UNIT-II

CULTURAL METHODS OF AZOSPIRILLUM, AZOTOBACTER AZOLLA ANABAENA AND CARRIER MATERIAL

Azospirillum:

It belongs to bacteria and is known to fix the considerable quantity of nitrogen in the range of 20- 40 kg N/ha in the rhizosphere in non- non-leguminous plants such as cereals, millets, Oilseeds, cotton etc. The efficiency of *Azospirillium* as a Bio-Fertilizer has increased because of its ability of inducing abundant roots in several pants like rice, millets and oilseeds even in upland conditions. Considerable quantity of nitrogen fertilizer up to 25-30 % can be saved by the use of *Azospirillum* inoculant. The genus *Azospirillum* has three species viz., *A. lipoferum*, *A. brasilense* and *A. amazonense*. These species have been commercially exploited for the use as nitrogen supplying Bio-Fertilizers.

One of the characteristics of *Azospirillum* is its ability to reduce nitrate and denitrify. Both *A. lipoferum*,and *A. brasilense* may comprise of strains which can actively or weakly denitrify or reduce nitrate to nitrite and therefore, for inoculation preparation, it is necessary to select strains which do not possess these characteristics. *Azospirlliumlipoferum* present in the roots of some of tropical forage grasses uch as Digitaria, Panicum, Brachiaria, Maize, Sorghum, Wheat and Rye.

Azospirillum :Dobereiner's malic acid broth with NH4Cl (1g per liter)

Composition of the 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

Azotobacter

It is the important and well known free living nitrogen fixing aerobic bacterium. It is used as a Bio-Fertilizer for all non leguminous plants especially rice, cotton, vegetables etc. *Azotobacter* cells are not present on the rhizosplane but are abundant in the rhizosphere region. The lack of organic matter in the soil is a limiting factor for the proliferation of *Azotobaceter* in the soil.

Field experiments were conducted in 1992, 1993 and 1994 during the pre-kharif wet seasons to find out the influence on rice grain yield by the combined use of N- fixing organisms and inorganic nitrogen fertilizer which recorded increase in was yield.

Waksman medium No.77 (N-free Mannitol Agar Medium for *Azotobacter*)

Mannitol	:	10.0 g
Ca CO3	:	5.0 g
K2HPO4	:	0.5 g
Mg SO4.7H2O	:	0.2 g
NaCl	:	0.2 g
Ferric chloride	:	Trace
MnSO4.4H2O	:	Trace
N-free washed Agar	:	15.0 g
Ph	:	7.0
Distilled Water	:	1000 ml

The broth is prepared in flasks and inoculum from mother culture is transferred to flasks. The culture is grown under shaking conditions at 30±2°C as submerged culture. The culture is incubated until maximum cell population of 1010 to 1011 cfu/ml is produced. Under optimum conditions this population level could be attained with in 4 to 5 days for *Rhizobium*; 5 to 7 days for *Azospirillum*; 2 to 3 days for and 6-7 days for *Azotobacter*. The culture obtained in the flask is called **starter culture**. For large scale production of inoculant, inoculum from starter culture is transferred to large flasks/seed tank fermentor and grown until required level of cell count is reached.

Inoculum preparation

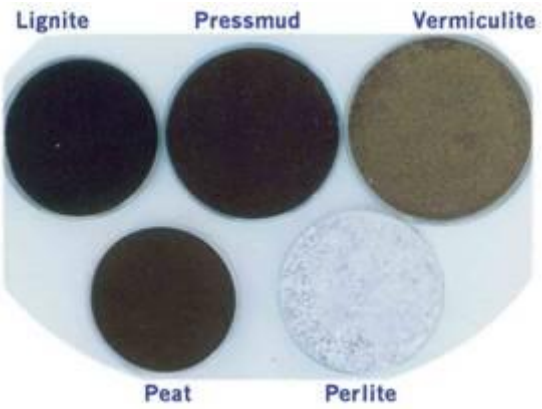
- Prepare appropriate media for specific to the bacterial inoculant in 250 ml, 500 ml, 3 litre and 5 litre conical flasks and sterilize.
- The media in 250 ml flask is inoculated with efficient bacterial strain under aseptic condition
- Keep the flask under room temperature in rotary shaker (200 rpm) for 5- 7 days.
- Observe the flask for growth of the culture and estimate the population, which serves as the starter culture.
- Using the starter culture (at log phase) inoculate the larger flasks (500 ml, 3 litre and 5 litre) containing the media, after obtaining growth in each flask.
- The above media is prepared in large quantities in fermentor, sterilized well, cooled and kept it ready.
- The media in the fermentor is inoculated 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.
- The cells are grown in fermentor by providing aeration (passing sterile air through compressor and sterilizing agents like glass wool, cotton wool, acid etc.) and given continuous stirring.
- The broth is checked for the population of inoculated organism and contamination if any at the growth period.
- The cells are harvested with the population load of 109 cells ml-1 after incubation period.
- 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 4o C number of viable cells begins to decrease.

