IV. Inoculant for Biofertilizers

1. *Rhizobium* Inoculant

1.1. Introduction

Bradyrhizobium strains are slow growing, gram negative, soil bacteria. The genus *Bradyrhizobium* represents a heterogeneous group of nodulating bacteria within which the taxonomic relationships are poorly understood (Jordan 1984). The *Bradyrhizobium* genus, currently consists of six species, *Bradyrhizobium japonicum* (Jordan, 1982), *Bradyrhizobium elkanii* (Kuykendall et al., 1992), *Bradyrhizobium liaoningense* (Xu et al., 1995), *Bradyrhizobium yuanmingense* (Yao et al., 2002), *Bradryrhizobium betae* (Rivas et al., 2004), and *Bradyrhizobium canariense* (Vinuesa et al., 2004).

Based on 16S rRNA gene sequences, the genus *Bradyrhizobium* was classified into a clade in the Proteobacteria along with oligotrophic soil or aquatic bacteria such as *Rhodopseudomonas palustris*, *Rhodoplanes roseus*, *Nitrobacter winogradskyi*, *Blastobacter denitrificans*, and the pathogen *Afipia* spp. (Saito et al., 1998; Sawada et al., 2003; van Berkum and Eardly, 2002; Willems et al., 2001).

1.2. Isolation of *Rhizobium* Strains

1.2.1. Collection and preservation of root nodules in field trips

Equipment for collection and preservation of root nodules

Sampling vials with desiccated silica gels: For field trips of more 2 days, the root nodules collected must be prevented from decomposing and prevented from invasion by soil microorganisms which interfere with subsequent isolation procedures. Therefore, the root nodules collected are preserved in a vial with desiccated silica gels shown in Fig 1. If the gel color turns pink, the desiccant must be changed for blue one as soon as possible.

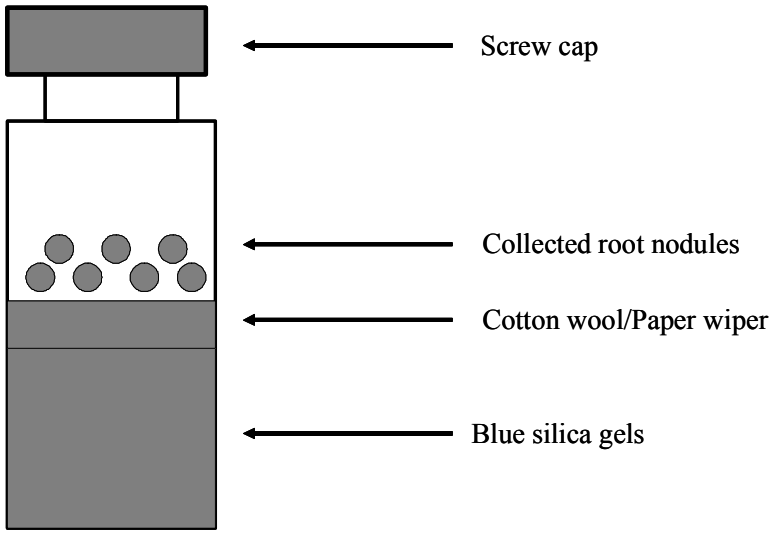

Fig.1: A preservation vial for root nodules

A sturdily built shovel: It is very difficult to collect root nodules from wild legumes, because soil growing the wild legumes is very hard. Therefore, the sturdily built shovel is essential for digging up legume roots.

Field book and pencil, Detailed road maps, Topographical maps, Compass and GPS, Camera, Knife,

Forceps, Small plastic Ziplocs bags for soil and root sample, Paper towels, Permanent marker, Large plastic bags for herbarium specimen collection.

Consideration points before sampling

a) Site identification: Country, prefecture, nearest town, kilometers from the nearest town and direction. If possible, record latitude and longitude by using GPS.

b) Host plants identification: It is highly recommended that you are accompanied by a specialist in legume plant taxonomy. Collect a sample of the legume plant for herbarium specimen to identify genus, species and cultivar. Take pictures of the leguminous plants.

In case of leguminous crops such as soybean and mung bean grown in farmers' fields, ask the farmers the variety of the leguminous crop and confirm history of the fields concerning inoculation of rhizobia.

c) Soil identification: Collect soils in small plastic Ziplocs bags and examine soil type, texture and pH.

Collection and preservation of root nodules

Excavate whole plants to retrieve root nodules.

