AmbujaNeotia
THE NEOTIA UNIVERSITY
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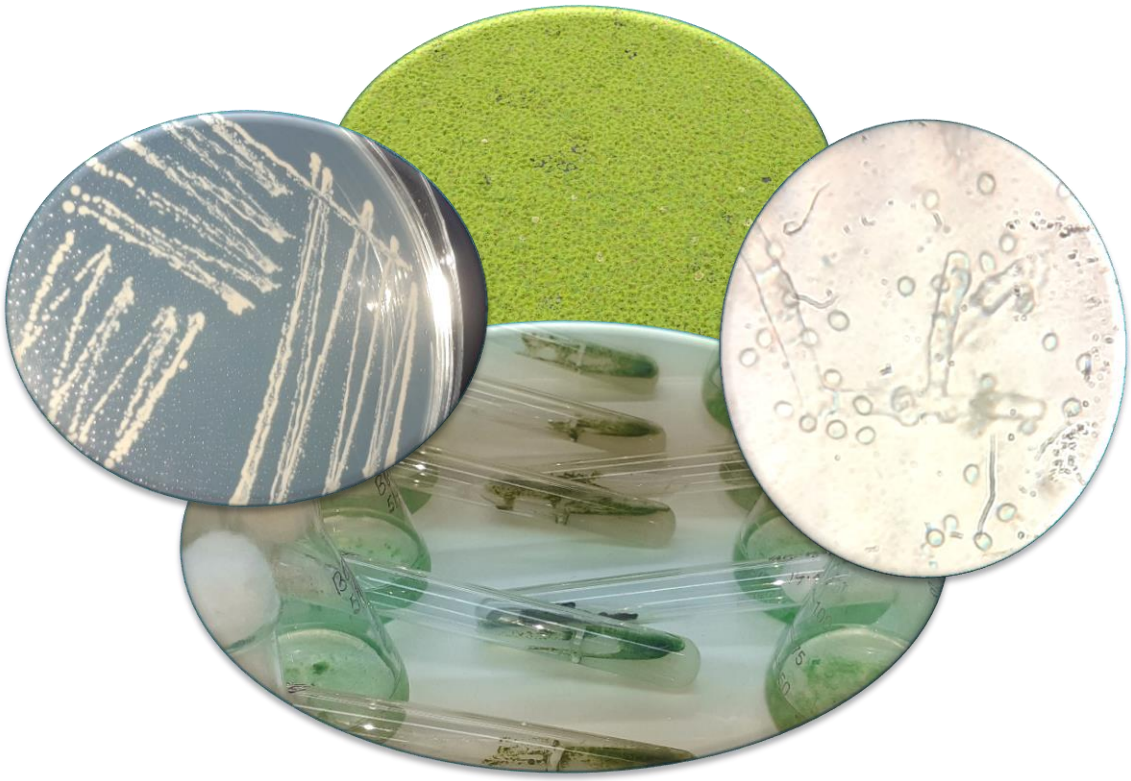
PRODUCTION TECHNOLOGY OF BIOAGENTS AND BIOFERTILIZERS

PRACTICAL MANUAL

COURSE NO EL-AGP 801



Department of Soil Science & Agricultural Chemistry
School of Agriculture and Allied Sciences
The Neotia University
Jhinger Pole, Diamond Harbour Rd, Sarisha, West Bengal 743368

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1. MASS MULTIPLICATION AND INOCULUMS PRODUCTION OF BIOFERTILIZERS

Introduction of Production Technology

The procedure involves isolation, mass culture production and preparation of inoculants along with inoculants quality control.

The individual organism can be mass multiplied using specific media either as small scale or as large-scale commercial production procedure using fermenters. ▪ The desired growth of organisms is then mixed with carrier materials and sealed in culture packets. ▪ The entire procedure is carried out under aseptic condition to avoid contamination from other undesired organisms. ▪ The quality of inoculant is regularly checked prior to distribution of individual biofertilizer culture.

Need for Basic Facilities-

There should be modern microbiological laboratory with sufficient space, electrical outlets, sinks, airlines, and temperature control and storage facilities. The rooms should be maintained properly to make it contamination free. The basic equipment are laminar air flow, autoclave, BOD incubator, dry sterilizing oven, rotary shaker, fermenter, sealer, balances, microscopes, and high grade chemicals and glass wares. Supply of gas, water and electricity without interruption should be ensured. The production of biofertilizers should be handled by technically qualified and experienced persons.

Production Technology

Isolation and selection of efficient and competitive strains. ▪ Media for broth culture. ▪ Multiplication of mother culture in the appropriate broth. ▪ Selection and preparation of broth material. ▪ Mass production and packing of the liquid/Carrier based /Granular /Encapsulated formulations biofertilizers. ▪ Storage and dispatch to farmers.

STEP 1: ISOLATION
STEP 2: MASS PRODUCTION
STEP 3: PREPARATION OF CARRIER MATERIALA AND BROTH CULTURE
STEP 4: PREPARATION OF INOCULANTS PACKET

2. ISOLATION AND PURIFICATION OF *TRICHODERMA* BIOPESTICIDES AND ITS PRODUCTION

Objectives:

- To learn the sampling and isolation technique of soil inhabiting biocontrol agents from soil.

Materials required:

1 Conical flask with 90mL sterile distilled water • 10 gm of soil sample • 5 numbers of test tube containing 9ml distilled water each • 100µL micropipettor with sterile tips • Sterile and molten media (PDA for fungi/NA for bacteria) • Sterile Petri dishes • Ethanol and absorbent cotton • Vortex • Test tube rack • Marker pen
All the ingredients for preparation of TSM

Procedure

For isolation of BCAs (soil inhabiting Bacteria and fungi) from soil

- Collection of rhizospheric soil samples Collect the soil sample from rhizosphere region of different crop rhizosphere. Nearly ½ Kg soil should collected in plastic bags from each rhizosphere region. Spread the samples to be dried and use for further study.
- PREPERATION OF TSM(Trichoderma Specific Media)

