ANNAMALAI **UNIVERSITY**

FACULTY OF AGRICULTURE

DEPARTMENT OF AGRICULTURAL MICROBIOLOGY

II. Diploma in Agriculture **IV - SEMESTER**

PRACTICAL MANUAL CUM RECORD

CAG – AGM 227 - BIOFERTILIZER TECHNOLOGY (0 + 2)

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EX.NO	DATE	TITLE OF THE EXPERIMENT	SIGNATURE
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2.		**ISOLATION OF N_2 FIXING BACTERIA - *Azospirillum* FROM PADDY ROOT SAMPLES**	
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13.		**METHODS OF APPLICATION OF DIFFERENT BIOFERTILIZERS**	
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Ex.No: 1	ISOLATION OF ROOT NODULE BACTERIUM *Rhizobium* FROM ROOT NODULES
Date:	

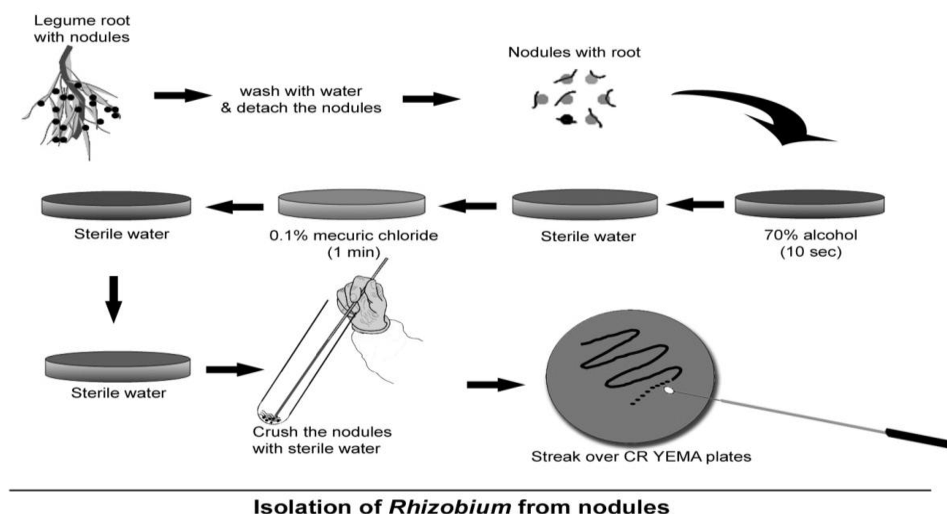
Introduction

Symbiotic associations between *Rhizobium* – legume plants are the primary biological contributors of fixed nitrogen in soil based ecosystem and most studied one also. Symbiotic N_2 fixation is dependent upon the infection of the host root by the appropriate microbial symbiont and the subsequent development of the required enzymes. *Rhizobium* is the microsymbiont, which infects the roots of legume and nodulate. Rhizobium is a common name of the nodulating microsymbiont which consists of six genera as *Rhizobium, Bradyrhizobium, Mesorhizobium, Sinorhizobium, Allorhizobium, Azorhizobium* with about 36 species.

Rhizobia are aerobic, gram-negative, non-sporulating rod shaped bacteria which form specialized structures on roots called "nodules". The size and morphology of the nodules vary with the plant species. The nodules on clover are relatively small and round or oval shaped. On the other hand, cowpea, common bean, and soybeans, the nodules are relatively large, round, and firmly attached to the root. On alfalfa, peas and vetch, the nodules are usually longer and finger-like.

Materials required

- Well developed legume nodule
- Mercuric chloride (0.1%) and Alcohol (70%)
- Forceps, Glass rod , Petri dishes and sterile water blanks
- Congo red yeast extract mannitol agar medium
- Inoculation needle



Isolation of *Rhizobium* from nodules

Procedure

- Uproot the plant and wash the roots gently and thoroughly under running tap water to remove soil particles
- Remove the nodules along with root portion without damaging it.
- Immerse intact, undamaged nodules for 5 – 10 seconds in 70 % ethanol
- Rinse the nodules in sterile water
- Surface sterilize the nodules by soaking in 0.1% acidified mercuric chloride or 2.5 – 3.0 % sodium hypochlorite solution for 1 – 2 minutes
- Wash the nodules in 5 - 6 changes of sterile water using sterile forceps
- Crush the sterilized nodules with a blunt ended sterile glass rod in a large drop of sterile water in a petri dish / test tube
- Using sterile inoculation needle transfer one loopful of nodule suspension and streak it over the sterile solidified CRYEMA medium already poured in Petri plate.
- Simultaneously, aliquots of serial dilutions prepared from the nodule suspension may be used for plating with YEMA either by spread plate method or pour plate method
- Incubate the plates at 28°C for 3 – 5 days.
- Appearance of circular, raised and white translucent colonies indicates *Rhizobium.* Red colored, small colonies are *Agrobacterium.*

Purification

A loopful of *Rhizobium* colony is taken in the inoculation needle and streaked on fresh yeast extract mannitol agar plates for purification. The purified cultures of *Rhizobia* are maintained on agar slants of the same medium. After isolation, the strains are purified and then authenticated.

Result:

Ex.No: 2	ISOLATION OF N_2 FIXING BACTERIA - *Azospirillum* FROM PADDY ROOT SAMPLES
Date:	

Introduction

The bacteria of the genus *Azospirillum* are gram negative, associative symbiotic micro aerophilic nitrogen fixing organism . "Dobereiner' who first isolated the organism. The cells of *Azospirillum* are comma or spiral shaped having abundant accumulation of poly β- hydroxybutyrate (70 %) in its cytoplasm. The important species of *Azospirillum* are *A. brasilense*, *A.lipoferum*, *A.amazonense*, *A.halopraeferens* and *A.irakense*.

A. Isolation of *Azospirillum*

Principle

When root sample containing *Azospirillum* is introduced into the N free malic acid medium, *Azospirillum* starts utilizing malic acid present in the medium as carbon source which resulted in the colour change from yellowish green to blue colour due to the change in pH of the medium from acidic to alkaline.

Materials required

- Freshly collected root samples
- 80 % ethanol or 0.1% Mercuric chloride
- Sterile water blanks
- N-free malic acid semisolid medium in test tubes
- Sterile forceps and petridishes

N-free semisolid malic acid medium

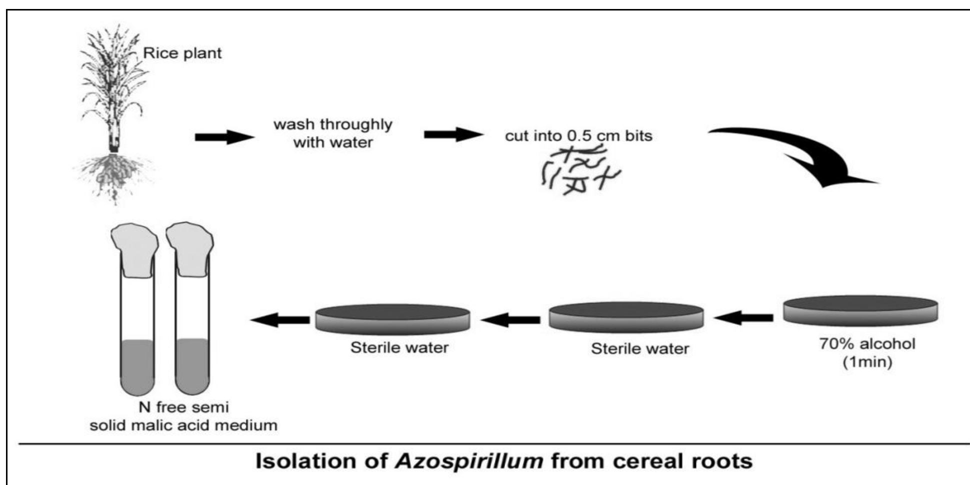
Malic acid	:	5.0g
Potassium hydroxide	:	4.0g
Dipotassium hydrogen orthophosphate	:	0.5g
Magnesium sulphate	:	0.2g
Sodium chloride	:	0.1g
Calcium chloride	:	0.2g
Fe-EDTA (1.64% w/v aqueous)	:	4.0 ml
Trace element solution	:	2.0 ml
BTB (0.5% alcoholic solution)	:	2.0 ml
Agar	:	1.75 g
Distilled water	:	1000 ml
pH	:	6.8

