# PRACTICAL MANUAL

# SOIL BIOLOGY AND FERTILITY

## Course No. FNR-218; Credit Hrs. 3(2+1)

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**COLLEGE OF HORTICULTURE AND FORESTRY**
**RANI LAKSHMI BAI CENTRAL AGRICULTURAL UNIVERSITY**
**JHANSI - 284003**

## Syllabus (FNR-218; 3(2+1)

Study of forest soil profile; Estimation of pH and EC – Organic carbon – available N, P, K, Ca, Mg, S and micronutrients – Determination of CEC and exchangeable cations; Interpretation of soil and plant analysis data for fertilizer recommendation. Basic sterilization techniques; culturing and maintenance of microorganism occurring in soil; Staining methods; Study of decomposition of forest litter by $CO_2$ – evolution method; Estimation of nitrification rate in soil; Isolation of legume bacteria and Azotobacter; Preparation and inoculation techniques for mycorrhizae and biofertilizers.

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**Roll No. .....**

**Batch .....**

**Session .....**

**Semester .....**

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Date: Course Teacher

# Index

## Experiment No. 1

## Objective: Study of forest soil profile

A soil profile is a single vertical cross section of soil extending from the surface into the underlying unweathered parent material. The soil profile is composed of horizons (horizontal layers of soil) which may be characterized by physical, chemical, and biological properties. Horizons may be divided into major categories corresponding to the surface soil (O and A horizons), the "subsurface" soil (E and B horizons), and the substrate (C and R horizons).

**Materials required:**

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**1. What is soil profile? Explain with diagram.**

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**2. Explain different horizons in forest soil.**

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**3. Write procedure to dig a pit to study soil profile.**

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**4. Write observations of soil profile you have studied practically.**

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**5. What precautions you will take while studying soil profile?**

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## Experiment No. 2

## Objective: Estimation of pH and EC

pH is negative logarithm of H ion activity and Electrical conductivity is a measure of salts in soil i.e. Salinity of soil. pH and EC are measured in pH meter and EC meter, respectively.

**Materials required:**

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**1. Determine pH of the given soil sample. Write its procedure.**

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**2. Determine EC of the given soil sample. Write its procedure**

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**3. Write observations from the practical you have carried out.**

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**4. Write precautions in determining pH and EC.**

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## Experiment No. 3

## Objective: Estimation of soil organic carbon

**Materials required:**

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**1. Name the reagents required for the analysis of soil organic carbon**

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**2. Determine soil organic carbon from the given sample. Write procedure with calculation.**

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**General calculation**

- Weight of soil (W) = ---------- g
- Normality of $K_2Cr_2O_7$ used = ---------- N
- Volume of $K_2Cr_2O_7$ added in soil sample = ---------- ml
- Volume of 0.5 N Fe $(NH_4)2.6H_2O$ used for blank (B) = ---------- ml
- Volume of 0.5 N Fe $(NH_4)2.6H_2O$ used for soil sample (S) = ---------- ml

% Organic Carbon = (B-S) x 0.003 x 100 / 2 W =

% Organic matter = % O.C. x 1.724 =

**3. What precautions needed to be taken while analyzing SOC?**

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## Experiment No. 4

## Objective: Estimation of available nitrogen

Estimation of available nitrogen is done by following **Alkaline Potassium Permanganate method**. The know weight of the soil is treated with an excess of alkaline $KMnO_4$ and distilled. The ammonia gas evolved is absorbed in boric acid and titrated with standard sulphuric acid using mixed indicator.

**Materials required:**

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**1. Name the reagents required for the analysis of available soil nitrogen**

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**2. Determine available nitrogen from the given sample. Write procedure with calculation.**

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**General calculations:**

- Weight of soil sample (W) = 20g
- 0.02 N $H_2SO_4$ used during the back titration = Z ml= (Y – X) ml =
- Blank reading = X ml =
- Reading of soil sample = Y ml =
- 1 liter 1N $H_2SO_4$ = 14 g N 1 ml =
- 1N $H_2SO_4$ = 0.014 g N
- 1 ml 0.1N $H_2SO_4$ = 0.0014 g N
- 1 ml 0.02N $H_2SO_4$ = 0.00028 g N

Available N (%) = Z x 0.00028 x 100 /20 =

Available N in ppm = % x 10000 =

Available N (kg/ha) = % x 22400 =

**3. What are the precautions needed to be taken while analyzing available nitrogen?**

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## Experiment No. 5

## Objective: Estimation of available phosphorus

Phosphorous occurs in the soil both in organic and inorganic forms. Organic fraction is present in humus. The solubility of calcium phosphate is controlled by the activity of $Ca^{2+}$ in soil solution and pH. The 0.5 N $NaHCO_3$ buffered to pH 8.5 increases the $HCO_3$- activity in the soil and decreases the calcium activity.

**Materials required:**

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**1. Name the reagents required for the analysis of available soil phosphorus**

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**2. Determine available phosphorus from the given sample. Write procedure with calculation.**

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**General calculations:**

- Weight of soil sample taken = 2.5 g
- Vol of 0.5 N $NaHCO_3$ solution added = 50ml
- First dilution = 20 times
- Volume of filtrate taken = 5 ml
- Final volume made = 25 ml
- Second dilution = 5 times
- Transmittance of test sample = T
- Absorbance of test sample = A
- Concentration of P for standard curve = X ppm =

ppm of available P in the soil = X x 100 =

Available P (kg/ha) = ppm x 2.24 =

**3. What precautions needed to be taken while analyzing available phosphorus?**

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## Experiment No. 6

## Objective: Estimation of available potassium

Potassium is an essential plant nutrient and farmers need to be able to predict how much soil K is plant available in order to optimize fertilizer applications and crop production. Available K is estimated by the method 1 N Neutral Ammonium Acetate method.

**Materials required:**

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**1. Name the reagents required for the analysis of available soil potassium**

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**2. Determine available potassium from the given sample. Write procedure with calculation.**

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**General calculations:**

- Weight of soil taken = 2.5 g
- Volume of 1 N $NH_4OAc$= 25 ml
- Dilution factor = 25/2.5= 10 times
- K concentration in blank= X ppm =
- K content in sample (Y) = X x 10 (DF) ppm =

Available K (Kg/ha) = Y x 2.24

**3. What are the precautions needed to be taken while analyzing available potassium?**

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## Experiment No. 7

## Objective: Estimation of available calcium and magnesium

Chelating agents like EBT, EDTA has tendency to form complex with metal cations especially with Ca and Mg. Buffer solution is added to increase the pH at 10 so that EBT will form complex with Ca and Mg. EDTA has more tendency to form complex, when the solution is titrated with EDTA, all the complex form with EBT will be broken and replaced by EDTA. Thus, knowing the amount of EDTA needed to form complex will give the amount of Ca and Mg present in the sample.

**Materials required:**

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**1. Name the reagents required for the analysis of available calcium and magnesium**

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**2. Determine available Ca and Mg from the given sample. Write procedure with calculation.**

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**General calculations:**

- Weight of soil taken = 10 g
- Volume of Extracting solution = 50 ml
- Dilution factor (DF) = 50/10= 5 times
- Titrate value of the sample = X =
- Titrate value of the blank = B =

- Reading, R = X – B =

(Ca + Mg) meg/100 g soil = (R x 0.01 x DF)/(100/10) =

**3. What are the precautions needed to be taken while analyzing available Ca and Mg?**

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## Experiment No. 8

## Objective: Estimation of available sulphur

Plants absorb sulphur in the form of sulphate ions ($SO_4^{2-}$). A large number of extractants like water, monocalcium phosphate [$Ca(H_2PO_4)_2$], Calcium Chloride ($CaCl_2$), potassium dihydrogen orthophosphate ($KH_2PO_4$), Morgan's reagent ($CH_3COONa$-$CH_3COOH$), ammonium acetate + acetic acid ($CH_3.COONH_4$ + $CH_3COOH$) and NaCl have been used for extraction of available S. Among different extraction procedures used, 0.15% $CaCl_2$ extraction method suggested by Williams and Steinbergs (1959) is most widely used.