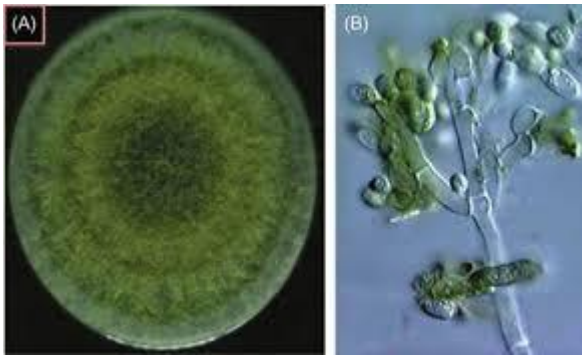
$MgSO_4$	0.2g
K_2HPO_4	0.9g
KCl	0.15g
NH_4NO_3	3g
Glucose/ Dextrose	3g
Chloremphenicol	0.25g
Fenaminosulf	0.305g
PCNB	0.20g
Rose Bengal	0.15g
Agar	18g
Distilled water	1000ml
pH	7

- Isolation of Trichoderma spp.

i. Mix 10 gm of soil aseptically into 90 ml of sterile distilled water. Mix it thoroughly using Vortex mixture or mix the bottle by inverting it 20 times. It will give 1:10 dilution (W/V). Label the bottle 10-1
ii. Using a fresh pipette, transfer 1 mL from the stock solution to the second test tube containing 9ml of distilled water.. Mix as before. Label the second bottle 10 -2 .
iii. Repeat the process up to 10^{-6}
iv. Label the Petri dishes: 10-2 , 10-3 , 10-4 , 10-5 and 10-6 , respectively.
v. Transfer liquid from the dilution blanks to the Petri dishes. Use a separate pipette for each blank, not for each plate
vi. One at a time, add a tube of molten TSM(*Trichoderma specific media)* to each Petri dish. After adding the agar, gently swirl the dishes in pattern for 30 seconds to mix the bacteria with the agar.
vii. After the agar has thoroughly solidified, incubate the plates at required temperature.
viii. Observe the culture plate at 4-5 days of incubation and identify the target

Observation:

After keeping the culture in BOD incubator for 250C white mycelium growth will arrive after 24hrs which will eventually turns to green that mean fungal spores are there.



Trichoderma 6days old culture

Conclusion

We must check the potentiality of the isolated fungi by techniques of dual culture method in laboratory. If the antagonist is the potential one then it will make more than 50% inhibition. After selection of an efficient antagonist, we can go for mass multiplication and field level trial .

3. ISOLATION AND PURIFICATION OF *PSEUDOMONAS* BIOPESTICIDES AND ITS PRODUCTION

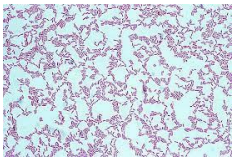
Objective: To learn the techniques of mass production of Pseudomonas sp.
Materials required • Pure culture of Pseudomonas sp. • Distilled water • Conical flasks • Autoclave • Cotton plugs • Inoculation loop • Talcum powder • Rubber bands • Peptone • Dipotassium phosphate (K2HPO4) • Magnesium sulphate (MgSO4) • Glycerol

Procedure:

1) Prepare king's B medium by mixing necessary components
   Peptone: 20.0g
   K2HPO4:1.5g
   MgSO4:1.5g
   Glycerol: 10ml
   Distilled water:1000 ml
   pH:7.2
2) Dispensed the prepared medium into conical flasks and sterilized at 15 lb pressure for 15 minutes in an autoclave.
3) Make serial dilution mentioned in Experiment 1.
4) Add King's B medium into petri plates having 10-2,10-3,10-5,10-6 dilutions
5) Incubate inside BOD incubator at 370C

Observation

After 16hrs whitish colonies will be found. If we go for gram staining pinkish b acterial cell will be found.



Conclusion

We must check the potentiality of the isolated the bacteria by techniques of dual culture method in laboratory. If the antagonist is the potential one then it will make more than 50% inhibition. After selection of an efficient antagonist, we can go for mass multiplication and field level trial

4. ISOLATION AND PURIFICATION OF *BACILLUS* BIOPESTICIDES AND ITS PRODUCTION

Introduction It's a gram +ve bacteria, Rod shaped, Flagellated, Cell size 4 to 10 micro meters, it can form endospores (To survive extreme environment conditions of temperature. Optimum temperature range is 25 to 35 degree Celsius. They produce colonies which are dry, flat and irregular with lobate margins.

Objective

Objective of this experiment is Isolation and Purification of Bacillus Biopesticides and Its Production.

Materials Required

Soil Samples, recently collected and not air dried one, Autoclave, Laminar Air flow chamber, BOD Incubator, Sterilized pipettes and Petri dish, Balance, Non Absorbent Cotton, Test tubes, Beakers (500ml), conical flasks (250 ml), Measuring Cylinder, Shaker and Squeeze bottle with Dis. Water, 90 ml sterile water blanks.

Equipments: Autoclave, Hot air Oven, Laminar air flow chamber and BOD incubator.

Procedure

1. First prepare Nutrient Agar Medium as per requirements.
Beef extract-3.00 g/l
Peptone-5.00 g/l
Sodium chloride (NaCl)-5.00g/l
Agar-20.00 g/l
Distilled water 1000 ml g/l
pH 7.0 ± 0.2

2. Pour Nutrient Agar Medium into sterile Petri plates and allow them to solidify.

3. Weigh the soil sample 10g soil sample into the 90 ml water blank, shake for 20-25 minutes on the magnetic shaker.

4. Make serial dilutions of this sample through sterile water blanks as mentioned in Experiment number 1

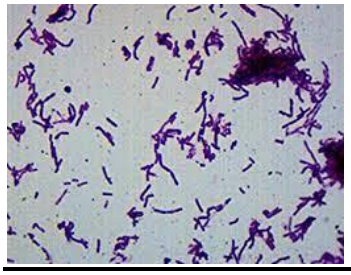
5. Add 1ml of each dilution on to the agar plates, rotate the plates for even spreading of dilusion upto 10-6 and incubate at 28 degree C for 3 to 4 days in inverted position.

Methodology

Spread Plate Method; The spread plate technique is used for the separation of a dilute, mixed population of the microorganisms so that individual colonies can be isolated. In this technique, a small volume of dilute microbial mixture is transferred to the center of an agar plate and spread evenly over the surface with a sterile L-shaped bent glass rod, while the petridish is spun, at some stage, single cells will be deposited with the bent glass rod on the agar surface. Incubate the agar plate at 37ºC for 24 hours, in the inverted position.

Observations;

After incubation colonies will come and the cell take purple colour on staining.



Mass Multiplication

The nutrient broth is prepared in fermentor and sterilized at 15 lb pressure for 15 minutes. Then the mother culture is added @ 1lit/100 lit of the medium and incubated at room temperature for 2 days. The medium containing the bacterial growth of B.subtilis is used for mixing with talc powder.

