Biofertilizer Manual

Biofertilizer Project

Forum for Nuclear Cooperation in Asia (FNCA)

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By

FNCA Biofertilizer Project Group

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Cover photographs (clockwise, from left to right):
1) 'BIO-N', an *Azospirillum* based biofertilizer manufactured by BIOTECH and used for corn in the Philippines
2) Groundnut demonstrated by the field test in Vinh Puc Province, Vietnam Agriculture Science Institute, Viet Nam (left: without biofertilizer, right: with biofertilizer)
3) The field demonstration of biofertilizer for corn at Muara Field Station, Department of Agriculture, Bogor, West Java, Indonesia

Photo credits: 1) BIOTECH, U.P.L.B College, the Philippines; 2) Japan Atomic Industrial Forum, Japan; 3) Ms. Soertini Gandanegara, National Nuclear Energy Agency (BATAN), Indonesia

Preface to Manual on Biofertilizer Production and Application

by Dr. Sueo Machi, the FNCA Coordinator of Japan

The proper feeding of the rapidly growing populations in developing countries is the most important challenge for mankind. Presently, about 800 million people in the world are suffering from chronic malnutrition due to shortage of suitable foods. In this context, improving agriculture to increase yield of crops without deteriorating the environment should be an ultimate goal. Continuous and excess use of chemical fertilizers and other agrochemicals to increase yield may lead to ground water contamination and depletion of soil nutrients, eventually resulting in reduction of crop yield.

Biofertilizers from microorganisms can replace chemical fertilizers to increase crop production. In principle, biofertilizers are less expensive and are more environmentally-friendly than chemical fertilizers.

In the Forum for Nuclear Cooperation in Asia (FNCA) project on "Biofertilizer", experts from member countries cooperate through exchange of experiences and information including showing field demonstration on biofertilizers for a variety of crops.

In the production of biofertilizer, radiation processing has been tested and proposed for sterilization of carriers for the biofertilizer microorganisms. Ionizing radiation from existing irradiation facilities in member countries should be able to provide a simple, reliable and less expensive method to sterilize carriers.

The stable isotope of nitrogen, N-15, can be effectively utilized as a tracer to accurately quantify the efficiency of biofertilizer microorganisms such as *Rhizobium* to fix atmospheric nitrogen. This may be extended to other suitable isotopes for other major plant nutrients.

I am confident that this manual is useful for the extension of biofertilizer usage to a large number of farmers for improvement of crop yield and other benefits, while minimizing environmental pollution from agrochemical inputs.

Acknowledgments

This manual is written for scientists and technicians involved in biofertilizer research, production and application in FNCA member countries. We would like to thank the Ministry of Education, Culture, Sports, Science and Technology (MEXT) of Japan and respective Ministries or Agencies of China, Indonesia, Korea, Malaysia, the Philippines, Thailand and Viet Nam for the sponsorship. The publication of the manual would not be possible without the efforts and assistance of our supporting staff. We are grateful to Dr. Pan Jiarong (China), Ms. Soertini Gandanegara (Indonesia), Dr. Tadashi Yokoyama, Dr. Issay Narumi, Dr. Takuji Ohyama, Dr. Masanori Saito, Dr. Keishi Senoo (Japan), Dr. Jang Sun Suh (Korea), Dr. Khairuddin bin Abdul Rahim (Malaysia), Dr. Achara Nuntagij (Thailand), Dr. Pham Van Toan (Vietnam) and their associates. Thanks are also extended to Dr. Kaushal Tewari (Japan), Dr. Mercedes U. Garcia, Ms. Juliet A. Anarna (the Philippines), Dr. Omsub Nopamornbodi, Ms. Supaporn Thamsurakul, Dr. Panlada Tittabutr, Ms. Somchai Patiyuth (Thailand) as co-authors. Special thanks to Dr. Sueo Machi, the FNCA Coodinator of Japan for his creative ideas and advice on the publication of the manual, Dr. Pham Van Toan for his contribution as the Editor-in-Chief and Dr. Khairuddin bin Abdul Rahim for assistance in English editing. Finally, we are grateful to Ms. Yuko Wada and Ms. Mari Miura for their untiring effort and assistance to complete the manual.

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I. Introduction

1. The Biofertilizer Manual

This biofertilizer manual is the product of the Biofertilizer Project in FNCA (Forum of Nuclear Cooperation in Asia). Eight countries, China, Indonesia, Japan, Korea, Malaysia, Philippines, Thailand, and Viet Nam participate in this project. The Editor-in-Chief is Dr. Pham Van Toan, the Project Leader of Viet Nam. The activities of this project are introduced in FNCA homepage (http://www.fnca.jp/english/index.html) in English.

This project is aimed at improving and disseminating biofertilizer technology to increase the yields of grain legumes and other crops which are important food and animal feed sources in Asia, and to enhance environmental friendly sustainable farming practices by reducing excessive amount of chemical fertilizer application.

The project formulation meeting was held in Bangkok, Thailand in August, 2001. In this meeting, members agreed that this project deals with biofertilizers involving microorganisms which promote nutrient acquisition of the plants, such as N_2 fixation by rhizobia or free living bacteria, arbuscular mycorrhizal fungi and phosphorous solubilizing bacteria which improve phosphorous nutrition, and other microorganisms that help nutrient uptake. These biofertilizers can be expected to reduce the use of chemical fertilizers. Sometimes the term biofertilizer is used for various types of materials such as composts, agro-waste, and some liquid cultures of unidentified miscellaneous microbes. However, we do not include them in this project, because the evaluation of effectiveness of such products and their quality control is quite difficult as compared with biofertilizers from identified microbes under controlled conditions.

We agree the definition of biofertilizer proposed by Prof. Dr. Zulkifli Hj. Shamsuddin, University Putra Malaysia, in Inaugural Lecture of 17th June 2005. “Biofertilizer is a substance which contains living microorganisms which, when applied to seed, plant surfaces, or soil, colonizes the rhizosphere or the interior of the plant and promotes growth by increasing the supply or availability of primary nutrients to the host plant(Vessey, 2003) (Vessey, J.K. 2003. Plant growth promoting rhizobacteria as biofertilizers. Plant Soil 255, 571-586). This definition separates biofertilizer from organic fertilizer. The latter contains organic compounds which directly, or by their decay, increase soil fertility. Likewise the term biofertilizer should not be used interchangeably with the terms, green manure, manure, intercrop, or organic-supplemented chemical fertilizer. Not all plant growth promoting rhizobacteria (PGPR) can be considered biofertilizers. Bacteria that promote plant growth by control of deleterious organisms are biopesticides, but not biofertilizers. Similarly bacteria can enhance plant growth by producing phytohormones and are regarded as bioenhancers, not biofertilizer. Interestingly, some PGPR can promote growth by acting as both biofertilizer and biopesticide or bioenhancer.”

The production and use of biofertilizer is proposed, to improve yield of crops by using root nodule bacteria (rhizobia), mycorrhizal fungi, and other microorganisms that are able to increase availability of plant nutrients from the soils. For this purpose, the most effective microorganisms for each specific crop will be identified, for example, by measuring N_2 fixation activity by using nitrogen-15 isotope as tracer and using other methods too. Ionizing radiation is used to sterilize the carriers of the rhizobia and other biofertilizer

microorganisms.

These microorganisms are selected by pot and field experiments, cultured and packed with carrier materials, and provided commercially for the agricultural crops and reclamation of forest and devastated lands. Quality control is extremely important, especially for the population of infected effective microbes and other contaminants, which may often give adverse effects. The carrier sterilization by ionizing radiation is one of the best ways to keep biofertilizers in storage for a long period.

The working plan for the project was formulated as the following: Selection of effective microorganisms (2002), Improvement of inoculant (2003), Improvement of soil microbial activities (2004), Field trials (2005), Economic analysis including assessment of impact on cost savings (2006).

This biofertilizer manual is written by project members and other experts to share information and experiences of biofertilizer use in Asian countries, their effectiveness, efficient production processes, storage and application on different crops. The field demonstration was proposed in the 2002 Workshop. Each country carried out the field experiments using various biofertilizers. Some examples of field experiments are shown in this manual.

This manual has the following chapters: 1) Introduction, 2) General methods to evaluate microbial activity, 3) Carriers for biofertilizers, 4) Inoculant for biofertilizers including rhizobia, non-symbiotic nitrogen fixers, mycorrhiza, phosphorous solubilizers, and 5) Quality control of biofertilizers, from advanced basic information to practical methods in each participating country.

We try to write as easy as possible for scientists and technicians involved in biofertilizers in Asia. However, we admit that this manual may be quite difficult for farmers. It is our hope that scientists and technicians will translate some part of this manual into the respective mother language in some brochures or pamphlets for farmers. Each chapter was written by expert of this field. Please feel free to contact FNCA for enquiries on this manual.

II. General Methods to Evaluate Microbial Activity

1. Isotopic Methods

1.1. N_2-fixing Activity

1.1.1 Introduction

Leguminous plants comprise a large group, of about 18,000 species including annual grasses and perennial trees. Although only small numbers of leguminous species are selected as leguminous crops, they are very important as food and animal feed world wide (Somasegaran and Hoben, 1994). Soybean (*Glycine max*) production (217 million t/yr) accounts for a half of leguminous crops due to the nutritional value both for human and livestock. The common bean (*Phaseolus vulgaris*) is an important dietary protein source in many of the Latin American countries. Pigeon pea (*Cajanus cajan*) is a major food legume (pulse) in India, while chick pea (*Cicer arientinum*) is a widely grown grain legume in the world. Table 1 shows the annual production of major leguminous crops in the world in 2002. Total leguminous crop production is comparable to the production of major crops such as maize, paddy rice, wheat and potatoes. The yields of leguminous crops are generally lower than maize, paddy rice, wheat and potatoes (Table 1). The average yield of soybean in Asia is very low (1,385 kg/ha) compared with world average (2,266 kg/ha), although the yields of other leguminous crops are comparable with world average. The potential yield of leguminous crops is considered to be much higher than the world average yield. For example, the highest record of soybean yield was 7,800 kg/ha in Japan and over 5,000 kg/ha yield is obtained in well managed experimental field in Japan.

Table 1: Annual production and average yield of major leguminous corps in comparison with major crops （FAO）

Leguminous crops	Production (1,000 Mt)			Yield (kg/ha)		
	World	Asia	% in Asia	World	Asia	% in Asia
Soybean	179,917	23,720	13	2,266	1,385	61
Groundnut in shell	34,075	23,022	68	1,381	1,693	123
Castor bean	1,120	983	88	1,000	956	96
Dried beans	18,334	7,673	42	683	536	78
Dried broad bean	3,728	1,654	44	1,524	1,580	104
Dried peas	9,872	2,156	22	1,698	1,186	70
Chick peas	7,808	6,824	87	789	777	98
Lentils	2,938	2,131	73	811	772	95
Green beans	5,646	3,880	69	6,767	6,585	97
Green peas	9,059	5,708	63	8,972	10,426	116
Major crops						
Maize	602,589	165,915	28	4,343	3,884	89
Paddy rice	576,280	523,030	91	3,916	3,998	102
Wheat	572,879	252,615	44	2,720	2,629	97
Potatoes	307,440	120,575	39	16,131	15,566	96

Leguminous crops require a large amount of N for seed protein synthesis The N is derived from symbiotic N_2 fixation with soil microorganisms in addition to soil mineralized N and fertilizer N. Promotion of N2 fixation by inoculation of highly efficient rhizobium strain and improvement of soil management and cropping practice is very important to increase seed yield of leguminous crops especially in Asia. Evaluation of N_2 fixation in leguminous crops is essentially important to select effective rhizobia and to improve fertilizer application and crop management in compatible with N_2 fixation. A survey of the quantities of N_2 fixed revealed the principal crop legumes to be ranked in the following descending order; soybean, lupin, field pea, faba bean, common bean, lentil and chick pea (Unkovich and Pate, 2000).