Carefully remove soil around root nodules.

Exposed root nodules can be collected with forceps.

All nodules from a single host plant represent one unit of collected material and are stored in the same vial.

Root nodules from different plants of the same species should not be combined because they may represent different soil environments even if only several metres apart.

The vials containing dried root nodules are kept in a refrigerator at 4 ^{o}C until isolations of bradyrhizobia.

1.2.2. Isolation of *bradyrhizobia* from dried root nodules

(1st day)

Wash dried root nodules under tap water to remove soil contamination and transfer to sterile water.

Keep the root nodules in sterile water in a refrigerator at 4 ^{o}C overnight to absorb water.

(2nd day)

The root nodules are surface sterilized by immersion in 70 % ethanol for 30 s, and in a 3 % sodium hypochlorite solution for 3 min. The root nodules are subsequently washed at least five times with sterile water. Each root nodule is crushed in a microfuge tube with 100 µl of 15 % glycerol solution. Ten µl of turbid suspension with 15 % glycerol solution is streaked onto the surface of a yeast extract mannitol agar (YMA) medium (Somasegaran and Hoben 1994).

(The turbid suspensions with 15 % glycerol solution in a microfuge tube keep in freezer at -30 ^{o}C until isolations of bradyrhizobia are complete. If first isolations do not succeed, the glycerol solutions are restreaked onto fresh plate to obtain pure cultures.)

(2 weeks later)

The plates are incubated at 28 ^{o}C for 2 weeks in an inverted position. Well-separated single colonies are restreaked onto fresh plate to obtain pure cultures.

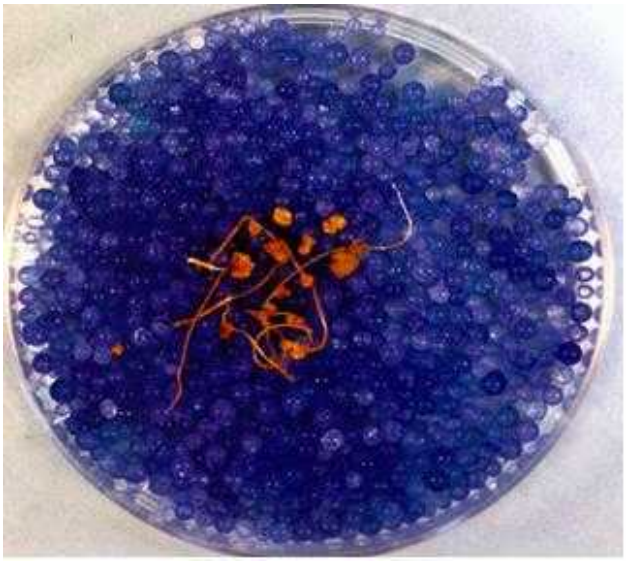

Fig. 2: Dried root nodule in blue silica gels

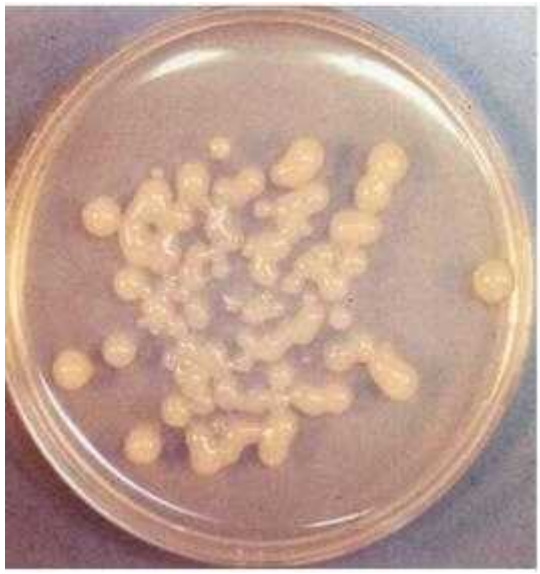

Fig.3: Well separated *Bradyrhizobium* colonies isolated from a dried root nodule originated in Wild *Vigna* plant