5. ISOLATION AND PURIFICATION OF *METARHYZIUM* BIOPESTICIDES AND ITS PRODUCTION

Introduction

Fungi represent a diverse group of insect pathogens. ▪ The insects attacked by the fungus die shortly after the fungus begins to develop in the haemocoel. These fungi usually attach to the external body surface of insects in the form of microscopic bodies (usually asexual, mitosporic spores also called conidia).

Objective

Objective of this experiment is Isolation and Purification of Metarhyzium Biopesticides and Its Production.

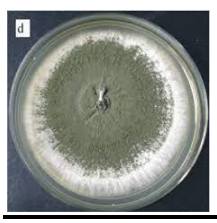
Materials Required: Soil Samples, recently collected and not air dried one, Sterilized pipettes and Petri dish, Balance, Non Absorbent Cotton, Test tubes, Beakers (500ml), conical flasks (250 ml), Measuring Cylinder, Shaker and Squeeze bottle with Dis. Water, 90 ml sterile water blanks. Equipments: Autoclave, Hot air Oven, Laminar air flow chamber and BOD incubator.

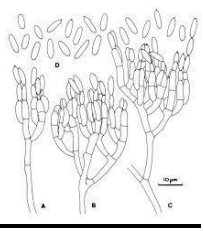
Procedure:

1. First prepare Potato Dextrose Agar medium as per requirements.
2. Pour Potato Dextrose Agar medium into sterile Petri plates and allow them to solidify.
3. Sieve the soil through 2 mm sieve, weigh two 10g samples, keep one sample in an oven over night at 35 degree C. Weigh this sample to find out the percentage of moisture in soil.
4. Add the other 10g soil sample into the 90 ml water blank, shake for 20-25 minutes on the magnetic shaker.
5. Make serial dilutions of this sample through sterile water blanks
6. Add 1ml of each dilution on to the agar plates, rotate the plates for even spreading of inoculum and incubate at 28 degree C for 3 to 4 days in inverted position

The composition of *Metarhyzium* isolating medium-
Starch (Potato) 20g
D-Glucose 20g
Urea 3g
MgSO4.7
KCl 0.15g
KH2PO4 0.08g
ZnSO4.7H2O 0.01g
Agar 20g
Distilled Water 1000 ml

Observation





Mass production of Metarhizium anisopliae (green muscardine fungus)

Metarrhizium anisopliae is a widely distributed soil inhabiting fungus. The spore of *M. anisopliae* can be formulated as dust and sprayable formulation.

Materials required

Coconut water, Flat glass bottles, Cotton plug, Pressure cooker, Injection syringe, Bunsen burner Laminar flow chamber, Mixer grinder **Methodology**

- Take glass bottles containing 40 ml of coconut water. Plug these bottles with cotton plug and sterilize in autoclave for 20 minutes at 15 psi.
- The bottles are inoculated with 1 ml suspension containing spores of the fungus with the help of a sterile injection syringe.
- Sterilize the bottles with the help of burner. Spores are inoculated in bottles with the help of syringe in a laminar flow chamber.
- Keep inoculated bottles till the surface of medium is fully covered by the olive green sporulated fungus.
- Whole culture is grinded thoroughly in mixer.
- Keep culture in cool and dry place in packets.

6. ISOLATION OF *BEAUVERIA BASSIANA*

Introduction- The insect disease caused by the fungus is a muscardine which has been called white muscardine disease. When the microscopic spores of the fungus come into contact with the body of an insect host, they germinate, penetrate the cuticle, and grow inside, **killing the insect within a matter of days**.

Materials required—

Soil Samples, recently collected and not air dried one, Sterilized pipettes and Petri dish, Balance, Non Absorbent Cotton, Test tubes, Beakers (500ml), conical flasks (250 ml), Measuring Cylinder, Shaker and Squeeze bottle with Dis. Water, 90 ml sterile water blanks. Equipments: Autoclave, Hot air Oven, Laminar air flow chamber and BOD incubator.

METHODS-

I. soil samples were collected from a depth of about 20 cm. Before collection, the surface layer of soil was removed. A total of five soil samples was taken from each collection site and placed in a plastic bag and transported to the laboratory. The five collected soil samples of each site were placed together in a plastic bag and mixed completely

II. One gram of a given soil sample and 10 ml of the sterilized distilled water were mixed in 15-ml test tubes, which were vortexed for 10 min to obtain homogenous solution.

III. Then, a serial dilution from 10^{-1} to 10^{-7} for each soil sample was prepared to isolate a single colony of fungi. Two hundred fifty microliters of the obtained soil extracts from each tube was spread on selective medium and incubated at 28 °C for 2 weeks.

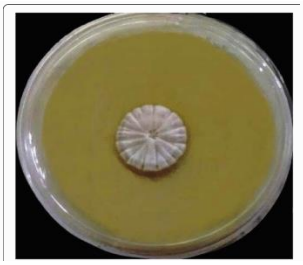
Selective media for B. bassiana

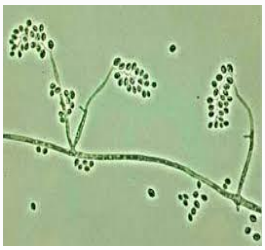
DOC2
Peptone-3g
CuCl2-0.2g
Crystal violet – 2mg
Agar-15g
Chloramphenicol-0.5mg
Oat-50gm
Distilled watr-1l

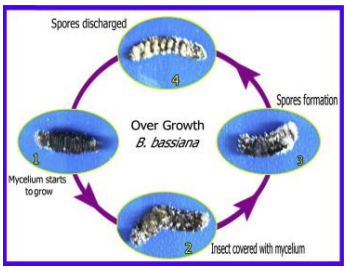
Observation-

After keeping the culture in BOD incubator for 25^0C white mycelium growth will

arrive.





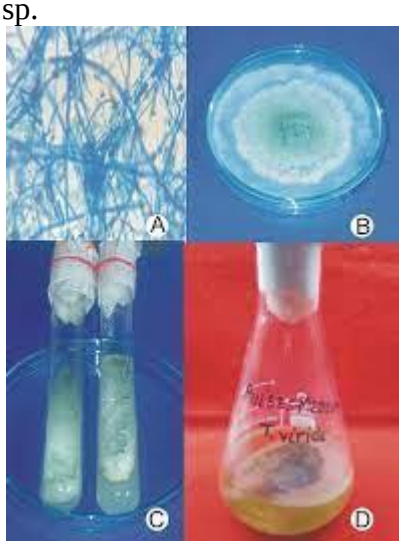


Conclusion

The selected colonies from a single colony were transferred on another for further investigation. All purified fungal isolates were stored in 15% glycerol at − 20 °C.

