Semester – IV

18MBU414A 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

COURSE OBJECTIVES

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

COURSE OUTCOME

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.

EXPERIMENTS

1. Mass production and application of *Rhizobium* spp. from root nodules
2. Mass production and application of *Azotobacter* spp.
3. Mass production and application of *Azospirillum* spp.
4. Mass production and application of phosphate solubilizing bacteria- quantification, infectivity and potential of bacteria
5. Mass production and application of mycorrhizae.
6. Mass production and application of *Bacillus thuringiensis*.
7. Mass production and application of *Trichoderma viridae*- Inhibition studies.
8. Mass production and application of *Beauveria bassiana*
9. Isolation and identification of potash solubilizing microbes.
10. Isolation and identification of zinc solubilizing microbes.

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.

PRACTICALS

Mass production and application of *Rhizobium* sp. from root nodules

Aim

To isolate, identify and mass culture of *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 ℃ 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 ℃ for 9 days.
- Sufficient nutrients should be supplied at regular intervals of 24 hrs.

Results

Mass production and application of Azotobacter sp.

Aim

To isolate, identify and mass production of *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 ℃ 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 ℃ for 3 days.
- After 3 days the cultures were mixed with carrier material for biofertilizer production.

Results

Mass production and application of *Azospirillum sp.*

Aim

To isolate, identify and mass culture of *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 ℃ for 48 hours.
- Characteristic growth of *Azospirillum* is indicated by the formation of white pellicles 2-4mm below the surface of the medium.

Aim

Mass production and application of phosphate solubilizing bacteria

To isolate, identify and mass culture of 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 ℃ for 7 days.
- Colonies showing clear zones were picked from Pikovskaya's (PVK) agar medium for studying colony morphology**.**

Mass production and application of mycorrhizal biofertilizer

Aim

To isolate, identify and mass culture of mycorrhiza

Principle

Mycorrhizae are mutualistic symbiotic associations formed between the roots of higher plants and fungi. It is an Greek word, mykes: mushroom or fungi; rhiza: root. Fungal roots were discovered by the German botanist A B Frank in the last century (1855) in forest trees such as pine. In nature approximately 90% of plants are infected with mycorrhizae. 83% Dicots,79% Monocots and 100% Gymnosperms. Convert insoluble form of phosphorous in soil into soluble form.

Isolation

sample + sterile water

Hot water

Filter and sieve

(719µm → 250µm → 50µm → 45µm)

Spores separated from soil particles

Mix with carrier material

Use when required as biofertilizer

Mass production

Spores + antibiotic solution
(streptomycin of 220 ppm concentration for 15 min)

Wash spores with mercuric chloride

Wash with distilled water

Inoculate the plant pots (Guinea grass or Bahiya grass)

Keep in green house for 3 - 4 weeks

Uproot the plants