**Materials required:**

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**1. Name the reagents required for the analysis of available sulphur.**

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**2. Determine available sulphur from the given sample. Write procedure with calculation.**

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**General calculations:**

- Weight of the soil taken = 10g
- Volume of extractant used = 50ml
- First dilution = 5 times
- Volume of the aliquot taken = 20ml
- Final volume = 25ml
- Second dilution = 1.25 times

- Total dilution = 5 ×1.25 =6.25 times
- Absorbance reading = A =
- ppm of S from standard curve against A value = Y =
- ppm of S in soil = Y × 6.25 =

Kg/ha = ppm × 2.24 =

**3. What are the precautions needed to be taken while analyzing available sulphur?**

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## Experiment No. 9

## Objective: Estimation of available micronutrients

Micronutrient cations are extracted by shaking the soil with DTPA extracting solution containing 0.005M DTPA, 0.1M TEA (Triethanol amine) and 0.01M $CaCl_2.2H_2O$ buffered at pH 7.3. During this extraction, TEA gets protonated as HTEA+ as a result of which micronutrient cations from the solid phase come into solution and are chelated by DTPA. Buffering of the extractant in the slightly alkaline pH range and inclusion of soluble $Ca^+$ through $CaCl_2.2H_2O$ helps avoiding dissolution of $CaCO_3$ and thus excludes from the estimation of the occluded micronutrients, which do not form a part of the pool that is available for the absorption by plant roots. After that the contents of the micronutrient cations in the soil extract are estimated on atomic absorption spectrophotometer.

**Materials required:**

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**1. Name the reagents required for analysis of available Fe, Mn, Zn and Cu micronutrients from soil.**

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**2. Determine available micronutrients like Fe, Mn, Zn and Cu from the given sample. Write procedure with calculation.**

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**General calculations:**

- Weight of soil taken = 20 g
- Volume of DTPA Extractant = 40 ml
- Dilution factor (DF) = 40/20= 2 times
- Fe/Cu/Mn/Zn concentration in sample= X ppm =

Fe/Cu/Mn/Zn content in sample (Y) = X x 2 (DF) ppm =

**3. What are the precautions needed to be taken while analyzing available micronutrients?**

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## Experiment No. 10

## Objective: Determination of cation exchange capacity (CEC)

The Cation Exchange Capacity (CEC) is the capacity of soil to hold an exchangeable cation. The higher the CEC of soil, the more cations it can retain. Soil differ in their capacities to hold exchangeable K+ and other cations. The cations exchange capacity depends on amount and kind of clay and organic matter present. High clay soil can hold more exchangeable cations than a low clay soil. CEC also increases as organic matter increases. Clay mineral usually range from 10 to 150 meq/100g.

**Materials required:**

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**1. Name the reagents required for the analysis of CEC.**

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**2. Determine CEC from the given sample. Write procedure with calculation.**

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**General calculations:**

- Let D = Weight of oven dry soil in the weight of air dry soil taken for analysis =
- V = Total volume of the final solution containing the ammonium ion =
- T = Volume of standard acid (0.02N) used for titrating the ammonia nitrogen after correction for the blank =
- N = Normality of standard acid = 0.02 N

- If 20 ml of ammonium solution is distilled, V ml contains 0.01 T V 20 D milli equivalents = 1 T V 2000
- Thus, Cation exchange capacity = NTVx100 / 20D meq/100 gm of soil.
- If 10 ml ammonium solution is distilled,

Cation exchange capacity = NTVx100 / 10D meq/100 gm of soil =

**3. What are the precautions needed to be taken while analyzing CEC?**

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## Experiment No. 11

## Objective: Interpretation of soil and plant analysis data for fertilizer recommendation

Fertilizer recommendations contain several important factors, including fertilizer form, source, application timing, placement, and irrigation management. Another important part of a fertilizer recommendation is the amount of a particular nutrient to apply. The optimum fertilizer amount is determined from extensive field experimentation conducted for several years, at multiple locations, with several varieties, etc. Although rate is important, rate should be considered as a part of the overall fertilization management program.
The goal of fertilizer rate is to determine the amount of fertilizer needed to achieve a commercial crop yield with sufficient quality that is economically acceptable for the grower.

**Materials required:**

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**1. What is the importance of soil and plant analysis?**

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**2. What is interpretation of soil and plant analysis?**

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**3. How can you interpret soil and plant analysis data for fertilizer recommendation?**

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**4. What are the recommended doses of nitrogen, phosphorus and potassium?**

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**5. What are the precautions you to keep in mind while interpreting the analyzed data for fertilizer recommendation?**

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## Experiment No. 12

## Objective: To study basic sterilization techniques

Sterilization is necessary for the complete destruction or removal of all microorganisms (including spore-forming and non-spore forming bacteria, viruses, fungi, and protozoa) that could contaminate pharmaceuticals or other materials and thereby constitute a health hazard. Since the achievement of the absolute state of sterility cannot be demonstrated, the sterility of a pharmaceutical preparation can be defined only in terms of probability.

Classical sterilization techniques using saturated steam under pressure or hot air are the most reliable and should be used whenever possible. Other sterilization methods include filtration, ionizing radiation (gamma and electron-beam radiation), and gas (ethylene oxide, formaldehyde).

**Materials required:**

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**1. Define following terms.**

**Sterilization**

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**Cleaning**

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**2. Explain different types of sterilization techniques.**

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**3. What is the principle of autoclaving and dry heating?**

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**4. At what temperature generally the sterilization is done using autoclave and hot air oven?**

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**5. What precautions should be followed while performing autoclaving?**

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**6. How does the laminar flow safety hood work?**

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**7. What is agar and agar slants? Where do we use these?**

**Agar:** ……

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**Agar slants:** ……

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**8. Why petri plates are incubated in inverted position?**

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**9. What precautions should be taken while using pipettes?**

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**10. How inoculating loop and needle can be sterilized?**

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**11. How petri plates can be sterilized?**

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## Experiment No. 13

## Objective: Culturing and maintenance of microorganism occurring in soil

Determining an accurate count of environmental microorganism is critical to assessing the health of a soil ecosystem. This can be accomplished by culturing microorganism colonies with appropriate dilutions.

**Materials required:**

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**1. Define following terms**

**Culture:** ……

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**Media:** ……

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**Growth:** ……

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**Colony:** ……

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**Broth culture:** ……

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**2. Write uses**

**Laminar Air Flow:** ……

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**BOD:** ……

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**Autoclave:** ……

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**2. Explain serial dilution technique with diagram:** ……

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**3. Describe isolation and enumeration of microorganisms from soil by the serial dilution – agar plating method.**

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**4. How colony of microorganisms are counted?**

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**5. What precautions should be maintained during isolation of microorganisms from soil?**

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## Experiment No. 14

## Objective: Staining and microscopic examination of microbes

A variety of staining techniques can be used with light microscopy, including gram staining, acid fast staining, capsule staining, endospore staining and flagella staining. The gram stain is the most important staining procedure in microbiology. It is used to differentiate between gram positive organisms and gram-negative organisms. Hence, it is a differential stain.

**Materials required:**

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**1. How Gram positive and Gram-negative bacteria can be differentiated using gram staining protocol?**

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**2. Describe four different reagents required for Gram staining**

**a. Primary Stain:** ……

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**b. Mordent:** ……

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**c. Decolorizing agent:** ……

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**d. Counter stain:** ……

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**Observations:**

| Culture | Gram reaction | Arrangement of cells |
|---|---|---|
| Yeast cells | | |
| Bacterial cells | | |

**Result:** ……

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**4. Describe gram staining technique.**

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**5. Draw the observations of gram staining under microscope.**

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| Yeast cells | | |
| Bacterial cells | | |

**Result:**

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**6. What precautions needed to be taken while carrying out gram staining?**

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## Experiment No. 15

## Objective: Study of decomposition of forest litter by $CO_2$ – evolution method

Belowground storage and cycling of carbon in forest ecosystems are important components of the global carbon cycle. The majority of carbon in terrestrial ecosystems is stored in forests, and much of that carbon is found in the mineral soil and forest floor. Because of the magnitude of belowground carbon pools, there

is a need to determine the impact of human activities on soil carbon storage and turnover, and the degree to which forestry practices contribute to atmospheric enrichment with green-house gases such as carbon dioxide and methane. Carbon dioxide efflux can be estimated using the soda lime technique described by Edwards (1982) and modified by Nay *et al.* (1994).

**Materials required:**

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**1. Name the reagents required for the estimation of $CO_2$ evolution.**

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**2. Explain decomposition of forest litter. How $CO_2$ evolution is related with decomposition and global warming?**

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**3. Estimate $CO_2$ evolution from a particular forest area. Write its procedure and calculation.**

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**General calculations:**

- Weight of granular soda lime = A =
- Weight of redried granular soda = B =

$CO_2$ evolution = B – A = C x 1.41 =

**4. What are the precautions needed to be taken while estimating $CO_2$ evolution?**

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## Experiment No. 16

## Objective: Estimation of nitrification rate in soil

Nitrification is the aerobic conversion of ammonium ($NH_4^+$) into nitrite ($NO_2^-$) and nitrate ($NO_3^-$) by nitrifying bacteria. There are several chemoautotrophic bacteria such as *Nitrosomonas, Nitrosospira*, *Nitrosococcus*,

and *Nitrosovibrio* involved in the first step of the process where ammonium is oxidized to nitrite. In soil systems, *Nitrobacter* and *Nitrosospira* - like bacteria are involved in the second step where nitrite is converted to nitrate.

