2. Non-Symbiotic Nitrogen Fixers

2.1. Introduction

2.1.1. *Azospirillum*

Eighty percent (80 %) of the atmosphere is nitrogen gas (N_2). Unfortunately N_2 is unusable by most living organisms. Plants, animals, and microorganisms can die of nitrogen deficiency, if surrounded by N_2 they cannot use. All organisms use the ammonia (NH_3) form of nitrogen to manufacture amino acids, proteins, nucleic acids, and other nitrogen-containing components necessary for life. Biological nitrogen fixation (BNF) process changes inert N_2 to useful NH_3. This process is mediated in nature only by bacteria and certain species of actinomycetes. In the free-living system, plants gain benefit when the bacteria die and release nitrogen to the environment, or when the bacteria are loosely associated with the roots of plants. In legumes and a few other plants, the bacteria live in small club-like growths on the roots called nodules. Within these nodules, N_2 fixation occurs, and the NH_3 produced is directly absorbed by the plant. Nitrogen fixation by legumes is a close/symbiotic relationship between a *Rhizobium* bacterium and a legume host plant.

Biological nitrogen fixation takes many forms in nature from the 1) symbiotic forms including blue-green algae (nostoc), 2) lichens, actinomycetes, non legume 3) associative symbiosis and the 4) and free-living soil bacteria. These types of N_2 fixation contribute significant quantities of NH_3 to natural ecosystems. Nitrogen fixation by legumes can be in the range of 25-75 pounds of N per acre per year in a natural ecosystem and several hundred pounds in a cropping system.

Symbiotic N_2 fixation occurs through associations of plant roots with nitrogen-fixing bacteria. The symbioses are 1) between many leguminous species and *Rhizobiu*m or *Bradyrhizobium*, forming nodules on roots; 2) between a small number of non-leguminous genera and *Frankia.*

The utilization often associative BNF technology in grass and cereal crops was found to be useful in the development of profitable agriculture technologies. The findings of several authors (Rinaudo et al., 1971; Dobereiner et al., 1972; Dobereiner and Day, 1976; von Bulow and Dobereiner, 1975) revealed existing associations of tropical grasses with nitrogen-fixing bacteria, that which under favorable conditions, may be contributing significantly to the N economy of these plants.

Azospirillum as a "biofertilizer" is particularly important in agricultural systems where fertilizer inputs are either impractical (rangelands), undesirable (organic farming), or not possible (subsistence agriculture) (Pacovsky, 1990). Experiments on inoculation of crops with *Azospirillum* or other diazotrophs (Haahtela et al., 1988) often resulted in enhanced plant growth or nitrogen content under environmental conditions (Patriquin et al., 1983; Bashan et al., 1987), improve nutrient assimilation (Kapulnik et al., 1985), alter root size (Okon and Kapulnik, 1986) and function (Sarig et al., 1984).

Numerous studies have shown greater N_2 fixation activities in inoculated plants than in uninoculated controls (Boddey and Dobereiner, 1984; Okon et al., 1985). In a study conducted by Okon (1985) it was reported that higher N_2 fixation rates were observed near or at flowering stage particularly under conditions of high temperature and soil moisture. In addition to N_2 fixation, inoculation with

Azospirillum results in the following benefits (Okon, 1985b):

1. Promotion of root hair development and branching;
2. Increased uptake of N, P, K and microelements;
3. Improved water status of plants and,
4. Increased dry matter accumulation and grain yield.

Inoculated plants when examined under the electron microscopes revealed invasion of the cortical layer (Umali-Garcia et al., 1985).

From the roots of "talahib" a native grass (*Saccharum spontaneum* L.) several nitrogen-fixing bacteria were isolated which when used as inoculants were found capable of enhancing shoot growth, root density and yield of rice, corn and sugar cane. The bacteria were found to possess at least 57 % of the characteristics of the genus *Azospirillum*. Several reports from field tests in different regions of the country confirmed the significant contribution of these nitrogen-fixing bacteria to yield improvement of corn, rice and a few vegetable species. These associative N_2 fixing bacteria were found to be capable of producing growth regulators like gibberellins and cytokinins, which were thought to contribute to the stimulated plant growth. The bacteria belong to the genus *Azospirillum* and are the most promising microorganisms that colonize roots of economically important grasses and cereals.

Azospirillum species are described as Gram negative, rod-shaped, 1mm in diameter, very motile. Cells are about 1.0 um x 3.5 mm in size single flagellum when grown in MPSS broth while lateral flagella when grown on MPSS agar at 30 ºC. They also form wrinkled, dark pink colonies when grown on MPSS agar. A formation of a white veil or bacteria band, is visible when inoculated into an Nfb and Dobereiner's liquid medium.

Azospirillum utilizes glucose, lactate, succinate, fructose, malate, pyruvate, fumarate, as carbon source, reduced nitrate and does not require biotin. The N source used by *Azospirillum* for their growth:

- Ammonium
- Nitrate
- Amino acids
- Elemental N

Azospirillum spp. are highly adaptable, being able to grow under:

- Anaerobic conditions (nitrate used as eletron acceptor)
- Microaerobic (elemental or ammonia used as N source)
- Fully aerobic conditions (ammonia, nitrate, amino acid or combined N only)

Preliminary field experiments in Batangas, Pangasinan, Laguna, Bulacan and Cagayan Valley showed when BIO-N inoculated corn produce a comparatively high yield in the presence of 1/3 to 2/3 of the required N fertilizer. In most of the test sites, the inoculated but unfertilized plots gave rise to consistently and significantly taller and greener plants than the uninoculated unfertilized control, particularly at sixty days after planting.

2.1.2. Other Associative Nitrogen Fixing Bacteria

Associative nitrogen fixing bacterium is defined as the bacterium that not only lives on

rhizospherial environment, but also fixes N_2 from the atmosphere and contributes passively to the plant growth.