There are several purposes of evaluation of N_2 fixation. For initial screening of rhizobia species or for experimental purposes leguminous plants are normally cultivated in test tubes, growth pouches, Leonard jars, or sterilized sand or vermiculite culture under controlled environment. In greenhouse experiments, plants are often cultivated with sterilized soil pots or using hydroponics system. Evaluation of N_2 fixation in experimental fields and farmers fields is also very important for final selections of effective strains and to improve agricultural management for enhancing N_2 fixation and seed yield. The % Ndfa (percentage of N derived from atmospheric N_2) for a legume is not a trait determined by a legume genotype and rhizobia alone, but rather is a product of the interaction between the soil N environment and total legume growth (Unkovich and Pate 2000).

There are two approaches for evaluating N_2 fixation in leguminous crops. One is a point measurement of N_2 fixation activity at the moment of analysis, such as acetylene reduction assay, $^{15}N_2$ fixation activity and H_2 evolution. Relative ureide method is a kind of real time assay, and we can estimate the relative dependence on N_2 fixation by root nodules and from N absorption by the roots rather than N_2 fixing activity itself. The other approach is to estimate the cumulative amount of N derived from N_2 fixation, which means time-integrated measurement of N_2 fixation.

When leguminous plants are cultivated with N free medium and depend only on N_2 fixation, all of assimilated N is derived from N_2 fixation. Therefore, total N content in a plant is equal to the amount of fixed N. The N balance method is very convenient method for estimating the total amount of fixed N in field experiments using targeted leguminous crops and the non-fixing reference (control) plants such as non-nodulating isolines or other species.

The ^{15}N dilution method is considered as a most accurate estimation for the amount of N derived from N_2 fixation and N from fertilizer and soil. This is also a cumulative method. In this section, various methods of evaluating N_2 fixation by legume crops using stable isotope ^{15}N are introduced. Also in Section 1.3., other conventional methods are described. These methods can be adapted not only for legume grain crops, but also for forage legumes, tree legumes and non-leguminous symbiotic N_2 fixers as well.

Detailed methods are shown in good manuals and books such as “Handbook for Rhizobia” (Somasegaran and Hoben 1994), “Maximising the Use of Biological Nitrogen Fixation in Agriculture” (Hardarson and Broughton 2003) “Nitrogen fixation by legumes in tropical and subtropical agriculture” (Peoples and Herridge 1990),. Please see these references for more details.

Abbreviations: Ndfa - N derived from atmospheric dinitrogen, Ndfs - N derived from soil, Ndff - N derived from fertilizer

1.1.2. Total N accumulation

The total N accumulation in a whole plant or a shoot in which most of all N is derived from N_2 fixation indicates the amount of N originating from N_2 fixation. This is a cumulative evaluation for N_2 fixation from planting until harvest. This is not the isotopic method. However, understanding the N accumulation method and the N balance method will help for better understanding of the more complex ^{15}N dilution method.

Total N accumulation method is applicable for the sterile culture, greenhouse experiments and field experiments. For example, for the initial assessment of the N_2 fixation activity with many isolated strains of rhizobia, the easiest way is to cultivate the inoculated leguminous crops in an N-free medium in seed pouches, Leonard jars or pots with sand or vermiculite under controlled environment in a chamber or in the greenhouse. For the pot experiments, commercial vermiculite is one of the best medium because it does not contain mineral N and free from rhizobia since the medium has been heated in preparation. The commercial vermiculite should be washed thoroughly with tap water before use to remove alkali and salts. When the top of pot is covered with aluminum foil and treated carefully, plants can be maintained without contamination by rhizobia in the environment. N-free medium such as in the nutrient solution (originally from Dr. J.E. Harper) below is good for supplying nutrients and water for hydroponics or vermiculite pot experiments (Fujikake et al. 2002).

The culture solution containing mineral nutrients as following concentration (mg L^{-1}): K_2SO_4, 109. K_2HPO_4, 8.5. KCl, 0.935. $CaCl_2.2H_2O$, 183.0. $MgSO_4.7H_2O$, 123. H_3BO_3, 0.367. $CuSO_4.5H_2O$, 0.032, $MnSO_4$, 0.189. $ZnSO_4.7H_2O$, 0.144. $(NH_4)_6Mo_7O_{24}$, 0.004. $CoSO_4$, 0.028. $NiSO_4.6H_2O$, 0.0035. $FeSO_4.7H_2O$, 13.9 solubilized with EDTA・Na_2, 18.6.

We have devised a two-layered pot culture for soybean plants (Fig. 1) (Ohyama et al. 1993, Fujikake et al. 2002). Seedlings are inoculated with rhizobia and cultured in vermiculite for about 10 days until primary leaves are opened. Then the seedlings are transplanted to the two-layered pot. The roots grow both in the upper pot filled with vermiculite and in the lower pot with culture solution. Nutrient solution is periodically supplied both in the upper and lower pots every 2 or 3 days. Soybean plants can grow healthy until mature stage due to the sufficient supply of oxygen in the upper pot and water and nutrients from the lower pots. Using hydroponics or vermiculite medium alone, it was difficult to grow soybean as well as the plants grown in the field with good management.

When legumes are grown with N-free medium, the N availability by N_2 fixation is the limiting factor for the plant growth. Therefore, the plant biomass production (FW or DW) of whole plant, shoots or seeds, can be used as a semi-quantitative index for N_2 fixation activity. Nodule DW (FW) or leaf chlorophyll concentration may be used as an indicator for N_2 fixation activity. Nodule number is a less reliable indicator. Nodule evaluation is quick, convenient, and inexpensive. The degree of nodulation is determined by nodule weight, number, size, or distribution on the root system.

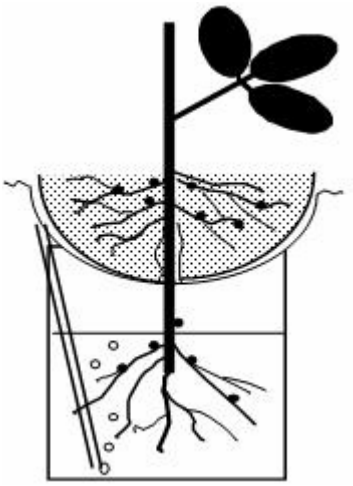

Fig. 1 A: Tow-layered pot for soybean culture (Fujikake et al. 2002)

Fig. 1B: Photograph of soybean cultivation with two-layered pot

However, a more accurate estimation can be obtained by total N accumulation in whole plants. Plant samples are dried and ground into a powder and digested by Kjeldahl digestion and the N concentration can be measured by distillation method or colorimetric method (cf. Appendix 1). Total N accumulation in the harvested plant, or total N accumulation in the harvested plant minus an average seed N for young plants is the cumulative N_2 fixation from planting until harvest time. Appropriate harvesting time may be decided according to the purposes of the experiment and the cultivation methods, e.g. 30 days old plants in seed pouches, at initial flowering stage in pot culture, or at seed maturing stage in the field.

1.1.3 N difference method

Fig. 2 shows the basic concept of the "N difference method". This term is often used synonymously as the "N balance method", although "N balance method" actually means comprehensive analysis of input and output of N in plant-soil system, as will be discussed later.

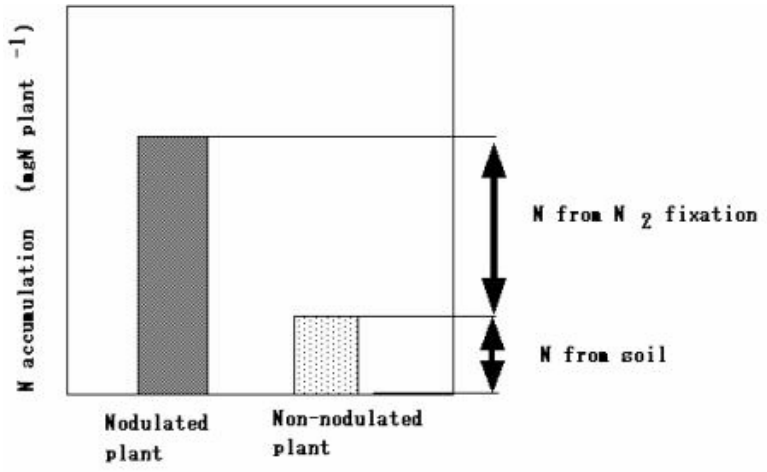

Fig. 2: Concept of N difference method

This method is a cumulative evaluation in field experiments. Since this method is based on the assumption that the legumes and the reference plants assimilate the same amount of soil N or soil + fertilizer N, the nodulated legume crops and non-nodulated reference crops should be planted in the adjacent site of the same field (Fig.3).

Non-nodulating isogenic mutant line is the best reference plant if it is available. The amount of fixed N_2 is estimated by the subtraction of total N in non-nodulated reference plant from total amount of N in a nodulated plant. The complete recovery of the root system in the field is normally very difficult. A whole shoot is used instead of a whole plant that includes the root system. To recover as much N as possible in the plant, plants are usually harvested at R7 stage just before the leaves start to fall, as in the case of soybean. The shoots or whole plants are dried, and after the dry weight is measured, samples are ground into a fine powder, and the N concentration is determined by Kjeldahl digestion method (see Appendix 1).

Fig. 3: Nodulating (left) and non-nodulating (right) isolines of soybean planted in the same field at the same time

The N difference method is based on the assumption that the same amount of N is absorbed in the nodulating plant and the non-nodulating plant. If the reference plant absorbs the same amount of N as in nodulating legumes at harvest time, any plant species such as wheat or corn can be used as a reference plant. However, plant growth, structure and development and the N absorption activity of the root system may be quite different among plant species. Therefore, non-nodulating legume mutant is considered the best reference plant, especially in experiments where both plants are harvested successively throughout their growth stages.

It should be acknowledged that the amount of absorbed N by reference plants is not always equal to the amount of the absorbed N in nodulating plants. It has been suggested that reliable results could be obtained if available soil N is low and % Ndfa accordingly high (Unkovich and Pate 2000). In very fertile soils or by an application of heavy dose of N fertilizer, N may not be a limiting factor. In addition, N absorption by reference plants may compensate the lack of N_2 fixation by scavenging N from the soil. As a result, the difference in the growth and amount of accumulation of N between nodulated plants and non-nodulated plants become smaller or the same. In this case, the amount of Ndfa or % Ndfa is often estimated to be lower than the actual value. On the other hand, when plants are grown in soils very poor in N, the growth of the reference plant will be very poor and the root development will be smaller than the nodulating plants. In this case, the amount of N derived from N_2 fixation is estimated to be higher than the true value. Although there are many restrictions for accurate measurement of N_2 fixation, N difference method is valid and convenient because this method is the easiest and the cheapest way to evaluate the amount of N derived from N_2 fixation and N absorption in experimental plots or farmers fields.

In the case of soybean, T202 (nodulating) and T201 (non-nodulating) isolines have been used for

this method. T201 is a non-nodulating line detected by Williams and Lynch (1954) by their crossing experiments using Lincoln x (Lincoln x Richard). Also, many non-nodulating lines are made by crossing T201 and the local soybean varieties.