Trace element solution

Sodium molybdate	: 200 mg
Manganous sulphate	: 235 mg
Boric acid	: 280 mg
Copper sulphate	: 8 mg
Zinc sulphate	: 24 mg
Distilled water	: 200 ml

Procedure

- Prepare semisolid malic acid medium in test tubes in 5 ml quantity and sterilize 121°C (15 psi) for 15 min.
- Collect fresh root samples from any graminaceous plant
- Wash the roots in tap water to remove the adhering soil particles.
- Using sterilized knife/ blade, cut the roots into small bits of 1-2 cm size.
- Surface sterilize the root bits by immersing them in either 80% ethanol or 0.1% mercuric chloride for 1 min.
- Wash the root bits with sterile distilled water 3-4 times to remove the excess ethanol or mercuric chloride.
- Using sterile forceps transfer aseptically 2-3 root bits to the test tubes containing N-free semisolid malic acid medium.
- Incubate the tubes under room temperature 28 ± 2°C for 2-3 days
- Maintain one tube as control without root bits



Isolation of *Azospirillum* from cereal roots

Observation

- At the end of incubation time observe the tubes for the change of the color of the medium from yellowish green to blue and for the formation of subsurface white pellicle.
- Collect the positive tubes and take a loopful of the growth from the pellicle and streak on the Malate medium in Petri dish; purify and maintain in slants.

Result:

Ex.No: 3	ISOLATION OF N_2 FIXING BACTERIA - *Azotobacter*
Date:	FROM SOIL SAMPLES

Azotobacter

Azotobacter is the non-symbiotic, free living, aerobic nitrogen fixing bacterium. In general, cells are gram negative, polymorphic, form cyst and accumulate poly β–hydroxybutyrate (PHB) and produces abundant gum. In addition to N fixation, they secrete plant growth hormones *viz*, IAA, GA and growth factors *viz*., thiamine, riboflavin etc. and produces some antifungal antibiotics also. Totally six species are identified based on their pigmentation. Among them, *A. chroococcum* is the dominant species found in tropical soils.

A. chroococcum	-	Produces black pigments (melanin)
A. vinelandii	-	Produces yellow pigments
A. beijerinckii	-	Produces green yellow fluorescent pigments
A. insignis	-	Produces yellow – brown pigments
A. macrocytogenes	-	Produces pink pigments
A. paspali	-	Produces pink to green fluorescent pigments

Isolation

Beijerinck was the first to isolate and describe *Azotobacter*. *Azotobacter* cells are not present on the rhizoplane but are abundant in the rhizosphere region. Lack of organic matter in the soil is a limiting factor for the proliferation of *Azotobacter*. They depend on the energy derived from the degradation of plant residues.

Materials Required

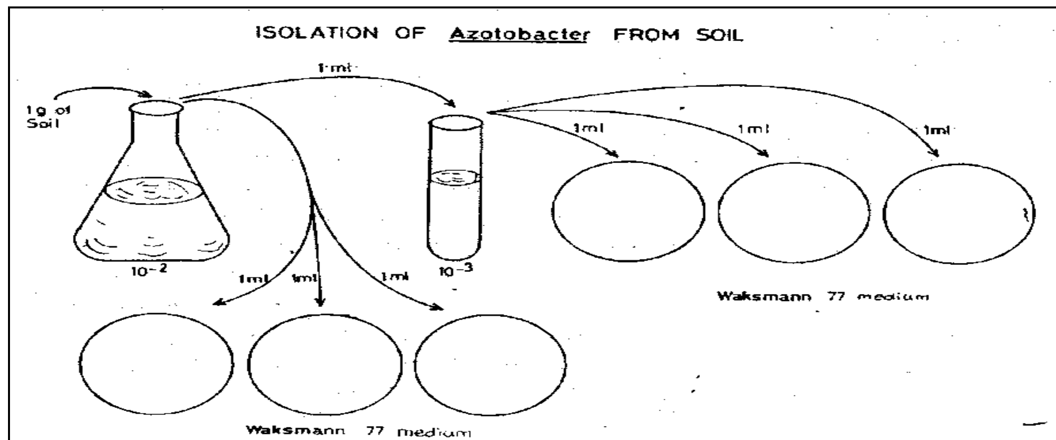
- Organic matter rich soil sample
- Sterile water blanks
- Petri plates
- Waksman No.77 medium

Waksman No.77 Medium

Mannitol	:	10.0 g
$CaCO_3$	:	5.0 g
K_2HPO_4	:	0.5 g
$MgSO_4.7H_2O$	:	0.2 g
NaCl	:	0.2 g
Ferric chloride	:	Trace
$MnSO_4.4H_2O$	:	Trace
N-free washed Agar	:	15.0 g
pH	:	7.0
Distilled Water	:	1000 ml

Procedure

1. Weigh one g of sample and put in the 100 ml water blank and mix thoroughly
2. Shake for 15 min for complete dispersion (*10^{-2}dilution)*
3. Transfer one ml of the suspension to 9 ml water blank *(10^{-3} dilution)*
4. Transfer 1 ml of appropriate dilutions (10^{-3}) to Petri dishes
5. Maintain 2 or 3 replications for each dilution
6. Pour melted and cooled media (just before solidification) of about 15 ml and mix well by shaking clock wise and anti clock wise for 3 or 4 times and allow it for complete solidification
7. Incubate the plates in inverted position at room temperature for 3-4 days for appearance of *Azotobacter* colonies.
8. Determine the moisture content of the soil as described earlier.



Results and Observation

Azotobacter produces raised, gummy colonies on agar surface and aged cultures show yellowish brown/black colouration due to pigment production.

Ex.No: 4	**ISOLATION OF ENDOPHYTIC N_2 FIXING BACTERIUM -**
Date:	***Gluconacetobacter* FROM SUGARCANE**

Introduction

Gluconacetobacter diazotrophicus is an endophytic nitrogen fixing bacterium first isolated from the sugarcane growing regions of Brazil. *G. diazotrophicus* strains were isolated from samples of sugar rich crops like sugar cane, sweet potato, pine apple and wild cane. It is found to occur in large numbers in the root, stem and leaf tissues. In addition to nitrogen fixation, these organisms also produce indole acetic acid, which serves as growth promoter. These organisms can tolerate up to 30% sucrose concentration and pH as low as 3.0. It is considered highly suitable for sugarcane environment. It can be isolated from sugar rich crops like sugar cane, sweet potato, pine apple and wild cane.