1.2.3. Isolation from fresh nodule

Fresh roots of legume crops collected from field are cleaned with tap water to remove all soil and organic particles. Forceps are used to hold the nodules, and the roots with attached nodules are cut, 2-3mm on each side of the nodules. Intact and, undamaged nodules are immersed for 10 second in 95 % ethanol or isopropanol (to break the surface tension and to remove air bubbles from the tissue); transferred to a 2.5-3 % (v/v) solution of sodium hypochlorite or chlorox (commercial bleach) 1:1 (v/v) and soaked for 4-5 min. The segments are then rinse in five changes of sterile water using sterile forceps for transferring. Forceps may be sterilized quickly by dipping in alcohol and flaming. Sterile glass or plastic Petri dishes may be used as containers for the alcohol, sodium hypochlorite, and water. Alternatively, nodules may be placed into a 125 ml Erlenmeyer flask. The sterilizing and rinsing fluids may be changed as required, leaving the nodule in the flask each time. Mercuric chloride solution (0.1% weight/volume) or solution of hydrogen peroxide (3% W/V) can be used for the sterilization of nodule.

The nodule is crushed in a sterile tube with sterile glass rod and sterile water. The slurry is than diluted and then streaked on the surface of YMA (Yeast-Mannitol Agar) plates containing Congo red. The inoculated Petri plates is incubated at 25-28 ^{o}C for 3 to 10 days, depending on the strain and species until colonies appear. The colony of rhizobia is mucoid, round and show little or no Congo red absorption. The isolate from a single rhizobial colony is then purified and confirmed as *Rhizobium* by demonstrating nodule-forming ability on test host legume under bacteriologically controlled condition (Authentication of isolates).

The second method is isolation by needle. The needle method of isolation is especially useful with freshly harvested nodules 2 mm or larger in diameter. The nodule is initially washed in water, then in alcohol, and then held with forceps and briefly passing the nodule through a flame. This surface-sterilized nodule is placed on a small piece of sterile filter paper (2 x 2 cm) in a sterile Petri dish. A new piece of filter

paper should be used for each nodule. The same Petri dish can be used for several nodules. The blunt-tipped forceps are dipped into 95 % alcohol and flamed momentarily. While holding the nodule with the forceps and resting the nodule on sterile filter paper, a small section was quickly sliced off with a flamed, hot scalpel. While still holding the nodule with the forceps on the filter paper, the tip of a sterile inoculation needle (with a 1-mm loop) is inserted into the cut surface. The loop is then loaded with inoculum, and then streaked directly onto a YMA plate containing CR and a YMA plate containing BTB. When using the needle method, the nodule can also be held in the fingers of one hand while inserting the needle with the other hand. The heels of the hands are braced together to steady them.

1.3. Rhizobial Inoculant Production

1.3.1. Production of broth culture

Rhizobia are relatively easy to grow in liquid medium. Since rhizobia are not competitive with other microorganisms, it is very important to sterilize the whole of growth vessel and medium as well as ensuring inoculation of the fermenter with rhizobial starter culture under sterile environment. The purpose of the production is to have high density of rhizobia in the broth culture. This can be influenced by culture medium, rhizobial strain, temperature and aeration.

Rhizobia are aerobic bacteria and need oxygen for growth. Long experience in rhizobial inoculant production has shown that rhizobia need aeration of 5-10 litre of air for 1 litre medium in 1 hour. Optimum temperature for rhizobial growth is 28-30 ^{o}C.

The medium supplies energy, nitrogen, certain mineral salts and growth factor. General Yeast Manitol (YM) medium is used in rhizobial broth culture. Compositions of YM broth are as follows:

Ingredient	g l^{-1}
K_2HPO_4	0.5
$MgSO_4.7H_2O$	0.1
NaCl	0.2
Manitol	10.0
Yeast extract	0.5
Distilled water	1.000 l

Adjust pH to 7.0 if necessary

Some laboratories use sucrose, corn steep liquor, proteolyzed pea husks, malt sprout extract and unsupplemented instead of adding mannitol.

Normally yeast extract is used as growth factor supplement for rhizobia. Alternatively, fresh starch-free cakes of baker's yeast can be used.

