the water sample.

S.No.	Burette reading		Amount of hypo rundown
	Initial	Final	

Calculate the amount of dissolved oxygen by using the following formula:

$$\text{D.O. mg/lit} = \frac{\text{Vol. of Hypo} \times \text{Normality of hypo} \times 1000 \times 8}{\text{Vol. of sample (50 ml)}}$$

Result: The give water sample contain ___ mg/lit of dissolved oxygen.
Waters with low dissolved oxygen content (<4%) are considered as most polluted waters.

12. ENUMERATION OF SOIL MICROBIAL POPULATIONS

(a) ISOLATION OF SOIL FUNGI

Aim: To isolate the fungi from soil by dilution plate method.

Principle: At ultimate dilution, individual fungal cell will gives rise to a single colony.

Requirements: Sterilized petridishes, pipettes, test tubes, conical flasks, measuring cylinder, cotton, sterile distilled water etc.

Procedure: Prepare 250 ml of Czepeck-Dox medium by using the following composition.

Sucrose	: 30.0 g
Sodium Nitrate	: 2.0 g
Dipotassium Phosphate	: 1.0 g
Potassium Chloride	: 0.5 g
Ferrous sulphate	: 0.01 g
Agar Agar	: 20.0 g
Distilled water	: 1000 ml
pH	: 4.5

The medium was sterilized by autoclaving at 121^{o}C and 15 lbs pressure for 15 minutes. After sterilization, about 20 ml of the medium was dispersed into each of the sterilized petriplate. The preparation of plates to be done in front of a spirit lamp in an inoculation chamber to avoid contamination. Allow the plates to solidify.

Preparation of serial dilutions: Take one gram soil sample in 250 ml conical flask and add 100 ml of sterile distilled water to prepare 10^{-2} (1:100) dilution. From 10^{-2} dilution take one ml and to 9 ml of sterile distilled water in a test tube to get 10^{-3} (1:1000) dilution. Likewise, prepare up to 10^{-4} dilutions in separate test tube.

Plating: Take 0.5 ml of soil suspension from 10^{-3} and 10^{-4} dilutions and add to each of the two media plates. Spread the inoculum on the surface of the medium by using a spreader a bent glass rod. Maintain three replicates for each dilution. Incubate the plates at room temperature for 24 hrs. After incubation count the number of fungal colonies. Count the number of colonies per plate in all the petriplates. Tabulate the results and represent your result as number of fungal colonies or colony forming units per plate.

Dilution	Replicates	No. of colonies per plate	No. of CFU's/Plate (av. of 3 plates)
10^3	1		
	2		
	3		
10^4	4		
	5		
	6		

Result: Number of CFU's are more in 10^{-3} dilution than 10^{-4}. Number of fungi decreased with increase in dilution.

Precaution: Care to be taken while preparing the serial dilutions. While transfering take 1 ml of suspension only after thorough shaking (which avoid loss of soil particles during transfer).

(b) ISOLATION OF BACTERIA FROM SOIL

Aim: To isolate the bacteria from the given soil sample by dilution plate method.

Principle: At ultimate dilution, individual bacterial cell gives rise to a single colony.

Requirements: Sterilized petriplates, pipettes, test tubes, conical flasks, measuring cylinders etc.

Procedure: Prepare 250 ml of Walkimoto agar medium by using the following composition:

$Ca(NO_3)^2 .4H_2O$ – 1 g
$FeSO_4 . 7H_2O$ - 1 g
Peptone – 5 g
Sucrose – 15 g
Di-sodium hydro orthophosphate – 2 g
Distilled water - 1000 ml
pH 7

The medium was sterilized by autoclaving at 121^{o}C and 15 lb pressure for 15 min. After sterilization, when the medium was about to solidify (at 45^{o}C) add 50 mg/l of streptomycin sulfate and mix the contents thoroughly. Then disperse about 20 ml of the medium into each of the sterilized petriplate, in front of a spirit lamp, in an inoculation chamber. Allow the plates to solidify.

Preparation of serial dilutions:

Take one gram of soil sample into (250 ml) conical flask and add 100 ml of sterile distilled water (1:100). From this, take one ml and add 9 ml of sterile distilled water in a test tube (1:1000). Repeat the same until the final dilution reaches upto 1: 10,00,000.

Plating: Take 0.5 ml of soil suspension from 10^{-5} (1 : 1,00,000) dilution and spread over each petriplate containing the medium, maintain 3 plates for each dilution. Similarly, take 0.5 ml soil suspension from 10^{-6} (1:10,00,000) dilution and spread on each petriplate containing medium. Maintain 3 plates (replicates) for each dilution. Incubate the plates at room temperature for 24 hrs. After incubation, observe the plates for bacterial colonies. Count the number of colonies per plate in all the plates and report your results as number of colony forming units per plate at each dilution.

Dilution	**No. of the Plate**	**No. of colonies per plate**	**No. of CFU's/Plate (av. of 3 plates)**
10^5	1		
	2		
	3		
10^6	1		
	2		
	3		

Result: Number of CFU's are more in 10^{-5} dilution than 10^{-6}. The number of bacteria decreased with increase in dilution.

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ESTIMATION OF SOIL ORGANIC MATTER CONTENT OF SOIL SAMPLE

Aim: To estimate the amount of organic matter content in the given soil sample by Walklay-Black method.

Principle: The oxydisable organic matter of soil was determined by chromic acid oxidation with spontaneous heating. The organic carbon (matter) was determined by measuring the quantity of dichromate ion, an oxydizing agent, consumed in the process of organic matter oxidation.