Materials

- Sample - Sugarcane (roots, bud, leaf and stem)(*Modified roots of sweet potato, fruit of pineapple, stem of wild cane can also be used*)
- 70% alcohol
- Sterile pestle and mortar
- LGI Semisolid medium

Semi-Solid LGI medium (g/l)

Dipotassium hydrogen phosphate	0.200 g
Potassium dihydrogen phosphate	0.600 g
Magnesium sulphate	0.200 g
Calcium chloride	0.020 g
Sodium molybdate	0.002 g
Ferric chloride	0.010 g
Bromothymol blue	5.0 ml
(0.5% solution in 0.2N KOH)	
Cane sugar	100.0 g
Agar	1.8 g
Distilled water	1000ml
pH	6.0

Semisolid diluted Cane Juice Medium

Semisolid LGI medium	250ml
Sugarcane juice	250ml
Distilled water	500ml
Agar	1.8 g

Semisolid Acetic LGI Medium

- Semisolid LGI medium was acidified with glacial acetic acid to pH 4.5.
- Agar concentration was increased to 2.2 g l^{-1}

Procedure

- Collect the sugar rich plant tissues like root/stem/leaf
- Wash thoroughly 1 g of the sample in running tap water
- Rinse the washed materials in 70% alcohol for 15 seconds to remove the surface contaminants
- Immediately wash in sterile distilled water for about 3-4 times to remove alcohol from the tissues
- Crush the surface sterilized samples in a sterile pestle and mortar
- Inoculate 0.01 ml of the crushed suspension directly into various enrichment media *viz.*, semisolid diluted cane juice medium/ semi solid LGI /acetic LGI medium in test tubes
- Incubate the tubes at room temperature without disturbance for about 3-4 days.
- Wait for the formation of sub surface pellicles.
- Latter pellicle will move to surface and becomes orange
- Streak pellicle over LGI agar plates
- Small moist dark orange colonies will develop after one week
- Pick up the colonies from the plates and streak it over LGI agar tubes

Results

- Observe the development of pellicle in semisolid medium
- Observe colony development in the Solid LGI medium
- Stain the culture and observe under microscope
- Tabulate the results

Ex.No: 5	ISOLATION OF PHOSPHATE SOLUBLIZING BACTERIA - PHOSPHOBACTERIA FROM SOIL SAMPLE
Date:	

Introduction

Phosphorus is one of the essential and vital nutrients for plant growth. Phosphorus nutrition is absolutely essential for the activities of food synthesis, cell reproduction, growth of shoot and root, flower formation, fruits and seed setting. The inorganic forms of the element in soil are compounds of calcium, iron and aluminum. The organic forms are compounds of myoinositol phytins, phospholipids and nucleic acids. A large portion of phosphatic fertilizer applied to soil is fixed in soil by conversion in the form of insoluble phosphates such as calcium, magnesium, aluminium and iron phosphates, which are not available to plants. Only 20 -25 per cent of phosphorus applied in the form of fertilizers is available to plants. These insoluble and unavailable forms of phosphorus in soil remain unutilized by plants.

A different group of microorganisms such as bacteria and fungi, which convert insoluble inorganic phosphate compounds into soluble form, e.g., *Pseudomonas striata*, *Bacillus megaterium* var. *phosphaticum, Bacillus subtilis*, *Penicillium digitatum* and *Aspergillus awamori*. These organisms can be isolated from soil by serial dilution and plating technique.

Materials Required

- Rhizosphere soil
- Pikovskaya's / Sperber's Hydroxy Apatite Medium
- Sterile water blanks
- Sterile Petriplates
- Sterile pipettes

Pikovskaya's Broth

Glucose	:	10.0 g
$Ca_3(PO_4)_2$	:	5.0 g
$(NH_4)_2SO_4$	:	0.5 g
KCl	:	0.2 g
$MgSO_4 . 7H_2O$	:	0.1 g
$MnSO_4$	:	Trace
$FeSO_4$	:	Trace
Yeast Extract	:	0.5 g
Distilled Water	:	1000 ml

Sperber's Hydroxy Apatite Medium

Soil Extract : 100 ml

Glucose : 1.0 g

Agar : 2.0 g

a. Sterilize in 100 ml lots, cool and add 5 ml of 10% KH_2PO_4 and 10 ml of 10% $CaCl_2$ to each flask and shake well to get milky white change. Adjust pH to 7.0 with sterile N/10 NaOH.
b. Prepare soil extract by mixing soil and water @1:10 and autoclaving for 30 min. After autoclaving, the solution is filtered and the volume is made upto the quantum of water used initially

Procedure

1. Prepare soil or rhizosphere soil suspensions by taking 10 g or 1g sample in 100 ml sterile water blank
2. Prepare serial dilution up to 10^{-4} by transferring 1 ml aliquot to 9ml water blank with a sterile pipette. (*Since the PSB population is comparatively less in numbers, dilution upto 10^{-4} is sufficient*)
3. Transfer 1.0 ml aliquot from 10^{-3} and 10^{-4} to sterile petridishes. Dilutions should be plated in triplicate for greater accuracy.
4. Pour approximately 15 to 20 ml molten and cooled medium (45°C) to each petri dish and mix the inoculum by gently rotating the petri dish.
5. Incubate the plates at room temperature for 3-5 days.
6. Observe & calculate the number of colonies per gram sample by applying the formula

$$\text{No. of colony forming units (cfus) / g of sample} = \frac{\text{Mean no. of cfu x Dilution factor}}{\text{Qty of sample on dry weight basis}}$$

Observation

- Observe for clear halo zone around bacterial colonies surrounded by turbid white background after 3-5 days of incubation.
- Count the number of colonies with clear halo zone in each plate and arrive at the average number of phosphate solubilizing bacteria in each case.
- Calculate the population of phosphobacteria per g of the soil and express on dry weight basis.
- Measure the diameter of the clearing zone.

Result

Ex.No: 6	ISOLATION OF PINK PIGMENTED FACULTATIVE METHYLOTROPHS (PPFM) FROM PHYLLOSPHERE BY LEAF IMPRINT TECHNIQUE
Date:	

Introduction

Bacteria of the genus *Methylobacterium*are facultative methylotrophs coming under the group of alpha proteobacteria. They are sometimes referred to as PPFMs (Pink pigmented facultative methylotrophs) because of their distinctive pigmentation. *Methylobacterium*strains are commonly found in soils as well as on the surface of leaves of a wide variety of plants. These bacteria are capable of growing on methanol and methylamine as well as on a variety of C2, C3 and C4 compounds. These organisms flourish well on leaf surface by utilizing methanol present in leaf surfaces. Hence this bacteria can be isolated from leaf surface as a phyllosphere bacterium.

Ammonium Mineral Salt Medium (Green and Bousifield, 1982)

NH_4Cl	:	0.50 g
K_2HPO_4	:	0.70 g
KH_2PO_4	:	0.54 g
$MgSO_4.7H_2O$	:	1.00 g
$CaCl_2.2H_2O$	:	0.20 g
$FeSO4.7H_2O$	:	4.00 mg
$ZnSO4.7H_2O$	:	100.0 g
$MnCl_2.4H_2O$	:	30.0 µg
H_3BO_4	:	300.0 µg
$CoCl_2.6H_2O$	:	200.0 µg
$CuCl_2.2H_2O$	:	10.0 µg
$NiCl_2.6H_2O$	:	20.0 µg
$Na_2MoO_4.2H_2O$	:	60.0 µg
Agar	:	15.0 g
Distilled water	:	1000 ml
pH	:	6.8
Before plating add methanol	:	0.5%

Isolation of *Methylobacterium*

(Phyllosphere region- Leaf imprint technique - Corpe, 1985)

1. Prepare ammonium mineral salt (AMS) plates and allow to solidify
2. Collect fresh leaf samples of Cotton, Sorgum and Rice
3. Place the upper surface of the leaf on the AMS plate and gently press the leaf
4. Make imprint of the lower surface of the leaf also
5. Incubate the plates at 28±2°C for 5 days.
6. Observe for the development of pink pigmented colonies

Purification of *Methylobacterium* isolates

1. *Methylobacterium* culture will be purified by streak plate method on AMS medium.
2. Transfer the purified colony to AMS slant; after sufficient growth store in refrigerator for further investigation.

Results

Ex.No: 7	MASS PRODUCTION OF BACTERIAL BIOFERTILIZERS & LIQUID BIOFERTILIZERS
Date:	

Introduction

Biofertilizers are defined as preparations containing living cells or latent cells of efficient strains of microorganisms that help crop plants' uptake of nutrients by their interactions in the rhizosphere when applied through seed or soil. They accelerate certain microbial processes in the soil which augment the extent of availability of nutrients in a form easily assimilated by plants. Several microorganisms and their association with crop plants are being exploited in the production of biofertilizers.N_2 fixing organism such as *Azospirillum, Rhizobium, Azotobacter, Gluconacetobacter* and PO_4 solubilizing bacterial genera *Pseudomonas*, *Bacillus* and PO_4 mobilizing Arbuscular mycorrhizal fungi are presently used as biofertilizers for commercial application .