7. MASS PRODUCTION OF TRICHODERMA VIRIDE

Procedure and laboratory techniques involved in mass multiplication of Trichoderma sp.



Objective: To learn the techniques of mass production of Trichoderma sp.

Material required Pure culture plate of Trichoderma sp., cork borer, distilled water, conical flasks, autoclave, jaggery, yeast extract, cotton plugs, streptocyclin, inoculation needle, muslin cloth, talcum powder, carboxy methyl cellulose, rubber bands.

Procedure: The whole process will be done by two steps

Step1: Preparation of mother culture

1) Prepare Molasses yeast medium by mixing 5gm yeast powder and 30 gm molasses into 1000 ml water.
2) Dispensed the prepared medium into conical flasks and sterilized at 15 lb pressure for 15 minutes in an autoclave.
3) Inoculate the cooled media with 10 days old fungal disc of Trichoderma and incubate for 10 days for fungal growth.

Step2: Mass multiplication

1) Molasses yeast medium is prepared in fermentor and sterilized as described earlier.
2) Then after the medium is cooled, the mother culture is added to the fermentor @ 1.5 lit / 50 lit of the medium and incubated at room temperature for 10 days.
3) The fungal biomass collected from fermentor is mixed with talc powder at 1:2 ratio.
4) The mixture is air dried in shade and mixed with carboxy methyl cellulose (CMC) @ 5 g / kg the product.
5) It is packed in polythene bags and should be used within 4 months.

8. IDENTIFICATION OF IMPORTANT BOTANICALS

Few plants have acquired mechanisms such as repellency or insecticidal action to protect themselves from pest attack. As such plant products are regarded as an effective substitute for chemical pesticide. The eco-friendliness, easy availability and renewable nature fulfill the criteria of the preparation of different pesticides from different plants.

1. Azardirachta indica- it finds an important place in plant origin pesticide by virtue of activities exhibits against the wide range of insect pest. The extraction Azardiractin found to be effective against feeding deterrent, repellent also insects having biting and chewing type mouthparts,aphid,maize weevil)

2. *Lonchocarpus spp*- Rotenone (Against Aphid,leafhopper, red spider mite)

3. *Chrysanthemum cinerariaefolium*- Pyrethrum(Againts Ants,Termites)

Chrysanthemum cinerariaefolium
Pirette / Pelitre

4. *Nicotiana tabacum*-nicotine(Against Aphid,Mite,Bug)

5. Citras trees-d-Limonene(Against Aphid,mites,wasp,Cricket)

6. *Shoenacaulon officinale*- Sabadilla dust(Against Bugs, Blister beetles, caterpillar)

7. *Ryania speciosa*-Ryania(Against-Caterpillar, beetles bugs Aphid)

8. *Adenium obesum*-Chacals Baobab(Against Cotton Pest)

9. QUALITY CONTROL OF BIOPESTICIDES

Introduction

• Pesticide use has certainly contributed towards improving agricultural production, in terms of both yield and quality, thus increasing agricultural income, particularly in developed countries.

• However, careless use of pesticides without adhering to the safety norms and recommended practices has posed serious health risks to humans, other living organisms, and the environment, from on-farm workers' exposure and release of chemicals into the air and water, to commodities containing pesticide residues.

• There has been a growing demand for food safety and quality in recent decades, as reflected in the tight safety regulations on imports of products and strict regulations on the amount of pesticide residues on commodities.

• Moreover, increasingly high standards regarding product quality are continuously being set. Public awareness about the adverse effects of pesticides on the safety of foods and on the environment has increased in recent years, and the search for alternatives to widely used chemical pesticides, including biopesticides, has become a priority Regulation of Biopesticides

• Bio-pesticides are potential alternatives to synthetic chemical pesticides. It was known that, biopesticides are living natural enemy organisms and/or their products including plant and microbial products and/or their byproducts and they could reduce pest populations

• In the present decade, biopesticides are widely acceptable and demanded for sustainable agriculture and for production of safer foods.

• It was significantly considered that, biopesticides are eco-friendly, target- specific, easily biodegradable and safer alternatives

• Regulatory measures: As the bio-control agents are living organisms, it is very important to have effective regulatory measures. The quality control of commercial bio-agents must be strictly enforced by the Government. In this connection, the Directorate of Plant Protection Quarantine and Storage, Department of Agriculture and Cooperation, Ministry of Agriculture, GOI have issued guidelines/data requirements for registration of bio-pesticides in the country. As per this, all the units have to meet the Indian standards and technical specifications to be eligible for registration under the Insecticides Act, 1968.

Why Quality Control is required?

The registration data must include its

a) Composition and description

b) Biological properties and quality control standards at different stages c) Specificity of containers/packaging, delivery system, label information for market products,

d) Tolerance limits, residues, safety to non-target organisms

e) Taxonomic identification

f) Detail production technology

g) Efficacy and biological impact

h) Contamination, and shelf-life under storage and regular use Quality control (QC)

• Quality control needs to be ensured at all levels of mass production of natural enemies, viz. host/prey insects, natural enemy and plants or their products.

• Quality control is needed in all the sectors, i.e. rearing, culturing, preserving, storage methods, containers, transportation and releasing techniques.
• Therefore, Quality control (QC) is of paramount importance in order to ensure that products are delivered that complies with pre-determined specifications and delivers the efficacy within the prescribed conditions for use.
• Quality control does not only refer to the final end-use product, but also to the production and the production processes.
• Definitions of quality control used for microbials will preferably be similar to the ones used for natural enemies and other beneficial biocontrol agents

Quality Control Objectives

➢ Properties of incoming raw materials comply with the manufacturer's specifications.
➢ There is consistency between production runs and products.
➢ End-use products meet criteria set by registration authorities.
➢ Product performance meets the end-user's perception of quality in relation to price, and leads to repeat purchases of the product. Quality control is important and should be an integral part of the mass production. Quality control can be divided in
a) Production control
b) Process control
c) Product control Methods QC
1. Quality Control (Production, Process and Product)
2. Product Quality Control of Microbial Pest Control Products (MPCP) 3. Identity of the Microbial Pest Control Agent
4. Number of Infective Propagules
5. Microbial purity
6. Presence of Toxins
7. Physical, Chemical and Technical Characteristics
8. Efficacy
9. Quality Control and Shelf-Life

10. ISOLATION AND PURIFICATION OF *AZOSPIRILLUM* BIOFERTILIZERS AND ITS PRODUCTION

Azospirillum is an aerobic or microaerophilic, motile, helical, or fibroid, gram negative bacterium. Being an associative symbiotic, this bacterium brings many benefits to many non leguminous crop like cereals, millets, forage crops, and vegetable crops.