Requirements: Soil sample, conical flasks (500 ml), Pipettes, Burettes, measuring cylinders etc.

Preparation of Reagents:

i) 1 N $K_2 Cr_2 O_7$ $\rightarrow$ dissolve 49.04 g of $K_2 Cr_2 O_7$ in 1000 ml H_2O

ii) Diphenyl amine $\rightarrow$ dissolve 0.5 g of di phenyl amine in 20 ml of distilled water and 100 ml of conc. H_2SO_4.

iii) 0.5 N Ferrous Ammonium Sulphate $\rightarrow$ Dissolve 196.1 g of salt in 800 ml of distilled water containing 20 ml of conc. H_2SO_4 and dilute it to 1 litre.

iv) 85% Orthophosphoric acid $\rightarrow$ Take 85 ml of orthophosphoric acid and dilute it to 100 ml with distilled water.

Procedure:

1. Take 0.5 g of soil sample in 500 ml conical flask. Maintain a blank without soil sample.
2. Add 10 ml of 1 $NK_2Cr_2O_7$ to each flasks and mix the contents thoroughly.
3. Add 20 ml of conc. H_2SO_4 to each flasks and allow the contents to react for 30 min.
4. Then make up the volume of the contents in each flask to 200 ml by adding 170 ml of distilled water.
5. Then add 10 ml of 85% orthophosphoric acid and 0.2 g of sodium flouride salt to each flasks.
6. Then add 1.5 ml of diphenyl amine indicator to each flasks.
7. The resulting solution will be bluish brown in colour.
8. Then titrate the total contents of the flask against 0.5 N Ferrous ammonium sulphate.

The end point will be brilliant green colour.
Tabulate the results and calculate the percentage organic matter content in the soil by using the following formula.

$$\% \text{ organic matter} = 10 \left(1 - {}^{T}/_{S}\right) \times 1.34$$

Where 10 = Volume of $K_2Cr_2O_7$ T = Sample titre value
1.34 = Black's constant
S = Blank reading

S.No.		Burette readings		Amount of acid run down (ml)
		Initial (ml)	Final (ml)	
1	Blank	0		
2	Sample	0		

Result: The percentage of organic matter in any soil is usually ranges from 2-6. In peat soils it may reach upto 9 or 10.

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14. ESTIMATION OF MICROBIAL ACTIVITY BY CO_2 EVOLUTION METHOD

Aim: To estimate the microbial activity in soil by CO_2 evolution method.

Principle: Carbon dioxide is liberated during respiration by microorganisms. Microbial activity can be estimated indirectly by the estimation of CO_2 liberated.

Requirements: Conical flasks, pipettes, burettes, glass vials, soil sample.

Reagent Preparation: i) 0.1 N $Ba(OH)_2 \rightarrow$ Dissolve 19 g of $Ba(OH)_2$ in 1000 ml dis. water
ii) 0.1 N HCl – add 1 ml of conc. HCl to 116 ml of dis. water.

Procedure: Take 100 g of soil sample in 250 ml conical flask. Maintain a flask without soil sample to serve as control.

- Add 15 ml of distilled water to the soil sample to maintain 50% water holding capacity.
- Add 2 g of glucose to the soil sample.
- Take 15 ml of 0.1 N $Ba(OH)_2$ in 2 glass viols, and hang them in each conical flask using a thread with rubber stopper.
- Make the flasks **air tight** by applying molten wax on the rubber stoppers.
- Incubate them at room temperature for one week.
- After incubation titrate the contents of the glass vials against 0.1 N HCl using phenophthelene as indicator.
- Appearance of pink colour is the end point.
- Tabulate the results and calculate the amount of CO_2 liberated by using the following formula.

$$\text{Amount of } CO_2 \text{ liberated} = \frac{\text{(Difference in titre values) X Normality of the acid X 22 X 15}}{\text{Size of the sample}}$$

$$= \text{mg of } CO_2/\text{g soil}$$

Where 22 is the equivalent wt of CO_2
15 is the volume of $Ba(OH)_2$

S.No.		Burette readings		Amount of acid run down (ml)
		Initial (ml)	Final (ml)	
1	Blank	0		
2	Soil Sample	0		

Result: The amount of CO_2 liberated is calculated. It is due to microbial respiratory activity in the soil.

*****

15. ESTIMATION OF CATALASE ACTIVITY

Aim: To estimate the catalase activity in the soil.

Principle: Soil microorganisms produce enzyme catalase, that catalyses the reaction of converting hydrogen peroxide into water and oxygen. In titremetry, $KMnO_4$ reacts with H_2SO_4 and resulted in the release of nascent oxygen as one of the end products. This nascent oxygen reacts with H_2O_2 remained unreacted by the soil enzyme and dissociate it to H_2O and oxygen. The amount of nascent oxygen necessary is directly proportional to the amount of H_2O_2 present.

Requirement: Conical flasks, pipettes, burette, measuring cylinder, volumetric flasks etc.