Recently, a chemical mutagen EMS (ethyl methane sulfonate) is used for inducing mutation for plants. Non-nodulating mutant can be relatively easily induced by this treatment on the seeds. For example, several hundreds of legume seeds are put in 0.5 % EMS solution for 24 h and washed thoroughly with water. The concentration and treatment period should be optimized for the seed characters and the plant species. The treated seeds (M_1) are inoculated with compatible rhizobia and cultivated to obtain M_2 seeds. The harvested seeds (M_2) are inoculated with compatible rhizobia and cultivated in a vermiculite or sand bed without N supply to distinguish non-nodulating plants easily. After selecting the plants exhibiting chlorosis (yellow leaf color) and inferior growth due to N deficiency by the lack of nodulation by eyes, the plants are transplanted to several pots and cultivated with an adequate level of N fertilizer. At flowering time nodulation trait can be checked and non-nodulating plants are carefully grown with sufficient supply of N fertilizer to get good M_3 seeds. M_3 seeds are inoculated and cultivated again. If the non-nodulating trait is genetically dominant, about 75% of the M_2 plants will exhibit the non-nodulated trait. If it came from recessive gene defects, all the M_2 plants exhibit nodulating trait. In this case, the M_3 seed harvested from M_2 parents will show 25% of non-nodulating trait. The genetic trait should then be fixed by self-crossing further for several generations.

The N balance method is usually used to have the same meaning as the N difference method, but it is treated as different by Peoples and Herridge (1990). The N balance method requires the estimation of all possible external N inputs (e.g. in rainwater, dust, animal droppings, by ammonia absorption, or through weathering) and outputs (denitrification, volatilization of nitrous oxides, ammonia, leaching, erosion, and removal of crop or animal products) within a given soil-plant system. It is difficult to determine all the N inputs and outputs, especially changes in soil N with precision within short period. For this reason, experimentation over several years is required.

N fertilizer equivalent method

One of the evaluation method based on total N analysis of the legume crops is "N fertilizer evaluation" (Peoples and Herridge 1990). This method assesses the amount of N_2 fixed by a legume by growing N fertilized non-N_2 fixing plants in plots alongside the unfertilized N_2 fixing test legume. The N fertilizer level at which the yields of the non-fixing plants match those of the legume is equivalent to the amount of N_2 fixed (Fig.4).

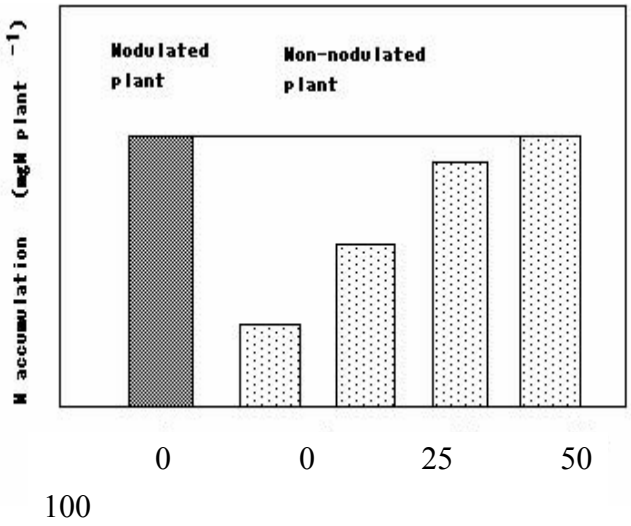

Fig. 4: Concept of N fertilizer equivalence

1.1.4. ^{15}N dilution method

It is generally accepted that the ^{15}N dilution method is a most reliable estimation for evaluating % Ndfa as well as total amount of Ndfa. By this method, Ndfs and Ndff can be also calculated at the same time. This is a cumulative estimation like the N balance method and this method is based on the assumption that both nodulating plants and non-nodulating reference plant absorb N from fertilizer and N from soil with the same ratio which means that the ratio S1:F1=S2:F2 in Fig. 5.

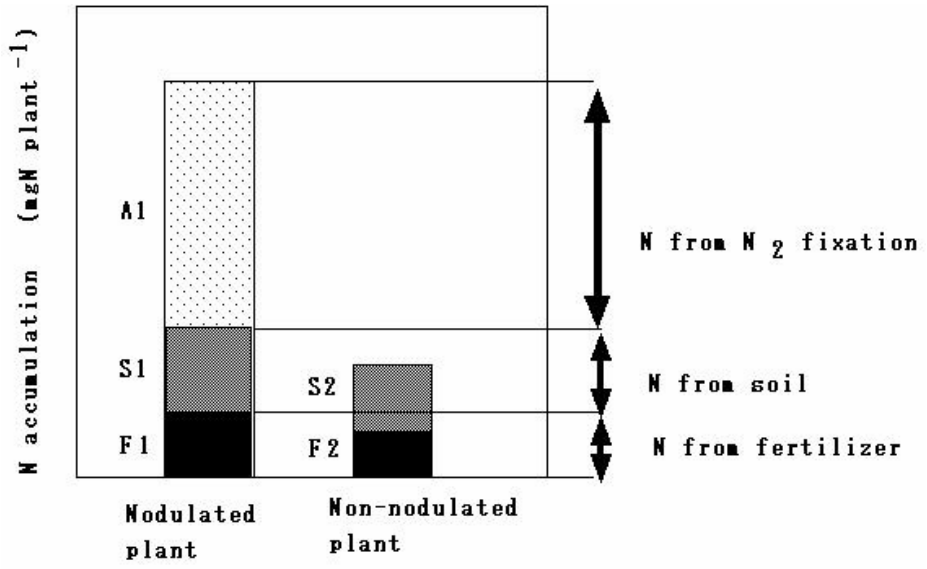

Fig. 5: Concept of ^{15}N dilution method for the evaluation of Ndfa, Ndfs and Ndff

A1; Amount of Ndfa in nodulating plant, S1; Amount of Ndfs in nodulating plant

F1; Amount of Ndff in nodulating plant, S2; Amount of Ndfs in non-nodulating plant

F2; Amount of Ndff in non-nodulating plant

The ^{15}N dilution method provides both yield-independent and time-integrated estimate of % Ndfa, and it is used to evaluate the effect if any treatment on N_2 fixation. ^{15}N labeled fertilizer is applied to nodulating and non-nodulating plants separately. After harvesting at maturing stage just before leaf fall, plants are dried and the DW is measured and N concentration and ^{15}N abundance are determined. Emission spectrometry or mass spectrometry can be used for ^{15}N abundance measurement of the samples. The amount of Ndff is directly calculated from the data of ^{15}N abundance and N content in plant. The ratio Ndff:Ndfs (F2:S2)in non-nodulating plant is calculated and the amount of Ndfs (S1) can be estimated as following equation:

$$S1 = F1 \times S2 / F2$$

Then the amount of Ndfa is calculated in the following equation, where TN indicates total N in nodulating plant:

$$A1 = TN - F1 - S1$$

The percentage of Ndfa, Ndfs and Ndff can be calculated as follows:

$$\% \text{ Ndfa} = 100 \times A1 / TN$$

$$\% \text{ Ndfs} = 100 \times S1 / TN$$

$$\% \text{ Ndff} = 100 \times F1 / TN$$

In this method even if the reference plant cannot develop the same size of the root system, the estimation can be made if both nodulated and reference roots absorb the Ndfs and Ndff at the same ratio. Of course, if the developmental patterns of roots are quite different spatially or timely, both plants will not absorb the same ratio of Ndfs and Ndff. For example, when roots develop faster in the reference plants and absorb more N in earlier period, this reference plants will absorb more basal dressing of N fertilizer than soil mineralized N. Therefore, non-nodulating isoline may be the best selection for the reference plants for ^{15}N dilution method as same as in N-balance method.

It has been widely accepted that ^{15}N dilution method is most accurate estimation for measuring % Ndfa of legumes in a field, but it should be remembered that the results obtained by ^{15}N dilution method may also have some errors. The main weakness of the methodology is the difficulty in establishing a stable and uniform ^{15}N enrichment of soil mineral N in space and time. The differences in patterns of soil mineral N uptake between plant species make it difficult to get a reliable estimate of legume ^{15}N uptake from the soil using a non-N_2 fixing plant of the same or other species (Unkovich and Pate 2000, Peoples and Herridge 1990).

In practice, ^{15}N is usually applied in a square or rectangle microplot that separated the root systems by using plastic or wooden barrier in the field. High application of N fertilizer depresses nodulation, so the amount of N application should be low (1-2 g N/m^2). ^{15}N abundance of samples is analyzed either by GC-MS or optical emission spectrometry. GC-MS analysis is very precise and reliable to the three orders under the decimal point, so low ^{15}N abundance of ^{15}N fertilizer (3-5 atom %) can be used. However, GC-MS is very expensive and need a well-trained technician in its operation. Optical emission spectrometry is more popular and less expensive. However, for optical emission spectrometry the quantitative analysis of ^{15}N abundance of sample should be over 0.5 atom % excess, preferably over 1 atom % (0.63 atom % excess). Therefore, ^{15}N abundance of fertilizer about 10 atom % is normally recommended.

Since the prerequisite for this estimation depends on the equal ratios of Ndff and Ndfs in nodulating and non-nodulating plants, the reference plant should be carefully chosen. The non-nodulating isoline like the T201 is the best. However, if non-nodulating mutants are not available, different species of plants such as upland rice are usually used. The growth pattern of the reference plants until sampling time should be similar to the nodulating plants. In addition, it is desirable that the reference plants develop root system temporarily and specially similar to the nodulating plants, and that for both plants absorb fertilizer N and soil mineralized N in similar manner. The mineral N such as ammonium and nitrate in the soil from fertilizer N and soil N may be unevenly distributed in soil and these can dramatically change during cultivation.

Instead of application of ^{15}N labeled soluble chemical fertilizers such as ammonium sulfate or urea which are relatively easy to be lost by leaching or plant uptake in the early period, ^{15}N labeled manure, soil residues or controlled release N fertilizers such as the 100-day type coated urea will give more reliable result, because the release of N from fertilizer is similar to the soil mineralized N. However, ^{15}N labeled manures or soils are not commercially available, and it is necessary to prepare in advance. The ^{15}N labeled coated urea is also expensive at present.

The A-value method is similar to ^{15}N dilution method except that the reference non-fixing plants receive higher rate of N fertilizer to obtain satisfactory growth (Hardarson et al 1991). However, it was criticized that this method has no advantage over the ^{15}N dilution method.

Example of the ^{15}N dilution method for field soybean

An example of ^{15}N dilution method for assessing N_2 fixation in soybean plants in the field is as follows. Soybean plants are planted in an experimental field at 75 cm row distances and 15 cm planting distances as shown in Fig. 6. Four replication plots for ^{15}N application are made surrounded by plastic board (75 cm x 120 cm) at the depth of 25 cm which prevent the absorption of non-labeled fertilizer outside. ^{15}N labeled fertilizer (e.g. ammonium sulfate 2 g N m^{-2} (5 atom % excess) is applied in the plot. Four nodulating soybean plants and four non-nodulating isolines are planted alternatively in the same row. At R7 stage before leaves fall, plants are harvested to minimize the loss of leaves. Dry weight is measured and the dried plants are ground into a fine powder, and the N concentration and ^{15}N abundance of the sample are determined.