Objective

Objective of this experiment is isolation of Azospirillum from root surface of crops and study about its morphology.

Materials Required: 1. Rhizospheric Soil Samples Recently collected and not air dried one. 2. Sterilized pipettes and Petri dish, Balance, Non Absorbent Cotton, Test tubes, Beakers (500ml), conical flasks (250 ml), Measuring Cylinder, Shaker and Squeeze bottle with Dis. Water, 90 ml sterile water blanks.

Equipments: Autoclave, Hot air Oven, Laminar air flow chamber and BOD incubator. Procedure 1. First prepare Dobereiner's Medium as per requirements. 2. Pour Dobereiner's Medium into sterile Petri plates and allow them to solidify. 3. Weigh the soil sample 10g soil sample into the 90 ml water blank, shake for 20-25 minutes on the magnetic shaker. 4. Make serial dilutions of this sample through sterile water blanks as mentioned under bacteria. 5. Add 1ml of each dilution on to the agar plates, rotate the plates for even spreading of inoculum and incubate at 28 degree C for 3 to 4 days in inverted position.

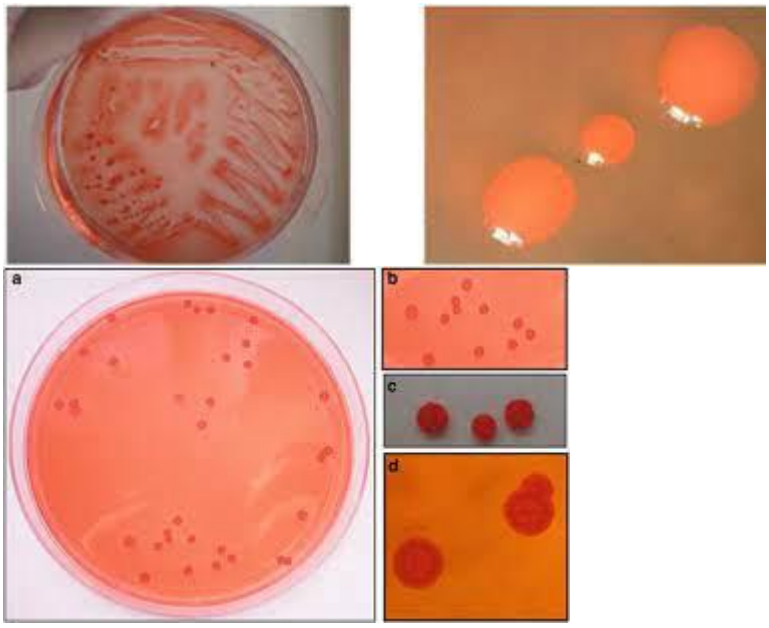
Dobereiner's medium which is the selective media for *Azospirillum* composed with

Dobereiner's medium (*Azospirillum)*	**g/l**
Malic acid	5.00
KOH	4.00
Yeast Extract	5.00
Mn SO_4H_2O (1%)	1.00
$MgSO_47H_2O$ (10%)	1.00
NaCl (10%)	2.00
K_2HPO_4 (10%)	4.00
$NaMoO_4$ (0.1%)	0.20
$CaCl_2$ (10%)	1.00
$FeSO_4.7H_2O$ (5%)	1.00
1 m NH_4Cl	5.00
Bromthymol Blue	3.00 ml

Methodology -Spread Plate Method

The spread plate technique is used for the separation of a dilute, mixed population of the microorganisms so that individual colonies can be isolated. In this technique, a small volume of dilute microbial mixture is transferred to the center of an agar plate and spread evenly over the surface with a sterile L-shaped bent glass rod, while the petridish is spun, at some stage, single cells will be deposited with the bent glass rod on the agar surface. Incubate the agar plate at 37ºC for 24 hours, in the inverted position.

Observation



Colonies like above pictures will come and on Gram staining bacterial cell will uptake pink colour

Conclusion-

If the isolated bacteria from the culture fulfils all the criteria for desired benefits it will go for mass production

11. ISOLATION AND PURIFICATION OF *AZOTOBACTOR* BIOFERTILIZERS AND ITS PRODUCTION

Objective

- The most widely studied of the free living, or non-symbiotic, biological agents of bacteria fixation is undoubtedly the gram negative soil bacterium (*Azotobacter*).
- The objective is isolation of *Azotobacter* from soil and study about its morphology.

Materials Required:

Soil Samples, recently collected and not air dried one, Autoclave, Laminar Air flow chamber, BOD Incubator, Sterilized pipettes and Petri dish, Balance, Non Absorbent Cotton, Test tubes, Beakers (500ml), conical flasks (250 ml), Measuring Cylinder, Shaker and Squeeze bottle with Dis. Water, 90 ml sterile water blanks.

Equipments: Autoclave, Hot air Oven, Laminar air flow chamber and BOD incubator.

Procedure

1. First prepare Jensen medium as per requirements.
2. Pour Jensen medium into sterile Petri plates and allow them to solidify.
3. Sieve the soil through 2 mm sieve, weigh two 10g samples, keep one sample in an oven over night at 150 degree C. Weigh this sample to find out the percentage of moisture in soil.
4. Add the other 10g soil sample into the 90 ml water blank, shake for 20-25 minutes on the magnetic shaker.

Make serial dilutions of this sample through sterile water blanks as mentioned under

Jensen's nitrogen free medium (*Azotobacter*)	**g/l**
Sucrose	20.00
Dipotassium hydrogen ortho-phosphate (K2HPO4)	1.00
Magnesium sulphate heptahydrate (MgSO4.7H2O)	0.10
Sodium chloride (NaCl)	0.50
Sodium molybdate di hydrate	0.001
Calcium chloride dihydrate (CaCl2.2H2O)	0.01
Calcium carbonate (CaCO3)	2.00
Agar	20.00
Distilled water	1000 ml
pH	7.0 ± 0.2

Observation

After incubation raised and circular colony will be found and on Gram staining –ve rod shaped bacteria will be found.