Reagent Preparation:

(i) 0.05 N $KMnO_4$ $\rightarrow$ dissolve 790 mg of $KMnO_4$ in 500 ml of distilled water.
(ii) 20% H_2O_2 $\rightarrow$ 16 ml of 30% H_2O_2 in 8 ml of distilled water.
(iii) 12% H_2SO_4 $\rightarrow$ Take 12 ml of conc. H_2SO_4 and add 88 ml of distilled water in a volumetric flask.
(iv) **Phosphate buffer** 6.8

Prepare Sol. A and Sol. B
Sol. A:- dissolve 35.61 g of Na_2HPO_4 in 1000 ml of distilled water.
Sol. B:- dissolve 31.2 g of NaH_2PO_4 in 1000 ml of distilled water.
Mix 49 ml of Sol. A and 51 ml of Sol. B and dilute it to 200 ml.

Procedure: Sterilize the phosphate buffer before use. Take 24 ml of sterilized phosphate buffer in to two conical flasks. Add 1 ml of 20% H_2O_2 to each flask. Then add 1 g of soil sample to one conical flask. The flask without soil serve as control. Incubate the flasks for 48 hrs. After incubation decant the contents and titrate the supernatant of the flask against 0.05 N $KMnO_4$ after adding 1 ml of 12% H_2SO_4. End point is appearance of pink colour. Tabulate the results and calculate the H_2O_2 oxidized during the incubation, by using the following formulas:

H_2O_2 present in the sample = Acid run down during titration for 25 ml x 0.85
H_2O_2 oxidized in soil during 48 h = H_2O_2 present in control – H_2O_2 present in soil sample

S.No.		Burette readings		Amount of acid run down
		Initial (ml)	Final (ml)	
1	Control	0		
2	Sample	0		

Result: H_2O_2 present in control is more than that in sample. In soil, due to enzyme activity of catalase the added H_2O_2 was oxidized. Therefore, the amount of H_2O_2 present in soil is less that of control.

*****

16. ESTIMATION OF UREASE ACTIVITY

Aim: To estimate the activity of the enzyme urease in soils by hydrolysis of urea.

Requirements: Conical flasks, pipettes, burette, measuring cylinder, funnel, filter paper etc.

Reagent preparations: (1) 1% Urea solution: 1 g of Urea dissolved in 100 ml of distilled water.
(2) 0.1 N H_2SO_4 :- Take 2.7 ml of H_2SO_4 in a volumetric flask, make up the volume to 1000 ml by adding distilled water.

Procedure:

- Take 5 g soil sample in to a 150 ml conical flask.
- Add 0.5 ml toluene to the flask to arrest the microbial activity.
- Incubate the flask for 2 hours.
- Maintain a flask without soil sample to serve as control.
- After the solvent get evaporated, add 50 ml of 1% Urea solution to each flasks.
- Incubate the flasks for 2 h and after incubation filter the contents.
- From the filtrate, take 10 ml aliquots and titrate against 0.1 N H_2SO_4 after adding few drops of methyl red indicator.
- End point is appearance of red colour.
- Tabulate the results and calculate the amount of ammonia liberated by using the following formula:

$$\text{Amount of } NH_3 \text{ liberated} = \frac{T \times N \times \text{eq. wt.}}{\text{Sample size (10)}} \text{ mg / g soil}$$

Where T = difference in titre value
N = Normality of acid
eq.wt. of NH_3 = 17.1

S.No.		Burette readings		Amount of acid run down (ml)
		Initial (ml)	Final (ml)	
1	Control	0		
2	Soil Sample	0		

Result: Amount of acid run down in control is very less, compared to sample. Because, in control there is no enzyme activity. In sample, due to enzymatic action, some portion of the urea was hydrolysed. The liberated ammonia reacts with the CO_2 to form Ammonium carbonate. Ammonium carbonate react with sulphuric acid to give the end product Ammonium sulphate.

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17. ISOLATION OF *RHIZOBIUM* FROM LEGUME ROOT NODULES

Aim: To study the colony characters of *Rhizobium* by isolating from root nodules.

Principle: *Rhizobium* is the gram negative rod shaped bacteria, that forms nodules in the roots of legume plants. It helps in fixing the atmospheric nitrogen.

Requirements: Sterilized petriplates, conical flasks, pipettes, measuring cylinders, glass rod etc.

Procedure: Gently uproot a legumenous plant, wash the root system under running tap water to remove the soil. Separate the nodules from the roots with the help of a sharp blade (nodule should contain a portion of root attached). Wash the nodules in running tap water and surface sterilize them by keeping them in 0.1% ($HgCl_2$) mercuric chloride or 5% sodium hypochloride solution for 5 minutes. Transfer the nodules with sterile forceps into a sterile petriplate containing 10 to 20 ml of 95% ethanol and keep them for 2 minutes. Remove the excess of alcohol on the surface of the nodules by washing them in sterile distilled water. Take 2-3 nodules into a sterile test tube and crush them with the help of a glass rod, in presence of 1 m of sterile distilled water. Thus, bacterial suspension is prepared.

Prepare 100 ml of yeast mannitol agar medium by using the following composition:

Dipotassium hydrogen orthophosphate	- 0.5 g
Mannitol	– 10 g
Magnesium sulphate	– 0.2 g
Yeast	– 1.0 g
Sodium chloride	– 0.1 g
Distilled water	– 1000 ml

After sterilization, 2.5 ml of 1% congo red solution was added to the medium. Then disperse about 20 ml of the medium into each sterilize a petriplate. Allow them to solidify. Take a loopful of bacterial suspension from the test tube and streak on the petriplate. Incubate the plates at room temperature for 2-3 days. After incubation, small, round, translucent, white, slimy colonies are identified as *Rhizobium* colonies.

Note: Congo red is used to differentiate *Rhizobium* from its closely related soil microorganism – *Agrobacterium*. Agro-bacterial colonies take up the dye in and appear red in colour.