**Materials required:**

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**1. Describe nitrification process with equation involved.**

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**2. Write factors affecting nitrification rate.**

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**3. Write procedure of estimating nitrification rate in soil? Calculate the nitrification rate.**

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**General calculations:**

- Weight of soil sample taken = W =
- $NO_3^-$ content in core sampler 1 = A =
- $NO_3^-$ content in core sampler 2 (after 2 – 3 days) = B =

Nitrification rate = A – B =

**4. Write importance of nitrification in soil?**

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**5. What are the precautions you need to keep in mind while estimating nitrification rate?**

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## Experiment No. 17

## Objective: Isolation of *Rhizobium* and *Azotobacter*

Beijerinck, first of all, isolated a bacterium from root nodules of legumes in 1888 and named it *Bacillus radicicola*, now placed under the genus *Rhizobium*. They infect and live in symbiotic association with leguminous plants, forming nodules in them and fix atmospheric nitrogen.

*Azotobacter* is a free living, aerobic, nitrogen fixing bacterium and is known as non-symbiotic nitrogen fixer. *Azotobacter* is heterotrophic and depends on the energy derived from degradation of plant residues.

**Materials required:**

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**1. What are the media commonly used for isolating rhizobium and *azotobacter*?**

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**2. Explain the procedure for isolation of *rhizobium* from root nodules.**

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**3. Explain the procedure for isolation of *azotobacter* from soil.**

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**4. Write some characteristics of colony of *rhizobium* and *azotobacter* on petri plates.**

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**5. Draw colonies of *rhizobium* and *azotobacter* observed on petri plates.**

**6. Write precautions needed to be taken while isolating microorganisms.**

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## Experiment No. 18

## Objective: To study inoculation techniques of mycorrhizae and biofertilizers.

**Biofertilizers** and mycorrhizae increase the nutrients of host plants when applied to their seeds, plant surface or soil by colonizing the rhizosphere of the plant. **Biofertilizers** are more cost-effective as compared to chemical fertilizers.

**Materials required:**

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**1. Explain mycorrhizae and biofertilizers?**

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**2. Explain the inoculation technique for mycorrhizae.**

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**3. Explain the procedure for preparation of biofertilizers.**

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**4. What are the advantages of biofertilizers.**

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# APPENDICES

## STUDY OF FOREST SOIL PROFILE

A soil profile is a single vertical cross section of soil extending from the surface into the underlying unweathered parent material. The soil profile is composed of horizons (horizontal layers of soil) which may be characterized by physical, chemical, and biological properties. Horizons may be divided into major categories corresponding to the surface soil (O and A horizons), the "subsurface" soil (E and B horizons), and the substrate (C and R horizons).

Fully developed forest soils are natural bodies with a vertical sequence of layers. At the top is an organic surface layer or "forest floor" (O horizon) with subdivisions of fresh, undecomposed plant debris (Oi horizon, formerly called **L**); semi-decomposed, fragmented organic matter (Oe horizon, formerly called **F**) and humus; and amorphous organic matter without mineral material (Oa horizon, formerly called **H**). Below this surface layer is a mineral surface horizon (**A**); a subsurface mineral horizon often leached (**E**); a subsurface mineral horizon with features of accumulation (B horizon); a mineral horizon penetrable by roots (**C**); and locally hard bedrock (R). The E, B, C, and R horizon may be lacking, or the B horizon may be modified by groundwater or stagnant water.

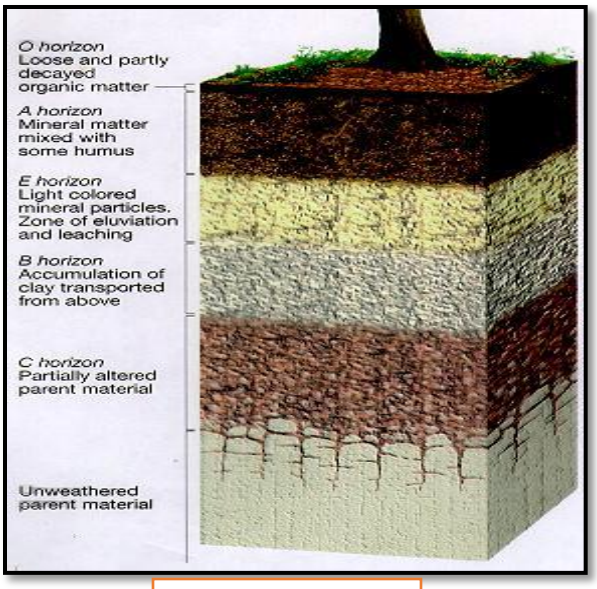
Agricultural soils associated with rangelands and grasslands often have similar vertical sequences. However, if they are being cultivated (arable land)-or have been in the past-they may lack the **O** horizon (unless peat soils are being used), and the **A** horizon may have been mixed with parts of the **E** and even the **B** horizon, resulting in a plow layer (Ap horizon). The B and/or C horizons may have been broken up by deep cultivation. The soils may have been so degraded by past human actions that they are no longer cultivatable. Such soils may still be classified as agricultural soils and used, for example, for grazing or non-cropping production.

**Procedure:**

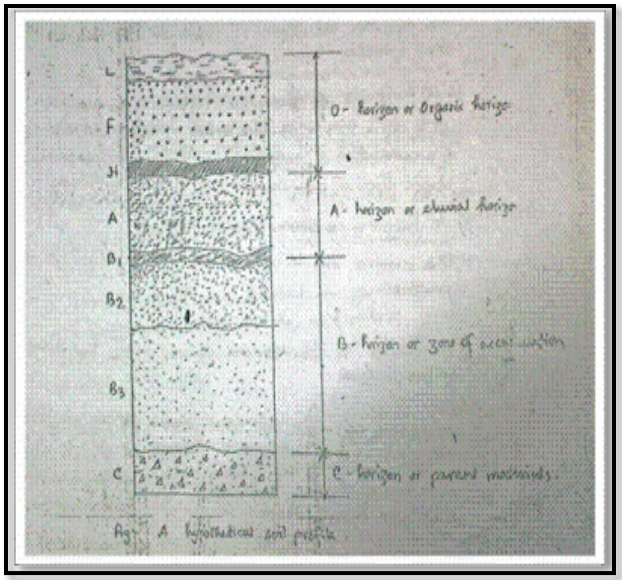
- Field visit to specific crop area must be carefully selected.
- Make a vertical section of a soil careful depth 120 cm (0-15, 15-30, 30-60, 60-90 and 90 120) to selected area with the help of spade for various depths.
- Such a section is called a profile and individual layers are known as horizons.
- Every horizon well developed; undisturbed soil has its own distinctive profile characteristics.
- Record certain observations physically in the field and collect soil samples for physico-chemical analysis in the laboratory.

**Precautions:**

- Soil sample collected must be free from particles which are larger than that of soil (>2mm), sieving must be done carefully




Soil profile



Forest soil profile

**ESTIMATION OF PH AND EC**

**Equipment and Material Required:** Mechanical shaker, pH meter, EC meter, Plastic bottles, distilled water, beaker/conical flask, pH buffer solutions, weighing balance, aluminium can box, filtration apparatus, dish, oven, wash bottle, measuring cylinder

**Procedure For pH:**

- Calibrate the pH meter, using 2 buffer solutions, one should be the buffer with neutral pH (7.0) and the other should be chosen based on the range of pH in the soil.
- Take the buffer solution in the beaker. Insert the electrode alternately in the beakers containing 2 buffer solutions and adjust the pH.
- The instrument indicating pH as per the buffers is ready to test the samples
- Weigh 10g of soil sample/10 ml water into 50 or 100 ml beaker, add 20ml of distilled water
- Allow the soil to absorb water without stirring, then thoroughly stir for 10 second using a glass rod.
- Stir the suspension for 30 minutes and record the pH on the calibrated pH meter.