Nitrogen fixing bacteria in rice root system were classified as in Table 1. The common associative N_2 fixing bacteria in rice rhizosphere are *Alcaligenes faecalis, Enterobacter cloacae, Klebsiella oxytoca, Klebsiella planticola, Azospirillum brasilense* and *Azospirillum lipferum*. Some strains have been isolated from *Alcaligenes faecalis* and *Azospirillum brasilense*. But only *Azospirillum, Enterobacter cloacae, Alcaligenes faecalis* and *Klebseilla pneumoniae* have been proved as safe strains and used for biofertilizer. Generally, the number of associative N_2 fixing bacteria in paddy is larger than that in dry land, as large as 10^3-10^7 cells/g soil. Associative N_2 fixing bacteria live mainly in the rhizosphere.

Table 1 Classification of associative nitrogen fixing bacteria in rice root system

1. Autotroph	*Rhodobacter(rhodopseudomonas)*
-Photosynthetic N-fixing bacteria	*Rhodospirillum*
2. Heterotroph	
-Autofixing Bacteria/aerobic	*Azotobacter, Azotomonas*
-Autofixing Bacteria/ slightly aerobic	*Derxia, Methylomonas*
-Autofixing Bacteria/ anaerobic-aerobic	*Bacillus*
-Autofixing Bacteria/anaerobic	*Clostridium, Desulfotomaculum,Desulfovibrio*
-Associative nitrogen fixing bacteria/aerobic	*Beijerinckia*
-Associative nitrogen fixing bacteria/slightly anaerobic	*Alcaligenes, Arthrobater,Azospirillum, Flavobacterium, Pseudomonas*
-Associative nitrogen fixing bacteria/oxidative-reductive	*Enterobacter, Klebsiella*

Types and number of soil microorganisms depend primarily on the components of root exudates and chemical characteristics of root residues. In terms of rhizobacteria, the carbon sources needed for survival of rhizobacteria must be provided by plant roots because the capacity of degrading organic matter for rhizobacteria is very weak. In addition, there are some growth regulators and antibiotics in root exudates, which regulate the growth of associative N_2 fixing bacteria. On the other hand, plant roots have selectivity for types of microbes, such an effect is surveyed on different varieties of rice and difference in nitrogen fixation activity of one strain of associative nitrogen fixing bacteria inoculated to different varieties of rice is found. Rice root exudates and residues supply associative nitrogen fixing bacteria with organic acids and sugars for carbon source and growth regulators such as GA3. While IAA is determined if rice roots were incubated with associative nitrogen fixing bacteria.

In general, either beneficial or adverse effect of rhizobacteria on plant root is surveyed. Associative N_2 fixing bacteria belong to PGPR (Plant Growth-Promoting Rhizobacteria) (1) Associative N_2 fixing bacteria provided rice with N sources such as ammonia exudates; (2) Associative N_2 fixing bacteria enhance the growth of rice roots by exudation of growth regulators such as GA3 and IAA; (3) Most associative N_2 fixing bacteria have nitratase, which goes into plant roots after inoculation and assist in nitrate reduction in plant and increase the N level, hence enhancing N_2 fixation; (4) Associative N_2 fixing bacteria can enhance plant mineral uptake; (5) Associative N_2 fixing bacteria can enhance the

growth of lateral roots.

2.2. Isolation of Microbial Strains

2.2.1. Isolation of endophytic bacteria from grass/rice roots

There have been several methods that were developed for isolating nitrogen-fixing bacteria from rhizosphere and roots of grass hosts. The following are some techniques that may be used to isolate nitrogen-fixing bacteria. Field grown roots of intended hosts or its relatives are target materials. Any of the fallowing methods may be used:

1. Collect roots from healthy looking host plants from diverse soil environment.
   Place in clean plastic bags and seal. Keep bag inside Styrofoam container provided with small amount of ice to keep temperature from rising while in transit.
2. Select young and mature roots.
3. Surface sterilize with 50 % laundry bleach.
4. Wash 3-5 times with sterile distilled water
5. Divide the roots into young and mature and further divide each of these into 3 sets;
   1 set to be kept intact (A) and the other 2 for grinding (B).

Set A Use of whole/intact roots

Note: When intended to isolate for root endophyte it is necessary to surface sterilize the roots. When isolating for general population of root – associated bacteria, there is no need to surface sterilize the roots. Sterilization may be roughly conducted by slowly shaking the roots for 20 min. in 10-15 % chlorox bleach (by volume) followed by 3X rinse in sterile water.

1. Cut roots into 1-2 cm length.
2. Inoculate into N-free defined semi solid medium.
3. Incubate at 28-30 ºC for 2-3 days; observe for pellicle formation below surface of medium.
4. Using a wire loop, get sample from tubes with good growth.
5. Streak onto N-free defined agar medium.
6. Incubate plates inside at 28-30 ºC.
7. Examine for colonies everyday, pick individual colonies when fully grown.
8. Transfer individual colonies into semi-solid tubes.
9. Run ARA on individual tubes.
10. Mark tubes with high nitrogenase activity and set aside for purification and identification.

Set B Crush roots

1. Divide into 2 portions; one portion to be used in stab culture (B-1), the other as inoculum for rice or intended trap host (B-2).

Set B-1 Use of crushed roots

1. By means of a long fine forcep or wire loop get sample from grind roots and stab into semi-solid N-free defined medium.

2. As in A, observe for growth of pellicle below surface of medium daily
3. With the use of a wire loop obtain sample of the pellicle and streak into plates of N-free medium.
4. When colonies form, pick individual colonies and stab each into semi-solid defined medium.
5. Run ARA as in A and compare nitrogenase activity
6. Keep tubes with high nitrogenase activity and set aside for purification, identification and further evaluation.