Conclusion

On confirmation of the bacteria is Azotobactor, mass production is done.

12. ISOLATION OF *RHIZOBIUM* FROM LEGUME ROOTS NODULE.

Introduction

The Most important system by which molecular nitrogen is fixed biologically results from a symbiotic association between a soil bacterium and a legume plant. Rhizobium, infect legume seedlings through root hairs and stimulate the formation of tumor like nodules on the roots. The nodules comprised of both plant and microbial tissue, are capable of using atmospheric nitrogen.

Root Nodules

Objective

The Objective of this method is to provide a procedure for the isolation of Rhizobium species from nodules found on the roots of legume plants and study about their morphology.

Materials Required:

Root systems of nodule-bearing legume plants freshly collected ones, Sterilized water, Sterilized Petri dishes, Ethanol (70%), Sterilized forceps, Scalpel or Razor blade, Inoculating loop, YEMA medium

Equipments: Autoclave, Hot air Oven, Laminar air flow chamber and BOD incubator.

Procedure

1. Wash the soil from the roots and nodules of legume plant chosen for study. Note the appearance, distributions and abundance of nodules.
2. Carefully out, do not tear a nodule from the roots so that small portions of the root remains attached. If possible, select a plum firm nodule preferably pinkish in colour. Wash under running water to insure the removal of all soil particles.
3. Put the nodule into a Petri dish containing 0.1 % $HgCl_2$ and leave it immersed for 5 minutes.
4. Transfer the nodule to a sterile Petri dish containing sterile water. Use sterile forceps to make the transfer. Wash the nodule in the sterile water.
5. Transfer the nodule to another Petri dish containing 70% ethanol for 3 minutes. Remove to another Petri dish of sterile water and rinse thoroughly, and then transfer to a second plate of sterile water for final rinsing.
6. Add 0.1 ml of sterile water to each of six sterile Petri dishes. Remove the nodule from rinse water to Petri dish No.1 and Crush it with flamed forceps. Mix the nodule tissue with the water.
7. Transfer two loops full of the suspension in the No.1 Petri dish to Petri dish No.2 and mix it with the sterile water previously added. Repeat this loop dilution progressively for plate nos.3, 4, 5 and 6.
8. Add 15 ml of YEMA medium (melted in a boiling water bath and held at 42 degree C for pouring) to plates Nos. 2 to 6 inclusive. Mix the agar and dilutions thoroughly by swirling, and incubate at 28 degree C for 7 days.

At the end of one week representative colonies of Rhizobium should have developed and

1. isolation can be made for further use or storage. Select the mucoid, or flat, watery colonies that are most abundant in the loop dilution plates. Choose an isolated colony among the domain, Rhizobium-like colonies derived from a single nodule, and transfer aseptically to several slants of the same medium. Label, incubate, and store for further testing.

YEMA medium Composition; (Yeast Extract Mannitol Agar)

Mannitol	10 g/l
K2HPO4	0.5 g/l
MgSo4.7H2O	0.2 g/l
NaCl	0.1g/l
Yeast Extract	0.5 g//l
Agar	20 g/l
Dis.Water.	1000ml

Methodology:

Pour Plate Method

In pour plate method, successive dilutions of the inoculum (serially diluting the original specimen) are added into sterile petriplates to which is poured melted and cooled (42ºC - 45ºC) agar medium and thoroughly mixed by rotating the plates which is then allowed to solidify.

Observations:

Observe the plates for umber of colonies of ***Rhizobium*** bacteria from each dilution and select the plates for enumeration which contain colonies in the range of 30-300.

13. ISOLATION AND PURIFICATION OF P-SOLUBILIZERS BIOFERTILIZERS AND ITS PRODUCTION

Introduction ▪ Phosphorus solubilizing microorganisms (bacteria and fungi) enable P to become available for plant uptake after solubilization. 21 ▪ Several soil bacteria, particularly those belonging to the genera Pseudomonas and Bacillus and fungi belonging to the genera Penicillium and Aspergillus possess ability to bring insoluble soil phosphates into soluble forms by secreting acids such as formic, acetic, propionic, lactic, glucolic, fumaric and succinic.

Objective Objective of this experiment is isolation of PSB from Soil and study about its morphology.

Materials Required & Equipments ▪ Rhizospheric Soil Samples Recently collected and not air dried one. ▪ Pikovskaya's (PVK) medium ▪ Sterilized pipettes and Petri dish, Balance, Non Absorbent Cotton, Test tubes, Beakers (500ml), conical flasks (250 ml), Measuring Cylinder, Shaker and Squeeze bottle with Dis. Water, 90 ml sterile water blanks. Equipments: Autoclave, Hot air Oven, Laminar air flow chamber and BOD incubator.

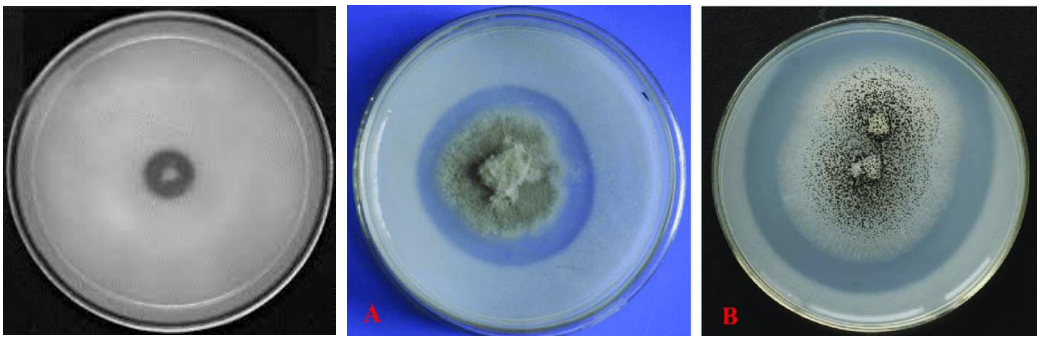
Procedure ▪ First prepare Pikovskaya's (PVK) medium as per requirements. ▪ Pour Pikovskaya's (PVK) medium into sterile Petri plates and allow them to solidify. ▪ Weigh the soil sample 10g soil sample into the 90 ml water blank, shake for 20-25 minutes on the magnetic shaker. ▪ Make serial dilutions of this sample through sterile water blanks as mentioned under bacteria. ▪ Add 1ml of each dilution on to the agar plates, rotate the plates for even spreading of inoculum and incubate at 28 degree C for 3 to 4 days in inverted position.