I. Mass Production of bacterial biofertilizers

Biofertilizers are carrier based preparations containing efficient strain of nitrogen fixing or phosphate solubilizing microorganisms. Biofertilizers are formulated usually as carrier based inoculants. The organic carrier materials are more effective for the preparation of bacterial inoculants. The solid inoculants carry more number of bacterial cells and support the survival of cells for longer periods of time.

The mass production of carrier based bacterial biofertilizers involves three stages.

1. Mass culturing of microorganisms in fermentor
2. Processing of carrier material
3. Mixing of broth culture with the carrier and packing

1. Mass culturing of Microorganisms in Fermentor

Although many bacteria can be used beneficially as a biofertilizer the technique of mass production is standardized for *Rhizobium*, *Azospirillum*, *Azotobacter* and phosphobacteria and *Gluconacetobacter*

The media used for mass culturing are as follows:

Rhizobium	:	Yeast extract mannitol broth.
Azospirillum	:	Dobereiner's malic acid broth with NH_4Cl (1g/lit)
Azotobacter	:	Waksmann No.77 broth
Phosphobacteria	:	Nutrient broth
Gluconacetobacter	:	LGI broth

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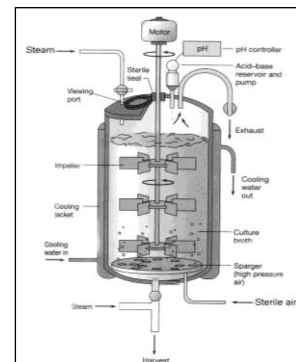
- Prepare appropriate broth in 50 ml flasks and inoculate the mother culture in to the flasks.
- Grow the culture under shaking conditions at 30±2°C until maximum cell population of 10^{10} to 10^{11} cfu/ml is reached.
- Under optimum conditions this population level could be attained within 4 to 5 days for *Rhizobium*; 5 to 7 days for *Azospirillum*; 2 to 3 days for Phosphobacteria and 6-7 days for *Azotobacter & Gluconacetobacter.* The culture obtained in the flask is called **starter culture**.
- Use the starter to inoculate the broth in large size flasks of 250 ml, 500 ml, 3 liters and 5 liters and grow until required level of cell count is reached.
- For large scale production of inoculant, inoculum from starter culture is transferred to large flasks/seed tank fermentor.

Fermentor

Bacterial biofertilizers are normally mass cultured in fermenters. Fermentor is the vessel which maintains the controlled environmental conditions for the growth of microorganisms and provides access for inoculation, sampling, aeration and cleaning. It should be made of stainless steel to withstand high pressure and also to resist corrosion. High quality fermentor will have smooth surface inside.

Basic functions of a fermentor



- To provide a controlled environment for the growth of microorganisms in order to obtain a desired product.
- Fermentor vessel should be capable of being operated aseptically for a number of days.
- Should withstand high pressure. The power consumption should be as low as possible. The vessel should be designed to require minimal use of labour for operation.
- The vessel should be constructed to ensure smooth internal surfaces without cracks and crevices.

Sterilization of growth medium in the Fermentor

- Prepare required quantity of growth medium and adjust to the required pH
- Pour the medium into the fermentor vessel after closing the sampling valve
- Keep the air outlet valve open

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- Bring the growth medium to boiling under maximum heat by using steam generator
- Close the air outlet valve and allow the pressure to build up inside the vessel
- Maintain a pressure of 15 1b / in^2 at 121°C for 20 minutes
- Switch off the fermentor and cool the medium by circulating cool water.

Mass culturing in Fermentor

- Spray the inoculation port with alcohol and flame thoroughly
- Allow the port to cool, inoculate the media in the fermentor vessel with the log phase culture grown in 5 litre flask. Usually 1 -2 % inoculum is sufficient, however inoculation is done up to 5% depending on the growth of the culture in the larger flasks.
- Turn on the air pump, open the air outlet valve
- Regulate the air flow to 3-10 lit of air per hour per lit. of the medium. The sterile air provides aeration as well as agitation for the growth of culture
- Draw samples and analyze for growth, periodically if necessary
- Once the culture reaches full growth turn off the air supply and harvest the broth with the population load of 10^9 cells ml^{-1} after incubation period through the sampling port.
- There should not be any fungal or any other bacterial contamination at 10^{-6} dilution level
- It is not advisable to store the broth after fermentation for periods longer than 24 hours. Even at 4^oC number of viable cells begins to decrease.

2. Processing of carrier material

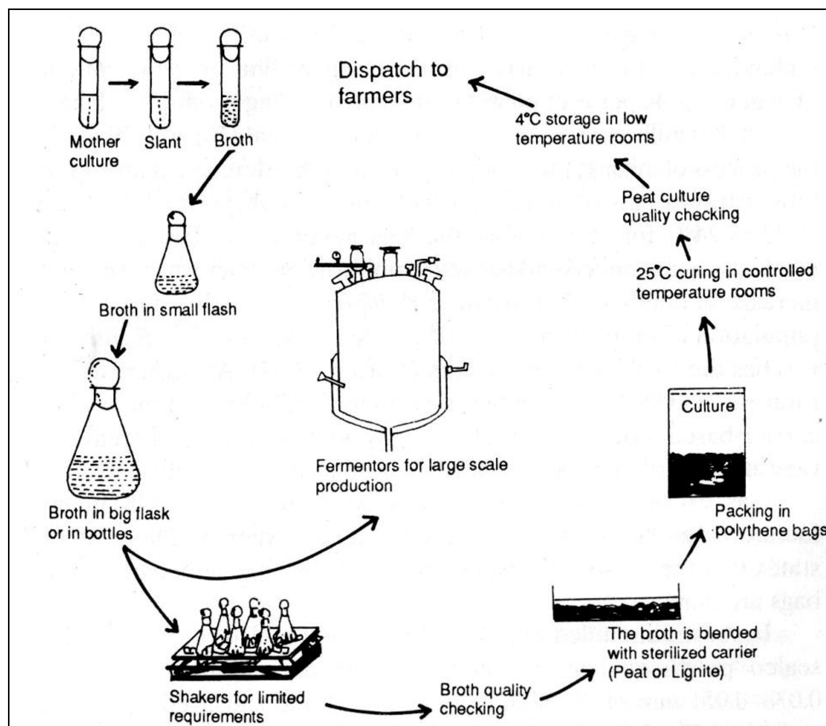
The use of ideal carrier material is necessary in the production of good quality biofertilizer. Peat soil, lignite, vermiculite, charcoal, press mud, farmyard manure and soil mixture can be used as carrier materials. The neutralized peat soil/lignite are found to be better carrier materials for biofertilizer production.

Characteristics of an Ideal carrier

- Cheaper in cost
- Should be locally available
- High organic matter content
- Should not be toxic
- Water holding capacity of more than 50%
- Easy to process, friability and vulnerability.
- Amenable for mixing

a. Preparation of carrier material:

- Powder the carrier material (peat or lignite) to a fine powder so as to pass through 212 μ IS sieve.
- Neutralize the pH of the carrier material with the help of calcium carbonate (1:10 ratio) , since the peat soil / lignite are acidic in nature (pH of 4 - 5)
- Sterilize the neutralized carrier material in an autoclave to eliminate the contaminants. For large scale production gamma irradiation and sun drying method is followed.



Schematic representation of mass production of bacterial biofertilizers

3. Mixing of broth culture with the carrier and packing

Add the bacterial culture drawn from the fermentor to the neutralized and sterilized carrier material to the moisture content of 35 to 45% on wet basis. The carrier and broth can be mixed either manually (by wearing sterile gloves) or mechanically.