Set B-2 Use of trap host

1. Prepare N-free liquid mineral medium suitable for the test plant e.g. rice.
2. Sterilize dehulled seeds.
3. Pre-germinate in sterile agar plates.
4. When radicle/embryonic roots come out, select ones that do not show contamination.
5. Carefully transfer loopfuls of the colonies obtained from grind preparation from B into each of the tubes containing semi-solid medium.
6. Transfer a germinant into the semi inoculated tube.
7. Place in the dark for a week or until roots have developed.
8. Run ARA on each tube.
9. Get tubes with high ARA values.
10. Collect the roots from tubes in # 8 and add a drop of sterile water.
11. Streak loopfuls of juice from #9 into N-free agar plates containing defined medium.
12. Pick individual colonies and transfer individual colonies into tubes containing N-free semi-solid medium.
13. Incubate and when obvious growth develops, run ARA again.
14. Separate tubes with high ARA and set aside for future purification and identification.

Formula for Acetylene Reduction Assay

$$\text{Nmol } C_2H_4/\text{sample} = \frac{e-b-i}{S} \times c \times v \times r \times \frac{1}{t} \times \frac{1}{x}$$

Where:

e = peak height or area of analyzed sample (mm)

b = peak height or area of sample without acetylene (mm)

i = peak height or area of sample with acetylene, uninoculated (mm)

S = peak height of ethylene standard (mm)

v = volume of incubation chamber (ml)

t = time of incubation

c = concentration of ethylene standard in nmoles

Formula:

$$\text{Nmole } C_2H_4 \text{ per hour per tube} = \frac{e-b-i}{S} \times c \times v \times r \times \frac{1}{t}$$

Where:

e = peak height of ethylene produced from the sample tube (mm)

b = peak height of ethylene produced from control tube (media without acetylene) (mm)

i = peak height of ethylene produced from control tube (media with acetylene) (mm)

s = peak height of ethylene standard (mm)

c = concentration of ethylene standard (nmol)

= 4.47 based on:

- concentration of ethylene in tank = 218.8 ppm
- at STP C_2H_4 occupies 24.4 li

r = ratio of area of internal standard without sample to area of internal standard with sample = 1

v = volume of incubation chamber

t = time with incubation in acetylene

Dobereiner's medium

Composition	**g/l**
Malic acid	5 g
KOH	4 g
Yeast Extract	5 g
Mn SO_4H_2O (1%)	1 ml
$MgSO_47H_2O$ (10%)	1 ml
NaCl (10%)	2 ml
K_2 HPO_4 (10%)	4 ml
$NaMoO_4$ (0.1%)	0.2 ml
$CaCl_2$ (10%)	1 ml
$FeSO_4.7H_2O$ (5%)	1 ml
1 m NH_4Cl	5 ml
Bromthymol Blue	3 ml

Preparation of Bromthymol blue

- 0.5 g in 53 ml of 95% ETOH and add 47 ml of distilled water.

Preparation of 5M NH_4Cl

- Weigh 24 g ammonium chloride
- Dissolve in 100 ml distilled water

Preparation for 5M KOH

- Weigh 35.05 g potassium hydroxide
- Dissolve in 125 ml distilled water

Preparation of Dobereiners medium

1. Prepare stock solution of the 8 chemical reagents and label each bottle.
2. Pipette the aliquot (volume) of the stock and complete volume to 1000 ml.
3. Add 3 ml Bromthymol Blue to the liquid medium,
4. Adjust pH using 1.0 N KOH until its greenest color is attained (pH 6.5-6.8).
5. Dispense medium into desired bottles properly covered and Sterilize at 15 psi for 15 minutes.

Nfb Medium

Composition	**g/l**
Dl-Malic acid	
K_2HPO_4	0.5
$MgSO_4.7H_2O$	0.2
NaCl	0.1
$CaCl_2$	0.02
Distilled Water	1 l
Trace element	2.0 ml

Preparation of Trace Element Solution

Composition	**g/l**
$Na_2MoO_4.2H_2O$	**0.2**
$MnSO_4$	0.235
H_3BO_3	0.2
$CuSO_4.7H_2O$	0.24
Distilled Water	1 l

- Bromthymol blue (0.5 % aqueous) 2.0 ml solution (dissolved in 0.2 N KOH)
- Fe EDTA (1.64 % solution) – 4.0 ml
- Vitamin solution – 1.0 ml

Preparation of Vitamin solution:

Composition	**g/l**
Biotin	0.01
Pyridoxin	0.02
Distilled Water	1 l

- pH adjusted to 6.8 with KOH
- Semi solid agar – 1.75 g
- Solid agar - 18 g

MPSS Medium

Composition	**g/l**
Peptone	5.0
Succinic acid (free acid)	1.0
$(NH_4)SO_4$	1.0
$MgSO_4.7H_2O$	1.0
$FeCl_3.6H_2O$	0.002
$MnSO_4.H_2O$	0.002
Agar	18
Distilled Water	1 l
pH	7.0

BMS Agar

Composition	g/l
Peeled, sliced potatoes	200
Dl-Malic acid	2.5
KOH	2.0
Raw cane sugar	2.5
Vitamin solution	1.0 ml
Bromthymol blue	2 drops
Agar	18
Distilled Water	1 l

The potatoes are placed in a gauze bag, boiled in 1 liter of H_2O for 30 min., then filtered through cotton, saving the filtrate. The malic acid is dissolved in 50 ml of water and the bromthymol blue added. KOH is added until the malic acid solution is green (pH 7.0). This solution, together with the cane sugar, vitamins and agar, is added to the potato filtrate.

The final volume is made up to 1 l with distilled water. The medium is boiled to dissolve the agar, then sterilized by autoclaving.