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 The following points are to be considered in the selection of ideal carrier material.

- Cheaper in cost
- Should be locally available
- High organic matter content
- No toxic chemicals
- Water holding capacity of more than 50%
- Easy to process, friability and vulnerability.

Preparation of carrier material



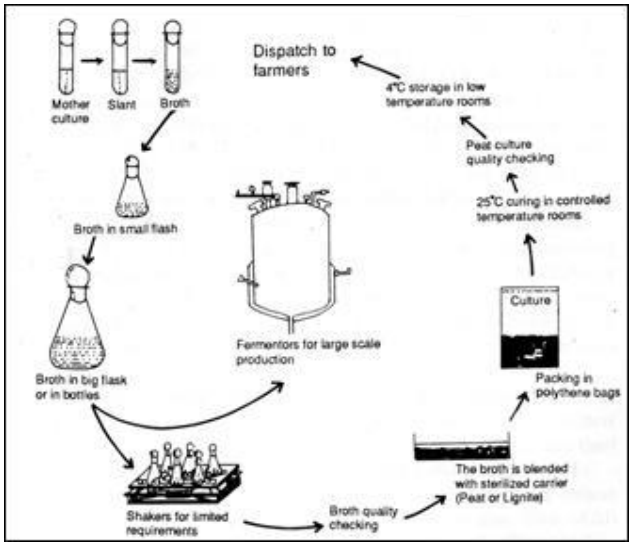
- The carrier material (peat or lignite) is powdered to a fine powder so as to pass through 212 micron IS sieve.
- The pH of the carrier material is neutralized with the help of calcium carbonate (1:10 ratio) , since the peat soil / lignite are acidic in nature (pH of 4 - 5)
- The neutralized carrier material is sterilized in an autoclave to eliminate the contaminants.

Mixing the carrier and the broth culture and packing

Inoculant packets are prepared by mixing the broth culture obtained from fermentor with sterile carrier material as described below:

Preparation of Inoculants packet

- The neutralized, sterilized carrier material is spread in a clean, dry, sterile metallic or plastic tray.
- The bacterial culture drawn from the fermentor is added to the sterilized carrier and mixed well by manual (by wearing sterile gloves) or by mechanical mixer. The culture suspension is to be added to a level of 40 – 50% water holding capacity depending upon the population.
- The inoculant packet of 200 g quantities in polythene bags, sealed with electric sealer and allowed 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).



Schematic representation of mass production of bacterial biofertilizers

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 of biofertilizerpacket

- 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.
- The population of inoculant in the carrier inoculant packet may be determined at 15 days interval. There should be more than 109 cells / g of inoculant at the time of preparation and107 cells/ g on dry weight basis before expiry date.

Azolla

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* fronds consist of sporophyte with a floating rhizome and small overlapping bi-lobed leaves and roots. Rice growing areas in South East Asia and other third World countries have recently been evincing increased interest in the use of the symbiotic N2 fixing water fern *Azolla* either as an alternate nitrogen sources or as a supplement to commercial nitrogen fertilizers. *Azolla* is used as biofertilizer for wetland rice and it is known to contribute 40-60 kg N ha-1 per rice crop. The agronomic potential of *Azolla* is quite significant particularly for rice crop and it is widely used as biofertilizer for increasing rice yields. Rice crop response studies with *Azolla* biofertilizer in the People's Republic in China and in Vietnam have provided good evidence that *Azolla* incorporation into the soil as a green manure crop is one of the most effective ways of providing nitrogen source for rice.

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

- 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.
- Observations
- Note the *Azolla* mat floating on the plot. Harvest the *Azolla*, drain the water and record the biomass.

II. 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 tranctor 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.