**For EC**

- Take 40 g soil/ 40 ml water into 250 ml Erlenmeyer flask, add 80 ml of distilled water, stopper the flask and shake on reciprocating shaker for one hour. Filter through Whatman No.1 filter paper. The filtrate is ready for measurement of conductivity.
- Wash the conductivity electrode with distilled water and rinse with standard KCl solution.
- Pour some KCl solution into a 25 ml beaker and dip the electrode in the solution. Adjust the conductivity meter to read 1.412 mS/cm, corrected to 25˚C.
- Wash the electrode and dip it in the soil extract.
- Record the digital display corrected to 25˚C. The reading in mS/cm of electrical conductivity is a measure of the soluble salt content in the extract, and an indication of salinity status of this soil. The conductivity can also be expressed as mmhos/cm

**Precautions**

- Calibration of the instrument is must in order to avoid any error in result
- Handle the electrode carefully as these are made of glass and delicate and sensitive

## ESTIMATION OF SOIL ORGANIC CARBON

**Equipment and material required**: Volumetric flask, Pipette and Burette, Glass rod, Analytical balance and conical flask
**Reagents:**

- **Potassium dichromate solution:** 1 N. Dissolve 49.04 g pure crystals of potassium dichromate in water and dilute to 1 litre.
- **Ferrous Ammonium sulphate solution:** 0.5 N. Dissolve 139 g $FeSO_4.7H_2O$ in water and add 15 ml conc. $H_2SO_4$ and dilute to 1 litre. Ferrous ammonium sulphate $Fe(NH_4SO_4)_2.6H_2O$ can also be used and if that is to be used dissolve 785 g of the salt in 4 litres of water containing 100 ml conc. $H_2SO_4$.
- **Phosphoric acid:** 95%
- **Diphenylamine:** Dissolve 0.5 g diphenylamine in a mixture of 100 ml conc. $H_2SO_4$ and 20 ml water.
- **Sulphuric acid:** Not less than 96%.

**Procedure:**

- Weigh 1.0 g soil sample in a 500 ml beaker.
- Add 10 ml 1N $K_2Cr_2O_7$ by means of a pipette and 20 ml concentrated $H_2SO_4$ by measuring cylinder.
- Mix thoroughly and allow the reaction to proceed for 30 minutes on asbestos sheet.
- Dilute the reaction mixture with 200 ml of water and 10 ml $H_3PO_4$ by measuring cylinder and add 7-8 drops of diphenylamine indicator.
- Titrate the solution with std. 0.5 N Fe $(NH_4)_2(SO_4)_2$ to a brilliant green colour.
- A blank without soil should be run simultaneously.
- Calculate the organic carbon content of a given soil sample.

**Precautions**

- Handle acids carefully and titration should be carried out effectively as it is sensitive
- Preparation of reagents should be accurate to get accurate result

## ESTIMATION OF AVAILABLE NITROGEN

**Equipment and Apparatus Required:** Micro-Kjeldahl distillation assembly, Erlenmeyer flask (150 ml capacity), distillation flask, pipettes, burette
**Reagents Required:** 0.32% $KMnO_4$ solution, 2.5% NaOH solution, 4% Boric acid, 0.02N $H_2SO_4$
**Procedure:**

- Weight 20 g of soil sample of the soil and put into distillation flask.
- Moist the sample with distilled water and fix the distillation assembly.
- Add 100 ml of 0.32% KMnO4 for solution and 100 ml of 2.5% NaOH solution into distillation flask.
- Take 150 ml conical flask and add 20 ml boric acid and 3-4 drops of mixed indicator. Keep this conical flask at the bottom of receiving tube of distillation assembly.
- The boric acid is back titrated with 0.02N sulphuric acid. At the end point, the blue colour just disappears to pink.
- Blank samples should be run as per above procedure without soil.

**Precautions:**

1. Always add exactly measured quantity of acid and base.
2. Standards should be prepared accurately
3. The extract should be handled properly

## ESTIMATION OF AVAILABLE PHOSPHORUS

**Equipment and Apparatus Required:** Erlenmeyer flask (150 ml capacity), mechanical shaker, Whatman No. 1 filter paper, Spectrophotometer.
**Reagents required:** 0.5 M $NaHCO_3$, 1.5% ammonium molybdate solution, Darco G-60, stannous chloride solution
**Procedure:**

- Weigh 2.5 g soil and transfer it to a 100 ml conical flask.
- Add a pinch of Darco G-60 and 50 ml 0.5 M $NaHCO_3$.
- Shake on mechanical shaker for half an hour and then filter it.
- Take 5 ml of filtrate in 25 ml volumetric flask and add 5 ml of ammonium molybdate to it.
- Add 10 ml of distilled water and add 1 ml dilute stannous chloride solution.
- Make volume to the mark by adding distilled water.
- Put the test solution in another colorimetric tube and record per cent transmittance or absorbance.

**Precautions**

- Always add exactly measured quantity of acid and base.
- Standards should be prepared accurately
- The extract should be handled properly

## ESTIMATION OF AVAILABLE POTASSIUM

**Equipment and Apparatus Required:** Erlenmeyer flask (150 ml capacity), volumetric flask (1000 ml), funnels, pipettes , Whatman No. 1 filter paper, Flame photometer

**Reagents Required**: 1N neutral ammonium acetate, standard stock solution of K (1000 ppm K), working standard solution of K (100 ppm).

**Procedure:**

- Weigh 5 g soil in a 100 ml plastic shaking bottle.
- Add 25 ml of 1 N $NH_4OAc$ (pH 7.0) with a pipette (1:5::soil: solution ratio)
- Keep it as such for about 10-15 minutes.
- Now filter the suspension through Whatman No.1 filter paper in a beaker.
- Prepare a standard curve involving 0, 5, 10, 15 & 20 ppm K solution.
- Take reading of the standard solutions and sample in Flame photometer

**Precautions:**

1. Always add exactly measured quantity of acid and base.
2. Standards should be prepared accurately
3. The extract should be handled properly

## ESTIMATION OF AVAILABLE CALCIUM AND MAGNESIUM

**Equipment and Apparatus required:** weighing balance, mechanical shaker, pipettes, burette, whatman no. 1 filter paper and conical flask

**Reagents required:**

- **Extractant solution:** 77.08 g Ammonium acetate ($NH_4OAC$) and make up to 1 litre of distilled water in a vol. flask
- **Buffer solution:** 67.5 g of Ammonium Chloride ($NH_4Cl$) in 400 ml of distilled water to this and 570 ml of concentrated Ammonia solution and dilute to 1 litre in a vol. flask
- **Erichrome Black T (EBT) indicator:** 1 g of EBT indicator in a 400 ml vol. flask make up using ethanol alcohol
- **0.01 N EDTA:** 2 g of EDTA and 0.05 g of Magnesium Chloride ($MgCl_2.6H_2O$) dissolve in water in 1 litre vol. flask

**Procedure:**

- Weigh 10 g of soil in 150 ml conical flask
- Add 50 ml of Extractant Ammonium Acetate
- Shake the solution in a mechanical shaker for 5 mins and filter using whatman no. 1 filter paper
- Take 10 ml of the aliquot in 150 ml conical flask
- Add 5 ml buffer solution and 1-2 drops of EBT indicator
- Titrate with 0.01 N EDTA till the colour changes from purple to blue

**Precautions:**

- Use mask as the smell of buffer solution is suffocating
- Titrate carefully as the colour change is very mild
- Prepare reagents carefully

## ESTIMATION OF AVAILABLE SULPHUR

**Equipment and material required:** Weighing balance, spatula, measuring cylinder, conical flask, volumetric flask, glass rod, magnetic stirrer, beaker, reagent bottle, Wash bottle, filter paper Whatman no.1, colorimeter or spectrophotometer.

**Reagents required:**

- **Extracting solution:** Dissolve 1.986 g of calcium chloride dihydrate ($CaC1_2.2H_20$) in distilled water and dilute to one litre.
- **Barium chloride ($BaCl_2$):** 30 to 60—mesh crystals: Grind barium chloride crystals until they pass through 30--mesh sieve and are retained on a 60—mesh sieve.
- **Gum acacia solution (0.25%):** Dissolve 0.25 g gum acacia in distilled water and dilute to 100 ml.

- **Standard sulphur solution (100 ppm S):** 0.5434 g of the reagent grade potassium sulphate ($K_2SO_4$) in distilled water and dilute to one litre.