After mixing, pack the inoculants in 200 g quantities in polythene bags, seal with electric sealer and allow for curing for 2 -3 days at room temperature (curing can be done by spreading the inoculant on a clean floor/polythene sheet/ by keeping in open shallow tubs/ trays with polythene covering for 2 -3 days at room temperature before packaging). Curing improves the cell count to 10^9 to 10^{10} cells /g. After curing it is then packed in low density polythene bags. Theinoculants may be allowed for curing even after packing for 3- 4days at room temperature.

Specification of the polythene bags

- The polythene bags should be of low density grade.
- The thickness of the bag should be around 50 – 75 micron.
- Each packet should be marked with the name of the manufacturer, name of the product, strain number, the crop to which recommended, method of inoculation, date of manufacture, batch number, date of expiry, price, full address of the manufacturer and storage instructions etc.,

Storage

- The packet should be stored in a cool place away from the heat or direct sunlight.
- The packets may be stored at room temperature or in cold storage conditions in lots in plastic crates or polythene / gunny bags.

Product specifications

- There should be more than 10^8 cells / g of inoculant at the time of preparation and10^7 cells/ g on dry weight basis before expiry date.
- It should not have any contaminant at 10^{-5} dilution

General procedure for the production of carrier based inoculants

- Prepare the starter culture in small size flasks
- Prepare the seed culture in large size flasks or in seed tank fermentor
- Inoculate the seed culture into the fermentor @ 1-3 % and multiply the culture in fermentor for large scale production with the respective growth medium to the population level of 10^9 cells/ml broth
- Mix the broth with prepared carrier material (neutralized and should pass through 212 micron IS sieve) to 40-50% moisture level
- Pack it with low density printed poly bags (50-75 micron)
- The final product should contain the population load of 10^8 cells/g product at preparation

II. Mass production of liquid biofertilizers

All the bacterial biofertilizers except *Rhizobium* are produced in liquid formulations also. Liquid biofertilizers are produced through three step process.

1. **Preparation of starter culture and seed culture**

   Prepare the starter culture from the mother culture in the respective growth medium as given for carried based inoculants

2. **Mass culturing in fermentor**

- Do the mass culturing similar to carried based inoculants in the fermentor.

- Harvest the broth once the population reaches the cell load of 10^{10} cell per ml broth

2. **Preparation of liquid formulation**
   - Fill the harvested culture in the sterile plastic container of one liter or 500 ml capacity
   - Add Glycerol @ of one ml per liter broth to arrest the metabolic activities of the cell so as to avoid bursting of the container under storage
   - Seal the mouth with sterile caps and store under room temperature

Observations:

Ex.No: 8	ESTIMATION OF ARBUSCULAR MYCORRHIZAL (AM) INFECTION IN ROOTS
Date:	

Introduction

'Mycorrhiza' (Fungus root) is the mutual association between plant roots & fungal mycelia. Frank, the German Botanist (1985) was the first person to use the term "Mycorrhiza". Arbuscular Mycorrhiza (VAM) is an endomorphic mycorrhizae, characterized by the presence of arbuscules and vesicles. They are grouped under Glomeromycota associated as on obligate symbiont with majority of agricultural crops, growing under broad ecological ranges. This fungal association is characterized by the movement of plant produced carbon to the fungi and fungal acquired nutrients (through the external hyphae extending from the root surface in the soil) to the plants.

Principle

In order to view and quantify symbiotic fungal association in roots, contents of root cells need to be cleared, and then fungal walls need to be stained to view arbuscular mycorrhizal (AM) fungal structures to assess the percentage of root length colonized by AM fungi.

Materials Required

- 10% w/v KOH
- Ammonia/hydrogen peroxide solution (3ml 20% NH_2OH in 30ml 3% H_2O_2)
- 5% HCl (10ml v/v glacial HCl in 190ml Distilled water)
- 0.05% Tryphan blue stain (10 ml glacial acetic acid, 200ml glycerol, 0.2g tryphan blue, 190ml distilled water)
- Lactoglycerol (1:1:1 lactic acid, glycerol, and water)
- 20 ml glass scintillation vials
- Slides & cover slips (50x75x1 mm slides and 48x65 mm No 1 cover slips)
- Mounting medium - typically Polyvinyl-Lacto-Glycerol (PVLG)
- Compound microscope

Procedure

I. Examination of AM colonization in roots

AM fungi colonize the root cortex both externally and internally. The internal structures *viz.,* vesicles and arbuscules could be viewed only after clearing the roots as well as staining.

Root clearing and staining Technique

Collect the representative root samples from growing roots (preferably thin roots) and wash gently in running tap water to remove adhering soil particles.

Clearing of roots: Cut the roots into 1 cm pieces and transfer to boiling tubes. Add 10% KOH solution till the roots are immersed. Plug the tubes with cotton, cover it with paper heat at 90°C for about 1 hour. Alternatively cook the samples in autoclave at 100-120 ^{0}C for 15 minutes. Care should be taken to avoid smashing of roots. Delicate roots can be heated for a short time with care. This boiling with KOH leads to clearing of root contents. Wash the roots till no brown colour appear in washed water.

Bleaching: Bleaching is necessary for heavily pigmented root material which is not cleared adequately in KOH alone. Immerse the roots in alkaline H_2O_2 solution at room temperature for 10 min. to 1 hr. till the roots are bleached. (Alkaline H_2O_2 is prepared by adding 3 ml of NH_4OH, 30 ml of 10%. H_2O_2 to 567 ml of tap water.) Wash the roots thoroughly in water to remove all H_2O_2.

Acidification: Immerse the roots with 2% HCl and retain it for 3-5 minutes and decant. After acidification should not wash the roots in water, since the stain requires an acidic medium for the reaction.

Staining: Add equal volume of 0.05% tryphan blue stain, autoclave for 10 minutes at 120°C or heat at 90°C for 15-20 minutes or left unheated for several hours.

Destaining: If roots are stained excess, immerse the roots in lactophenol for destaining.

Microscopic Observation: Arrange about 10 segments in a microscopic slide, put a drop of water, cover with cover slip. Give gentle pressure over the cover slip so as to expose the structures inside the roots .Examine the roots under the microscope for the presence of arbuscules and vesicles. All the fungal structures will be stained dark blue .100 segments should be observed for each sample. Calculate the root colonization using the following formula.

Calculation:

$$\% \text{ AM colonization} = \frac{\text{No. of root bits with infection}}{\text{Total no. of root bits examined}} \times 100$$

Result:

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Ex.No: 9	MASS PRODUCTION OF ARBUSCULAR MYCORRHIZAL (AM) FUNGAL BIOFERTILIZER
Date:	

Introduction

The commercial utilization of mycorrhizal fungi has become difficult because of the obligate symbiotic nature and difficulty in culturing on laboratory media. Production of AM inoculum has evolved from the original use of infested field soils to the current practice of using pot culture inoculum derived from the surface disinfected spores of single AM fungus on a host plant grown in sterilized culture medium. Several researches in different parts of the world resulted in different methods of production of AM fungal inoculum as soil based culture as well as carrier based inoculum. Root organ culture is being used for the production of soil less culture.

As a carrier based inoculum, pot culture is widely adopted method for production. The AM inoculum was prepared by using sterilized soil and wide array of host crops were used as host. The sterilization process is a cumbersome one the inert materials such as vermiculite, perlite , montmorillonite clay etc. are utilized for the production.

Mother Culture

Mother culture can be produced from efficient spores using funnel techniques as well as multiplication in small sized pots .3-4 cycles will be made for developing the mother culture. Mother culture should have 100 % root colonization and the minimum of 8-10 spores per gram of the inoculum. Mother culture can also be maintained in maize roots continuously.