Rodriuez Medium

Composition	g/l
K_2 HPO_4	0.5
MgSO4.7H_2O	0.2
NaCl	0.1
Yeast Extract	0.5
$FeCl_3.6H_2O$	0.015
Malic Acid	5
KOH	4.8
Agar	18
Distilled Water	1 l
pH	7.0

Maintenance of Isolates:

1. Semi-solid –N free Dobereiner's medium
2. Solid slants

   NA (Nutrient agar) slants + mineral oil

   Tryptic soy agar slants
3. Freeze dried/ (lyophilization)

Temperature: 8-20 ^oC

With monthly transfer for 1 & 2

Nutrient Agar

Composition	g/l
Peptone	5
Beef extract	3
NaCl	1
Agar	18
Distilled Water	1 l

Characterization

Gram reaction (Hucker method)

1. Prepare a smear. Air dry and heat fix.
2. Stain with crystal violet solution for 1 min.
3. Wash with tap water.
4. Stain with the iodine solution for 1 min.
5. Decolorize with 95 % ethanol until no more stain comes away.
6. Wash with tap water.
7. Counterstain with safranin solution for 2 min.
8. Wash with tap water.
9. Blot dry.

a. Flagellar stain (Kodaka's method)

1. Prepare a smear. Air dry.
2. Stain for 5 minutes
3. Wash. Air dry.

b. Metabolism of glucose

1. Inoculate into Hugh and Leifson medium using two tubes per sugar.
2. Seal the surface of one set of the tubes with water agar to a 5mm height.
3. Incubate at 30 °C for 24-48 h.
4. Observe results. Change of color from purple to yellow indicates acid production. If acid is produced in open tube only metabolism is oxidative, if in both tubes, metabolism is fermentative. Fermentation is aerogenic when it is accompanied by gas production and anaerogenic when there is no gas produced.

Spermosphere Model

It consists of a seed germinating in the dark, releasing exudates in a C – and N – free medium, and this is then inoculated with soil dilutions and incubated under acetylene. In this system, the seedling provides the bacteria with the actual C source through the root exudates thus avoiding bias in the C nutrition and N made available by the N_2 fixers are then utilized by the growing seedlings. This set-up keeps the medium N- free and is highly selective.

Rhizosphere soil is the source of bacterial inocula. Roots with adhering soil (about 10g) are ground in a mortar, serially diluted in 100 ml of sterile distilled water and to be considered as 10-1 dilution. This homogenate is further serially diluted.

Rice seeds are decorticated and surface by successively soaking in saturated Ca $(CIO)_2$ (2h with shaking) rinse in sterile water, soak in hydrogen peroxide (11 volumes, 20 min), rinse again in sterile water. The seeds are then planted on the surface of 5 ml semi-solid (0.3 % agar) N-free, C-free medium with the following composition. KH_2PO_4, 450 mg., KH_2PO_4, 100 m; $MgSO_4.7H_2O$, 200 mg;

NaCl, 100 mg; anhydrous $CaCl_2$, 30 mg; $FeCl_3$, 10 mg; 0.1 g; water, 1 liter; pH 6.8. The medium is contained in a test tube.

When the coleoptiles are 1 cm high, inoculation is done with 0.5 ml of soil dilution. Earlier inoculation may cause death of seedlings and delay in inoculation allows identification of contamination by insufficiently sterilized seeds.

Roots pooled from 10 test tubes of the highest dilution positive for ethylene production are homogenized serially diluted, plated on modified Watanabe's medium (Watanabe and Barraquio, 1979) and incubated under 1% acetylene. Individual colonies are picked out, assayed for acetylene reduction in Watanabe's medium. Purification of all the isolates is done in nutrient agar.

Solutions from Gram strain

Solution I: Crystal violet solution

Crystal violet	10 g
Ammonium oxalate	4 g
Ethanol	100 ml
Water (distilled)	400 ml

Solution II: Iodine solution

Iodine	1 g
Potassium iodide	2 g
Ethanol	25 ml
Water (distilled)	100 ml

Solution III: 95 % Ethanol

Solution IV: Counterstain

2.5% Safranin in ethanol	10 ml
Water (distilled)	100 ml

Solutions from Flagellar stain

Solution A

Phenol 5%	10 g
Fannic acid	2 g
Saturated potassium Aluminum sulfate – H_2O	10 ml

Solution B

Saturated crystal violet in ethanol

Mix 10 parts solution A with 1 part solution B_1 filter

2.2.2. Isolation of Associative Nitrogen Fixing Bacteria from rice roots

For isolation of different species of associative nitrogen fixing bacteria, different medium are used. Follows are some examples for isolation and identification of *Alcaligenes faecalis* and *Enterobacter cloacae* from rice roots

Media:

(1) Ashby N-free Sucrose medium. Sucrose 20.0 g, K_2HPO_4 0.5 g, $MgSO_4.7H_2O$ 0.2 g, NaCl 0.2 g, $CaSO_4.2H_2O$ 0.2 g, $CaCO_3$ 5.0g, 5% $Na_2MoO_4.2H_2O$ 0.002 g, $MnSO_4.4H_2O$ 0.002 g, distilled water 1000 ml, agar 20.0 g. Autoclave 20-30 min at 1 kg/cm^3.

(2) Dobereiner N-free malate semi-solid medium. Mannitol 10.0 g, Sucrose 10.0 g, K_2HPO_4 0.5 g, $MgSO_4.7H_2O$ 0.2 g, NaCl 0.2 g, $CaCO_3$ 5.0 g, 5 % agar 1.5 g, traces of $FeSO_4$,$MnSO_4.4H_2O$ 0.002 g and malate, distilled water 1000 ml. Autoclave 20-30 min at 1 kg/cm^3.

(3) Mixture medium. Mixture of medium (1) and (2), but concentration of sucrose and malate should be maintained at the level of (1) or (2).

All above media were placed in glass flasks and autoclaved at 1 kg/cm^3 for 30 mins, the pH maintained at 6.8-7.0.

(4) Benzoate medium. Benzoformyl 10 g, K_2HPO_4 0.2 g, $MgSO_4.7H_2O$ 0.2 g, NaCl 0.2 g, $CaSO_4.2H_2O$ 0.2 g, 5 % Na_2MoO_4 1 ml, 1% FeSO4 1 ml, 1 % $MnSO_4$ 1 ml, agar 18-20 g, distilled water 1000 ml, pH 6.8-7.0. Autoclave 20-30 min at 1 kg/cm^3.