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18. ANATOMICAL STRUCTURE OF LEGUME ROOT NODULES

Aim: To observe the internal structure of the root nodule, about the nature of *Rhizobium* inside the nodule.

Requirements: 1% saffranin, root nodules, microscopic slides, microscope.

Procedure: Gently uproot the legume plant, wash the roots under running tap water to remove the soil.

- ✈ Separate the nodules from main root system with help of a sharp blade. (Each nodule should contain a root portion attached).
- ✈ Take thin free hand sections of root nodule, at the region of attachment between root and nodule.
- ✈ Transfer the very thin section on to a clean glass slide with the help of fine brush.
- ✈ Mount the section with lactophenol, after staining with saffranin.
- ✈ Place the cover slip, gently and observe under the compound microscope.

Result: The cross section of nodule shows outer epidermis with cuticle and inner corticular tissue. At the centre of the nodule, a zone of bacteriods is present. The bacterioids are yellow coloured cells, 2-3 of which are usually covered by a peribacteriod membrane. From the root vasculature is seen travelling on either side of the central bacterial zone.

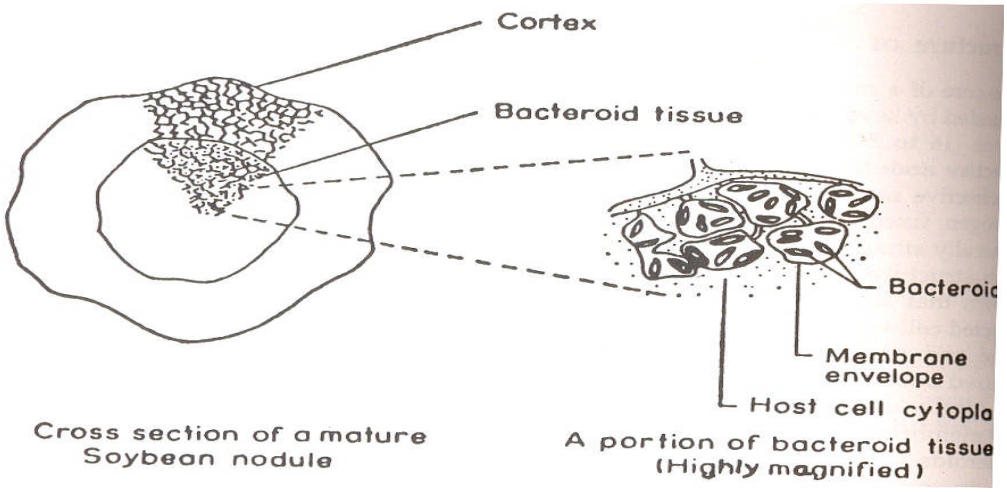

Fig. T.S. root nodule

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19. ISOLATION OF *AZOSPIRILLUM LIPOFERUM*

Aim: To isolate *Azospirillum lipoferum* from monocot roots.

Principle: On nitrogen free sodium malate agar medium. *A. lipoferum* produce white mycelial mat.
A. lipoferum is a nitrogen fixing bacterium, present in the roots of monocotyledonous plants.

Requirements: Screw cap glass test tubes, conical flasks, measuring cylinders, etc.

Procedure: Prepare 100 ml of semi-solid sodium malate agar medium by using the following composition.

Malic acid	– 5 g	$CaCl_2$	– 0.02 g
KH_2PO_4	– 0.5 g	$FeSO_4$	– 0.05 g
KOH	– 4 g	Sodium molybdate	– 0.002 g
$MgSO_4$	– 0.2 g	$MnSO_4$	– 0.01 g
NaCl	– 0.1 g	Agar	– 2 g
Distilled water	– 1000 ml		

Sterilize the medium after the addition of bromothymol blue dye (0.5 g in 100 ml of 20% alcohol). The prepared semi solid medium was dispersed aseptically into 10 screw cap test tubes. Sterilize them once gain. Gently uproot the *Cynodon dactylon* (grass) plant and wash the root system under running tap water to remove the soil. The roots were cut into 2.5 cm bits using a sharp blade. Surface sterilize these root bits in freshly prepared phosphate buffer (pH 7).

Introduce the root bit into screw cap test tubes with the help of a sterile forceps. Incubate the tubes for 24-48 hrs. After incubation, observe for the presence of white coloured colony growth.

Result: White colony was observed on the surface of the medium. *Azospirillum* is a flagellated slightly bent rod shaped bacterium.

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19. ISOLATION OF *AZOTOBACTER* FROM SOIL

Aim: To isolate *Azotobacter* spp. from soil by dilution plating method.

Principle: *Azotobacter* is a free living Nitrogen fixing bacterium. It produces soft flat milky and mucoid colonies on Jensen's medium.

Requirements: Sterilized petriplates, test tubes, pipettes, measuring cylinder, conical flasks etc.

Procedure: Prepare 100 ml of Jenson's medium by using the following composition:

Sucrose – 20.0 g
K_2HPO_4 – 1.0 g
$MgSO_4$, $7H_2O \rightarrow$ 0.5 g
NaCl - 0.5 g
$FeSO_4$ – 0.1 g
$CaCO_3$ – 2.0 g
Agar – 15.0
Distilled water – 1000 ml

Sterilized the medium at 120°C and 15 lb pressure for 15 min. in an autoclave. Disperse about 20 ml of the medium into each sterilized petriplate in front of a spirit lamp, in an inoculation chamber. Allow the medium to solidify.