Important for broth culture production is the maximum number of rhizobia per volume of media. It is recommended to choose medium with ingredients that are easily available locally, cheap and easy to

prepare. International Center for Agricultural Research in the Dry Areas (ICARDA) summarized some commonly used compositions of rhizobia growth media as the following:

Ingredient	Composition by authors (g l^{-1})			
	Waksman 1928	VanSchreven 1963	Date 1976	Fred and Burton 1976
Mannitol	10.0	-	10.0	2.0
Sucrose	-	15.0	-	10.0
K_2HPO_4	0.5	0.5	0.5	-
K_3PO_4	-	-	-	0.2
KH_2PO_4	-	-	-	0.4
$MgSO_4.7H_2O$	0.2	0.2	0.2	0.2
NaCl	0.1	-	0.2	0.06
$CaCO_3$	3.0	2.0	-	0.2
$CaSO_4.2H_2O$	-	-	-	0.04
$FeCl_3.6H_2O$	-	-	0.1	-
Yeast water	100.0	100.0	100.0	-
Yeast extract	-	-	-	0.5
Paraffin oil	-	0.5	-	-
$(NH_4)_2HPO_4$	-	-	-	0.1
Water	900	900	900	1000

For production of broth culture, vessels or fermenters in different sizes are often used. It is important that all equipment are sterilizable and that inlet air is also sterile. In most ASEAN countries simple autoclavable vessels of glass or stainless steel with capacity of 2-20 L are used in broth culture production. For sterilizing the inlet air, a compressor or a small aquarium pump with sterile filters are used. Vessels are filled with media to one-third to two-third and sterilized in an autoclave at 121 ^oC. After the medium is cooled to room temperature, liquid starter culture is inoculated to the vessel with the ration of 1-3 % (v/v) of media. Time required for growing rhizobia range from 3 to 7 days, depending on the rate of growth of rhizobial strains.

Starter cultures are best prepared in Büchner flasks. Two 1 L Büchner flasks are connected at the arms with latex tube, one filled with YMA medium and another with distilled water. After sterilization, the agar surface is inoculated with the desired rhizobial strain aseptically and then incubated at 28 ^oC until the growth of rhizobium is observed. Pour water from the second flask to the first flask via the connecting latex

tube. Ensure suspending maximum rhizobium cell from agar surface to the water by shaking. This technique has an advantage over a broth culture, because an experienced operator can see evidence of contamination, if any, on the agar surface and discard contaminated preparations.

During rhizobia growth in both starter and broth culture it is very important to look for contaminants and control the rhizobial density.

1.3.2. Production of sterile carrier-based inoculant

The production requires a completely sterile carrier in sterile package. The simplest way is to mix the sterile carrier with bacterial liquid culture. Each pre-sterilized carrier bag is injected aseptically with culture by means of a syringe fitted with a sterile needle. For mass production, an automatic dispensing machine (auto syringe) may be used. The area of puncture must be disinfected with ethanol. Quantity of broth should be adequate to wet 40 % of the carrier by carrier weight. If the carrier material contains nutrient available for the incorporating bacteria to grow (e.g. mineral soil), injection into the carrier package of starter culture of the bacterial cells together with sterile water for moisture adjustment will be sufficient. The puncture hole is then immediately sealed with preprinted self-sticking label. The bags are then kneaded by hand or by shaker until the liquid inoculum has been uniformly absorbed in to the carrier. The final moisture of inoculant should be 45-50 %. After the injection, the carrier package should be placed in temperature-controlled area for appropriate period to let the bacterial cells grow up to maximum population. The inoculant is ready for use after 2 weeks.

1.3.3. Production of non-sterile carrier-based inoculant

In this production method broth culture is mixed or sprayed onto the carrier in a mixer under non-sterile conditions. The proportions of broth and carrier are governed by the nature and moisture holding capacity of the carrier. The carrier mixture is then cured for a period of one to six days. It should be covered to prevent the desiccation. During this period the moisture equilibrate and any heat of wetting is dissipated. Following the curing period, during which some multiplication of rhizobia takes place, the inoculant is passed through a coarse sieve or hammer mill to remove lumps and make a consistent product. The finished inoculant can be packaged in moisture retaining polyethylene bags and ready for testing and application.

1.3.4. Production of liquid inoculant (For scale 1,500 ml)

Step 1. Inoculate a loop full of rhizobia into 500 ml Erlenmeyer flask containing sterilized 150 ml of YM broth.

Step 2. Culture on rotary shaker at 28 °C, 200 rpm until late log phase (cell concentration about 10^8 cells/ml). This culture will be used as starter culture for liquid inoculant production.

Step 3. Inoculate 150 ml of starter culture into 2,000 ml Erlenmeyer flask as simple fermenter containing sterilized 1,500 ml of a modified YEM media (G5 media*), which is used as basal media for liquid inoculant formulated with selected appropriate additive for each genus of rhizobia**.

Step 4. Culture at 28 °C by using air continuously pump through a 0.45 μm filter into the medium until reach maximum cell concentrations of 10^9 cells/ml.