Isolation

Rice roots are washed clean and surface-sterilized by mercury hydrochloric for 2 mins after removal from immediately immersed into 95 % alcohol. There are cut into pieces of 1 cm after washed 6 times with axenic water and incubated in mixture medium at 33 ^oC for 1-2 days. Those with high reduction activity of acetylene are selected for further incubation at medium (1), (2) and （3）.

Identification

Those with high reduction activity of acetylene are incubated in medium (4). Bacteria in medium 4 are inoculated into common N-free liquid medium.

(1) Carbon source determination. Add 1 % of different carbon sources into N-free liquid medium, inoculate bacteria into medium and incubated at open air and 3 % oxygen air for 7 days respectively, to check if the bacteria grow well or not with a photoelectric nephelometer.

(2) GC contents in Bacteria DNA are determined by Tm value method.

Table 2 Physiological and Biochemical features of *Alcaligenes faecalis* and *Enterpbacter cloacae*

Tested Items	*Alcaligenes faecalis*	*Enterpbacter cloacae*
Grams-staining	Negative	Negative
Flagella	Peritrichous	Peritrichous
Oxidation/ferment	Alkaline produced	Ferment gas produced
Oxidase	+++	-

Catalase	-	-
O2 pattern	Microaerobic	Facultative anaerobic
Methyl red test	-	+++
V.P. Test	-	-
Formed indole	-	-
H_2S production	-	-
Reduction of nitrate	+++	+++
Gelatin Hydrolysis	+	+
Urease	+	-
Arginine bihydrolase	-	++
Ornithine deaminase	-	+
Phenylalanine deaminase	-	-
Lysine decaroxylase	-	-
Growth with KCN	-	+
Litmus milk	Alkaline produced	Acid produced, aggregate Reduction
Acid produced from lactose		+
Acid produced from lactose		+
Gas produced from glycerin		+
GC % of Mole in DNA	62.9-63.93	55.5

Table 3 Utilization of carbon sources by *Alcaligenes faecalis* and *Enterobacter cloacae*

Carbon sources	*Alcaligenes faecalis*	*Enterpbacter cloacae*
Malate	+++	
Lactate	+++	+++
Succinate	+++	+++
Benzoate	+++	-
Acetate	++	+++
Pyruvate	++	-
Citrate	-	+++
Malonic acid	-	++
Formate	-	-
Tartarate	-	+++
Muconic acid	-	++
Gluconic acid	-	+++
Glutamate	-	++
Alanine	-	-
Cysteine	-	+++
Praline	-	++
Serine	-	±
Esculin	-	+
Salicin	-	++
Myco-inositol	-	+
Glucose	-	+++
Fructose	-	+++
Galactose	-	++
Mannose	-	++
Xylose	-	++
Arabinose	-	++
Lactose	-	++
Sucrose	-	++
Maltose	-	++
Mycose	-	++
Glycerin	-	+++
Mannitol	-	++
Sorbose	-	++
Dulcitol	-	-

Raffinose	-	-
Adonitol	-	-
Cellobiose	-	++
Melibiose	-	+

2.2.3. Evaluation of N_2 Fixation of Associative Nitrogen Fixing Bacteria

(1) Incubation. Use semi-solid mediums with 0.2 % agar. Ashby N-free sucrose medium for *Enterobacter cloacae,* and Von Bulow and Dobereiner N-free malate medium for *Alcaligenes faecali.* 13 ml incubation vials are used and filled with 3ml medium. Autoclaved at 33 ^{o}C at 1 kg/cm^3 for 20-30 mins. Inoculate and incubate at 33 ^{o}C.

(2) Acetylene Reduction method. Replace plug of vial with a rubber plug after incubation and inserted 1 ml ethylene, incubate at 33 ^{o}C for 1 hour. 0.5 ml of gas inside the vial was taken and concentration of ethylene is analyzed. Acetylene reduction activity is expresses as μmol ethylene formed per h per ml inoculum.

(3) ^{15}N dilution methods.

For ^{15}N dilution method, ^{15}N enriched soil, medium and fertilizer could be used. As we know, the calculation of N_2 fixation by ^{15}N dilution method depends on the difference in nitrogen sources between none-nitrogen fixation system and nitrogen fixation system. For the former, there are only two N sources, soil (medium) and fertilizer, but for latter, there are three sources, soil (medium), fertilizers and atmosphere nitrogen. The ^{15}N abundance in plant N will be diluted by that fixed from atmosphere.

For this method, two treatments should be included, inoculated treatment and none-inoculated treatment. These treatments are all conducted the same except for inoculation of associative nitrogen fixing bacteria.

Calculation of nitrogen fixation can be done in two ways.

a. Without yield and nitrogen data. Only Nitrogen fixation % is calculated

$$15N\% = \frac{100}{2R+1}$$

R is m/e (Mass/Electrons) determined with mass spectrometer.

$$\text{Nitrogen fixation \%} = \left(1 - \frac{^{15}\text{N atom excess \% in N fixation system}}{^{15}\text{N atom excess\% in N none-fixation system}}\right) \times 100$$

b. With yield data. Nitrogen fixation per area/volume could be obtained.

$$\text{\% Ndfa} = \left(1 - \frac{^{15}\text{N atom excess \% in plant of N fixation system}}{^{15}\text{N atom excess \% in in plant of N none-fixation system}}\right) \times 100$$

Because ^{15}N atom excess is different among parts of plants, so N contents, weight and % ^{15}N atom excess are needed.

$$^{15}\text{N atom excess \% in plant} = \left(\frac{\sum {}^{15}\text{N atom excess \% in part i} \times \%\text{N in part i} \times \text{weight in part i}}{\sum \%\text{N in part i} \times \text{weight in part i}}\right) \times 100$$

2.3. Inoculant Production

2.3.1. Associative Nitrogen Fixer

2.3.1.1. Incubation and fermentation

Medium: Modified Dobereiner medium.