Prepare serial dilutions of the soil sample. Take 1 g soil sample and transfer it to a conical flask containing 100 ml of sterile distilled water, mix the contents thoroughly. From this 10^{-2} dilution (1:100), take 1 ml and transfer it to a test tube containing 9 ml of sterile distilled water, this become 10^{-3} dilution (1:1000). From this, again, take 1 ml and mix it with 9 ml of sterile distilled water in a separate test tube, this become 10^{-4} dilution. Likewise, prepare up to 10^{-6} (1:1000000) dilutions. Take 1 ml from 10^{-5} dilution and spread it over media plate using a bent glass rod. Similarly take 1 ml from 10^{-6} dilution and spread it over a media plate. Similarly, prepare 2 plates each for 10^{-5} and 10^{-6} dilutions. Incubate them at 30°C for 3-4 days. After incubation observe the plates for the presence of *Azotobacter* colonies. Tabulate the results and count the number of CFU's in each plate.

Result: The *Azotobacter* colonies are characteristically flat, soft, milky white in colour and mucoid.

S.No.	Serial dilution	No. bacterial (av. of 2 plates) Colonies per plate
1	10^{-5}	
2	10^{-6}	

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20. PHOSPHATE SOLUBILIZING ACTIVITY OF MICROORGANISMS

Aim: To study the phosphate solubilizing ability of microorganisms in soil.

Principle: Some of the bacteria and fungi have the capacity to solubilize insoluble inorganic phosphates such as rock-phosphate, tricalcium phosphate and other phosphates by production of organic acids. Eg: *Pseudomonas striata, Bacillus polymyxa, Aspergillus niger* and *Penicillium digitatum*.

Requirements: Sterilized petriplates, conical flasks, measuring cylinders, pipettes, test tubes etc.

Procedure: Prepare 100 ml of Pikovskaya's medium by using the following composition:

Glucose	– 10.0 g	$MgSO_4,7H_2O$	– 0.1 g
$Ca_3(PO_4)^2$	– 5.0 g	$MnSO_4$	- trace
$(NH4)_2SO_4$	– 0.5 g	$FeSO_4$	- trace
KCl	– 0.2	Yeast extract	– 0.5 g
Distilled water	– 1000 ml	Agar	– 15.0 g

Sterilize the medium by autoclaving at 121^{o}C temperature and 15 lb pressure for 15 minutes. After sterilization, cool the medium and disperse about 20 ml into each sterilized petriplate in front of a spirit camp, in an inoculation chamber. Allow the medium to solidify.

Prepare serial dilutions using the soil sample. Take 1 g of soil sample, dissolve it in 100 ml of sterile distilled water to produce 10^{-2} dilution. Transfer 1 ml from 10^{-2} dilution into a test tube containing 9 ml of sterile distilled water to prepare 10^{-3} dilution, similarly prepare serial dilutions upto 10^6.

Take 1 ml of soil dilution from 10^{-5} dilution and spread it on a media plate in front of a spirit lamp, in an inoculation chamber. Similarly, inoculate another media plate with 1 ml of 10^{-6} dilution. Maintain two petriplates for each dilution. Incubate the plates at room temperature for 2-3 days. After incubation observe the plates for appearance of a transparent zone around the isolated bacterial colonies.

Result: A clear zone appeared surrounding the colonies, indicating that they are phosphate solubilizers.

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21. MICROSCOPIC OBSERVATION OF VAM COLONIZATION

Aim: To study the nature of VAM colonization in roots of higher plants.

Requirements: Microscope, Microscopic slides, Cover slips, needle, brush, blade etc.

Reagents: Preparation: (i) 10% Potassium hydroxide → Take 10 g of KOH dissolve in 100 ml
(ii) 10% HCl → Take 10 ml of HCl make up the volume to 100 ml by adding dis. water.
(iii) 0.1% Trypan blue → Take 100 mg of trypan blue dissolve it in 100 ml of dis. water.

Procedure:

- Gently uproot a monocot plant, wash the adventitious root system under running tap water to remove the soil.
- From the root system, select the fine roots and cut them into 1 cm bits using a sharp blade.
- Immerse these root bits in 10% KOH solution for 30 min.
- Wash the roots to remove the excess KOH and immerse them in (H_2O_2) hydrogen peroxide for 20 minutes.
- Wash the roots under running tap water to remove the hydrogen peroxide.
- Acidify the roots with 10% HCl for 10-15 minutes.
- Then stain the roots with 0.1% trypan blue: slightly boil the roots immersed in trypan blue, and leave them as such overnight.
- Take 8-10 root bits, place them on clean glass slide.
- Mount them with lactoglycerol.
- Place a rectangular cover slip over these root bits.
- Gently, press the cover slip over the root bits, for better spreading of the root tissue.
- Observe the root bits under compound microscope.
- Scan a minimum of 50 root bits for understanding the nature of VAM colonization.
- Observe the roots for various structures like, vesicles, arbuscules, external hyphae etc.

Result: VAM fungi produces external hyphae which are characterized by the presence of dimorphic nature. The broad and narrow hyphae. The broad mycelium is called knobby due to presence of protruberances at different places, and it is thicker than narrow mycelium. Vesicles and Arbuscules are internal structures. Arbuscule is the dichotomously branched hyphal structure produced inside the host cell. Vesicles are produced solitarly, both inter and intracellulary, which are thick walled storage structures.