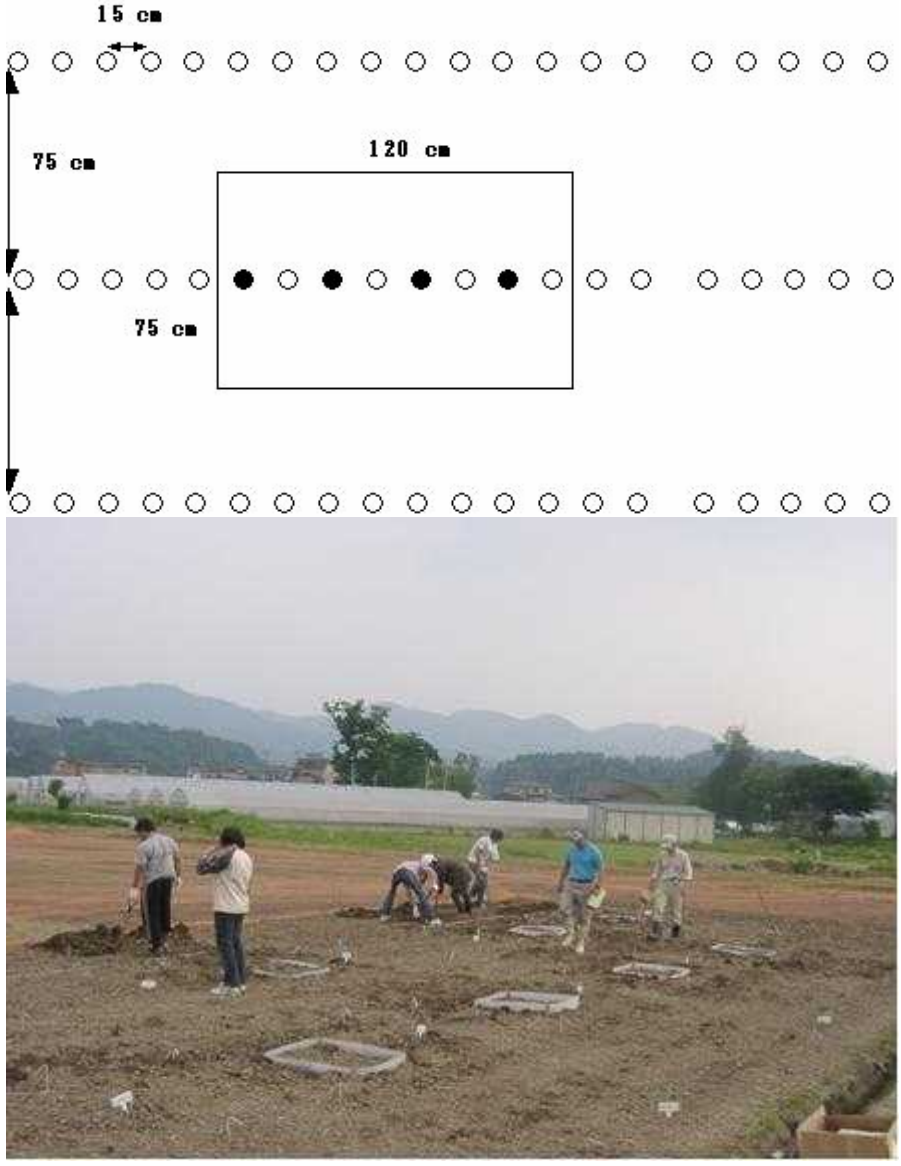

Fig. 6: An example of ^{15}N treatment for soybean experiment in the field

^{15}N labeled N fertilizer such as urea or ammonium sulfate (1-10 atom% ^{15}N) is applied to nodulating leguminous plants which use Ndfa (N derived from atmospheric N_2), Ndfs (N derived from soil) and Ndff (N derived from fertilizer) and its reference plants which use Ndfs and Ndff. If the rate of absorption from soil (Ndfs) and fertilizer (Ndff) are the same between the nodulated plants and the non-nodulated reference, the decrease in ^{15}N abundance in nodulated plants is attributed to Ndfa. This method is most reliable for field estimation of % Ndfa as well as quantitative analysis of the amount of N fixed.

$$\%Ndfa = \left(1 - \frac{^{15}N \text{ atom \% excess of nodulating plant}}{^{15}N \text{ atom \% excess of reference plant}}\right) X\ 100$$

$$\%Ndff = \frac{^{15}N \text{ atom \% excess of nodulating plant}}{^{15}N \text{ atom \% excess of fertilizer}}\ X\ 100$$

% Ndfs = 100 - % Ndfa - % Ndff

Total amount of Ndfa = Total N X % Ndfa / 100
Total amount of Ndff = Total N X % Ndff / 100
Total amount of Ndfs = Total N X % Ndfs / 100

Emission spectrometry analysis of ^{15}N

Dr. Kumazawa introduced emission spectrometric determination of ^{15}N through precise analysis of a trace amount of samples. Dr. Kumazawa and his coworkers applied this method for plant nutrition research. In this method sample is taken into a Pyrex glass tube with oxidizer (CuO) and water and CO_2 absorber (CaO) and evacuated, sealed, followed by heating for converting sample N to N_2 in the tube. Spectroscopic measurement of the ^{15}N abundance is based on the measurement of the intensity of the emission spectra of $^{14}N^{14}N$, $^{14}N^{15}N$ and $^{15}N^{15}N$ molecules by scanning from the wavelength from 299 nm to 297 nm. ^{15}N labeled compounds are available after condensation of ^{15}N by the exchange reaction between nitric acid and the oxides of nitrogen.

Fig. 7: Photograph of ^{15}N analyzer by emission spectrometry (JASCO Ltd N-150 analyzer)

The advantages of emission spectrometry for ^{15}N measurement as compared with mass spectrometry are as follows:

1) The amount of N required for the anaysis is only 1-2μg N. This is equivalent to about 0.1mg of dry

matter, or one spot of amino acid separated by two-dimesional thin layer chromatography (Ohyama and Kumazawa 1978b).

2) Low running cost and easy maintenance of emission spectrometry equipment as compared to mass spectrometry.

The disadvantages of this method are as follows:

1) Less precision in low ^{15}N abundance samples. For quantitative analysis, the data lower than 0.1 atom % excess is not reliable. Emission spectrometry cannot be used for determination of natural abundance of ^{15}N ($\delta^{15}N$).

2) The preparation of discharge tubes needs some practice and time consuming.

The authors analyzed the Kjeldahl digested solution directly put in a Pyrex tube. Please see the details of the method in Appendix 1.

Mass spectirometry analysis of ^{15}N (Manual FAO/IAEA)

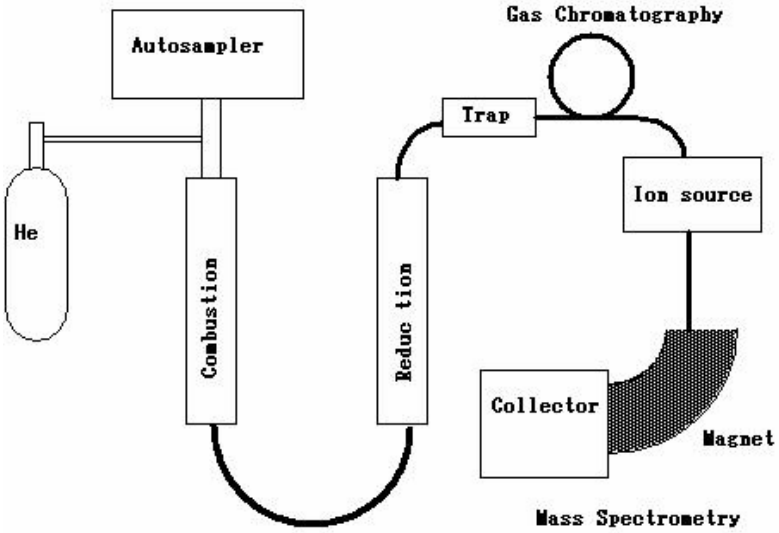

Fig. 8: Schematic diagram of an isotope ratio mass spectrometer combined with sample combustion system (FAO/IAEA)

Several mg of dried sample powder is weighed and put into an small tin cup. The tin cup is put into an autosampler. After purging the sample with He gas, the sample in tin cup drops into the hot combusion tube heated at 1000℃ filled with Cr_2O_3 as an oxidation catalyst. The combustion gases (CO_2, H_2O, N_2, SO_2 and N-oxides) are moved with a He carrier gas to the reduction tube at 600℃ which is filled with metallic Cu wires. The N-oxides are reduced to N_2 and the access of O_2 reacts with the hot Cu forming CuO. A trap filled with Mg-perchlorate removes the water out of the gas stream. CO_2 is absorbed by a chemical trap of Carbosorb. The gases are then separated by gas chromatography column and introduced to the mass spectrometer by a continuous flow interface. Mass spectrometry (MS) is an analytical technique in which atoms or molecules from a sample are ionized, separated according to their mass-to-charge ratio (m/z); the counts by different m/z are then recorded.

1.1.5. ^{15}N Natural Abundance Method ($\delta\ ^{15}N$ method)

In all the biological and non-biological materials including atmospheric N_2, the ^{15}N abundance is almost the same, i.e. about 0.37 atom %. However, the precise analysis of ^{15}N abundance by GC-MS analysis revealed that ^{15}N abundance is different among materials due to discrimination of ^{14}N and ^{15}N through some physical, chemical and biological processes. The ^{15}N abundance of atmospheric N_2 (0.3663 atom %) is used for standard and the difference of ^{15}N abundance is expressed as $\delta\ ^{15}N$ instead of ^{15}N atom % excess. It has been shown that ^{15}N abundance of soil mineral N is slightly higher (e.g. 0.368-0.373 atom %) than atmospheric N_2.

$$\delta\ ^{15}N = \frac{^{15}N \text{ atom \% of sample} - {}^{15}N \text{ atom \% of standard}}{^{15}N \text{ atom \% of standard}} \times 1000\ (‰)$$

$\delta\ ^{15}N$ derived from nitrogen fixation is 0 ‰

$\delta\ ^{15}N$ derived from fertilizer N is slightly lower between -5 and 0 ‰

$\delta\ ^{15}N$ derived from soil organic matter is higher between 5 and 15 ‰

$$\%Ndfa = \frac{\delta\ ^{15}N \text{ of reference plant} - \delta\ ^{15}N \text{ of nitrogen fixing plant}}{\delta\ ^{15}N \text{ of reference plant} - B}$$

where B indicates $\delta^{15}N$ value of nitrogen fixing plant totally dependent on atmospheric N_2.

^{15}N natural abundance method is usually used for initial survey of nitrogen fixing plants in natural habitat, and not for field estimation of nitrogen fixation. Isotopic fractionation during N_2 fixation is minimal but not zero and should be taken into account (Peoples and Herridge 1990). Therefore, the value of B should ideally be prepared for each new legume species studied. The use of the appropriate B value (e.g. in soybean it is –1.30 ‰ when analyzing only shoots, or a value of –0.79‰ if whole plants are harvested (Bergersen et al., 1989). There can be dynamic changes in $\delta\ ^{15}N$ of plant parts during organ development, so estimates of % Ndfa should be based on $\delta\ ^{15}N$ of whole plants or total shoot N and not of single leaves or individual plant parts (Peoples and Herridge 1990). There appeared to be no evidence for significant rhizobial strain-induced changes of $\delta\ ^{15}N$ values and B values in tropical legumes.