30 % Lactate 7.5ml, $(NH_4)_2SO_4$ 0.4 g, KH_2PO_4 0.4g, K_2HPO_4 0.1g, $MgSO_4.7H_2O$ 0.2g, NaCl 0.1g, $Fe_2(SO_4)_3.H_2O$ 0.01 g, $MnSO_4.4H_2O$ 0.01 g $Na_2MoO_4.2H_2O$ 0.01 g. diluted with distilled water to 1000 ml. Autoclave 20-30 min at 1 kg/cm^3.pH maintained at 6.5.

Conduct fermentation in specific facility such as Model MF-104 fermentation jar. Inoculated bacteria in 10 % incubated at 30 ^oC for 18-24 h.

2.3.1.2. Carrier preparation

(a) Carrier preparation

Biofertilizer from associative N_2 fixing bacteria come in three forms: liquid, solid and lyophilized. For liquid and lyophilized ones, only solution medium is used, but for solid form, carriers such as peat and chicken dung are needed. Peat and chicken dung are dried to just dryness and ground into small particles and sieved at 0.18mm.

(b) Carrier sterilization

There are two common methods of sterilization. Autoclave (High temperature +high pressure) is used popular due to low cost. Irradiation is a promising alternative method for carrier sterilization. All procedures are described previously.

2.3.1.3. Packaging and preservation of Biofertilizer with Associative N_2 Fixer

(a) Packing

Production and packaging of biofertilizer from associative nitrogen fixing bacteria is different, depending on its forms. For liquid biofertilizer, bacteria in solution medium is directly transferred into 1 kg, 5 kg or 10 kg plastic bottles or glass bottles after fermentation, sometimes in big plastic barrels. For lyophilized form, fermentation liquid is immediately freeze dried and then packaged into finger-shaped glass tubes under vacuum. For solid form, fermentation solution is mixed thoroughly with carriers after autoclaving (or irradiation), which is then packed into small polyethylene bags under axenic environment. The bacterium per g or ml is described as in Table 4. All small packages should be put into big paper boxes, tied tightly and labeled with product name, brand, standard number, producer, address, production date, log number and net weight after information sheets with product name, brand, standard number, available bacterium number, production date, period of validity, technical specification, manual and producer address sealed inside paper boxes.

(b) Transportation

Common vehicles can be used for transportation of associative N_2 fixing bacteria biofertilizer, as long the products are sheltered from sunshine and rain. But rain and sunshine shelf are needed. Temperature of transportation must not be over 35 ^oC, protection measures should be used if temperature under 0 ^oC.

(c) Storage

Biofertilizer with associative N_2 fixing bacteria should be stored under shade, dry and air circulated storeroom. It should not be stored in open areas. The best temperature is 10-25 ^oC, avoiding temperature below 0 ^oC and above 35 ^oC.

2.3.1.4. Specification of associative nitrogen fixing bacteria biofertilizer

Table 4 Technical specification of biofertilizer from associative nitrogen fixing bacteria

Indexes	Liquid	Solid	Freeze-drying
Appearance and smell	Ivory or light brown solution with some precipitation, no strange smell	Black brown or brown powder, moist and friable, no strange smell	Crystallised, no strange smell
Humid (%)		25-35	3.0
pH	5.5-7.0	6.0-7.5	6.0-7.5
% of particles after sieve 0.18mm	2	20	
Available living microorganism number/ ml,g	5.0×10^8	1.0×10^8	5.0×10^8
% of contaminated bacteria	5.0	15.0	2.0
Period of validity (months)	3	6	12

2.3.2. Mass Inocula Production

Key steps:

a. Select and dry carrier materials.

b. Grind carrier materials.

c. Sift carrier materials and select suitable sizes for granular and powdered inoculants.

d. Neutralize carrier materials.

e. Sterilize the carriers.

f. Examine the carriers for sterility after sterilization.

g. Inoculate carriers with broth cultures.

h. Plate inoculant for quality control.

The water holding capacity of a carrier determines the amount of liquid inoculum that can be added to it. Carriers vary greatly in their water holding capacity. The first step is to determine the inherent moisture level of the carrier. Weigh 10 g accurately on glass weighing dish and place it into the

oven at 70 ºC for 24 hours. Weigh and return to the oven. Another weighing at 48 h will confirm the endpoint of moisture loss.

$$\text{Moisture Content} = \frac{(W1 - W2) \times 100\ \%}{W2}$$

W1 = Weight of carrier before drying

W2 = Weight of carrier after drying 70 ºC

Carrier materials are chosen based on criteria mentioned earlier. The pH of an inoculant carrier should be around 6.5 – 7.0.

Test sterility of carrier materials, by aseptically removing a 10 g sample from each bag and transfer into 90 ml of sterile water in dilution bottles. Prepare serial dilutions from 10^{-1} to 10^{-4} Perform Miles and Misra drop plate method on specific media. Check the plates daily for 7 days for signs of growth and appearance of microorganisms which survived the sterilization.

The sterilized carrier materials in sealed bags are injected aseptically with a suitable amount of broth culture. Swap a small area in a corner of the carrier bag with 70 % ethanol. Cut open the bag and inoculate the desired amount of inoculum. Seal the hole with labeling tape. Work the broth into the peat by kneading the bags until the liquid inoculum has been uniformly absorbed by the carrier. Incubate at 30-32 °C for 1 to 2 weeks.