**Procedure:**

a) **Preparation of standard curve:** Pipette out 0.25, 0.5, 1.0, 2.5 and 5.0 ml of 100 ppm S solution in different 25 ml volumetric flasks. Add in every flask 10 ml of 0.15% $CaCl_2$, solution and 1.0 g of 30 - 60 mesh $BaCl_2$, crystals. Swirl for one minute to dissolve the crystals and add 1 ml of 0.25% solution of gum acacia. Make up the volume in every flask with distilled water and shake well. Within 5 to 30 minutes after the development of turbidity read the standards on a colorimeter at 420 nm using a blue filter. Plot a standard curve showing relationship between concentration of S and turbidity/absorbance readings.

b) **Analysis of test sample:** Weight 10 g air-dried soil and transfer it to a 150-m1 conical flask. Add 50 ml of 0.15 % CaCl solution and shake for 30 minutes on an electric shaker. Filter the Suspension through Whatman No.42 filter paper. Pipette out 20 ml of the filtrate in 25-m1 volumetric flask and proceed further as in case of standard curve. Run blank with all the chemicals, except the soil. Find out sulphate concentration from the standard curve.

**Precautions**

- Reagents should be prepared accurately
- Standard working solution should be prepared according to the need

## ESTIMATION OF AVAILABLE MICRONUTRIENTS

**Equipment and Apparatus Required:** Weighing balance, mechanical shaker, pipettes, burette, conical flask and Whatman No. 42 filter paper

**Reagents Required:** DTPA extracting solution i.e. 0.005 M DTPA, 0.01M $CaCl_2$, 0.1 M TEA

**Procedure:**

- Weigh 20 g soil in a 100 ml conical flask.
- Add 40 ml of DTPA extracting solution (pH 7.3) in 1:2 :: soil : solution ratio
- Shake for 30 minutes in a mechanical shaker
- Now filter the suspension through Whatman No.42 filter paper in a beaker/plastic bottle.
- Prepare a standard curve involving 0, 5, 10, 15 & 20 ppm Fe, Mn, Zn, Cu working solution from the given stock solution of 1000 ppm
- Take the reading in AAS

**Precautions**

- Shaking should be done properly and filtration should be done immediately after shaking
- Filtration should be done with filter paper of Whatman No. 42 as AAS is very sensitive
- Handle the instrument carefully as it contains flame

## DETERMINATION OF CATION EXCHANGE CAPACITY (CEC)

**Equipment and material required:** To estimate cation exchange capacity of soil, measuring cylinder, Mechanical shaker, volumetric flask, Whatman No. 1 filter paper

**Reagents required:**

- **Ammonium Acetate, 1N, pH 7:** Dissolve 77.08 gm of ammonium acetate in distilled water and makeup the volume to one litre. Adjust the pH at 7 with ammonia or acetic acid.
- **Potassium Chloride** (10%, pH 2.5 + 0.1): Dissolve 10 gm of potassium chloride in distilled water and make up the volume to 100 ml. Adjust the pH at 2.5 with 1N HCl.
- **Methanol or ethanol**, 60%: Dilute 60 ml methanol with distilled water and make up the volume to 100 ml.
- **Sodium hydroxide, 40%:** Dissolve 400 gm NaOH in water in a 1 litre volumetric flask. Let it cooled and make it upto the mark.
- **Methyl red indicator**: Dissolve 0.1 gm methyl red in 100 ml ethanol (98%).
- **Hydrochloric acid (0.02N):** Take 1.8 ml of concentrated HCl in one litre volumetric flask and make the volume with distilled water.

**Procedure:**

- Weigh 10.0 gm of 5 mm sieved air dried soil sample into 500 ml plastic conical flask. Add 250 ml of neutral ammonium acetate solution.

- Shake the contents on mechanical shaker at 110 rpm for an hour and keep it overnight. Next day again shake it for 1 hour.
- Filter the contents through Whatman No. 1 filter paper receiving the filtrate in a 250 ml volumetric flask.
- Transfer the soil completely on the filter paper and continue to leach the soil with the neutral solution (using 20-25 ml at a time).
- Allowing the leachate to drain completely before fresh aliquot is added. If it is not enough add some ammonium acetate to make up the mark. The solution is now ready for determination of individual cations.
- The residue left on the filter paper is intended for the determination of cation exchange capacity of soil. Wash the leached soil with 60% methanol or ethanol to remove the excess ammonia. Each time 10 ml of alcohol in interval is added, draining between each addition is necessary. Nessler's reagent does the test of ammonia. If Nessler's reagent is not available the test of ammonia is done by HCl.
- Collect the leachable washing of ethanol / methanol in watch glass and add few drops of concentrated HCl if it fumes that indicates presence of ammonia otherwise not. After washing, the soil is leached with acidified potassium chloride (10%, pH = 2.5+0.1) and the extract is collected finally in a 250 ml volumetric flask.
- 25–30 ml of KCl solution is added. Each time draining between each addition is necessary. The volume is adjusted to the mark by the replacing solution of KCl. It is shaken to make the concentration solution homogeneous.
- Transfer 20 ml of KCl leachate and 10 ml of distilled water in a Gerhardt tube, add 10 ml of 40% NaOH and distilled into 20 ml of HCl (0.02 N) containing 6 drops of methyl red indicator. The approximate distillate is collected about 150 – 200 ml. Titrate the solution until the yellow colour appears with 0.02 N NaOH.
- The changes from red to yellow, the end point being taken at the first appearance of the yellow colour. 20 ml of 10% KCl solution is distilled in the same manner for blank.
- Color differentiation must be observed and with the help of spectrophotometer, the same can be confirmed.

**Precautions**

- Titration should be doe carefully
- Handle carefully while using acids and fuming
- Handle the instrument carefully as it is sensitive.

## INTERPRETATION OF SOIL AND PLANT ANALYSIS DATA FOR FERTILIZER RECOMMENDATION

Fertilizer recommendations are only as good as the accuracy of the soil tests on which they are based. A fairly high degree of variability has been observed among testing laboratories. Therefore, good interpretation of analyzed data for the fertilizer recommendation is a crucial step.

| Nutrient | Low Levels | Medium Levels | High Levels | Optimal pH |
|---|---|---|---|---|
| | | **Kg/ha** | | |
| Nitrogen (N) | < 260 | 260 – 540 | > 540 | 6.5 – 8 |
| Phosphorus (P) | < 16 | 16 - 25 | > 25 | 6.5 – 8 |
| Potassium (K) | < 150 | 150 – 250 | > 250 | 6.5 – 8 |
| Sulfur (S) | < 10 | 10 - 20 | > 20 | 6.5 – 8 |
| | | **ppm** | | |
| Boron (B) | < 0.5 | 0.5 – 1.0 | > 1.0 5 | 5 – 7 |
| Copper (Cu) | < 0.25 | 0.25 – 0.5 | > 0.5 | 5 – 7 |
| Iron (Fe) | < 2.5 | 2.5 – 5.0 | > 5.0 | 5 – 7 |
| Manganese (Mn) | < 0.5 | 0.5 – 1.0 | > 1.0 | 5 – 7 |
| Zinc (Zn) | < 0.25 | 0.25 – 0.5 | > 0.5 | 5 – 7 |

**Conversion factors**

| | |
|---|---|
| $P_2O_5$ x 0.4364 = P | P x 2.2919 = $P_2O_5$ |
| $K_2O$ x 0.8302 = K | K x 1.2046 = $K_2O$ |
| CaO x 0.7147 = Ca | Ca x 1.3992 = CaO |
| MgO x 0.6030 = Mg | Mg x 1.6582 = MgO |

## BASIC STERILIZATION TECHNIQUES

**Sterilization** is a method of freeing up of an article from all living organisms. The common methods of sterilization are as below.

A. **Dry heat**: Sterilization by dry heat is carried out in many ways.

1. **Red heat**: Inoculation loop: forceps and spatula are sterilized by heating them in flame up to red-hot.

2. **Flaming**: Stoppers, culture tubes, flasks etc., are sterilized by passing them through the flame
3. **Hot air oven**: It is electrically heated and fitted with thermostatic arrangement with blower for ensuring rapid and controlled heating of the materials, equipments such as Petri plates, pipettes, test tubes, conical flasks are sterilized at a temperature of 1600 C for one hour. The time and temperature for sterilization of glass ware are as follows:

    120°C – for 8 hours.
    140°C – for or 2 ½ hours
    160°C for 2 hours is most commonly used.
    180°C – for 20 minutes.

B. **Moist heat**: For most types of media, cloth, rubber and other materials that would be destroyed by dry heat is sterilized by moist heat in an autoclave at 1210C for 20 minutes using steam under 15 pounds pressure. Water boils when vapour pressure equals the surrounding atmospheric pressure. This occurs at 1000C, when water is boiled in a closed vessel at increased pressure. Thus the temperature at which is boils and that of the steam will also rise. The temperature boiling time and pressure used are as follows.