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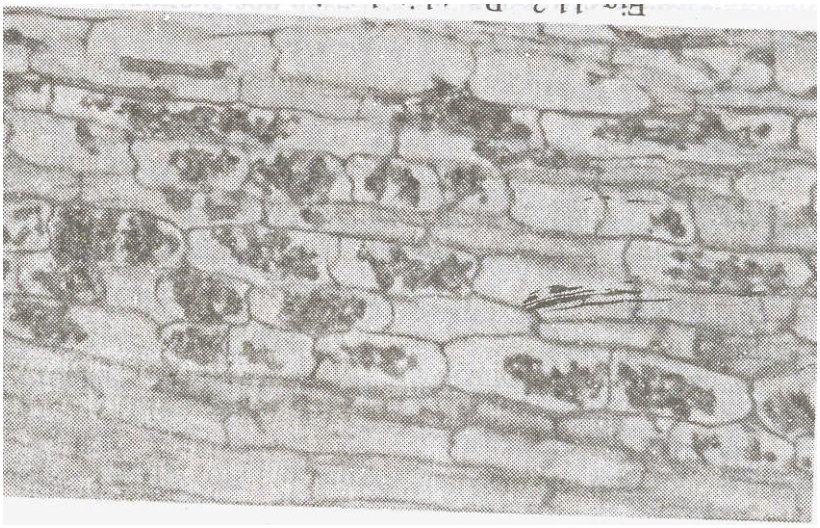

Root bit showing arbuscules

22. OBSERVATION OF PLANT DISEASE SYMPTOMS

Plants were prone to attack by number of diseases caused by pathogenic bacteria, fungi and viruses. Infection by the pathogens result in appearance of external morphological changes on plants called **symptoms**. The most common symptoms of plant diseases are rusts, smuts, powdery mildews, leaf spots, cankers and growth deformities. To observe these symptoms, diseased plants were collected during field trips.

Rusts: There are both 4,000 species of rust fungi distributed among 100 genera. They belongs to the order uredinales of class teliomycetes under Basidiomycotina. Rusts are obligate parasites. The symptoms caused by rust fungi are mainly due to the appearance of spore masses called sori, on infected parts. Rust fungi produce five types of spores viz., aeciospores, uredeniospores, teleuto spores, basidiospores and pycnidiospores. Of these uredeniospores are the dominant spores. These spores are reddish brown appear randomly on infected parts which gives the characteristic rusty appearance. These spores develop below the epidermis and expose to outside by rupturing the epidermis. Before exposing the spore mass below the epidermis, they appear like pus formation in the wound, hence the name pustules.

The rust pustules first appear on lower surface and later spread on upper surface of the leaves. The telial pustules are usually black in colour and appear not as pus but as crusts.

Rusts commonly occur on a number of weeds, crop plants and trees. Some important examples of rust diseases and the causal organisms are given below:

1) Wheat stem rust – *Puccinia graminis* var *tritici*

2) Groundnut rust – *Puccinia arachidis*
3) Blackgram rust – *Uromyces appendiculatus*

Smuts: Smut disease is very prevalent on sorghum, surgarcane, rice and other crops. Members belonging to the order : ustilaginales of teliomycetes under Basidiomycotina cause the smut diseases. These fungi usually attack the reproductive parts of the plants and produce characteristic black spore masses. There are about 75 genera and 1100 species of smut fungi. Some common smut diseases are:

(a) **Grain smut of sorghum** caused by *Sphacelotheca sorghi*.
The plant appear normal but the inflorescence was infected by fungus. As a result, in place of healthy grains, the infected grains with large black coloured spore mass, are seen.

(b) **Whip smut of sugarcane** caused by *Ustilaginoidea scitaminea*.
The entire inflorescence is transformed into long whip like structure, covered with black spore mass.
(c) **Smut of rice** caused by *Entyloma oryzae*. It is a leaf smut and symptoms appears like small black crusted sori on the entire lamina.

Powdery mildews:
These diseases are easily recognized in the field by the appearance of characteristic powdery mass on the aerial parts of the plants. Powdery mildews are caused by members of family Erysiphaceae belonging to ascomycotina. The symptoms are due to superficial growth of the pathogen on the surface and production of enormous number of hyaline conidia which appear as powdery mass.

They are common on a number of plants occur mostly during post rainy season. The common hosts are ***Croton, Euphorbia, Phyllanthus*, grapes, peas, pulse crops** and **others**.

Leaf spot diseases: Leaf spots are the most common necrotic symptoms formed due to death of the infected tissues. The characters of the spots vary with host pathogen combination. These diseases are early recognized in the field by their brown to reddish colour with or without margin. They vary in size and shape. Some appear like target spots with concentric rings.

The important leaf spot diseases are: **Tikka leaf spot of groundnut, leaf spot of chillies** etc. Two types of tikka spots are recognized in groundnut. **Earely leaf spots** caused by *Cercospora arachidicola* and the **late leaf spot** caused by *Cercosporidium personatum*.

Early leaf spots caused by *C. personatum* are round, 1-10 mm in dia, surrounded by a yellow halo on the upper surface. The spots are reddish brown to black on the upper surface and reddish brown on the lower surface. Leaf spots caused by *C. pesonatum* are smaller 1-7 mm 1 dia, dark black on upper surface and carbon black on lower surface. Sporulation is mainly on the lower

surface. The conidiophores are produced in groups or tufts forming stromata. These stroma are arranged in concentric rings on the lower surface of the leaf and can be seen with naked eye.