2.4. Application of biofertilizer from Associative Nitrogen Fixing Bacteria

2.4.1. Benefits of Biofertilizer

In general, biofertilizer from associative N_2 fixing bacteria could used especially for cereal crops such as rice and wheat, but also used for cash crops such as vegetables, fruits, flowers, tobacco, cotton, oilseed, tea and medicinal or herbal crops. BIO-N in Philippines is a microbial-based fertilizer for rice, corn and other agricultural crops like tomatoes, pepper, eggplant, okra, lettuce, pechay and ampalaya. It is a breakthrough technology that promises very significant impact on the country's farmers in terms of increasing farm productivity and income as well saving the country's dollar reserve due to decreased importation of inorganic nitrogenous fertilizers. It is mainly composed of microorganisms that can convert the nitrogen gas into available form to sustain the nitrogen requirement of host plants. The active organisms (bacteria) were isolated from the roots of Talahib *(Saccharum spontaneum* L.), a grass relative of sugar cane. These bacteria once associated with roots of rice, corn, sugar cane, and some vegetable plants can enhance their root development, growth and yield. In China and other FNCA-Countries, associative nitrogen fixing bacteria biofertilizer increased yield by 10-30 % and saved chemical N fertilizer by 15-25 %.It is reported that application of biofertilizer with associative N_2 fixing bacteria could enhance the maturation of crops, shorten vegetation period by 5-10 days and improved the soil quality and soil fertility. The benefits of biofertilizer with associative N_2 fixing bacteria can be seem as follows:

- Enhances shoot growth and root development.

- Improves yield of host plants.
- Replaces 30 – 50 % of the total amount of N requirement.
- Makes plants resistant to drought and pests.
- Reduces incidence of rice tungro and corn ear-worm attack.
- Increases yield and milling recovery of rice.

2.4.2. Application of Biofertilizer

2.4.2.1. Application in China

Cereal crops:

Liquid form is good for rice. At transplanting, immerse rice roots into liquid biofertilizer for 10-15 min before transplanting and spread on paddy soil at regreening stage at rate of 1.5-3.0 L per ha. For wheat, immerse seeds into liquid biofertilizer overnight before sowing, and spread onto wheat leaf at rate of 1.5-3.0 L per ha with water.

Vegetables:

Solid biofertilizer is spread, band-spread and hole applied as basal or top dressing. For leaf vegetables such as celery, spinach and cabbage, apply at rate of 3.75-15.0 kg per ha. For fruit vegetables such as cucumber, eggplant, tomato and melon apply at rate of 7.5 kg per ha. For root vegetable such as sweet potato, potato, ginger and garlic, apply at rate of 3.75-15.0 kg per ha.

Fruits:

10-20 g, 20-30 g or 30-50 g per plant will be applied to those respectively with plant yield less than 50 kg, 50-100 kg and over 100 kg.

Tobacco:

6.25 kg per ha is applied.

For those where biofertilizer with associative N_2 fixing bacteria applied, N fertilizer should be reduced by 20-25 %. Mixed application with organic manure should be encouraged, because organic manure will benefit microbes.

2.4.2.2. Application of BIO-N in Philippines

Corn:

- Place seeds in a suitable container and moisten with water. Pour sufficient amount of inoculants, 1 packet of BIO-N for every 3 kg of seeds.
- Mix thoroughly until the seeds are evenly coated; (a drop or 2 of sticker e.g. Tween 20 or APSA may be mixed with water to enhance adsorption of BIO-N on the seeds).
- Sow coated seeds immediately. Be sure not to expose the inoculated seeds to direct sunlight.
- Depending on the soil analysis, very marginal soils may require a basal application of at least a bag or two of 14-14-14 to a hectare as side dress.

NOTE:

The basal application of organic fertilizer is highly recommended to provide a whole array of other

nutrients for a balancing effect. Split application of the recommended inorganic macro-elements has been found effective, e.g. second application of 14-14-14 NPK is done before tasseling.

Rice:

- As solid inoculant for direct-seeded rice or for sowing on *dapog* bed

- Soak seeds overnight in clean water
- Pre-germinate the seeds in gunny sacks or suitable container.
- When radicles (embryonic root) come out, place germinants in suitable container.
- Pour required amount of BIO-N and mix thoroughly until germinants are evenly coated.
- Sow directly over field or on prepared beds.

- As Liquid Inoculant for *dapog* bed

Suspend the required amount of Bio-N in sufficient volume of clean water (e.g. 1 packet Bio-N to 1 gallon water) and evenly drench the seed/seedling-lined *dapog* bed.

- As slurry for transplant seedling

1. In a suitable container, mix BIO-N with clean water to form a slurry or thick preparation.
2. Prune the roots of seedlings into uniform length and dip for at least 30 min or 1 h before transplanting

2.4.3. Procedures for Growing Corn using Biofertilizer Inoculated Seeds in Indonesia

A) Seeds

- Use best seeds for certain locations as recommended by Department of Agriculture.

B) Land Preparation

- Land is ploughed with a tractor with depth 15-20 cm, and then hoed.
- Clear land from weeds and prepare seedbeds.

C) Seeds Inoculation

- Check the instruction on the biofertilizer pack. For example, one pack of biofertilizer for corn (200 g for 2000 m^2) and 3 kg of seeds.
- Inoculation is done step by step. Prepare one clean bucket or plastic bag to hold the seeds are being inoculated. Prepare slurry by mixing sticker with inoculant. If sticker is not available, use vegetable oil.
- Mix the slurry thoroughly with corn seeds and let them dry.
- When inoculating seeds, avoid making them too wet. See the procedure on the pack.

- Sweetcorn seeds are commonly coated with fungicide. Use a larger amount of inoculant and plant immediately after inoculation.
- Inoculated seeds are ready to sow. Put the inoculated seeds under shade.

D) Sowing

- Sow seeds at planting distance of 75cm x 25 cm
- To avoid seedlings from infestation of seed flies, insecticide (e.g. Furadan) is applied to seed holes.

E) Fertilization (see Fig. 1)

- Basal fertilizer, 66 kg N/ha (urea), 150 kg SP-36/ha and 100 kg KCl/ha are applied at 10 days after planting (DAP), banded in depth 5 cm and apply 7 cm in front of plant rows.
- Second N fertilization, 33 kg urea/ha is applied banded at 10 cm in front of plant rows

F) Weeding

- Weeding is done before fertilizer application.
- At the second N fertilizer application, soil and weeds are returned back to plant rows.