For sample collection and preparation attention should be given (1) to avoid contamination with ^{15}N enriched materials, (2) to prepare uniform dry matter samples to avoid variation due to tissue differences in ^{15}N abundance, and (3) to avoid losses of minute quantities of nitrogen during Kjeldahl digestion and distillation, or during concentration of distillates before analysis on the mass spectrometer (Peoples and Herridge 1990). The accuracy of the technique will ultimately depend on the levels of natural ^{15}N abundance of the soil. Low and /or variable soil d ^{15}N values will be unsuitable for assessing N_2 fixation.

1.1.6. $^{15}N_2$ fixation activity

The $^{15}N_2$ gas feeding method is the most direct method to quantify N_2 fixation activity and is very

useful in the study of N_2 fixation and N metabolism in the laboratory (Ohyama and Kumazawa 1978ab, 1979, 1980abc, 1981ab). Fig. 9 and Fig.10 show the ^{15}N feeding apparatus for soybean and sugarcane, respectively. Usually $^{15}N_2$ gas is mixed with O_2 and inert gas such as Ar by an apparatus as shown in Fig 9.

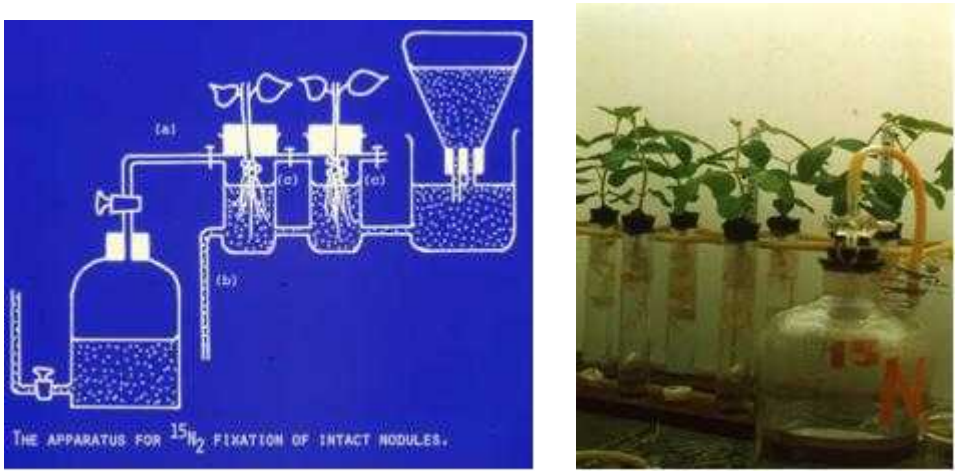

Fig. 9: Apparatus for $^{15}N_2$ fixation of intact soybean plants

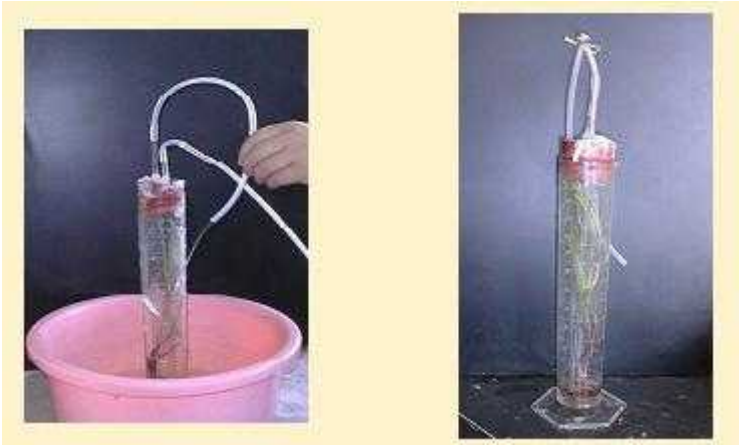

Fig. 10: Apparatus for $^{15}N_2$ fixation for sugarcane plants

Fig 11: Gas mixing apparatus for $^{15}N_2$ fixation studies

Although $^{15}N_2$ feeding experiment gives direct and most reliable evidence for N_2 fixation in any

N_2 fixing organisms, it is relatively difficult to estimate quantitative estimation due to the difficulty in maintenance of the labeled air composition including O_2, CO_2 and ^{15}N abundance for a long period. The use of $^{15}N_2$ in the field is also difficult as it involves high cost. Akao et al (1983) fed $^{15}N_2$ on soybean plants grown in a 1/2000 pot with soil for one or two weeks while maintaining O_2 concentration; and the % Ndfa estimated was 70.1 %.

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1.1.7. Appendix: Direct Analysis of ^{15}N Abundance of Kjeldahl Digested Solution by Emission Spectrometry (Bull. Facul. Agric. Niigata Univ. 57(1) 33-40, 2004)

ABSTRACT: Direct analysis of ^{15}N abundance of Kjeldahl digested solution was established. Plant sample is digested by Kjeldahl digestion method using H_2SO_4 and H_2O_2, and an aliquot of digested solution including ammonium nitrogen (about 2μgN) is directly taken into a Pyrex glass tube with the outer diameter 4mm. After water in the Pyrex tubes is evaporated, the glass tubes are evacuated by the vacuum system for discharge tube preparation and sealed with oxidation reagent (CuO) and water absorbent (heated CaO at 950℃). The sealed tubes are heated at 560℃ for 30 min in a muffle furnace to oxidize ammonium into N_2 gas. The ^{15}N abundance can be measured by emission spectrometry (JASCO N-150 analyzer). This procedure is the simplest and accurate method for the determination of ^{15}N abundance of total N in the samples.

Key words: ^{15}N, Emission spectrometry, Kjeldahl digestion

INTRODUCTION

^{15}N is a stable isotope and its natural abundance is 0.366 % (99.634 % ^{14}N), although there are small variations due to isotope discrimination by some physical, chemical and biological processes. Recently precise mass spectrometric analysis is used for measuring the natural abundance ($\delta^{15}N$) of biological materials for estimating the origin of N such as N_2 fixation, organic materials or chemical fertilizers.

Nitrogen is one of the most important plant macronutrient, and the studies on the fate of N fertilizer applied are very important. Also, the absorption, transportation and metabolism of nitrogen in plant are of a major interest in both fields of plant nutrition and physiology. Dr. Kumazawa introduced emission spectrometric determination of ^{15}N by revising the precise analysis of a trace amount of samples and he applied this method for plant nutrition researches [1-4]. In this method sample is taken into a Pyrex glass tube with oxidizer (CuO) and water and CO_2 absorber (CaO) and evacuated, sealed followed by heating for converting sample N to N_2 in the tube. Spectroscopic measurement of the ^{15}N abundance is based on the measurement of the intensity of the emission spectra of $^{14}N^{14}N$, $^{14}N^{15}N$ and $^{15}N^{15}N$ molecules by scanning from the wavelength from 299 nm to 297 nm. ^{15}N labeled compounds are available after condensation of ^{15}N by the exchange reaction between nitric acid and the oxides of nitrogen [5].

The advantages of emission spectrometry for ^{15}N measurement compared with mass spectrometry are as follows:

1. The amount of N required for the analysis is only 1-2μgN. This is equivalent to about 0.1mg of dry matter, or one spot of amino acid separated by two-dimensional thin layer chromatography [1,8].
2. Low running cost and easy maintenance of the equipment compared with mass spectrometry.

The disadvantages of this method are as follows:

1) Less precision in low ^{15}N abundance samples. For quantitative analysis, the data lower than 0.1 atom % excess is not reliable [6]. Emission spectrometry cannot be used for determination of natural

abundance of ^{15}N ($\delta^{15}N$).

2) The preparation of discharge tubes needs some practice and time consuming.

For the ^{15}N analysis of the total N in plant materials, a Pyrex glass tube with 8 mm diameter was originally used for making discharge tube by Dumas method[1]. Several mg of dry plant powder is taken into a 8 mm outer diameter Pyrex tube and air inside is evacuated and sealed with 0.5g of CuO and 1 g of CaO, and heated at about 560℃ for 2-6 hr for complete combustion of plant powder. This method needs skillful glass works and it is time consuming.

The alternative is Rittenberg method, in which ammonium sulfate solution by Kjeldahl digestion is converted to N_2 by the addition of NaOBr solution[1]. Relatively a larger amount of N (eg. 50-100μgN) is necessary for this analysis, and this method is also time consuming.

The condensation of ammonium in HCl solution was used for ^{15}N analysis, followed by diffusion from Kjeldahl digested solution by the addition of 10 M NaOH. The condensed solution was taken into a small capillary tube and dried under an infrared lamp[1].

The authors tried to use the Kjeldahl digested solution directly taking into a Pyrex glass tube with 4 mm outer diameter for discharge tube preparation. Although an aliquot of sulfuric acid remains in the bottom of the tube after evacuation, no interference occurred for emission and measurement due to the small amount of the existed sulfuric acid. So far, it is the easiest way to determine ^{15}N abundance of the total N in plant materials and other sources, such as animals or environmental materials.

MATERIALS AND METHODS

Sample preparation and grinding

Plant samples are thoroughly washed with tap water, then rinsed with de-ionized water and blotted by a paper towel. Plant samples or separated parts are packed in an envelope and dried in ventilation oven at 60-80℃ for several days until the dry weight becomes constant.

Dry plant materials are ground into a fine powder with special care not to cross contaminate. Special care should be paid not to cross contaminate (not to mix) the samples during grinding. The grinder should be cleaned up completely for each sample by washing or wiping the container. The amount of N in a tube for ^{15}N analysis is very low and a trace amount of N contamination will give a significant error. So care should be taken not to contaminate N from equipment, water, reagent, air, dust, hands or other environmental materials.

Kjeldahl digestion

Fig. 12 shows the outline of Kjeldahl digestion using H_2SO_4 and H_2O_2 for plant samples containing low nitrate (Fig. 12. left: NO_3-N is less than 5% of total N) and high nitrate (Fig.12. right) [7]. The concentration of NO_3-N in plant powder can be analyzed by Cataldo's method followed by hot water extraction[8,9]. About 50mg of fine dry powder with low nitrate, such as in seeds were put into a test tube (approximately 20 mm in outer diameter and 20 cm in tall). Then 1 mL of H_2SO_4 (precise analytical grade with least ammonium contamination) is mixed, and kept over night with a parafilm lid on it to protect