Step 5. Inoculate 20 ml aliquot of cell culture into sterile polypropylene bag and heat sealing. Liquid inoculant should be stored at appropriate temperature before used (4 °C for long-term storage).

Step 6. Use 20 ml of liquid inoculant for inoculation 1 kg of seed (for medium seed size, such as soybean), without using sticker, before sowing.

* G5 media composes of (g/l) 1.0 mannitol; 0.5 K_2HPO_4; 0.2 $MgSO_4.7H_2O$; 0.1 NaCl; 1.0 yeast extract; 1.0 glucose; 0.5 arabinose; 200 μM Fe-EDTA and 4 ml glycerol. pH is adjusted at 6.8 before autoclaving.

** Select **ONE type of additive** that is suitable for each genus of rhizobia from list below to formulate with G5 media.

Appropriate concentration of additive use for blending with G5 media

Genus of rhizobia	Concentration of additives (% w/v)			
	[1]Sodium alginate	[2]PVP	[3]Gum arabic	[3]Cassava starch
Azorhizobium	0.1	2.0	0.3	0.5
Bradyrhizobium	0.1	2.0	0.3	1.0
Mesorhizobium	0.1	2.0	0.1	Not appropriate
Rhizobium	0.1	5.0	Not appropriate	Not appropriate
Sinorhizobium	0.1	Not appropriate	Not appropriate	Not appropriate

[1] **In case of sodium alginate is selected.** Sodium alginate will be added to G5 media after maximum cell concentration was achieved due to alginate obstruct the cell growth during culturing. It can be prepared by

- Culture rhizobia in 2,000 ml Erlenmeyer flask (simple fermenter) containing **1,350 ml** of G5 media (without adding of sodium alginate) until the maximum cell concentration is reached.
- Separately prepare **150 ml** of 1% (w/v) sodium alginate in G5 media (apply heat to make solution). Then autoclave at 121 °C for 20 min.
- Add sterilized 150 ml of 1% (w/v) sodium alginate into 1,350 ml culture media and mix well by shaking before continue to Step 5.

[2] **In case of PVP is selected.** PVP is easily dissolved in media. It can be directly dissolved into 1,500 ml of G5 media and autoclaved at 121 °C for 45 min before rhizobial culturing.

[3] **In case of gum arabic or cassava starch is selected.** It can be directly dissolved into 1,500 ml of G5 media and autoclaved at 121 °C for 45 min before rhizobial culturing. However, heat is needed to make it dissolve in G5 media before autoclaving.

Information of additives

Additives	Company	Characteristic
Sodium alginate	Carlo	compound with adhesive property, useful in supporting long term survival of inoculant
Polyvinyl pyrrolidone PVP-40T (PVP)	Sigma	water soluble compound, high phenolic compound binding capacity, useful in reducing toxic substance from seed coat
Gum arabic	Carlo	biopolymer compound with emulsification and stabilization property, limit heat transfer
Tapioca Flour (Cassava starch)	Thai Better Food Co. Ltd.	biopolymer compound with stabilizing property, used as thickener and binder

1.4. Introduction to the *Rhizobium* Supplier and Field Experiments in Japan

~Tokachi Federation of Agricultural Cooperatives~ -The *Rhizobium* inoculant supplier in Japan-

Tokachi Federation of Agricultural Cooperatives (TFAC: Japanese abbreviation: Tokachi Nokyoren) in Hokkaido, which is the only organization producing and distributing the *Rhizobium* biofertilizers in Japan. The TFAC is located in Tokachi-Obihiro area in Hokkaido, the most northern prefecture in Japan, and it started the *Rhizobium* biofertilizer business since 1953.

Poster with the information of "Mamezo" products.

1.4.1. Production and Sales of *Rhizobium* Biofertilizers

In TFAC, 3 kinds of biofertilizers are produced and sold presently. These are "Mamezo" (rhizobia are mixed with peat and the natural organic matters), "R-Processing Seeds" (leguminous seeds inoculated with rhizobia), and "Hyper Coating Seeds" (leguminous grass seed coated by rhizobia within the capsule of calcium carbonate), with a catchphrase of "Environmental friendly agriculture". These biofertilizers are being used by about 80 % of farmers in Hokkaido.

This biofertilizer has advantages to enhance legume growth, and high seed yield and high nutritional quality (protein-rich). In the field of soybean cultivation with crop rotation system, 1.2 times of nodulation is possible and about 4 % increase of soybean yield is substantiated by inoculation in comparison with non-inoculated case in average.