Mass production

- Form a trench (1m x 1m x 0.3m) is and line with black polythene sheet to use this as a plant growth tub.
- Mix 50 kg of vermiculite and 5 kg of sterilized soil and pack in the trench up to a height of 20 cm
- Spread 1 kg of AM inoculum (mother culture) 2-5 cm below the surface of vermiculite
- Sow with surface sterilized Maize seeds (5% sodium hypochlorite for 2 minutes)

- Apply 2 g urea, 2 g super phosphate and 1 g muriate of potash for each trench at the time of sowing seeds. Further apply 10 g of urea twice on 30 and 45 days after sowing for each trench
- Apply 1 g of micronutrient mixture when there will be a symptom for deficiency
- Test the quality of the inoculum by estimating the AM colonization in root samples on 30th and 45th day
- Grow the stock plants for 60 days (8 weeks).
- Pull out the plants and cut the roots into small pieces
- Mix the roots thoroughly with vermiculite in the trench
- The inoculum produced consists of a mixture of vermiculite, spores, pieces of hyphae and infected root pieces.

Thus within 60 days 55 kg of AM inoculum could be produced from 1 sq m area. This inoculum will be sufficient to treat 550 m^2 nursery area having 11,000 seedlings.

Observations

- Take periodical sampling of carrier and liquid biofertilizers under storage and record the population
- Take periodical sampling of the AM inoculums and look for root colonization and spore load.

Result:

Ex.No: 10	ISOLATION OF BLUE GREEN ALGAE FROM SOIL
Date:	

The algae are abundantly present in the habitats wherein moisture and light are adequately available. Though different groups of algae are present, only certain groups of blue green algae (BGA) fix atmospheric nitrogen and they can contribute significantly to the fertility of soils. N_2 fixing blue green algae are grouped as cyanobacteria besides fixing nitrogen, these algae excrete vitamin B_{12}, auxins and ascorbic acid which may also contribute to plant growth.

Different groups of cyanobacteria

1. Filamentous, non heterocystous – *Spirulina*, *Oscillatoria*
2. Filamentous, heterocystous - *Anabaena*, *Nostoc*
3. Unicellular – *Microcoleus*, *Microcystis*

Isolation

I. BGA can be isolated from soil by serial dilution method using different media

- Weigh approximately 10 g rice field soil and put it in 100 ml sterile water blank and mix thoroughly
- Transfer aseptically, 10 ml of the soil mixture to another flask containing 90 ml sterile water to make 10^{-2} dilution
- Prepare BG 11 medium & pour into a series of petridishes
- Pipette out 1 ml of soil suspension from 10^{-2} dilution over the BG 11 medium plates and spread it
- Keep the plates in light chamber or partial sunlight and incubate for 3-4 weeks
- Observe for the blue green colour growth on the agar surface of the petriplates.

Chemical composition of BG - 11 medium

Ingredients		gms/Litre
Sodium nitrate	-	1.500
Dipotassium hydrogen phosphate	-	0.0314
Magnesium sulphate	-	0.036
Calcium chloride dihydrate	-	0.0367
Sodium carbonate	-	0.020
Disodium magnesium EDTA	-	0.001
Citric acid	-	0.0056
Ferric ammonium citrate	-	0.006
Final pH after sterilization (at 25°C)	-	07.1

II. BGA can also be isolated from soil using liquid medium

- Prepare Chu –10 liquid medium in 100 ml quantities
- Add 1 g sieved soil to each flask containing Chu-10 liquid medium, mix well
- Incubate in light or in partial sunlight
- Algal growth can be seen in 3-4 weeks after incubation, as floating biomass on the surface.

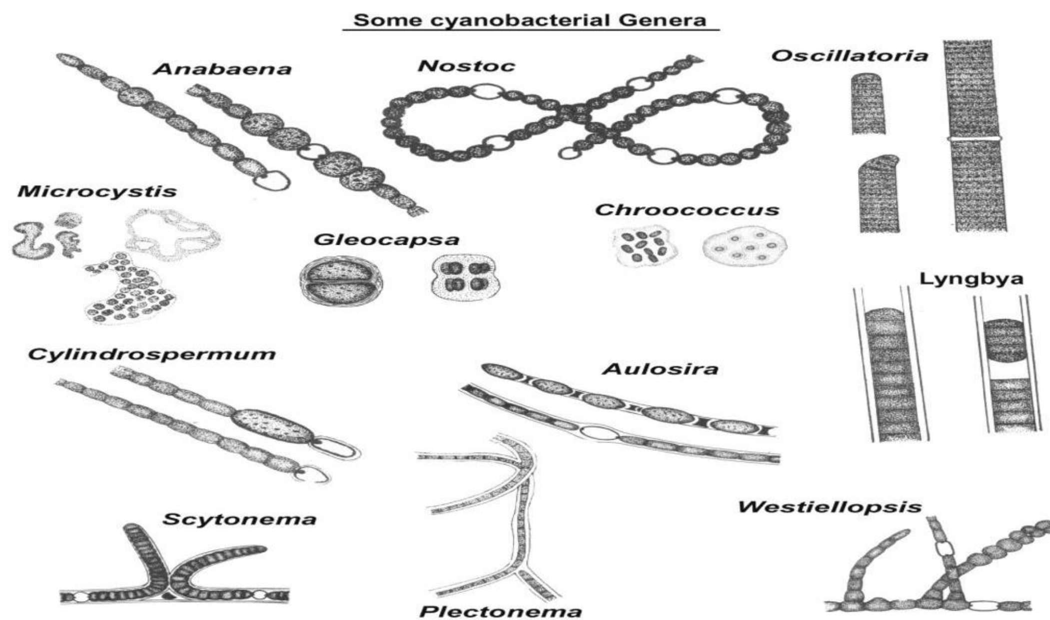
Result:

Ex.No: 11	MASS MULTIPLICATION OF BLUE GREEN ALGAE (BGA)
Date:	

Blue green algae

Blue green algae are free living Nitrogen fixing organisms .They may be single celled or filamentous organisms with and without heterocyst. *Anabena azollae* forms symbiotic association with water fern *Azolla*.

Blue green algal inoculation with composite cultures was found to be more effective than single culture inoculation.The blue green algal inoculum may be produced by several methods *viz.,* in tubs, galvanized trays, small pits and also in field conditions. However the large-scale production is advisable under field condition which is easily adopted by farmers.



I. Multiplication in trays

- Big metallic trays (6'x 3'x 6"lbh) can be used for small scale production
- Take 10 kg of paddy field soil, dry powder well and spread
- Fill water to a height of 3"
- Add 250 g of dried algal flakes (soil based) as inoculum
- Add 150 g of super phosphate and 30 g of lime and mix well with the soil
- Sprinkle 25 g carbofuran to control the insects
- Maintain water level in trays
- After 10 to 15 days, the blooms of BGA will start floating on the water sources

- At this stage stop watering and drain. Let the soil to dry completely
- Collect the dry soil based inoculum as flakes
- Store in a dry place. By this method 5 to 7 kg of soil based inoculum can be obtained.

II. Multiplication under field condition

Materials required

- Rice field
- Super phosphate
- Carbofuran
- Composite BGA starter culture

Procedure

- Select an area of 40 m^2 (20m x 2m) near a water source which is directly exposed to sunlight.
- Make a bund all around the plot to a height of 15 cm and give it a coating with mud to prevent loss of water due to percolation.
- Plot is well prepared and levelled uniformly and water is allowed to a depth of 5-7.5 cm and left to settle for 12 hrs.
- Apply 2 kg of super phosphate and 200 g lime to each plot uniformly over the area.
- The soil based composite starter culture of BGA containing 8-10 species @ 5 Kg / plot is powdered well and broadcasted.
- Carbofuran @ 200 g is also applied to control soil insects occurring in BGA.
- Maintain the water level to a height of 5 cm.
- After 15 days of inoculation, the plots are allowed to dry up in the sun and the algal flakes are collected and stored.