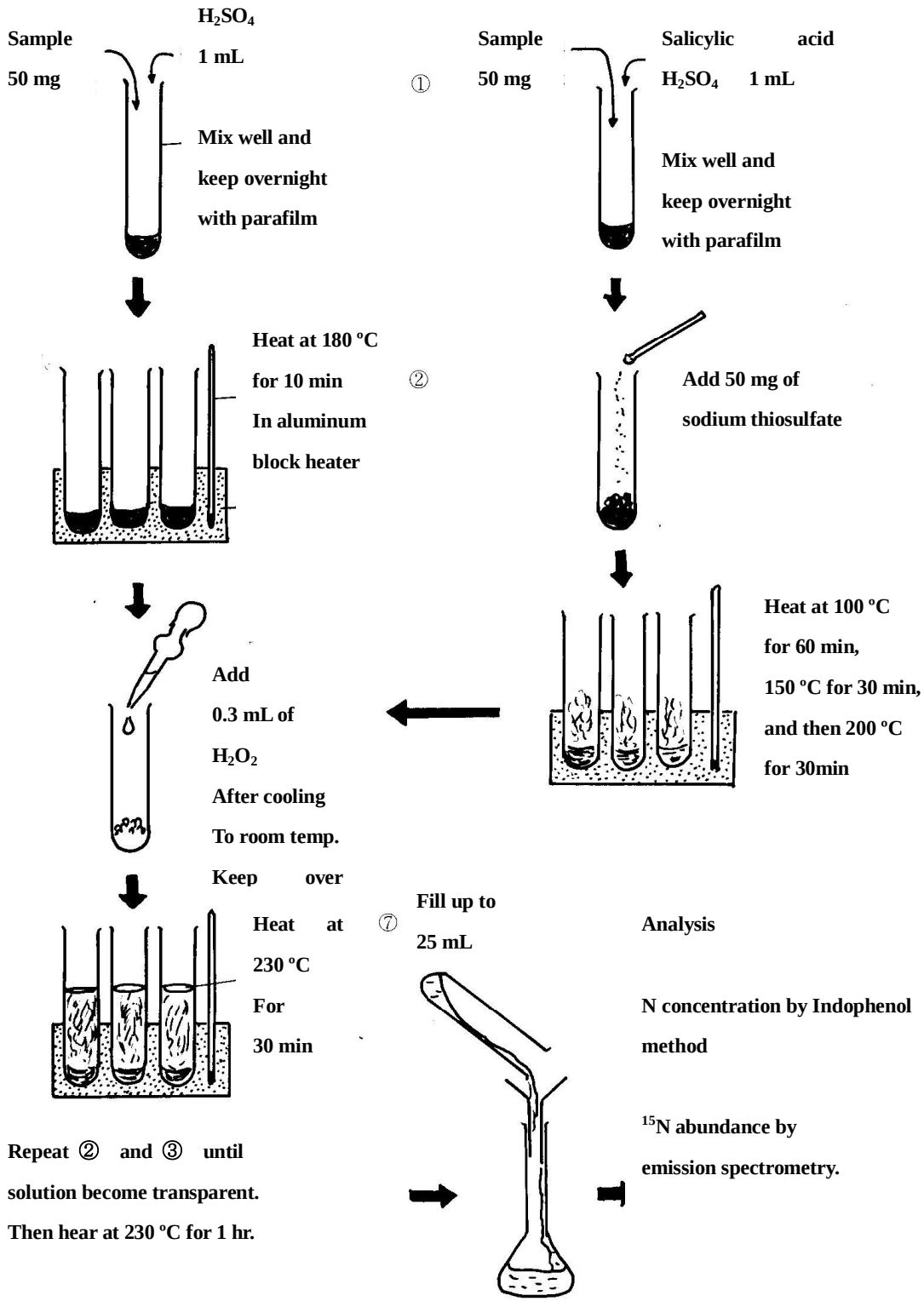


Fig. 12: Kjeldahl digestion of plant samples with low concentration of nitrate (left) and high concentration of nitrate (right)

ammonia or other N contaminant from the outside air and dust. Test tubes are set in an aluminum block and plant materials are digested at 180℃ for 10 min. Then the test tubes are taken out from the block and they

are kept at room temperature for 5 min for cooling. Then 0.3 mL of 30 % H_2O_2 are added to the test tubes. Vigorous agitation should be avoided due to insufficient cooling. The test tubes are heated at 230℃ for 30 min. Then 0.3 mL of 30 % H_2O_2 is added again. The heating and adding H_2O_2 for are repeated for several times until the solution becomes clear and transparent. Then the test tubes are heated at 230℃ for 60 min in order to degrade H_2O_2 completely. When H_2O_2 remains in the digested solution, it will interfere the Indophenol colorimetry for ammonium determination. The digested solution is filled up in a 25 mL volumetric flask.

In the case of plant materials that contain high levels of nitrate (NO_3^--N over 5% of total N) such as roots and stems, nitrate in the plant materials should be reduced to ammonium with reducing reagents (salicylic acid and sodium thiosulfate) under mild temperature conditions, unless nitrate will volatile from the acidic solution. 1 mL of salicylic-sulfuric acid (10 g of salicylic acid was dissolved in 300 mL of H_2SO_4) is used instead of H_2SO_4. Just before heating, about 50 mg of sodium thiosulfate is added. The temperature should be increased slowly, for example, 100℃ for 60 min, 150℃ for 30 min and up to 200℃ for 30 min, nitrate was then completely recovered[7]. Then the tubes are taken out to cool for 5 min, and 0.3 mL of H_2O_2 is added as same as mentioned before.

Determination of N concentration by indophenol method

Reagent

EDTA solution: Dissolve 25 g of EDTA (ethylenediamine-*NNN'N'*-tetraacetic acid)・2Na in about 800 mL of water. Adjust pH at 10 using 10 M NaOH solution. Add 20 mL of 0.25 % methyl red in 60 % ethanol solution as color indicator. Fill up to 1 L with water.

P-buffer (1 M of potassium phosphate buffer): Dissolve 136.09 g of KH_2PO_4 and 2.75 g of benzoic acid in 1 L of water.

Nitroprusside reagent: Prepare liquid phenol. Dissolve 500 g of phenol in water bath and add 47mL of water. (Stable for a half year under room temperature). Dissolve 10.25 mL of liquid phenol and 100 mg of sodium nitroprusside in 1 L of water. (Stable for 2 weeks in a refrigerator)

Hypochlorite solution: Dissolve 10 mL of sodium hypochlorite solution (available Cl 5%<), 10 g of NaOH, 7.06 g of Na_2HPO_4 ・$7H_2O$ and 31.8 g of $Na_3PO_4.12H_2O$ in 1 L of water.

1M NaOH: Dissolve 40 g of NaOH in 1 L of water.

Ammonium standard solution (100 μgN mL^{-1}): Dissolve 471.1 mg of ammonium sulfate in 1 L of 0.25 M sulfuric acid (stock solution). Dilute 2.5 mL of stock solution into 50 mL. Put 0, 0.5, 1.0, 1.5, 2.0 mL of the diluted solution (5μgN mL^{-1}) in 25 mL volumetric flask for calibration standard.

Procedure

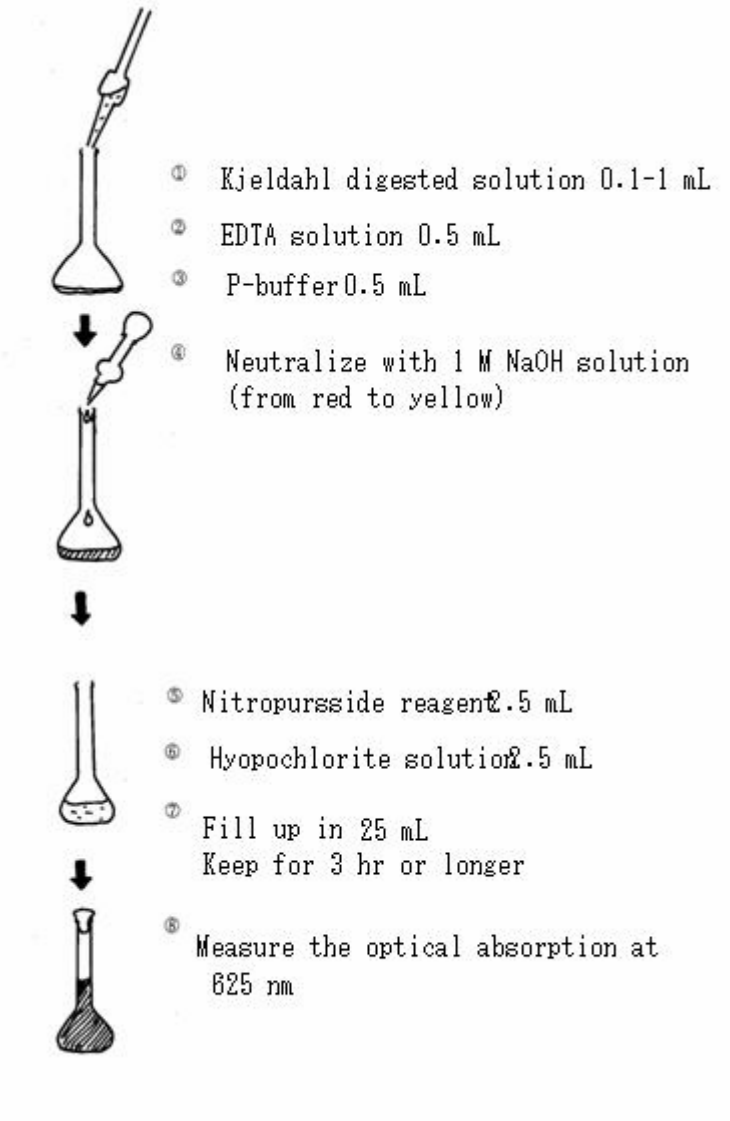


Fig. 13 shows the procedure for determination of the concentration of ammonium in Kjeldahl digested solution as mentioned above. 0.1-1 mL of digested solution is put into a 25 mL volumetric flask. The volume depends on the ammonium concentration estimated. 0.5 mL of EDTA solution and 0.5 mL of P-buffer are added to the flask in this sequence. The solution is neutralized with 1 M NaOH by the indication of methyl red color reagent changed from red to yellow, then 2.5 mL of nitroprusside reagent is added immediately and stirred well. Then 2.5 mL of hypochlorite solution is added, the flask is filled up to 25 mL with water. The volumetric flasks are kept at room temperature or at 30℃ incubator for 3 hr or longer. The optical absorption at 625 nm is measured. The ammonium concentration can be calculated from the calibration line with ammonium standard solution.

Fig. 13: Indophenol method for determination of ammonium concentration in the Kjeldahl digested solution

Determination of ^{15}N abundance of Kjeldahl digested solution by emission spectrometry

Preparation

Pyrex glass tubes: Pyrex glass tubes which have an outer diameter of 4mm and an inner diameter of 2mm are cut to 30 cm long pieces and submerged in a detergent solution overnight. The tubes are thoroughly washed with tap water, rinsed with de-ionized water, and dried in an oven. The center of the tube was melted and cut off by an oxygen burner to make two pieces of 15 cm long tubes one end of which is closed. About fifty tubes are wrapped with aluminum foil, and heated at 560℃ for 3 hr in a muffle furnace to clean them up. Tubes in the aluminum foil can be stored in a plastic bag keeping them away from adsorption of contaminated nitrogen such as ammonia from air or dirt.

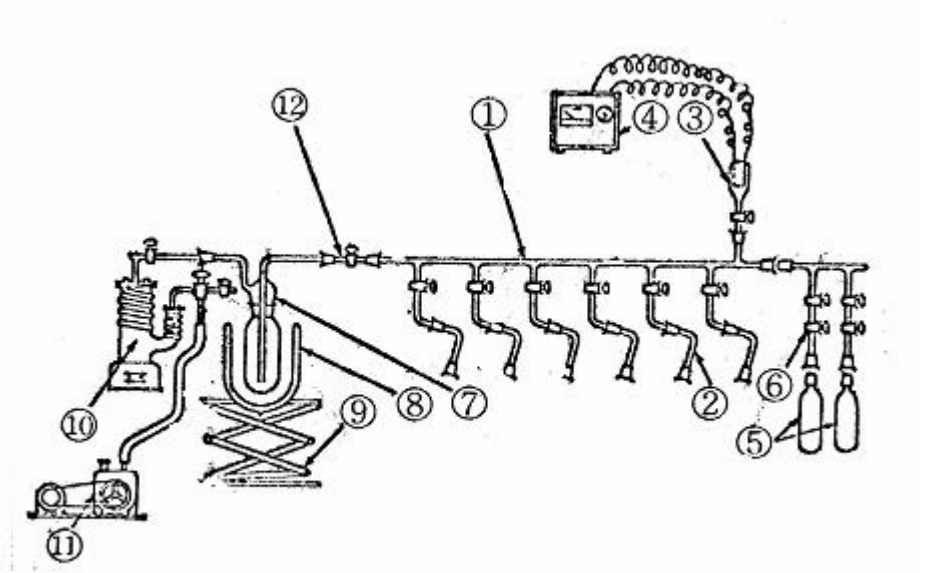
CaO reagent: Grind the calcium oxide blocks into a fine powder with mortar and pestle. Make a tablet (2 cm diameter and 1 mm thick) of CaO by pressing under the pressure at 4 ton cm^{-2}. Cut the tablet to 1 mm width sticks by a cutter knife. Heat the cut sticks in a crucible at 950℃ for 3hr in a muffle furnace to remove water and CO_2 and make them free from nitrogen contamination. CaO reagent should be kept at 950℃ furnace during preparation of the discharge tube.