Researchers at TFAC have commented that soils in Japan are well fertilized and rich in rhizobia. Therefore, only around 4 % yield increase is obtained. However, much more effective results could be expected in the Asian regions where the lands are infertile with low rhizobia population.

Factory building

1.4.2. Facility of biofertilizer production in TFAC

The TFAC facility with two production lines for producing the concentrated *Rhizobium* inoculant paste was completed in 1990. Microorganisms are cultured and propagated with sucrose and concentrated by a centrifuge, and then frozen for storage. The frozen culture is thawed and mixed with sterilized peat (carrier) to produce biofertilizers.

Cultivation plant

Control panel for the plant

TFAC uses peat imported from Canada as biofertilizer carrier and acetylene reduction method is adopted to measure the N_2 fixation efficiency. It was suggested that sterilization of peat by using Co-60 facility of Shihoro-Nokyo located near the TFAC may bring significant cost saving. The selling price of "Mamezo" is about US$5/40g pack for 10 a while the cost of microorganism itself is about US$0.6.

The most effective microorganisms should be identified for each area where the climate and soil conditions are different. However, sharing of the technologies of cultivation is possible and very useful.

The researcher in TFAC said that they have research programs to study the long-lived bacteria ("Mamezo" is valid for 1 year) or the bacteria overcoming indigenous microorganisms and also to develop intragenic recombination technology.

Plant test

* Tokachi Nokyoren URL:
http://www.nokyoren.or.jp/ (Japanese only)

1.4.3. Outline of *Rhizobium* inoculant production in Tokachi Federation of Agricultural Cooperation

The information was originated from "Historical Review of *Rhizobium* Technology" edited by Tokachi Federation of Agricultural Cooperation in 1997.

(1) *Rhizobium* inoculants:

Three types of *Rhizobium* inoculants are available. Peat carrier based inoculants, and preinoculated

seed by vaccum (*Rhizobium* inoculated seeds) and calcium carbonate coated preinoculated seeds. Tokachi Federation of Agricultural Cooperation (TFAC) provides *Rhizobium* and *Bradyrhizobium* for grain legumes (soybean, faba bean, bean, peanut, pea, azuki bean) as well as forage and green manure legumes (clover, alfalfa, chinese milk vetch). Recently TFAC provides peat based *Rhizobium* inculant mixed with *Azospirillum*.

(2) Inoculant carriers and inoculation methods:

In 1953 TFAC started supplying agar cultured *Rhizobium* inculant in test tubes or inoculant with sterilized soil carrier in paper bag. The former was better since the soil inculant can get contaminated and dry up easily. Since 1965, they produce mineral carrier (montmorillonite: perlite, 4:1 plus 20 % liquid *Rhizobium* inculant). At present TFAC peat import from Canada. 40 g of inoculant for 10a is available.

(3) Selection of effective strains:

TFAC takes great effort in selecting good strains as inoculants. Higher nodulation, and higher N_2 fixation activity are the most important criteria for selection. Moreover, compatibility for various varieties, stability and viability in package, and their survival in soil are also important criteria.

TFAC tests the strains by sand culture in a/5000 pots, seed pouch, or field experiments. From 1978 to 1980, 292 *Rhizobium* strains of soybean collected from 11 prefectures are tested for selection. Stock cultures of selected strains are kept in the freezer at –80 ºC with 20 % glycerol.

1.5. Inoculant Application

The effect of inoculant on growth and yield of legume crops depends on the quality of inoculant, soil properties and application techniques. General inoculant should be used according to specification on the package and used when a legume is introduced in to a new area or when the legume is known to have nodulation problem. The main purpose of inoculation is to nodulate the host legume with selected rhizobial strain. The inoculant should be of good quality at application time.

Commonly, two application methods are used in the inoculation of rhizobia to legumes. This is the direct inoculation, where the inoculant is placed in direct contact with the seed (seed–applied inoculant), and indirect inoculation, where by the inoculant is placed alongside or beneath the seed (soil–applied inoculant).