Leaf spot of chillies caused by *Cercospora capsici*. The symptoms appear like target spot as the spot grow in size. The leaf spots are mostly seen on older leaves than on young leaves. They are usually few in number per leaf. Sometime they coalse to form big spots. Usually 0.5 – 1 cm dia. The spots are round to irregular in shape appear on both surfaces of the leaf without any yellow hallow. The spots are reddish brown to black in colour with sporulation mostly from lower surface.

Citrus canker:- Among the bacterial diseases, canker disease is the common disease on citrus plants. This disease was reported from all over the world wherever the crop was grown. The aerial parts that show the characteristic canker symptoms are leaves, stem, thorns, fruits. On leaves the symptoms appear like rough, corky crater like appearance. The size is usually 8-10 mm or more in diameter. The lesions usually surrounded by a yellow hallow. The symptoms are same on all parts, however on fruits the lesions do not have the yellow hallow and the symptoms are restricted to skin only, that is the reason why there is no reduction in the quality of juice even in infected fruits.

Viral diseases on plants can be categorized into different types based on the type of symptoms they produce. The symptoms are: (1)chlorosis , (2) stunted growth, (3) malformation, (4) flower breaking.

Chlorosis: It is the general yellowing of leaves without giving any specific pattern. It occurs due to insufficient production of chlorophyll. **e.g.:** Rice yellow virus.

Mosaic: It is the appearance of irregularly distributed green and yellow colour patches on leaves. **e.g.:** Tobacco mosaic virus.

Stunted growth: Due to reduction in growth of various plants parts, the plant appear dwarf in nature. **e.g.:** Rice dwarf disease.

Colour breaking: It is due to discoloration of petals of the flower, resulting in the formation of white colour stripes on dark coloured petals. This increase the beauty of the flower. **e.g.:** Tulip flower breaking disease.

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23. ANATOMICAL STUDIES OF LEAVES SHOWING SYMPTOMS CAUSED BY FUNGI

Aim: To study the anatomical features of leaves infected by fungi. **e.g**.:

I. Blast of Rice caused *Pyricularia oryzae*
II. Tikka disease caused by *Cercosporidium personatum*.
III. Leaf spot of chillies caused by *Cercospora capsicii*.
IV. Groundnut rust caused by *Puccinia arachidis*

Requirements: Infected leaf material, pith material, blade, needle, brush, microscope etc.

Note: Before taking the section of the infected leaf material, it is essential to study the external morphological characters of the symptoms. Every pathogenic fungus produce characteristic symptoms. It is also essential to scrape off the leaf material at the region of infection with a blade and observe under the microscope. It will give the structure of conidiophores, conidia or any spores produced on the leaf surface.

Procedure:

- Take the diseased leaf and cut it into 1 cm^2 bits in the infected region.
- After making a vertical slit in the pith material, place the bit of diseased leaf in the slit.
- Using a sharp blade, take thin hand made sections.
- Select the thinnest section and place it on a clean glass slide using a brush.
- Stain the section with cotton blue; and remove the excess of stain.
- Mount the section using lactophenol/ lactoglycerol.
- Observe the section under compound microscope after gently placing a coverslip over it.

Observations:

I. Blast of Rice caused by *Pyricularia oryzae*.

(i) External symptoms: Leaf spots are elliptical with pointed ends, dark coloured margins with ash coloured centers, scattered on the entire lamina with the size range of 1-15 cm.

(ii) Internal structures:

a) 3-4 conidiophores arise from stomata on the upper side of the leaf.
b) Each conidiophore is simple, rarely branched, hyaline or olivaceous with sympodial growth.
c) Conidia are formed singly at the tip of the conidiophore and are developed sympodially.
d) Each conidium is pyriform to obclavate, 1 or 3 septate hyaline to pale olivaceous, 14-40 x 6-13 μm in size, with a prominent protruding basal hilum.

II. Tikka disease of Groundnut caused by *Cercosporidium personatum*.

i) External symptoms: Leaf spots on upper surface of the leaf are very dark, blackish, round or irregular 1-6 mm in diameter, with a yellow halo. On the lower surface of the leaf spot are carbon black in colour.

ii) Internal structures:

(a) Pinhead shaped stroma was observed on the leaf surface. It is formed by mass of hypha and measures about 40-65 μm diameter.

(b) Many conidiophores arise from stroma. Conidiophores are 25-54 μm long and 5-9 μm broad, 1-2 septate, and geniculate with prominent scars.

(c) Conidia are obclavate or cylindrical, measure 18-60 μm length and 6-10 μm in width, 1-7 septate, pale olivaceous, with prominent scar at base.

III. Leaf spot disease of chillis caused by *Cercospora capsici*.

i) External symptoms: Leaf spots appear round or irregular in shape, measure 0.5 – 1 cm in diameter, irregularly distributed on the leaf. Each leaf spot appears like target spot with concentric rings of dark colour with or without any yellow halo.

ii) Internal structures:

(a) Stroma is very small, produced on both sides of the leaf.

(b) Conidiophores, 4-12, divergent, arise from stroma.

(c) Each conidiophore is long, branched or unbranched, septate, geniculate, reddish brown, 45-75 μm long 2-5 μm width.

(d) Conidia are hyaline, acicular, multiseptate, 6-25 septa measuring 90-220 μm long.

IV. Rust of Groundnut caused by *Puccinia arachides.*

i) External symptoms: On leaves the disease appear like pustules distributed on the entire lamina giving the rusty appearance to the leaf.

ii) Internal structures:

(a) Uredosori are produced on both surface of the leaf.