Observations

- Observe the floating blue green algal mass.
- Weigh the mass of algal flakes in each harvest (From each harvest, 30 to 40 kg of dry algal flakes are obtained from the plot)

Ex.No: 12	MASS MULTIPLICATION OF AZOLLA
Date:	

Introduction

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*. *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 utilization of *Azolla* as dual crop with wetland rice is gaining importance in Philippines, Thailand, Srilanka and India.

The important factor in using *Azolla* as a biofertilizer for rice crop is its quick decomposition in soil and efficient availability of its nitrogen to rice. In tropical rice soils the applied *Azolla* mineralizes rapidly and its nitrogen is available to the rice crop in very short period. The common species of *Azolla* are *A. microphylla, A. filiculoides, A. pinnata, A. caroliniana, A. nilotica, A. rubra* and *A. mexicana*.

I. Mass multiplication of *Azolla* under field conditions

A simple *Azolla* nursery method for large scale multiplication of *Azolla* in the field has been evolved for easy adoption by the farmers.

Materials required

- 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 @ 8 kg to each pot.
- Apply super phosphate @ 100 g as top dressing fertilizer on 4th and 8th day after *Azolla* inoculation.

- Apply carbofuran (furadan) granules @ 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.
- Harvest the *Azolla* from 10-15 days at the rate of 8-10kg/plot daily, totally 50 kg of *Azolla* can be harvested.

II. Mass Multiplication of *Azolla* in Cement Tanks/polythene lined pits

Materials Required

- Cement tank /pits
- Cattle dung
- Super phosphate
- Furadan
- Fresh *Azolla* inoculum
- Silpauline sheet

Procedure

- Select a field and prepare thoroughly and level uniformly.
- Make the pit with the size of 10 sq. meter (12 feet x 9 feet) and make the floor of pit even by removing any roots and other plants.
- Spread out silpauline sheet of suitable size (for eg. 15 x 12 ft for a pit siz of 12 x 9 ft) even without any holes and fix the edges with mud or bricks.
- Pour appox. 30-35 kg (@3-3.5 kg/sq. m) of sieved fertile soil or make a soil bed of 1 to 2 cm thick evenly.
- Maintain water level to a height of 10 cm.
- Mix 4 kg (@ 400 g/sq.m) of cattle dung in 20 litres of water and sprinkle in the field.
- Apply 25 g super phosphate (@2.5g/sq.m) as basal dose.
- Inoculate fresh *Azolla* biomass @ 1 kg to each pot.
- Apply super phosphate @ 25 g as top dressing fertilizer on 4th and 8th day after *Azolla* inoculation.
- Apply carbofuran (furadan) granules @ 25 g/pit (@2.5g/sq.m) on 7th day after *Azolla* inoculation.
- Maintain the water level at 10 cm height throughout the growth period of two or three weeks.
- Under optimal maintenance and climatic condition *Azolla* grows rapidly and fill the pit within 10-15 days.

- There after the yield of *Azolla* is around 2-2.5 kg per day (@ 200-250g/sq.m/day). Totally 8-10 kg of *Azolla* can be harvested.
- Wash properly with water to get rid of foul smell.
- The same procedure can be followed for multiplication in cement tanks also.

Mass multiplication of Azolla in polythene lined sheet

Observation

- Observe the *Anabaena azollae* in the Azolla leaves

Ex.No:13	METHODS OF APPLICATION OF DIFFERENT BIOFERTILIZERS
Date:	

Biofertilizers are prepared as both carried based and liquid inoculants. Liquid formulation of biofertilizers is suitable for drip fertigation wherever crop is grown under precision farming.

1. Method of application of carrier based bacterial biofertilizers

Bacterial biofertilizers are applied as carrier based inoculants. Peat or lignite is used as carrier material. Carrier based bacterial inoculants are applied by the following methods.

1. Seed treatment or seed inoculation
2. Seedling root dip and
3. Main field application

Seed treatment

Mix one kg of the inoculant with approximately one liter of rice gruel to make slurry. Treat the seeds required for one hectare with the slurry so as to have a uniform coating of the inoculant over the seeds and then shade dry for 30 minutes. The shade dried seeds should be sown within 24 hours. For small seeds 600 g/ha can be used for seed treatment. For horticulture crops also it is recommended as 600 g/ha.

Seedling root dip

This method is used for transplanted crops. Mix one kg of the inoculant in 200 liters of water. Dip the root portion of the seedlings required for one hectare in the mixture for 15- 20 minutes before transplanting. For horticulture crops, it is recommended as 800 g/ha seedlings.

Main field application

Mix two kg of the inoculant with 25 kg of dried, powdered farm yard manure and then broadcast in one hectare of the main field just before transplanting.

Recommendation of biofertilizers

Rhizobium

It is recommended for all legumes as seed treatment.

Azospirillum / Azotobacter

Recommended for all crops, other than legumes. For transplanted crops apply *Azospirillum* **/** *Azotobacter* by seed treatment, seedling root dip and soil

application methods. For direct sown crops apply *Azospirillum* **/** *Azotobacter* through seed treatment and soil application methods.

Phosphobacteria

Recommended for all crops. Apply phosphobacteria by seed treatment, seedling root dip and soil application methods as that of *Azospirillum/Azotobacter*.

Gluconacetobacter diazotrophicus

It is recommended for sugarcane. For sett treatment it can be used @ 2kg/ha setts and soil application @ 2kg/ha at 30,60 and 90 days of planting.

Azophos

It is a combined formulation of *Azospirillum* and Phosphobacteria. Apply this formulation as *Azospirillum* / Phophobacteria with the same dosage.

Points to remember

- Bacterial inoculants should not be mixed with insecticide, fungicide, herbicide and fertilizers.
- Seed treatment with bacterial inoculant is to be done at the last when seeds are treated with other fungicides.
- Biofertilizer treated seeds should be sown immediately.
- Biofertilizer treated seeds should be dried under shade only.

II. Methods of application of liquid biofertilizers

Liquid biofertilizers can also be applied by the above three methods.

Seed treatment	: 50 ml/acre seeds
Seedling root dip	: 150 ml/acre seedlings
Soil application	: 200 ml/acre

Azospirillum, Phosphobacteria, Azotobacter and *Gluconacetobacter* are recommended based on above methods. Other than these methods, it can also be recommended for biofertigation and also by foliar sprays.

Biofertigation: All the bacterial inoculants are recommended through drip fertigation. It is recommended at the rate of 200 ml/acre.

Foliar application: PPFM alone is specifically recommended through this method @ 200 ml/acre twice in vegetative and flowering stages.

III. Method of inoculation of AM fungal inoculant

Nursery application - 100 g inoculum is sufficient for one meter square. The inoculants should be applied at 2-3 cm below the soil at the time of sowing. The seeds/cutting should be sown / planted above the AM inoculum.

For polythene bag raised seedlings (Forest trees, Coffee & Tea)

About 10 g of inoculum is sufficient for each plant raised in poly bags. Mix 10 kg of inoculum with 1000 kg of potting mixture and pack the potting mixture in polythene bags before sowing.

For out planting

Twenty grams of AM inoculum is required per seedling. Apply inoculum at the time of planting.

For existing trees

Fifty to one hundred gram of AM inoculum is required for inoculating one tree. Apply inoculum near the root surface at the time of fertilizer application.

IV. Method of inoculation of BGA in rice field

Blue green algae may be applied as soil based inoculum to the rice field following the method described below.

- Powder the soil based algal flakes very well.
- Mix it with 10 kg soil or sand (10 kg powdered algal flakes with 10 kg soil / sand).
- BGA is to be inoculated on 7-10 days after rice transplanting.
- Water level at 3-4" is to be maintained at the time of BGA inoculation and then for a month so as to have maximum BGA development
- A week after BGA inoculation, algal growth can be seen and algal mat will float on the water after 2-3 weeks. The algal mat colour will be green or brown or yellowish green.