Inoculant is applied to the seed in the following ways:

a) Dusting: With this method, the inoculant is mixed with the dry seeds directly. This may lead to poor adherence of rhizobia to the seeds; the method is least effective.

b) Slurry: Inoculant can be mixed with wetted seeds, or diluted with water and some stickers e.g. 25 % solution of molasses or 1 % milk powder. In some cases gum arabic, sucrose of methyl ethyl cellulose can be used as sticker.

c) Seed coating: The inoculant can be made into slurry and mixed with the seeds. The seeds are then coated with finely ground lime, clay, rock phosphate, charcoal, dolomite, calcium cacbonat or talc. The method has several advantages, such as protection of rhizobia against low pH soil, desiccation, acidic fertilizers, fungicides or insecticides.

In the indirect application method the inoculant is applied to the soil beneath or alongside the seed. The

method is used when seeds are treated with fungicide or insecticide, and when high amount of inoculant is needed to outcompete the indigenous rhizobial population. The simplest inoculation is to make the liquid formulation of the inoculant and spray to the soil or directly over the seeds after placement In this case high amount of inoculant is needed. Disadvantages of this method include loss in viability of rhizobia, short storage period and difficulty in the distribution of inoculant.

1.6. Field Experiments Related to New Inoculation and Fertilization Methods for Soybean in Japan

The average soybean seed yield in Japan is relatively low (less than 2 t ha^{-1}), although the seed yield is sometimes much higher up to 6 t ha^{-1} in a well managed fields under good climatic conditions. The following studies were conducted to obtain better seed yield by employing the different inoculation methods as well as by using deep placement of slow release N fertilizers such as coated urea and lime nitrogen.

We developed a new inoculation method using paper pot seedling transplantation (Figure 1). Seed inoculation is the most common way, but seed inoculation sometimes resulted in low occupancy rate of inoculated strain compared with indigenous strains. Also, the seed inoculation is usually less effective as compared to soil inoculation. We found that *Rhizobium* population increased 100 times in vermiculite medium for 25 days (Minagawa et al. 1997); therefore, we use a paper pot (paper cylinder with 12.5cm length and 2.5 cm diameter open bottom) filled with vermiculite for inoculation. A soybean seed was planted in each pot, and 1 mL of liquid culture of *Bradyrhizobium japonicum* (USDA110) was added. Ten-day old seedlings are then planted in the paper pots.

We compared ten-day old seedlings with *B. japonicum* inoculated paper pots (IPP), with non-inoculated paper pots (NIPP) and those grown in vermiculite bed without paper pots (DT). These were transplanted to sandy dune field of Niigata, rotated paddy field of Nagaoka and first cropping reclaimed field of Yamakita in 2001. In addition to a basal dressing application of 16 kgN ha^{-1} in a surface layer (Control), deep placement of 100 kg N ha^{-1} of urea (Urea), 100-day type coated urea (CU-100) and lime nitrogen ($CaCN_2$) treatments were applied at the depth of 20 cm using fertilizer injector (Figure 2).

Fig. 1: Paper pot inoculation method of soybean seed with liquid culture of *Bradyrhizobium japonicumu* USDA110

Fig.2: Fertilizer application by deep placement method

As shown in Table 1, it was observed that among the same N fertilizer treatments, the seed yield with IPP and DT tended to exceed those with NIPP in each respective fields; Nagaoka, Niigata and Yamakita. Among IPP method, significant higher seed yield was obtained with the deep placement of $CaCN_2$ and CU-100 compared to Urea and Control treatments in all the experimental fields. The effect was more pronounced in the first cropping reclaimed field of Yamakita, in which $CaCN_2$ gave 5 times higher in terms of seed weight than that of Control (Figure 3).

Among the three fields, the best yield was obtained in the Nagaoka field. Being a rotated paddy field, the Nagaoka field was fertile and with the addition of inoculum and fertilizers as CU-100 and $CaCN_2$, seed yield of about 6 t ha^{-1} was obtained. Despite the Yamakita field being infertile with no rhizobial population, deep placement of CU-100 or $CaCN_2$ in combination with inoculation enabled increase of yield by almost four times as much as that of Control without inoculation.