G) Pest Management

- Spray plants with suitable insecticide at the recommended dose as soon the symptom of infection appear.

H) Watering

- Corn needs sufficient water at sowing, flowering and grain filling.
- Drainage is made to avoid flooding

I) Harvesting

- Harvesting could be done at around 96 DAP for corn varieties, and 70 DAP for sweetcorn.

FERTILIZATION

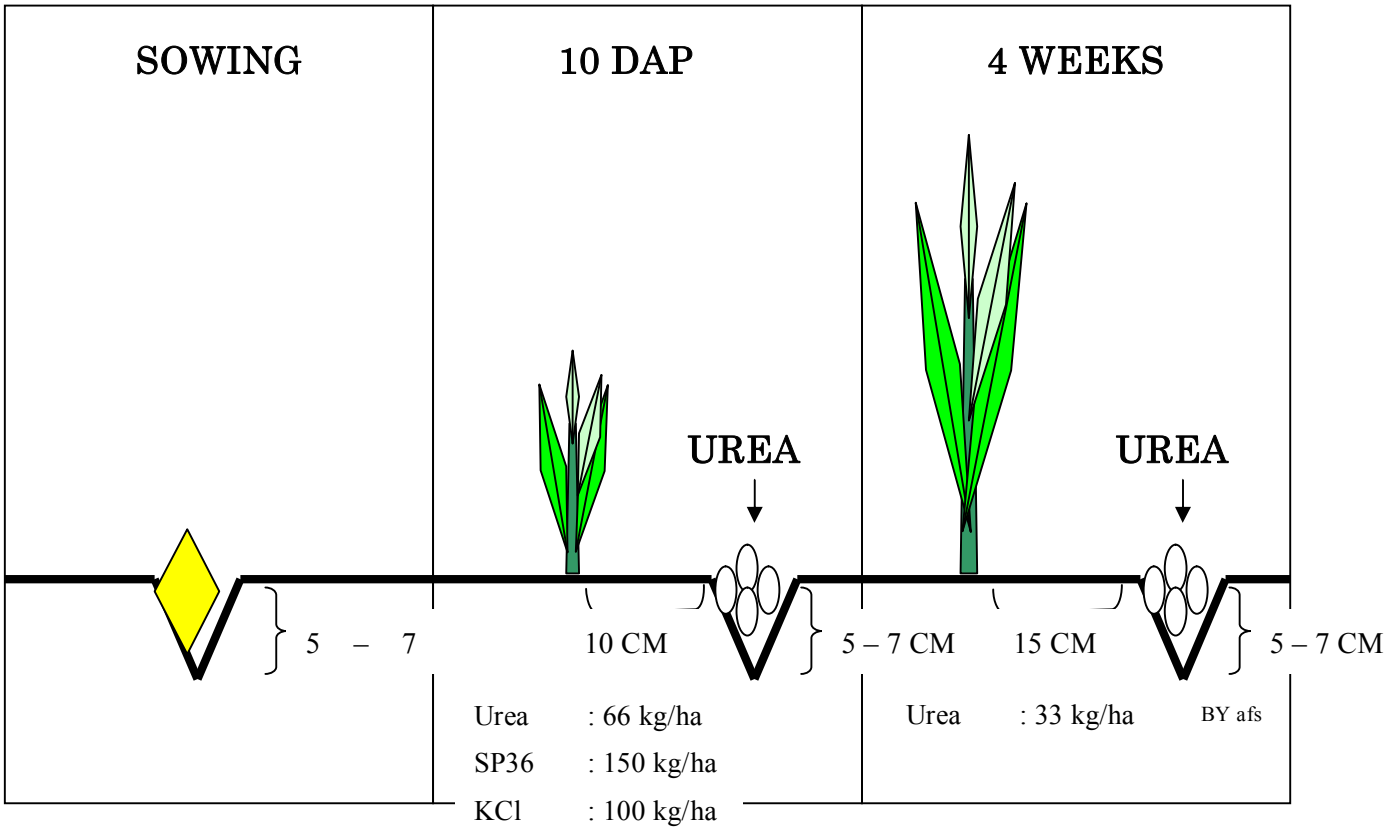

Fig. 1: Schematic diagram of fertilizer application (*)

(*). PT. Sanghyang Sri, Indonesia

2.5. Tips on Buying and Storage of Biofertilizers

1. Check biofertilizer package before buying. Make sure to buy biofertilizer for the specific crops (e.g. corn).
2. Ensure that the biofertilizer is fresh. Check the expiration date.
3. Keep the package in a cool place until ready to use. Storage in refrigerator is good. Cool temperature will not harm the bacteria but high temperatures can be damaging to the biofertilizer microorganisms.
4. It is best to inoculate seeds prior to planting. Bacteria die quickly on drying seeds.
5. Chemicals on seedsand applied to the soil (e.g. insecticides, fungicides) may be toxic to the bacteria.
6. Store the inoculated seeds in a cool protected place until planting. Keep them out of direct sunlight and protect them from excessive drying.
7. Leftover inoculant may be kept safely in the package provided it is closed tightly to prevent excessive drying. Leftover inoculant stored in a refrigerator at 4 ^{o}C or lower will remain effective for several months.

2.6. Cautions and Limitations of Biofertilizer

a. Never mixed with chemical nitrogen fertilizers;
b. Never apply with fungicides, plant ash etc. at the same time;
c. Never directly expose to sunlight;
d. Do not keep used solution overnight;
e. Store at room temperature, not below 0 ^{o}C and over 35 ^{o}C.

Biofertilizer with associative N_2 fixing bacteria only serves as supplement for nitrogen requirement of corn, rice and sugarcane. It is still necessary to apply 30-50 % of the recommended inorganic forms to meet the requirements for other nutrients such as phosphorous and potassium.

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