**Methods of sterilization**

I. **Chemical methods**: Disinfectants like alcohols, phenol, mercuric chloride etc., are used to surface sterilize any materials so that bacteria present on the outer surface are killed.

II. **Filtration**: Many materials for example sugars, blood sera, which are destroyed by heating at temperatures, which are normally used for sterilization. To sterilize such heat labile materials filters can be used. The filters remove bacteria by the sieve-like action of the pores in the filter and also by adsorption of microbes to the filters. The types of filters used are membrane filter, sintered glass filter, Seitz asbestos pad filter, the chamber land and selas candle type filter etc., All these filters are used by being attached to a suction flask to draw the liquid materials through them.

III. **Gaseous sterilization**: The use of ethylene oxide vapours under pressure in special equipment is a common method of cold sterilization: ethylene oxide is highly toxic for viral particles, bacterial and fungal cells and the heat-resistant endosperms.

**Sterilization:** a process of making an object free from all living organisms either by destroying or removing them from the object. This control of microorganisms is very important in microbiological research, preservation of food, prevention of diseases and in various industries.

**Cleaning:** is removal of visible soil (e.g., organic and inorganic material) from objects and surfaces. It is normally accomplished manually or mechanically using water with detergents or enzymatic products.

**Autoclave:** For sterilization, steam under pressure is generally employed using an instrument called autoclave. Autoclave can be used for sterilizing culture media, scalpel and other heat resistance instruments, glasswares, etc. but not for oils, powders and plastics. Autoclave was developed by *Chamberland* in 1884.

**Relation of Autoclave pressure with the temperature of steam Pressure**

| Pressure (Pounds/square inch-psi) | Temperature (°C) | Time (minutes) |
|---|---|---|
| 0 | 100 | - |
| 5 | 108 | >20 |
| 10 | 115 | 20 |
| 15 | 121 | 15 |
| 20 | 126 | 10 |
| 30 | 134 | 3 |

More the pressure, higher is the temperature and less is the time used for autoclaving. Generally, pressure of 15 pounds with temperature at 121˚C is employed for 15-20 minutes for autoclaving. Saturated steam heats an object about 2500 times more efficiently than dry heat at the same temperature. Steam condenses on the cooler surface of the object and transfer its heat energy to the object and sterilize it.

For autoclave, temperature at 121˚C is employed for 15-20 minutes (pressure of 15 psi). For Hot air oven, the most commonly used temperature is 160˚C for 2 hours.

**Working procedure of autoclave**

The culture media are wet (moist) sterilized because they are either liquids or semisolid at gels containing water. An autoclave which is a boiler with an inner chamber and a light fitting lid is employed for this. Water is taken at the bottom between the inner chamber and outer jacket and arrangement is made to heat the water to produce steam. The autoclave is provided with a safety valve and a pressure gauge. The principle of moist sterilization is to produce steam which raises the pressure in the

inner chamber. The steam temperature goes up with increasing pressure. A constant pressure is maintained by adjusting the heating. The most frequently adopted wet sterilization temperature is 1210C, which is achieved by maintaining a pressure of 151 bs/sq inch (1.05kg/sq.cm) for 15 minutes at this temperature are sufficient for sterilization.

**Precautions in using autoclave:**

- Autoclave should not be packed tightly otherwise steam won't be able to come in contact with every object in the autoclave.
- The air initially present in autoclave chamber should be removed before closing exhaust valve, otherwise temperature won't reach to 121˚C though the pressure would be 15 pounds.
- For larger sample of liquid, autoclave time should be increased so that centre of the liquid should reach to 121˚C.
- After autoclaving, steam should be released slowly otherwise liquid media would come out.

Laminar air flow biological safety cabinet contains HEPA (high efficiency particulate air) filters which remove, 99.97% of the particles having size more than 0.3 µm. Air is forced through these HEPA filters and a vertical column of sterilize air gets formed across the cabinet opening. It prevents the contamination of room and the workers from microorganisms. The cabinet also has UV light, which is switched on about 15-20 minutes earlier before and after the work is finished to make the working surface sterile. Laminar flow hood is employed in the research labs for conducting assays, preparing media and culturing microbes.

**Agar**, also called **agar-agar**, gelatin-like product made primarily from the red algae *Gelidium* and *Gracilaria* (division Rhodophyta). It is a complex polysaccharide. It is highly valuable to microbiologists, although it provides no nutrient support for bacteria. It is commonly used in the laboratory setting primarily because of the following reasons: It remains solid and only a few microbes can degrade it.

**Agar Slants**: Solid growth surface: easier to store and transport than plates.

Storage and incubation of petri plates should be done in an inverted position to prevent dropping down of water droplets formed by condensation during solidification of media on to the surface of the hardened agar surface. This can spread the microorganisms on the agar surface, resulting in confluent growth instead of discrete colonies.

**Precaution in using pipettes**

- Never do pipetting with mouth.
- For culturing, sterilized pipettes should be used.
- Never keep pipettes on working surfaces.

During inoculation, inoculating wire is sterilized by heating in blue (hottest) portion of the flame till it becomes red hot followed by cooling either by touching to petri dish cover or inner surface of culture tube to avoid killing of microbial cells. Only a small amount of culture is used for inoculation. This can be accomplished by touching a single colony on agar surface by inoculating needle or by taking a loopful of culture from broth medium. After inoculation, inoculating wire is again sterilized to destroy remaining microorganisms.

Disinfectants are those chemicals that destroy pathogenic bacteria from inanimate surfaces. Some chemicals when used at appropriate concentration for appropriate duration can be used for sterilization and are called sterilant liquids. Those chemicals that can be safely applied over skin and mucus membranes are called antiseptics.

An ideal antiseptic or disinfectant should have following properties:

- Should have wide spectrum of activity
- Should be able to destroy microbes within practical period of time
- Should be active in the presence of organic matter
- Should make effective contact and be wettable
- Should be active in any pH, stable, have long shelf life, speedy
- Should have high penetrating power
- Should be non-toxic, non-allergenic, non - irritative or non - corrosive
- Should not have bad odour, leave non-volatile residue or stain
- Should not be expensive and must be available easily

## CULTURING AND MAINTENANCE OF MICROORGANISM OCCURRING IN SOIL

**Culture:** A microbial culture is a collection of cells that have been grown in or on a nutrient medium.

A **medium** (plural, media) is a liquid or solid nutrient mixture that contains all of the nutrients required for a microorganism to grow. In microbiology, we use the word **growth** to refer to the increase in cell number as a result of cell division.

A single microbial cell placed on a solid nutrient medium can grow and divide into millions of cells that form a visible **colony.**

**Broth culture:** Broth cultures are liquid cultures used to grow bacteria in laboratories. To create a broth culture, a scientist begins with a sterile liquid growth medium. The medium is inoculated with bacteria and placed in an incubator at the appropriate temperature. After a certain amount of time has passed, the broth becomes cloudy from the increased number of microbes.

**Isolation and enumeration of microorganisms from soil by the serial dilution –agar plating method**

- The serial dilution plating method or viable plate counting method is one of the commonly followed procedure for isolation and counting of fungi, bacteria and actinomycetes which are the most prevalent microorganisms.
- This method is based upon the principle that when material containing microorganisms cultured each viable microorganism will develop into a colony, hence the number of colonies appearing on the plate represent the number of living organisms present in the sample.
- In serial dilution agar-plate method a known amount (10 ml or 10 g) of material is suspended or agitated in a known volume of sterile water blank (90 ml or so to make the total volume to 100 ml) to make a microbial suspension. Serial dilutions $10^{-2}$, $10^{-3}$….$10^{-7}$ are made by pipetting measured volumes (usually 1ml or 10ml) into an additional dilution blanks (having 99 ml or 90 ml sterile water).
- Finally 1 ml aliquot of various dilutions are added to sterile petri dishes (triplicate for each dilution) to which are added 15 ml (approximately) of the sterile, cool molten (45ºC) media (Nutrient agar for bacteria, Glycerol yeast agar for actinomycetes and Czapek-Dox agar or Sabouraud agar medium , supplemented with chloro tetracycline or streptopenillin, 10µg/ml, for fungi).