CuO reagent: Wire type CuO reagent (about 3mm in long and 0.5mm in diameter) in a crucible and heated at 560℃ for 3 hr to make it free from nitrogen contamination. Then it can be stored in a glass bottle with airtight cap.

Fig. 14: Evaporation of water in Kjeldahl digested solution in Pyrex glass tubes by round bottom flask with short neck

Fig. 15: Vacuum system for preparation of discharge tubes devised by Dr. Kumazawa

① glass tube ② attachment for tubes ③ vacuum gauge ④ vacuum meter
⑤ bottles for He and Ar ⑥ cocks ⑦ liquid nitrogen trap ⑧ liquid nitrogen container ⑨ lift
⑩ oil diffusion pump ⑪ vacuum pump ⑫ cock

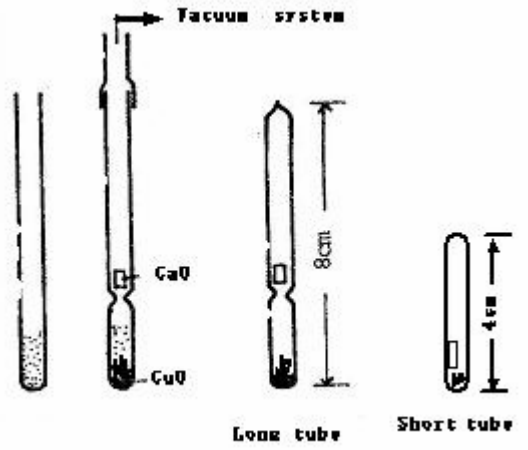


Procedure for making discharge tube

After N concentration was determined by indophenol method as mentioned before, an aliquot (usually 10-100μL depending on the N concentration) of Kjeldahl digested solution, which contains about 2 μgN, is put into a clean Pyrex glass tube with 15 cm long. The volume of the sample solution should not exceed 100 μL. Duplicated analysis is necessary for each solution, because ^{15}N abundance is often decreased by trace amount of N contamination. Therefore, when the difference of ^{15}N abundance (calculated ^{15}N atom %) between duplicated tubes is over 10% of the average ^{15}N abundance, the data should be omitted and reanalyzed. Sample letter or number should be recorded by white marker pen on the lower part

of the tubes, and the white letter remains after heating even at 560℃. A range of pressure at 2-6 Torr (270-800 Pa, about 1 to 3 μgN in a 4mm tube) is appropriate for discharge. When N is lower than 0.5 μg, a discharge emission is very weak and easy to fade out. On the other hand, when the N is over loaded in the tube, no discharge occurs at all. The use of the mixed gases of He and Xe is beneficial for extending the range of N amount in the discharge tube by preventing N_2 gas adsorption on the glass wall[1)], however, it is time consuming to make sure the purity of the mixed gases at each preparation time. Therefore, we do not use the mixed gases. Instead, when the N content is very low, we make a short discharge tube at 4 cm in long. When N concentration in the solution appeared to be low, the volume of fill up after digestion must be made to 10 mL instead of 25 mL (Fig. 12).

Fig. 16: Discharge tube preparation for long and short tubes about 4 cm in length



The Pyrex tubes with sample solution are bound with rubber band put into a 1 L round bottom flask with short neck and evaporated as shown in Fig.14. After evaporation about several hrs, most of the water will be transpired, but small drops of H_2SO_4 remains in the bottom of the tubes. These tubes are stored in a round bottle flask with tight lid, or some airtight container to prevent ammonium absorption in H_2SO_4 from air.

After addition of several wires of CuO in the tubes, the hot CaO sticks preheated at 950℃ for 3hr are put in the middle of the tubes. In total about 1cm long of CaO sticks are enough for each tube. The edge of the tube is heated with oxygen burner to smooth the collar of the edge not to damage the polyvinylacetate or silicon attachment tubes. Then CaO sticks are dropped down to the bottom, and the Pyrex tubes are attached to the attachment tubes of vacuum system with small amount of vacuum grease for making discharge tubes (Fig. 15). We do not use liquid N_2 trap, because neither water nor CO_2 was trapped in the liquid nitrogen trap (Fig. 15⑦) during 4 mm tube preparation. We make tubes under continuous vacuum conditions without closing the system. After evacuation of the air inside the tubes, the part of the outside tubes up to about 2cm-10 cm from the end are heated gently by oxygen gas burner from down up direction to remove the adsorbed water in the glass wall. About 2 cm long from the bottom of the Pyrex tube should not be heated, because sample N will be converted to N_2 and lost. After the pressure inside the discharge tubes reaches below 10^{-3} Torr (mg Hg), the tubes are cut off and sealed by oxygen gas burner, for about 4 cm (short tube for low N) to 8 cm (long tube for high N) from the bottom (Fig. 16).

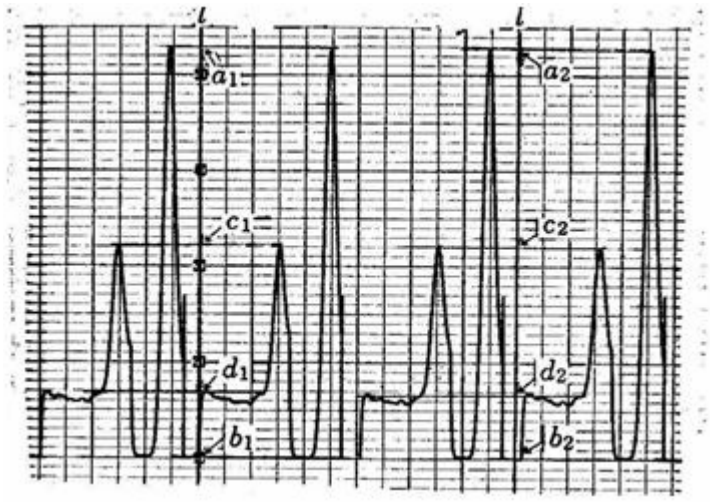
The tubes are wrapped in aluminum foil and heated at 560℃ for 30 min in a muffle furnace. In this step NH_4^+ in the tube is oxidized to N_2 and H_2O by CaO wire. After leaving overnight to ensure the absorption of water and impurities into CaO reagent, ^{15}N measurement can be done.

Fig. 17: Photograph of emission by JASCO N-150 analyzer

The position of the tube holder attachment was changed from the original position.

To obtain good emission, we use aluminum foil cap at the top of the discharge tube while measuring as shown in Fig.6. At least four times stable scans peaks were collected for each tube and the height of the $^{28}N_2$ (I_{28}) and $^{29}N_2$ (I_{29}) peaks are measured as shown in Fig. 18.

Fig.18: Measurement of the chart of ^{15}N emission spectrometry

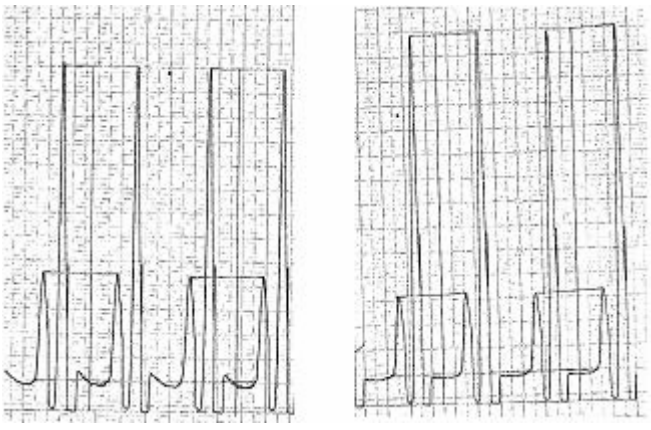


Four stable scans are recorded and the peak height of a pair of peaks are measured.

I_{28}=a-b, and I_{29}=c-d where I_{28} and I_{29} indicate peak intensity of $^{28}N_2$ and $^{29}N_2$, respectively. The average value of ^{15}N atom % calculated from two pair of peaks is calibrated to obtain theoretical ^{15}N atom % and ^{15}N atom % excess.

Fig. 19: Examples of the charts of ^{15}N emission spectrometry of discharge tubes prepared from Kjeldahl digested solution

Soybean roots (①left) and shoots (②right) treated with ^{15}N labeled urea in the field. Magnification factor R=16.



The emitted light should not touch the CaO reagent, because it will make base line high due to gas emission.

^{15}N abundance can be calculated as following equation:

$$^{15}N \text{ (atom \%)} = 100 / [2R(I_{28}/I_{29})+1]$$

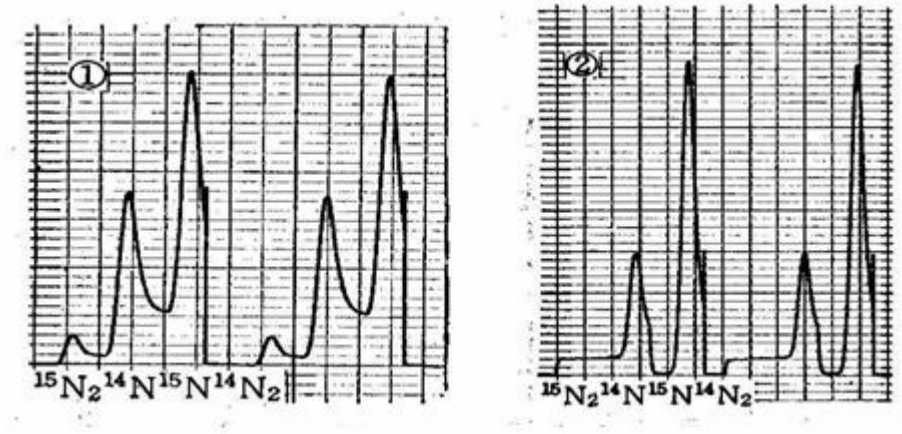
where, I_{28} and I_{29} are the peak height of $^{28}N_2$ and $^{29}N_2$ as shown in Fig. 7.

R is the magnification factor of I_{29}. One of the numbers of 32, 16, 8, 4, 2 or 1 can be selected for the

magnification factor R in N-150 analyzer.

The ^{15}N abundance obtained by this calculation is shifted from true value, therefore, a calibration curve should be made by using ^{15}N standard. The calibration curve is almost linear in the range of ^{15}N atom % of 1.0 - 15.0, but depart from linearity at higher atom %, [1)]. The ^{15}N abundance calculated in the above equation should be calibrated for fitting this curve to obtain the correct (theoretical) atom %. Atom % excess is calculated from theoretical atom % minus natural abundance of ^{15}N (0.366 atom %).