IV. Method of inoculation of *Azolla* to rice crop

The *Azolla* biofertilizer may be applied in two ways for the wetland paddy. In the first method, fresh *Azolla* biomass is inoculated in the paddy field before transplanting and incorporated as green manure. This method requires huge quantity of fresh *Azolla*. In the other method, *Azolla* may be inoculated after transplanting rice and grown as dual culture with rice and incorporated subsequently.

A. *Azolla* biomass incorporation as green manure for rice crop

- Collect the fresh *Azolla* biomass from the *Azolla* nursery plot.
- Prepare the wetland well and maintain water just enough for easy incorporation.
- Apply fresh *Azolla* biomass (15 t ha^{-1}) to the main field and incorporate the *Azolla* by using implements or tractor.

***B. Azolla* inoculation as dual crop for rice**

- Select a transplanted rice field.
- Collect fresh *Azolla* inoculum from *Azolla* nursery.
- Broadcast the fresh *Azolla* in the transplanted rice field on 7th day after planting (500 kg / ha).
- Maintain water level at 5-7.5cm.
- Note the growth of *Azolla* mat four weeks after transplanting and incorporate the *Azolla* biomass by using implements or tractor or during inter-cultivation practices.
- A second bloom of *Azolla* will develop 8 weeks after transplanting which may be incorporated again. By the two incorporations, 20 - 25 tonnes of *Azolla* can be incorporated in one hectare rice field.

Results

Ex.No: 14	TESTING QUALITY CONTROL OF BIOFERTILIZERS
Date:	

Quality control must begin with the maintenance of mother culture and broth culture before addition to the carrier and finished product

A.Rhizobium inoculant

(1) Mother culture

Repeated sub-culturing or longer period of storage may result in the loss of nodulation ability of Rhizobial isolates. Plant infection test is conducted by the seedling agar tube, Leonard jar assembly or by paper towel method to check the nodulation ability.

(2) Broth culture

Check for contamination by

(a) Streaking on glucose peptone agar plates. Slow growth or no growth ensures *Rhizobium*

(b) Performing Gram staining – absence of gram positive cells ensures purity of the culture

(3) Inoculant

Check the population level and presence of contaminants by serial dilution plate technique using yeast extract mannitol congo red agar medium (CRYEMA).

(4) BIS (Bureau of Indian Standards) specifications for *Rhizobium* inoculant

- Inoculation should contain a minimum of 10^8 viable *Rhizobium* cells/g of the carrier on dry weight basis at the time of manufacture and 10^7 cells on expiry date marked on the pocket.
- No contaminants at 10^6 dilution
- pH of inoculant should be 6.0 to 7.5
- Carrier material should pass through 10^6 micron size sieve
- Packing low density polythene bags of 50 - 75 μ
- Each packet should be marked with the details – Name of the product, leguminous crop for which intended, strain number, date of manufacture, date of expiry, method of application and storage instructions.

B. *Azospirillum* inoculant

The *Azospirillum* inoculant packets may be subjected to the quality control test-during preparation as well as during storage. The quality of the *Azospirillum* inoculant may be tested by the following methods.

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1. Determination of *Azospirillum* population in inoculant by MPN technique
2. Checking the presence of contaminating bacteria in the inoculant by serial dilution technique using nutrient agar medium.

Quality control specification for *Azospirillum* inoculant

- *Azospirillum* inoculant should contain a minimum of 10^9 viable *Azospirillum* cells/g of the dry carrier at the time of manufacture and 10^7 cells/g dry carrier at 15 days before the expiry date mentioned on the packets
- No contamination with other microorganisms
- pH of inoculant should be 7.2-7.5
- Carrier material should be neutral and pass through 100 micron sieve
- Each packet should be marked with strain number, date of manufacture, date of expiry, method of application etc.

A. Phosphobacterial inoculant

The quality control specifications prescribed for *Azospirillum* inoculant is followed for phosphobacterial inoculant.

Inference:

Ex.No: 15	BIS STANDARDS FOR ASSESSING THE QUALITY OF BIOFERTILIZERS
Date:	

Specification of Biofertilizers

1. Rhizobium

(i)	Base	Carrier based* in form of moist/dry powder or granules, or liquid based
(ii)	Viable cell count	CFU minimum $5x10^7$ cell/g of powder, granules or carrier material or $1x10^8$ cell/ml of liquid.
(iii)	Contamination level	No contamination at 10^5 dilution
(iv)	pH	6.5-7.5
(v)	Particles size in case of carrier based material.	All material shall pass through 0.15-0.212mm IS sieve
(vi)	Moisture percent by weight, maximum in case of carrier based.	30-40%
(vii)	Efficiency character	Should show effective nodulation on all the species listed on the packet.

***Type of carrier:** The carrier materials such as peat, lignite, peat soil, humus, wood charcoal or similar material favouring growth of organism.

2. Azotobacter

(i)	Base	Carrier based* in form of moist/dry powder or granules, or liquid based
(ii)	Viable cell count	CFU minimum $5x10^7$ cell/g of powder, granules or carrier material or $1x10^8$ cell/ml of liquid.
(iii)	Contamination level	No contamination at 10^5 dilution
(iv)	pH	6.5-7.5
(v)	Particles size in case of carrier based material.	All material shall pass through 0.15-0.212mm IS sieve
(vi)	Moisture percent by weight, maximum in case of carrier based.	30-40%
(vii)	Efficiency character	The strain should be capable of fixing at least 10 mg of nitrogen per g of sucrose consumed.

***Type of carrier: -** The carrier material such as peat, lignite, peat soil, humus, wood charcoal or similar material favouring growth of the organism.

3. Azospirillum

(i)	Base	Carrier based* in form of moist/dry powder or granules, or liquid based
(ii)	Viable cell count	CFU minimum $5x10^7$ cell/g of powder, granules or carrier material or $1x10^8$ cell/ml of liquid.
(iii)	Contamination level	No contamination at 10^5 dilution
(iv)	pH	6.5-7.5
(v)	Particles size in case of carrier based material.	All material shall pass through 0.15-0.212mm IS sieve
(vi)	Moisture percent by weight, maximum in case of carrier based.	30-40%
(vii)	Efficiency character	Formation of white pellicle in semisolid N-free bromothymol blue media.

***Type of carrier:-** The carrier material such as peat, lignite, peat soil, humus, wood Charcoal or similar material favouring growth of the organism.

4. Phosphate solubilising Bacteria

(i)	Base	Carrier based* in form of moist/dry powder or granules, or liquid based
(ii)	Viable cell count	CFU minimum $5x10^7$ cell/g of powder, granules or carrier material or $1x10^8$ cell/ml of liquid.
(iii)	Contamination level	No contamination at 10^5 dilution
(iv)	pH	6.5-7.5 for moist/dry powder, granulated carrier based and 5.0 – 7.5 for liquid based
(v)	Particles size in case of carrier based material.	All material shall pass through 0.15-0.212mm IS sieve
(vi)	Moisture percent by weight, maximum in case of carrier based.	30-40%
(vii)	Efficiency character	The strain should have phosphate solubilizing capacity in the range of minimum 30%, when tested spectrophotometrically. In terms of zone formation, minimum 5mm solubilization zone in prescribed media having at least 3mm thickness.

***Types of Carrier:-** The carrier material such as peat, lignite, peat soil, humus, wood Charcoal or **similar** material favouring growth of the organism.

5. Mycorrhizal Biofertilizers

i.	Form/base	Fine Powder/ tablets/ granules/ root biomass mixed with growing substrate
ii.	Particle size for carrier based powder formulations	90% should pass through 250 micron IS sieve (60 BSS)
iii.	Moisture content percent maximum	8 -12
iv.	pH	6.0 to 7.5
v.	Total viable propagules/ gm of product, minimum	100 /gm of finished product
V.	Infectivity potential	80 infection points in test roots/gm of mycorrhizal inoculum used