**Precautions:**

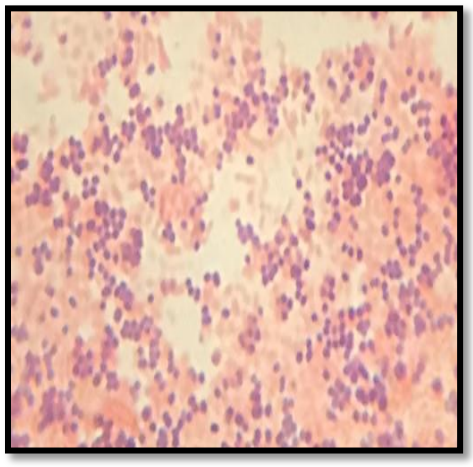
- Sterilization process should be done properly in order to avoid contamination
- Composition of each compound in the media should be weighed properly
- Mixing of the solution should be done properly in each dilution

## STAINING AND MICROSCOPIC EXAMINATION OF MICROBES

A variety of staining techniques can be used with light microscopy, including gram staining, acid fast staining, capsule staining, endospore staining and flagella staining. The gram stain is the most important staining procedure in microbiology. It is used to differentiate between gram positive organisms and gram negative organisms. Hence, it is a differential stain.

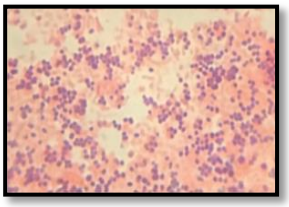
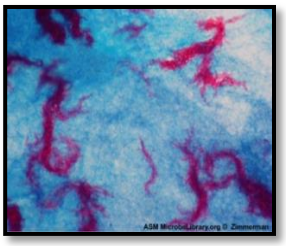
**Gram Staining:** The **Gram stain procedure** is a differential staining procedure that involves multiple steps. It was developed by Danish microbiologist Hans Christian Gram in 1884 as an effective method to distinguish between bacteria with different types of cell walls, and even today it remains one of the most frequently used staining techniques. The steps of the Gram stain procedure are listed below

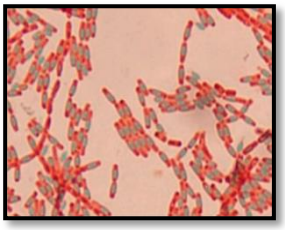
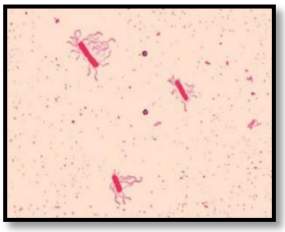
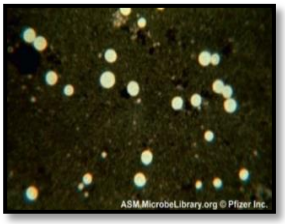
1. First, **crystal violet**, a **primary stain**, is applied to a heat-fixed smear, giving all of the cells a purple color.
2. Next, **Gram's iodine**, a **mordant**, is added. A mordant is a substance used to set or stabilize stains or dyes; in this case, Gram's iodine acts like a trapping agent that complexes with the crystal violet, making the crystal violet–iodine complex clump and stay contained in thick layers of peptidoglycan in the cell walls.
3. Next, a **decolorizing agent** is added, usually ethanol or an acetone/ethanol solution. Cells that have thick peptidoglycan layers in their cell walls are much less affected by the decolorizing agent; they generally retain the crystal violet dye and remain purple. However, the decolorizing agent more easily washes the dye out of cells with thinner peptidoglycan layers, making them again colorless.
4. Finally, a secondary **counterstain**, usually **safranin**, is added. This stains the decolorized cells pink and is less noticeable in the cells that still contain the crystal violet dye.



Gram-staining is a differential staining technique that uses a primary stain and a secondary counterstain to distinguish between gram-positive and gram-negative bacteria.

The purple, crystal-violet stained cells are referred to as gram-positive cells, while the red, safranin-dyed cells are gram-negative (Figure). However, there are several important considerations in interpreting the results of a Gram stain. First, older bacterial cells may have damage to their cell walls that causes them to appear gram-negative even if the species is gram-positive. Thus, it is best to use fresh bacterial cultures for Gram staining. Second, errors such as leaving on decolorizer too long can affect the results. In some cases, most cells will appear gram-positive while a few appear gram-negative (as in Figure). This suggests damage to the individual cells or that decolorizer was left on for too long; the cells should still be classified as gram-positive if they are all the same species rather than a mixed culture.

| Stain Type | Specific Dyes | Purpose | Outcome | Sample Images |
|---|---|---|---|---|
| ***Gram stain*** | Uses crystal violet, Gram's iodine, ethanol (decolorizer), and safranin | Used to distinguish cells by cell-wall type (gram-positive, gram-negative) | Gram-positive cells stain purple/violet. Gram -negative cells stain pink |  |
| ***Acid-fast stain*** | After staining with basic fuchsin, acid-fast bacteria resist decolonization by acid-alcohol. Non-acid-fast bacteria are counterstained with methylene blue. | Used to distinguish acid-fast bacteria such as *M. tuberculosis*, from non-acid-fast cells. | Acid-fast bacteria are red; non-acid-fast cells are blue. |  |

| | | | | |
|---|---|---|---|---|
| ***Endospore stain*** | Uses heat to stain endospores with malachite green (Schaeffer-Fulton procedure), then cell is washed and counterstained with safranin. | Used to distinguish organisms with endospores from those without; used to study the endospore | Endospores appear bluish-green; other structures appear pink to red. |  |
| ***Flagella stain*** | Flagella are coated with a tannic acid or potassium alum mordant, then stained using either pararosaline or basic fuchsin | Used to view and study flagella in bacteria that have them. | Flagella are visible if present |  |
| ***Capsule stain*** | Negative staining with India ink or nigrosine is used to stain the background, leaving a clear area of the cell and the capsule Counterstaining can be used to stain the cell while leaving the capsule clear | Used to distinguish cells with capsules from those without. | Capsules appear clear or as halos if present. |   |

**STUDY OF DECOMPOSITION OF FOREST LITTER BY $CO_2$ – EVOLUTION METHOD**

**Equipment and Apparatus Required:** Weighing balance, tin, bucket, phawda, hot air oven

**Reagents Required:** granular soda lime

**Procedure:**

- Weigh 20 g granular soda lime and put in an aluminium tin/can box.
- Put these tins on the surface of the soil litter in the forest
- Cover it by plastic bucket in inverted position
- Keep it covered for 24 hrs
- Lift the bucket and soda lime should be tightly capped
- Redry the soda lime for 24 hr at 100°C and weigh it
- Multiply the weight of the soda lime by 1.41 to account for the chemical water lost during the CO, fixation reaction
- Estimate $CO_2$ evolution using the acquired details

**Precautions**

- Bucket should be properly covering the tin/can box
- Don't take the sample from wet/waterlogged soil
- Weighing should be accurate as the difference in weight of soda lime will be little

**ESTIMATION OF NITRIFICATION RATE IN SOIL**

The in-situ technique involves collecting soil cores from field sites, destructively sampling one of the cores to determine the initial $NO_3^-$ concentrations and replacing the remaining cores (or soils in polyethylene bags) in the field and measuring the $NO_3^-$ accumulated after a specified time period. This technique enables nitrification to occur under field conditions, which include diurnal temperature fluctuations.

Ammonium is the substrate for autotrophic (and some heterotrophic) nitrification reactions, and it is typically added to soils through fertilizer addition (urea, $NH_4^+$, or anhydrous ammonia), manure addition, atmospheric deposition, or from the mineralization of plant or soil organic N. Once $NH_4^+$ is present, it could then be

- immobilized by the soil microbes, especially if an available carbon (C) source is present, such as fresh plant residues
- assimilated by plants
- volatilized if the soil is alkaline
- fixed by clay minerals
- nitrified to $NO_2^-$ and then to $NO_3^-$

Hence, decreases in $NH_4^+$ concentration over time cannot be solely attributed to nitrification because of the occurrence of other competing chemical or microbial processes in the soil. Instead, incubating soil and measuring the net increase in pool sizes of $NO_2^-$ and $NO_3^-$ would be a more useful indicator of the nitrification rate in soil.