Fig. 20: The charts of ^{15}N emission spectrometory of discharge tubes with high and low ^{15}N abundance



27.3 atom % excess (① left) and 0.08 atom % excess (② right).

We use a series of linear calibration lines for correction as follows. The atom % calculated was separated into five equations due to the calculated atom % (C). In these equations, natural abundance of ^{15}N (0.366 atom %) is deduced to get theoretical ^{15}N abundance (T) in atom % excess.

① If C (atom %) <2.9, T (atom % excess) = 0.9921 x C – 0.490

② If 2.9＜C (atom %) <3.5, T (atom % excess) = 0.9714 x C – 0.430

③ If 3.5＜C (atom %) <18, T (atom % excess) = 1.050 x C – 0.705

④ If 18＜C (atom %) <28, T (atom % excess) = 1.175 x C – 2.916

⑤ If 28＜C (atom %) <35, T (atom % excess) = 1.440 x C – 10.492

⑥ If 35＜C (atom %) T (atom % excess) can be obtained from a calibration curve.

RESULTS AND DISCUSSION

Chart of ^{15}N measurement by this method

Fig. 19 shows the examples of the chart of ^{15}N measurement by discharge tubes prepared from Kjeldahl digested solution of soybean roots (Fig. 19. left) and shoots (Fig.19. right) treated with ^{15}N labeled urea cultivated in the Nagaoka field in 2002. The discharge was stable and the back ground did not increase or disturb as shown in Fig. 20, although some sulfuric acid remained in the tube.

The appearance of peaks $^{14}N_2$, $^{14}N^{15}N$, and $^{15}N_2$ are different depending on the ^{15}N abundance. When ^{15}N concentration is high at 27.3 atom % excess, $^{15}N_2$ ($^{15}N^{15}N$:298.9 nm) peak can be seen with $^{15}N^{14}N$ (298.3 nm) and $^{14}N_2$ ($^{14}N^{14}N$:297.7 nm) peaks (Fig. 20 left). When ^{15}N abundance is low (0.08 atom % excess), $^{15}N_2$ peak is not detectable and $^{15}N^{14}N$ peak need to be multiplied (Magnification factor: R= 16) as shown in Fig. 20 right.

Usually peak height of $^{15}N^{14}N$ and $^{14}N_2$ ($^{14}N^{14}N$) are measured as shown in Fig. 18. The background of $^{14}N_2$ is baseline of the chart, however, the background of $^{15}N^{14}N$ is the front of the peak as shown by "d" in Fig. 18. The impurity gases such as CO (297.6 nm), H_2O (298.0 nm, 298.7 nm), O_2 and CO_2 (297.7 nm) increase and disturb the back ground level of $^{15}N^{14}N$. The height of background of $^{15}N^{14}N$ (d-b) should be lower than the peak height (c-d).

Calculation of N derived from labeled N and fertilizer efficiency

The percentage of N derived from labeled N can be calculated by the equation as follows:

100 x atom % excess of the sample / atom % excess of the labeled source.

For example, 10 atom % of ammonium sulfate (5 g N for one plant) is applied as a fertilizer. Then the ^{15}N abundance and total N of the harvested plant were 3 atom % and 4 gN, respectively.

The percentage of N derived from labeled N is 100 x 2.634 atom % excess / 9.634 atom % excess = 27.3 %. The amount of N from labeled source can be calculated by total N x %N from labeled N / 100. In this example the amount of N from labeled source is 27.3 x 4 / 100 =1.092 g. Therefore the fertilizer efficiency can be calculated by the equation that 100 x the amount of N from labeled source / amount of the applied N.

In this example: 100 x 1.092 / 5 = 22 (%)

Application to plant nutrition studies

We have used the direct analysis of Kjeldahl digested solution for ^{15}N emission spectrometry for studies on soybean, narcissus and tulip [9-11].

Sato et al. (1999) [9] reported the nitrate absorption and transport in non-nodulated and nodulated soybean plants with $^{13}NO_3^-$ and $^{15}NO_3^-$. The nodulated and non-nodulated soybean isolines were hydroponically cultivated, and radioisotope labeled $^{13}NO_3^-$ or stable isotope labeled $^{15}NO_3^-$ was added to the culture solution. The accumulation pattern of absorbed ^{13}N was observed by positron emitting tracer imaging system (PETIS) as well as bioimaging analyzer (BAS). The ^{15}N abundance of the 80 % ethanol soluble and insoluble fractions of plant part was digested by Kjeldahl digestion and digested solution was prepared for emission spectrometry in the method described in this paper. In the case of 80 % ethanol extract, 1 mL of extract was taken to the test tube and dried by heating in the aluminum block; then sulfuric acid or salicylic-sulfuric acid was added as shown in Fig. 1. The residue of the 80 % ethanol extract was dried in the oven and ground to a fine powder again, then 50 mg of powder was put in the test tube and digested with sulfuric acid.

Ruamrungsri et al (2000) [10] reported the ammonium and nitrate assimilation in daffodil (*Narcissus*) roots using ^{15}N labeled ammonium and nitrate. Ammonium was more rapidly absorbed in the roots than nitrate in 2 days after ^{15}N feeding. However, at 4-7 days after feeding, the amounts of N absorbed from ammonium and nitrate were almost equal.

Komiyama et al (2003) [11] reported the site of nitrogen accumulation in tulip roots during winter. Tulip plants absorb and accumulate N in the roots during winter. ^{15}N labeled nitrate and ammonium were supplied in a vertical split-root system (upper part of roots and lower part of roots). From the results obtained, tulip roots could absorb ammonium and nitrate either from the upper roots or from the lower roots and accumulate N in the absorption sites.

From the experiences of the above studies, we are convinced that the direct analysis of Kehldahl digested solution is the simplest and accurate method in which the chance of N contamination is least compared with other methods.

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1.2. P-solubilizing Activity

^{32}P and ^{33}P are two radioisotopes suitable for agronomic studies. The main characteristics of P isotopes used in plant nutrition studies can be summarised as follows:

Isotopes	Half-life (days)	Radiation characteristics		Typical application
		Type	Energy	
^{32}P	14.3	ß-	1.71 MeV	Exchangeable P in soil P avaiability from P fertilizer Plant root distribution Residual P fertilizer avaiability
^{33}P	24.4		0.248 MeV	Auto-radiography Diffusion in soils Double labeling with ^{32}P

The isotopic phosphorus composition (ration ^{32}P/total P) of any material is called specific activity (S.A). The determination of S.A. of sample requires 2 independent measurements:

1. Determination of activity of radioisotope by radioassay technique using appropriate detector (proportional detector, Geiger-Mueller detector, liquid scintillation counting, Cerenkov counting or sodium-iodide scintillation detector).
2. Determination of total nutrient content by any conventional chemical method, e.g. spectrophotometric method. ^{32}P in general is used to study the utilization of P fertilizer by crops in greenhouse and field experiments.

The fraction of P in plant material derived from ^{32}P or ^{33}P labelled fertilizer is termed Pdff. From isotope dilution principle Pdff = S.A. plant sample/S.A.labelled fertilizer or % Pdff = (S.A. plant sample/S.A. labelled fertilizer) x 100. Both the activity and total P content in the plant and fertilizer must be determined to measure Pdff.

Details of the method can be see in Zapata F. and Axmann H (1995): ^{32}P isotopic techniques for evaluating the agronomic effectiveness of rock phosphate material; Zapata F, Axmann H, Braun H (1986): Agronomic evaluation of rock phosphate material by means of radioisotope techniques; Zapata F (1990): Isotopic technique in soil fertilizer and plant nutrition studies and "Use of Isotope and Radiation methods in Soil and Water Management and crop Nutrition" (FAO/IAEA 2001)

To evaluate the P-solubilizing capability of microorganism, the liquid Pikovskaia medium is prepared, in which instead of $Ca_3(PO_4)_2$, $Ca_3(^{32}PO_4)_2$ is used. Tested microorganism will be grown in the medium under suitable condition for 7 to 15 days, depending on the microbial strains. The microbial biomass will then be separated by centrifugation. Specific activity (S.A.) of ^{32}P in the solution is determined and calculated in % of total P content in the medium.

The evaluation of the effect of P-solubilizing biofertilizer on the P uptake by plant is similar the using P isotopes in quantification of fertilizer P uptake from sources which cannot be labelled (indirect or reverse

dilution method). In this case ^{32}P in form of labelled KH_2PO_4 or NaH_2PO_4 solution are applied to soil at the rate of 7-18 x 10^6 Bq $^{32}P/m^2$ (for field experiment) or 4-7 x 10^6 Bq ^{32}P/kg soil (for greenhouse experiment). The experiment has 3 treatments as follows:

1. Soil + ^{32}P labelled solution
2. Soil + superphosphat + ^{32}P labelled solution
3. Soil + ^{32}P labelled solution + P-solubilizer

Plants are grown in the soil and plant materials are harvested after sowing or seeding time of 8 weeks to 3 months, depending on the plant. Specific activity of plant in each treatment is measured and calculated as follows:

- % Pdff (Phosphorus derived from fertilizer) = (S.A. plant sample/S.A. labelled fertilizer) x 100
- % Pdfl (Phosphorus derived from labelled source) = (S.A. sample/S.A. labelled source) x 100
- When S.A. labelled source = S.A. labelled soil, % Pdfl = % Pdfs (Phosphorus derived from soil) = (S.A. plant sample/S.A. labelled soil) x 100 and S.A. labelled soil = S.A. of plant in treatment 1

In the treatment 2 from the fraction utilization relationship %Pdfs/%Pdfl = %dfl/X can be calculated X (the amount superphosphat equivalent unit) in kg /ha.

In the same way on the treatment 3 X (the amount P.solubilizing biofertilizer equivalent unit) can also be calculated in kg/ha.

From the above results the P.solubilizing biofertilizer with the superphosphate can be compared quantitatively.

An example is given as the following:

Treatment:

1. Soil + ^{32}P labelled solution
2. Soil + superphosphat (60kg/ha) + ^{32}P labelled solution
3. Soil + ^{32}P labelled solution + P-solubilizer (2kg)

Results:

S.A. of harvested plant material per treatment:

1. S.A. plant = 823 Bq/mg P
2. S.A. plant = 398 Bq/mg P
3. S.A. plant = 512 Bq/mg P

Calculation:

Treatment 2:

% Pdfl = % Pdffs = 389/823 x 100 = 48.4 %

% Pdf - superphosphate = 100 – 48. 4 = 51.6 %

From the fractional utilization relationship 51.6/48.4 = 60 kg/X can calculate
the P derived from soil = (48.4 x 60): 51.6 = 56.3 kg

Treatment 3:

% Pdfs = 512/823 x 100 = 62.2 %
% Pdf- P.solubilizing biofertilizer = 100 – 62.2 = 37.8 %
From the fractional utilization relationship 37.8/62.2 = 2 kg/X can calculate
the P derived from soil equivalent to P.solubilizing biofertilizer =
(62.2 x 2): 37.8 = 3.29 kg

Quantitative comperation of P.solubilizer biofertilizer and superphosphate:

56.3 kg superphosphate = 3.29 kg P solubilizing biofertilizer
1 kg P solubilizing biofertilizer is equivalent to 17.11 kg superphosphate.

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2. Conventional Methods

2.1. N-fixing activity

2.1.1. