Semester – IV

17MBU414A BIOFERTILIZERS AND BIOPESTICIDES - PRACTICAL (3H – 1C)

Instruction Hours / week: L: 0 T: 0 P: 3 **Marks:** Internal: **40** External: **60** Total: **100**

End Semester Exam: 6 Hours

SCOPE

This course has been designed to provide the student knowledge about eco friendly product which play a crucial role in determining its future use and applications in environmental management. Provides detailed idea about biofertilizer production and plant disease.

OBJECTIVES

- To study about the biofertilizers in increasing soil fertility and usage of Biopesticides for plant disease.

EXPERIMENTS

1. Isolation and identification of *Rhizobium* spp. from root nodules – mass production and application.
2. Isolation and identification of *Azotobacter* spp. – mass production and application.
3. Isolation and identification of *Azospirillum* spp. – mass production and application.
4. Isolation and identification of phosphate solubilizing bacteria – mass production and application.
5. Isolation and identification of mycorrhizae.
6. Isolation and maintenance of *Azolla*.
7. Isolation and identification of *Anabena* and *Nostoc* – its mass cultivation.
8. Isolation, identification and maintenance of *Bacillus thuringiensis*.
9. Isolation and identification of *Trichoderma viridae* – its mass cultivation.
10. Isolation and identification of *Beauveria bassiana* – its mass cultivation.

SUGGESTED READINGS

1. Kannaiyan, S. (2003). Biotechnology of Biofertilizers, CHIPS, Texas.
2. Mahendra K. Rai (2005). Hand book of Microbial biofertilizers, The Haworth Press, Inc. New York.
3. Reddy, S.M. *et. al.* (2002). Bioinoculants for sustainable agriculture and forestry, Scientific Publishers.
4. Subba Rao N.S (1995) Soil microorganisms and plant growth Oxford and IBH publishing co. Pvt. Ltd. NewDelhi.
5. Saleem F and Shakoori AR (2012) Development of Bioinsecticide, Lap Lambert Academic Publishing GmbH KG.

Isolation and identification of *Rhizobium* sp. from root nodules – mass production and application

Aim

To isolate and identify *Rhizobium* sp. from root nodule of leguminous plants.

Background

Rhizobium is a soil habitat bacterium, which can able to colonize the legume roots and fixes the atmospheric nitrogen symbiotically. This belongs to bacterial group and the classical example of symbiotic nitrogen fixation. The bacteria infect the legume root and form root nodules within which they reduce molecular nitrogen to ammonia which is reality utilized by the plant to produce valuable proteins, vitamins and other nitrogen containing compounds. The site of symbiosis is within the root nodules. The morphology and physiology of *Rhizobium* will vary from free-living condition to the bacteroid of nodules. They are the most efficient biofertilizer as per the quantity of nitrogen fixed concerned. They have seven genera and highly specific to form nodule in legumes, referred as cross inoculation group. Rhizobium inoculum contains the viable cells of Rhizobium which fixes the atmospheric nitrogen when the roots of higher leguminous plants are injected by Rhizobium.

Materials required

Sample – Root nodules
Media – Yeast Extract Mannitol Agar (YEMA)
Chemicals – 0.1 acidified Kcl or sodium hypochlorite
Apparatus – Morter and pestle, petriplates, conical flasks, forceps.

Procedure

- The leguminous plants are uprooted and tested of any nodule is present in the root.
- The root nodule which are white brown to pink green in color and washed in water in order to eradicate the soil particles.
- Then a pinkish green nodule is selected and washed in distilled water.
- The washed root nodule is kept immersed in 0.1 acidified Kcl solution for 5 min.
- Then again wash the nodule to remove the disinfectant.
- Finally the nodule is immersed in ethyl alcohol and later washed with sterile H_2O.
- The Rhizobium is isolated either by washing the nodule in pestle and morter or by cutting the nodule and streaking.
- The washed juice is collected by a sieve and serially diluted and plated.

- The nodule is streaked in a solid media to obtain proper growth of the bacteria. The media used for the growth of Rhizobium is yeast extract mannitol agar medium and incubated at 30 °C 3 to 10 days depending on the nature of the Rhizobium.
- The correct strain of Rhizobia is identified by nodule formation, cultural tests, microscopic observation and staining techniques.
- The rhizobial cells from the culture identified are mass cultured for the preparation of inoculum.
- The selected rhizobial strain is cultured in YEMA medium for about 7 days in order to establish better growth.
- The Rhizobium culture is tested and the tested Rhizobial culture is transferred to a large container containing the sterile YEMA medium are incubated at 30 °C for 9 days.
- Sufficient nutrients should be supplied at regular intervals of 24 hrs.

Results

Isolation and identification of Azotobacter sp. – mass production and application

Aim

To isolate and identify *Azotobacter* sp. from soil sample.