(b) By rupturing the epidermis the uredosori exposed to the external environment.

(c) Each uredosorus consists of many uredospores.

(d) Each uredospore is sessile, reddish brown, oval, thin walled with attenuations, measuring 10-35 μm in diameter.

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24. ISOLATION OF PLANT PATHOGENIC FUNGI

Aim: To isolate the fungal pathogen from leaf infected with *Alternaria* sp.

Requirements: Infected leaves, sterilized petriplates, moist chamber, 0.1% $HgCl_2$, sterile water, blade, forceps, spirit lamp etc.

Procedure: Prepare potato dextrose Agar (PDA) medium by using the following composition.

Potato (Peeled) – 200 g
Dextrose – 20 g
Agar – 15 g
Distilled water – 1000 ml

Peel off the skin of potatoes, cut into small pieces (200 g) and boil in 500 ml of water. Filter through cheese cloth. Add dextrose and Agar to the filtrate. Bring the volume to 1 litre by addition water. Autoclave at 15 lb pressure for 15 minutes. Disperse about 20 ml of the medium in to each of the sterilized petriplate. Allow them to solidify.

Take the infected leaf and cut into 5 to 10 mm square pieces. Care to be taken that each piece should include both infected and healthy regions. Surface sterilize the pieces using 0.1% $HgCl_2$ solution for 50 seconds. Transfer them to ethanol and sterilize for 50 seconds. Wash the excess of ethanol on the pieces with sterile distilled water. Using a sterilized foreceps take one of the pieces and place it in the centre of the PDA plates in front of a flame. Similarly, transfer 4 to 5 pieces into each of 4 to 5 PDA plates. Incubate them at room temperature for 2 to 3 days.

Result: The fungus causing the leaf spot disease was identified as *Alternaria* sp. The spores are dyctiospore type with multicellular nature having both vertical and horizontal septa. They a dark brown to reddish brown in colour, thick walled with a prominent beak at the base and a scar at the apex.

After incubation, transfer a small portion of the fungal hyphae coming out of the infected leaf bit, on to a slide and observe under microscope to identify the fungal species.

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25 EFFECT OF FUNGICIDES ON GERMINATION AND GROWTH OF PATHOGENIC FUNGI

Aim: To study the effect of contact and systemic fungicides on germination and growth of pathogenic fungi.

Requirement: Fungicides – Bavistin, Dithane – M 45, Moist chambers, slides, PDA plates, *Alternaria* culture.

Procedure:

I. Effect of fungicides on germination of conidia:

- ✓ Collect the *Alternaria* leaf spot disease from the field.
- ✓ Bring the leaves to the laboratory and wash them under tap water.
- ✓ After gently blotting with papers, place them in a moist chamber for 24 hrs.
- ✓ Collect the spores developed at the leaf spot region with a brush.
- ✓ Transfer the spores on to clean glass slides.
- ✓ On the slide, place a drop of fungicide over the spores and incubate them in moist chambers for 24 hrs.
- ✓ To study the effect of fungicides; different concentrations, viz., 10, 20, 30 50 ppm. solutions are prepared using bavistin and Dithane M45.
- ✓ Maintain a slide with spores moistened with distilled water to serve as control.
- ✓ After 24 incubation remove the slides from moist chamber and observe them under microscope for the percentage of spore germination and count the number of germ tubes per conidia.

Tabulate and discuss the results.

Table I: Effect of fungicides on growth of the fungi by food poisoning technique.

I Step: Preparation of fungicide concentrations: (10, 20, 30 40 and 50 ppm)

Take 1 mg of bavistin (Carbondezim) and dissolve it in 10 ml of distilled water. It become 100 ppm concentration. From this, take 1 ml and add 9 ml of distilled water to prepare 10 ppm. Similarly take 2 ml and add 8 ml distilled water to prepare 20 ppm. In the same pattern, prepare 30, 40 and 50 ppm concentrations of the fungicide.

II Step: Preparation of Dox medium plates

Take all the ingredients of Dox medium to prepare 100 ml of medium. Add 90 ml of distilled water and 10 ml of pesticide concentration (10 ppm) and sterilize the medium. After sterilization, disperse the medium into 5 petriplates and labelled them as 10 ppm. Similarly prepare

5 plates each for 20 ppm, 30, 40 and 50 ppm concentrations. Maintain 5 plates without any pesticide to serve as control.

III Step: Inoculate all plates, at the centre with fungal spores of *Alternaria* using a inoculation needle. Incubate the plates for 2-3 days. After incubation, calculate the diameter of the colonies in each plate. Tabulate the results and comment on effect of pesticide of on fungal growth.

Table I. Effect of bavistin (carbendazim) on conidial germination of *Alternaria* after 24 h incubation.

Treatment	% germination	average number of germ tubes per conidium (av. Value of 25 conidia)	average germ tube length (μm)
Control			
Carbondazim			
10 ppm			
20 ppm			
30 ppm			
40 ppm			
50 ppm			
Dithane M 45			
10 ppm			
20 ppm			
40 ppm			

Table II. Effect of fungicide on growth of *Alternaria*

Pesticide conc.	Colony diameter (cm) average value of 5 plates
Control	
Carbondazim 10 ppm	
Carbondazim 20 ppm	
Carbondazim 30 ppm	
Carbondazim 40 ppm	
Carbondazim 50 ppm	

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