Pikovskaya's (PVK) Medium

C 6 H 12 O 6-10.00g/l, CaHPO 4-5.00 g/l,(NH4) 2 SO 4-0.50g/l, Sodium chloride (NaCl)-0.20g/l, MgSO4 .7H 2 O-0.10g/l, KCl 0.20g/l, yeast extract-0.50g/l, MnSO 4 .H 2 O-0.002g/l, FeSO 4 .7H 2 O-0.002g/l Distilled water-1000 m,l Agar-20.00g/l, pH 7.2 ± 0.2

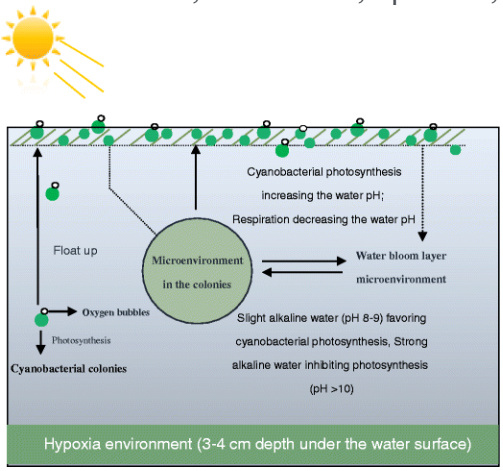
Observation

Colonies will be found as-



14. ISOLATION AND PURIFICATION OF *CYANOBACTERIA* BIOFERTILIZERS AND ITS PRODUCTION

Introduction ▪ Cyanobacteria can be found in almost every conceivable environment, from oceans to fresh water to bare rock to soil. 23 ▪ Aquatic cyanobacteria are probably best known for the extensive and highly visible blooms that can form in both freshwater and the marine environment and can have the appearance of blue green paint or scum.
Examples of **cyanobacteria**: ***Nostoc***, Oscillatoria, Spirulina, ***Microcystis***, ***Anabaena***.



Objective Objective of this experiment is isolation of Cyanobacteria from Water/Soil and study about its morphology.

Materials Required & Equipments ▪ Water Sample or Soil Samples Recently collected. ▪ BG 11 medium ▪ Sterilized pipettes and Petri dish, Balance, Non Absorbent Cotton, Test tubes, Beakers (500ml), conical flasks (250 ml), Measuring Cylinder, Shaker and Squeeze bottle with Dis. Water, 90 ml sterile water blanks. Equipments: Autoclave, Hot air Oven, Laminar air flow chamber and BOD incubator.

Procedure ▪ First prepare BG 11 medium as per requirements. ▪ Pour BG 11 medium into sterile Petri plates and allow them to solidify. ▪ Weigh 10ml water /10g soil sample into the 90 ml water blank, shake for 20-25 minutes on the magnetic shaker. ▪ Make serial dilutions of this sample through sterile water blanks. ▪ Add 1ml of each dilution on to the agar plates, rotate the plates for even spreading of inoculum and incubate at 28 degree C for 3 to 4 days in inverted position.

BG 11 Medium Composition
Magnesium Sulphate 0.025 g
Calcium Chloride 0.05g
Sodium Chloride 0.20g
Dipotassium Hydrogen Phosphate 0.35g
A5 trace elements stock solution 1.0 ml
Distilled Water 1000 ml

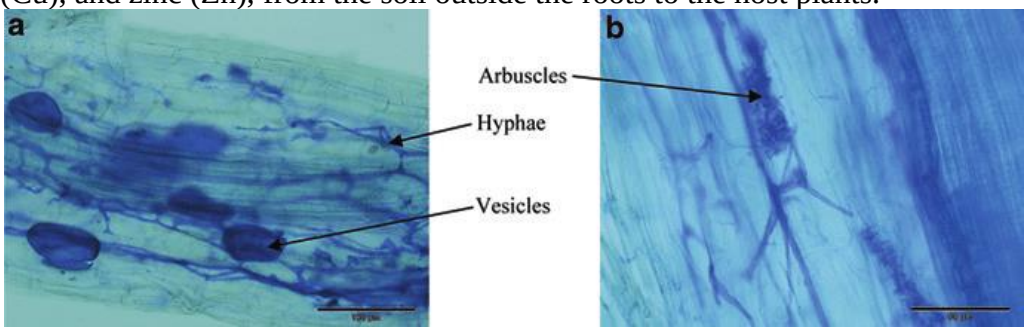
Methodology-Spread Plate Method

Observation
On BG 11 medium the Cyanobacteria Colonies on petriplate and mass multiplication

will be as the pictures below

15. ISOLATION OF AM FUNGI- WET SIEVING METHOD AND SUCROSE GRADIENT METHOD

Introduction ▪ Arbuscular mycorrhizal (AM) fungi (AMF, Glomeromycota) are important beneficial soil microorganisms establishing mutualistic associations with most food crops. These associations increase plant nutrient uptake and tolerance to root pathogens and drought. AMF are obligate biotrophs and colonize host roots to obtain sugars in exchange of mineral nutrients, absorbed and translocated through a fine network of extra radical mycelium (ERM) spreading from colonized roots into the soil. The function as auxiliary absorbing systems that transfer mineral nutrients, such as phosphorus (P), nitrogen (N), sulfur (S), potassium (K), calcium (Ca), iron (Fe), copper (Cu), and zinc (Zn), from the soil outside the roots to the host plants.



Objective Objective of this experiment is isolation of AM fungi from Soil by wet sieving method and sucrose gradient method and study about its morphology

Materials Required & Equipments ▪ Soil Samples Recently collected. Equipment: ▪ Sieves with various mesh size (1 mm, 100 µm and 50 µm. Other sizes such as 500µm and 250µm are preferable.), Sterilized Pipette or needle, Petri dish, Balance, Conical flask, Funnel, Glass tubes, Dissecting microscope, Compound microscope: etc.

Wet Sieving Method

Also known as wet sieving and decanting method (Gerdemann and Nicolson, 1963). Developed to isolate different size of spores. The soil near the root system is collected and an aqueous suspension is passed through different sieves to collect spores of different sizes.