Background

Azotobacter are free living bacteria which grow well on a nitrogen free medium. Azotobacter is one of the most dominant non-symbiotic nitrogen fixing heterotrophic bacteria. These bacteria utilize atmospheric nitrogen gas for their cell protein synthesis. Azotobacter is gram negative polymorphic bacteria. The species of Azotobacter are known to fix on an average 10 mg of N/g of sugar in pure culture on a nitrogen free medium. The population of Azotobacter is mostly influenced by other microorganisms present in soil. There are some microroganisms which stimulate the Aztobacter population in soil thereby increasing the nitrogen fixation by Azotobacter. On the other hand there are some microorganisms which adversely affect the Azotobacter population and hence nitrogen fixation process is hampered. Azotobacter naturally fixes atmospheric nitrogen in the rhizosphere. There are different strains of Azotobacter each has varied chemical, biological and other characters. The medium used for the growth of Azotobacter is required to have the presence of organic nitrogen, micronutrients and salts in order to enhance the nitrogen fixing ability of Azotobacter.

Materials required

Soil samples, petriplates, conical flask, L-rod, pipette, Ashbys medium.
Ashbys Mannitol medium (Nitrogen free medium)

Ingredients	**gm/L**
Sucrose	20
Dipotassium phosphate	1.000
Magnesium sulphate	0.500
Sodium chloride	0.500
Ferrous sulphate	0.100
Sodium molybdate	0.005
Calcium carbonate	2.000
Agar	15.000
Distilled water	1000ml

Procedure

- Soil samples were collected from soils and rhizosphere of plants like cotton, sunflower, onion, tomato and sugarcane.

- The collected soil samples were kept in polythene packets and brought to the laboratory for analysis.
- About 1gm of soil samples was added into 99 ml of the sterile distilled water and considered as stock solution.
- Then 1 ml of the stock solution is added to 9 ml of sterile distilled water to obtain 10^{-1} dilution, the sample was serially diluted up to 10^{-7} dilution.
- Then spread plate technique was performed using Ashbys mannitol medium for the isolation. 01 ml of the sample were inoculated on Ashbys mannitol agar medium and incubated at 28 °C for 3 days.
- After incubation the growth on the medium were presumed to be Azotobacter are the sub cultured on Ashbys mannitol agar medium for conformation.
- Isolated Azotobacter was identified on the basis of morphological, cultural and biochemical characteristics such as gram staining and motility.
- Then isolated Azotobacter was mass culture in Ashbys Mannitol broth medium and was incubated at 28 °C for 3 days.
- After 3 days the cultures were mixed with carrier material for biofertilizer production.

Results

Isolation and identification of Azospirillum sp. – mass production and application

Aim

To isolate and identify *Azospirillum* sp. from rhizosphere of cereals.

Principle

Azospirillum sp. occurs as free living in soil or in association with the roots of cereal crops, grasses and tuber plant. Azospirillum sp. is plants associated diazotrophs of the alpha subclass of Proteobacteria. Azospirillum medium with 0.17% agar is used for the cultivation of Azospirillum sp. Malic acid is used as the carbon source. Azospirillum sp. grows well in the presence of malic acid are not overgrown by other nitrogen fixers. Dipotassium phosphate provides buffering effect and other inorganic salt ingredients provide necessary growth nutrients. Agar at 0.17% concentration provides microaerophilic conditions necessary for nitrogen fixation by Azospirillum species.

Materials required

Soil samples, petriplates, conical flask, L-rod, pipette, Nitrogen free bromothymol blue medium.

Ingredients	**gm/L**
Malic acid	5.0
Potassium hydroxide	4.0
K_2HPO_4	0.5
Ferrous sulphate	0.05
Manganese sulphate	0.01
Magnesium sulphate	0.1
Sodium chloride	0.02
Calcium chloride	0.01
Na_2MoO_4	0.002
Bromo thymol blue	2.0 ml
Sodium molybdate	0.002
Agar	15
Final pH	6.5 – 7.0

Procedure

- The roots are separated from the plants and thoroughly washed in running tap water.
- Then transferred into 1 L flask containing 500 mL of sterile tap water and shaken for 30 min. The procedure is repeated three times, after which the same procedure is repeated with distilled water three times.

- The washed roots are transferred to sterile petri dish and are cut into pieces with sterile scissors.
- Half centimeter long root pieces are surface sterilized in 70% alcohol for 3-5 seconds.
- The root pieces are repeatedly washed in phosphate buffer (pH 7.0) and then they are plated in semi solid, nitrogen free medium.
- The plates are incubated at 35 °C for 48 hours.
- Characteristic growth of *Azospirillum* is indicated by the formation of white pellicles 2-4mm below the surface of the medium.

Isolation and identification of phosphate solubilizing bacteria – mass production and application

Aim

To isolate and identify phosphate solubilizing bacteria from rhizosphere soil.

Principle

Phosphate exists in both organic as well as inorganic forms in soil. Organic matter derived from dead and decaying plant debris is rich in organic sources of phosphorus. However, plants are able to utilize phosphorus from soil only in the free available form. Soil phosphates are rendered available either by plant roots or by soil microorganisms. Therefore, phosphate dissolving soil organisms play a part in correcting deficiency of crop plants. Yeast exract in the medium provides nitrogen and other nutrients necessary to support bacterial growth. Dextrose acts as an energy source. Different salts and yeast extract supports the growth of organisms. Phosphate solubilizing bacteria will grow on this medium and form a clear zone around the colony, formed due to phosphate solubilization in the vicinity of the colony.

Materials required

Rhizosphere soil samples, petriplates, conical flask, L-rod, pipette, Pikovskaya's agar medium.

Ingredients	**gm/L**
Yeast extract	0.500
Dextrose	10.00
Calcium phosphate	5.0
Ammonium sulphate	0.500
Potassium chloride	0.200
Magnesium sulphate	0.100
Manganese sulphate	0.0001
Ferrous sulphate	0.0001
Agar	15.000

Procedure

- Soil samples were collected from the rhizosphere at a depth of 15cm.
- The samples were then air-dried, powdered and mixed well to represent a single sample.
- Phosphate solubilizing bacteria were isolated from soil sample by serial dilution and spread plate method.
- 0.1ml of each dilution was spread on plates containing Pikovskaya's agar medium (PVK) and incubated at 27 – 30 °C for 7 days.
- Colonies showing clear zones were picked from Pikovskaya's (PVK) agar medium for studying colony morphology**.**

Isolation and maintenance of Azolla

Aim

To isolate and maintain the Azolla

Principle

Azolla is a free-floating water fern that floats in water and fixes atmospheric nitrogen in association with nitrogen fixing blue green alga *Anabaena azollae*. Rice growing areas in South East Asia and other countries have recently been evincing increased interest in the use of the symbiotic N2 fixing water fern *Azolla* either as an alternate nitrogen sources or as a supplement to commercial nitrogen fertilizers. *Azolla* is used as biofertilizer for wetland rice and it is known to contribute 40-60 kg N ha-1 per rice crop. The agronomic potential of *Azolla* is quite significant particularly for rice crop and it is widely used as biofertilizer for increasing rice yields. The common species of *Azolla* are *A. microphylla, A. filiculoides, A. pinnata, A. caroliniana, A. nilotica, A. rubra* and *A. mexicana*.

Materials

- One cent (40 sq.m) area plot
- Cattle dung
- Super phosphate
- Furadan
- Fresh *Azolla* inoculum

Procedure

- Select a wetland field and prepare thoroughly and level uniformly.
- Mark the field into one cent plots (20 x 2m) by providing suitable bunds and irrigation channels.
- Maintain water level to a height of 10 cm.
- Mix 10 kg of cattle dung in 20 litres of water and sprinkle in the field.
- Apply 100 g super phosphate as basal dose.
- Inoculate fresh *Azolla* biomass at 8 kg to each pot.
- Apply super phosphate at 100 g as top dressing fertilizer on 4th and 8th day after *Azolla* inoculation.
- Apply carbofuran (furadan) granules at 100 g/plot on 7th day after *Azolla* inoculation.
- Maintain the water level at 10 cm height throughout the growth period of two or three weeks.
- Observations
- Note the *Azolla* mat floating on the plot. Harvest the *Azolla*, drain the water and record the biomass.

Results

Isolation and maintenance of *Bacillus thuringiensis*

Aim

To isolate and maintain the organism *Bacillus thuringiensis* as biopesticides

Principle

Bacteria belonging to genus *Bacillus* are potent against many insect pests. They suppress pests by producing a toxin specific to the pest; causing a disease; preventing establishment of other microorganisms through competition; or other modes of action. An example of a bacterial pesticide is *Bacillus thuringiensis*, or "Bt". *Bacillus thuringiensis* (Bt) is a ubiquitous gram-positive, spore forming bacterium which produces parasporal crystals during sporulation (stationary phase of its growth cycle). *Bacillus thuringiensis* is a naturally occurring soil bacterium that is toxic to the larvae of several species of insects but not toxic to non-target organisms.

Procedure

- Soil samples were collected from soils. The collected soil samples were kept in polythene packets and brought to the laboratory for analysis.
- About 1gm of soil samples was added into 99 ml of the sterile distilled water and considered as stock solution.
- Then 1 ml of the stock solution is added to 9 ml of sterile distilled water to obtain 10^{-1} dilution, the sample was serially diluted up to 10^{-7} dilution.
- Then spread plate technique was performed using medium Hicrome Bacillus agar supplied L-serine selective medium and incubated at 27 °C.

Results