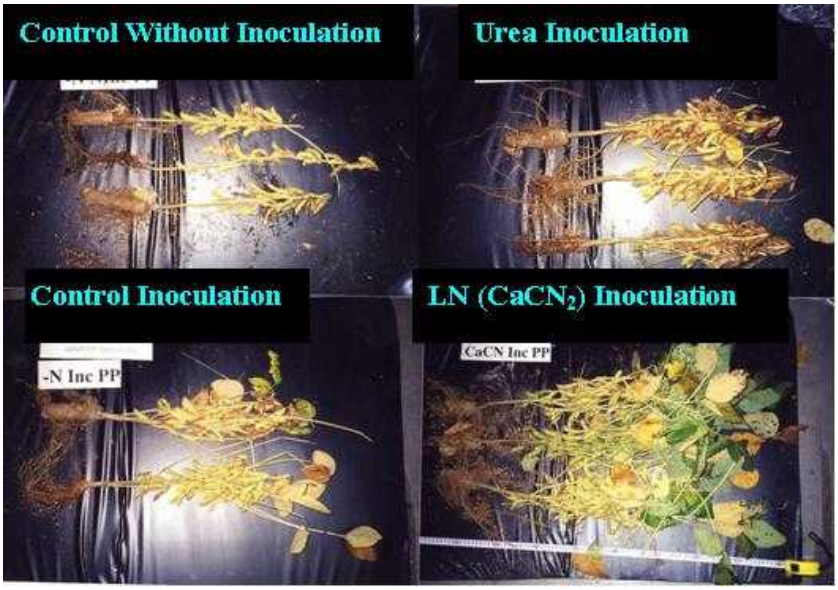

Fig. 3: Comparison of inoculation and urea or calcium cyanamide deep placement in reclaimed Yamakita Field where no indigenous *Rhizobium* was detected. Upper left (Non-inoculated without N deep placement), Lower left (Inoculated paper pot without N deep placement), Upper right (Inoculated paper pot with urea deep placement), Lower right (Inoculated paper pot with lime nitrogen deep placement)

Table 1: Seed yield of soybean with fertilizers and inoculation treatments at different fields in 2001

Inoculation Methods	Deep Placement	Seed yield (t/ha) in Experimental fields		
		Rotated paddy field	Reclaimed field	Sandy dune field
NIPP	Control	2.88 b	0.78 b	1.72 b
	Urea	4.53 a	2.86 a	2.46 a
	CU-100	4.29 a	3.58 a	2.49 a
	$CaCN_2$	4.60 a	3.40 a	2.50 a
DT	Control	3.14 b	1.94 b	1.91 b
	Urea	4.22 ab	3.36 a	2.62 a
	CU-100	5.35 a	3.97 a	2.71 a

	$CaCN_2$	5.41 a	3.56 a	2.67 a
IPP	Control	3.31 b	2.01 c	1.83 b
	Urea	4.67 b	2.90 b	2.73 a
	CU-100	6.04 a	4.00 a	3.05 a
	$CaCN_2$	6.12 a	4.19 a	3.32 a

NIPP; non-inoculated paper pot, DT; direct transplanting of inoculated seedlings, IPP; inoculated paper pot, Means followed by the same letter are not significantly different by 5% level in the same inoculation method in the same field. (From Tewari *et al.* 2002, 2003, 2004a)

The effect of placement (broadcasting and deep placement) of urea and coated urea fertilizers were analyzed for yield and quality of soybean seeds (Tewari et al. 2004b). It was found that the seed yield was higher in the deep placement treatment of coated urea (7.3 t ha^{-1}) than in the broadcasting treatment of coated urea (6.09 t ha^{-1}), although slow release fertilizer CU-100 was used in both treatments, signifying the importance of deep placement over broadcasting of fertilizer.

The % Ndfa (N derived from atmospheric N_2) estimated by the simple relative ureide method as well as ^{15}N dilution method was also higher in the plants with CU-100 and $CaCN_2$ compared to Urea and Control treatments as shown in Table 2 (Tewari et al. 2005). The results indicated that the deep placement of these slow release fertilizers promoted the N_2 fixation activity. Also *Ndff* (N derived from fertilizer) as well as N recovery rate was found to be highest with $CaCN_2$ followed by CU-100, which suggested that these fertilizers were efficiently utilized by plant without depression of N_2 fixation.

Table 2: Estimation of the amount of N originating from various N sources based on the ^{15}N dilution method (Tewari et al.2005)

Line	Fertilizer type	Ndfa (g $plant^{-1}$)	Ndfs (g $plant^{-1}$)	Ndff (g $plant^{-1}$)	Total N (g $plant^{-1}$)
Enrei	AS	2.24b	0.78c	0.09c	3.12b
	U	2.32b	1.07b	0.23b	3.62b
	CU	2.82b	1.05b	0.41[a]	4.28ab
	LN	3.61a	1.34[a]	0.48[a]	5.43a

Fertilizer type; AS (Ammonium sulfate), U (urea), CU (coated urea), LN (lime nitrogen)

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