**Skill enchancement-I**

**Unit 1: Biofertilizer**

**Symbiotic N2 fixers: Rhizobium- Isolation, characteristics, types, inoculums production and field application, legume/pulses plants**

1. **Rhizobial inoculants-** For the first Nobbe and Hiltner in 1895 introduced a laboratory culture of rhizobia with the name “Nitragin”. It was the first Rhizobial industry in the world. Later on much time was devoted for isolation and characterization of strains and optimization of growth conditions. Rapidly expanding knowledge of ***Rhizobium-*legume symbiosis** expanded rapidly and contributed towards better usage of this symbiosis for sustainable crop yield.
2. **Isolation-** Different species of rhizobia reside in soil as well as in root nodules of legumes. But, root nodules of legumes are preferred for getting contaminated-free rhizobia.

Healthy root nodules are taken out from the mature plant and washed with sterile distilled water to remove soil particle. Nodules are externally sterilized either by mercuric chloride or sodium hypochloride solution or 90% ethanol.

Clean nodules are crushed in sterile water and suspension is streaked on Petri plates containing YEMA (Yeast Extract Mannitol Agar), incubated at 28-30°C for 3-4 days. White, translucent, glistening and elevated (with entire margin) colonies appear on the surface of medium.

1. **Identification/characterization of Rhizobium-**

Both ***Rhizobium*** and *A****zotobacter*** grow on YEMA medium. One can characterize and identify using the following test-

1. **CRYEMA**- CONGO Red – Yeast Extract Mannitol Agar is prepred by mixing 2.5 ml congo red dye with 1 litre of YEMA medium. Bacteria colonies from YEMA are streaked over CRYEMA medium and incubated for a week at 28-30°C. ***Rhizobium*** utilizes congo red dye very slowly and form white, circular and raised colonies. In contrast, ***Azotobacter*** display colony characteristics like ***Rhizobium*** but the colony colour is similar to congo red.
2. **Microscopic obersvation** - Bacteria colonies on CRYEMA are stained with **carbol fuschin and observed microscopically. The ß-polyhydroxybutarate (PHB) of *Rhizobium* is stained. Thus the colonies are picked up to prepare *Rhizobium* inoculants.**
3. **Glucose- Peptone agar (GPA) test-** It is confirmative test of ***Rhizobium***. Master plate is prepared using the bacteria colonies on YEMA. Replica plating from master plate is done on petri plate containing GPA medium. ***Agrobacterium*** grows well on GPA medium but ***Rhizobium*** fails to grow on this medium.
4. **Salt tolerant test-** ***Agrobacterium*** is able to grow on YEMA medium containing 2% NaCl, whereas ***Rhizobium*** cannot grow on such medium.

**4.Inoculum Production**

For the production of inoculums following steps are follows-

1. Starter culture of ***Rhizobium***
2. Mass cultivation of ***Rhizobium***
3. Measuring Cell counts in Broth.
4. Prepration of carrier – based inoculums.
5. **Starter culture of *Rhizobium-***  Pure ***Rhizobium*** colony is transferred into sterile YEM broth. Inoculated YEM broth is incubated on a rotary shaker at 28-30°C. After 4 days sufficient number of cells are present in YEM broth. It is called **mother culture or starter culture.**
6. **Mass cultivation of *Rhizobium-*** for mass cultivation of *Rhzobium,* broth medium is prepared in large quantity and transferred in a large production fermentor. The pH is adjusted to 6.5 to 7.0 by using KOH or H2SO4.

**Following are the steps of mass cultivation-**

* **Sterilize the growth medium and inoculate with mother culture.**
* **Incubate for 3-4 days at 30-32°C**
* **Allow to grow the bacteria in a large fermentor**
* **Check the quality of broth.**
1. **Measuring Cell Counts in Broth-**

Rhizobial cell count of different strains vary ***R. japonicum*** counts (5 X 109 cells/ml) may be attained in 96 hours with a lag phase of 48 hours. ***R. meliloti*** cell count 5 x 109 cells/ml may be attained with a lag phase of 4 hours.

1. **Preparation of Carrier-Based Inoculum –**

A carrier is an inert material used for mixing with broth so that inoculants are easy to handled, packed, stored/transported and used.

A variety of carrier are used for example, peat, lignite, farmyard manure, charcoal powder, lignite, peat etc.

* The carrier is powdered and dried in sun.
* Screened through 100-200 mesh sieve and neutralized by mixing CaCO3 powder. If the carrier is neutral, then no need to mixing CaCO3.
* Carrier is autoclaved at 15 psi and dried.
* The harvested broth is mixed with carrier (kept in trays or tubes) with hand or mechanically. The moisture content is maintained to about 35-40% dry weight basis.
* After proper mixing, carrier containg inoclulant is left for 2-10 days by covering the trays with polythene at 22-24°C. During this period rhizobium multiplies in the broth. This process is called **curing**
* **Packing and Storage-**  the cured carrier are packed in polythene bags and kept at constant room temperature for about a week to facilitate the rhizobial cells to get established. After week, store the packets at about 4-15°C in a cold room so that rhizobial cells may remain viable for more than 6 months.
* **Each packet must furnish the following instructions-**
1. Product’s name
2. Name of host plants for which to be used
3. Name and address of manufactures
4. Type of carrier used
5. Batch name
6. Date of manufacture and date of expiry
7. Net quantity meant for 0.4 hectare
8. Instructions for storage.
9. The packet must be marked with ISI mark.
10. The manufactures must store the inoculants at about 15°C.
* **Field application –**

Water in container

* 50g sugar or gur (jaggery)
* Boil for 15 min
* Gum Arabic (200 g)
* Cool it

Sticker solution

* Rhizobial Culture
* Mix properly

Inoculum slurry

Fig 1: Field application- Procedure for seed inoculation with rhizobial culture.

* Sow it in field.

Seeds coated with rhizobial cells

* Add seeds, mix properly
* Dry seeds in shade, keep it covered