The wet sieving and decanting is one of the popular technique when compare to other techniques. This technique is used for sieving the coarse particles of the soil and retaining AMF spores and organic particles on sieves of different sizes.

10 g of soil was mixed with 100ml of water in the 500 ml conical flask. The soil mixture was agitated vigorously to free the AMF spores from soil and allowed to settle for 15-45 minutes and the supernatant was decanted through standard sieves. By using a dissecting microscope, spores were picked by means of pipette or needle.

Earlier, Gerdemann (1955) devised the first useful technique for extracting spores from soil. A soil sample was suspended in four times its volume of water, heavier particles were allowed to settle for a few seconds, then the liquid was decanted through a sieve with 1mm mesh. Whatever passed through this sieve was then poured through another sieve with 0.25 29 mm mesh. Material retained by this sieve was washed and transferred to a petridish, and the spores picked out by hand under a dissecting microscope.

Technique given by Gerdemann (1955) was slightly refined by Gerdemann and

Nicolson (1963) who used the following series of sieves: 1.0 mm ; 710 μm; 420 μm; 250 μm; 149 μm; 105 μm; 74 μm; and 44 μm.
They found that most of the desired spores fell in the 420- 149 μm range, and they used this fraction for their study.

Sucrose Gradient Method

Developed by Daniel and Skipper (1982); commonly used technique for AM spore extraction. Requires prior sieving and decanting. ▪ This gradient centrifugation method is result of many modifications, right from Ohms (1957), Mosse and Jones (1968), Mertz et al. (1979) etc. Spores were purified by re-suspending the sieving in the 40% sucrose solution and centrifugation was carried out. Centrifugation was carried out at 1750 rpm for 5 minutes. The supernatant was removed and poured into the sieves. The spores that hold on the sieves are carefully rinsed with tap water. The spores were collected by using dissecting microscope.

Procedure:

- 10 to 50 g of freshly collected soil sample is put into 1 to 2 liters of plastic beakers. Usually rhizosphere soils are rich in AM fungal spores. Beaker size can be changed depending on the soil sample size.
- Soil is suspended with about 500 ml to 1 litter of tap water. ▪ Soil macro-aggregates should be crushed with hand.
- After 10-30 seconds of settling down of soil particles, the upper layer of soil suspension is poured into the sieving.
- The procedure should be repeated until the upper layer of soil suspension is transparent.
- Usually AM fungal spores are collected on 100 μm. Some small spores are on 50 μm. To collect large spores such as *Gigaspora margarita*, 250 μm sieve is efficient.

Conclusion

Carriers for Bacterial inoculants Preparation of carrier material Mixing Broth with Carrier (Curing) Packing and Storage and Quality Standard is evaluated.

16. VA MYCORRHIZA PRODUCTION AND MARKETING

Introduction: Arbuscular mycorrhizal (AM) fungi are soil fungi that colonize roots of the majority of crop plants, forming a mutualistic symbiosis. The fungus takes up fixed carbon as hexose from the apoplast of the root cortex.

Materials required:

Petri plates, test tube, beaker, blade, sieving plate, seeds of desired plant, mother culture, jiggery, polypropylene bags, KOH solution, HCl solution, cotton blue solution, lactic acid

Autoclave, Hot Air oven, dissecting microscope, compound microscope.

Method:

i. Tanks or pits are constructed and mother culture is acquired from trusted organization to ensure complete purity.
ii. Seeds are then prepared by mixing them with jaggery and VAM fungi at suitable proportions.
iii. The tanks are then filled with the selected substrate.
iv. Tanks are then inoculated with treated seeds and more mother culture for better infection.
v. The tanks are then watered for 6 weeks and the plants were let to grow to promote maximum root growth.
vi. After 6 weeks the plants are cut from the roots and the roots are left in the substrate.
vii. Roots are then separated from the substrate by sieving.
viii. The separated roots are shredded into small pieces and mixed with the substrate again.
ix. The ready product is then packaged in packets of 250g & 500g respectively and marketed accordingly.


Observation:

i. Freshly collected root samples should be washed gently and be free from soil particles.
ii. Roots are treated with 10 % KOH solution for 30 min in a hot bath, depending on thickness of root structure.
iii. Treated roots are washed with water and treated with 2 % HCl solution.
iv. Acidified root samples are stained with 0.05 % cotton blue for a 15-20 mins.
v. The roots are destained with lactic acid or lacto-glycerol and are now observed first under a dissecting microscope with transmitted illumination and then observed under a compound microscope.

Blue coloured arbuscules will be found in the cortical cells of the host plant root.

17. MASS PRODUCTION OF AM INOCULANTS

Introduction • Being obligate symbionts AM fungi could be mass produced only in the presence of living roots. • Since AM fungal associations are universal and have been reported in almost all terrestrial plants, these can be reproduced on a wide range of host plants. • There are several techniques reported for mass production of AM inoculum.

A. In Vivo Culture ▪ AM fungi are grown on roots of green house plants and chopped mycorrhizal roots, often mixed with growth media containing hyphae and spores, are used as source of inoculum. ▪ Soil could be replaced by inert substances such as vermiculite, perlite, sand or a mixture of these for crude inoculum production. Mass Production of VAM

Method of Production:

1. Tank for mass multiplication of AM
2. Sprinkling of water in tank with vermiculite
3. Making of furrows to sow maize seeds
4. Sowing the seeds in furrows
5. View of the maize sown AM pit
6. Vermiculite contained raised AM infected maize plants

B. IN VITRO/ AXENIC CULTURE TECHNIQUES

I) **SOLUTION CULTURE** ▪ Involves growing infected roots in aqueous medium enriched with mineral nutrients required for the growth of the roots under controlled biotic and abiotic conditions.

II) **AEROPONIC CULTURE** ▪ Involves applying a fine mist of nutrient solutions to colonized roots for AM fungal inoculum production.

III) **ROOT ORGAN CULTURE** ▪ Use of a modified agar medium (MS rooting medium)/ liquid medium for creation of increased amount of roots from callus tissue and these roots are infected by AM spores or by surface sterilized root bits obtained from mycorrhizal plant.

18. VISIT TO BIOPESTICIDE LABORATORY AND FIELD VISIT TO IDENTIFY OF ENTOMOPATHOGENIC ENTITIES IN FIELD

Proposed site: 1. Nimpth KVK and Vivekananda Institute of Biotechnology

Proposed site:2 Nitrofix Laboratory , 25 Bansdroni Avenue ,Kolkata-70