The N mineralization and nitrification rate of soils could be used by producers or foresters to predict the amount of available N to crops or trees. For managed systems, this information could be used to adjust N application rates and timing and thereby match plant demand and minimize environmental impacts. Although most plants can readily absorb and utilize both $NH_4^+$ and $NO_3^-$, $NO_3^-$ is more readily transported to roots. Nitrate is, however, susceptible to leaching losses, especially in humid regions, and to denitrification losses when the soil is under anaerobic conditions and when a C source is available to the denitrifiers. In addition to regulating the N inputs to soil, nitrification can also be regulated with nitrification inhibitors such as nitrapyrin or dicyandiamide, or by applying smaller amounts of urea- or $NH_4^+$ based fertilizers several times during the growing season

**Procedure:**

- Select area from which you will collect sample
- Insert two core samplers in nearby place.
- Collect soil sample from one of the cores inserted in soil
- Estimate $NO_3^-$ content in the collected soil sample
- After 2-3 days, collect the remaining core sampler and estimate $NO_3^-$ content
- The difference in $NO_3^-$ content will give an idea of nitrification rate

**Precautions**

- Select the area of study in upland portion
- Avoid waterlogged soil sampling
- Insert core samplers properly in the soil for proper and accurate estimation

## ISOLATION OF *RHIZOBIUM* AND *AZOTOBACTER*

### *Rhizobium*

**Equipment and Apparatus Required:** Roots, weighing balance, mechanical shaker, conical flask, autoclave, hot air oven, laminar flow, inoculation needle, petri plates, bunsen burner, pipettes

**Reagents Required:** $K_2HPO_4$, $K_2SO_4.7H_2O$, NaCl, Mannitol, Yeast extract, Agar, Congo red 1% solution, Distilled water, ethyl alcohol, $HgCl_2$, $H_2O_2$

**Procedure:**

- Preparation of YEMA media

| | |
|---|---|
| $K_2HPO_4$ | 0.5 g |
| $K_2SO_4.7H_2O$ | 0.2 g |
| NaCl | 0.1 g |
| Mannitol | 10.0 g |
| Yeast extract | 1.0 g |
| Agar | 20.0 g |
| Congo red 1% solution | 2.5 ml |
| Distilled water | 1000 ml |

- Dissolve all the chemicals in distilled water and mix in agar solution. Make the volume to 1000 ml and autoclave it. Congo red solution is to be sterilized separately and added to the medium at the time of pouring in petri plates.
- Pour the autoclaved media after cooling (45-50°C) into sterile plates.
- Allow the medium to solidify.
- Uproot roots of leguminous plants and bring to the laboratory
- Wash the roots in running tap water to remove adhering soil particles
- Select healthy pink, unbroken and firm root nodules and wash in water

- Immerse the nodules in 0.1 % $HgCl_2$ or 3-5% $H_2O_2$ for 5 minutes for surface sterilization
- Repeatedly wash the nodules in sterile water for 3-4 times to get rid of the sterilizing agent.
- Place the nodules in 70% ethyl alcohol for 3 minutes (if treated with $HgCl_2$)
- Repeatedly wash the nodules in sterile water
- Crush a nodule in 1 ml of water with a sterile glass rod
- Make a uniform suspension of rhizobia with water
- Make serial dilutions of the nodules extract
- Prepare serial dilutions $10^{-3}$, $10^{-4}$, $10^{-5}$ and $10^{-6}$
- Spread 1 ml each of suspensions from various dilutions on YEMA media
- Incubate the plates at 26°C for 10 days

**Precautions**

- Pinkish nodules should be selected for isolation
- Nodules should be surface sterilized properly and should be free from all the surface borne microorganisms

### *Azotobacter*

**Equipment and Apparatus Required:** Soil sample, weighing balance, mechanical shaker, conical flask, autoclave, hot air oven, laminar flow, inoculation needle, petri plates, bunsen burner, pipettes, magnetic shaker

**Reagents Required:** Mannitol, $K_2HPO_4$, $MgSO_4.7H_2O$, NaCl, $K_2SO_4$, $CaCO_3$, Agar, Distilled water

**Procedure:**

- Preparation of Ashby culture media

| | |
|---|---|
| Mannitol | 20.0 g |
| $K_2HPO_4$ | 0.2 g |
| $MgSO_4.7H_2O$ | 0.2 g |
| NaCl | 0.2 g |
| $K_2SO_4$ | 0.1 g |
| $CaCO_3$ | 5.0 g |
| Agar | 15.0 g |
| Distilled water | 1000 ml |

- Dissolve mannitol, $MgSO_4.7H_2O$, NaCl, $K_2SO_4$, $CaCO_3$ in 200 ml distilled water. In another flask, dissolve $K_2HPO_4$ in 100 ml distilled water. Mix all the constituents and make the volume to 1000 ml and autoclave it 121°C for 15 minutes. Congo red solution is to be sterilized separately and added to the medium at the time of pouring in petri plates.
- Pour the autoclaved media after cooling (45-50°C) into sterile plates.
- Allow the medium to solidify.
- Add 10 g sieved (2 mm) soil to 90 ml sterile water and shake it for 15 – 20 minutes on a magnetic shaker.
- Prepare serial dilutions $10^{-2}$, $10^{-3}$, $10^{-4}$ and $10^{-5}$
- Spread 1 ml each of suspensions from various dilutions on solidified agar media
- Incubate the plates at 28°C for 3 days

**Precautions**

- Always use cooled and set agar medium for pouring of suspensions
- Evenly spread the suspension over medium

## PREPARATION AND INOCULATION TECHNIQUES FOR MYCORRHIZAE AND BIOFERTILIZERS

**1. Microorganism isolation and identification or procurement of well-known beneficial microorganisms like *Rhizobium, Azotobacter* etc.**

Rhizospheric soil samples should be collected from various farmlands. For each sample, 1 g soil should be suspended in 10 mL sterilized water, diluted to $10^{-7}$, and then 100 µL of each dilution should be spread on the agar medium. After incubation of the plates for 4 d at 30°C, colonies on the plates should be selected, purified by repeated culturing, and maintained on slants at 4°C. Then screening of beneficiary traits should be done to select best microbes. Lastly, molecular characterization should be done to know their identity.

These processes may require a lot of time, so we can procure already known microbes with good plant growth promoting traits from an institute where they have maintained the cultures.

2. **Carrier preparation:** Carrier materials may be peat, wheat husk, corn cob, composted cattle manure etc. Carrier materials should be first powdered and passed through a 100-mesh sieve before physicochemical characterization. The main criteria used to select carrier materials should be the ability to adjust the pH to neutral (pH 7.0), high water holding capacity, low cost, and wide availability. The pH of all of the materials should be adjusted to pH 7.0 with $CaCO_3$ before use. To adjust pH, materials should be thoroughly mixed with $CaCO_3$ powder.
3. **Carrier sterilization:** The packages of carriers should be sterilized by autoclaving. For the autoclaving treatment, the samples should be autoclaved for 40 min at 121°C. The sterilized bags should be placed into another large sterile cotton bag after cooling overnight in the autoclave. After sterilization, the packages should be dried for 12 h at 60°C in a blow-type oven or simple air dry.
4. **Inoculant preparation and incubation:** To obtain fresh colony of microbial culture, the bacteria should be grown on nutrient agar medium at 30°C for 5 d. The colonies must be recovered from plates in 5 mL sterile broth media. The bacteria can be mass produced in a broth media of 500 ml conical flask. The media along with bacteria can be mixed thoroughly with sterilized carrier materials. Population density should be counted on colony counter.

**Inoculation technique for mycorrhizae:** Modern inoculants are available in diverse forms such as granular, powder and liquids to accommodate a variety of equipment and application methods.

Seed treatment is best accomplished using either powdered or liquid mycorrhizal inoculants applied so that the inoculum adheres directly to the surface of the seed. Powdered inoculants work well with hairy-textured seeds such as wheat, barley, oats or many grass seeds. Seed adhesion is important not only to insure inoculum proximity to the germinating seed, but because excess powder falling off the seeds can accumulate in the seed box, possibly leading to mechanical problems with the auger and drill operation of the planting equipment.

A liquid inoculum is often preferred for corn, beans, alfalfa and similar smooth-surfaced seeds because it will adhere well. A “sticker” or tackifier product is necessary to apply powdered inoculants to these types of seeds to keep the powder attached to the seed surface. Inoculum application can also be accomplished using specialized seed-treating equipment, a service often provided by seed or agronomy suppliers.

**Advantages of biofertilizers:**

- Biofertilizers are means of fixing the nutrient availability in the soil.
- Since a bio-fertilizer is technically living, it can symbiotically associate with plant roots. Involved microorganisms could readily and safely convert complex organic material into simple compounds, so that they are easily taken up by the plants. Microorganism function is in long duration, causing improvement of the soil fertility. It maintains the natural habitat of the soil.
- It increases crop yield by 20-30%, replaces chemical nitrogen and phosphorus by 30%, and stimulates plant growth. It can also provide protection against drought and some soil-borne diseases.
- It has also been shown that to produce a larger quantity of crops, biofertilizers with the ability of nitrogen fixation and phosphorus solubilizing would lead to the greatest possible effect.
- Biofertilizers also promote healthy soil, leading to greater farming sustainability.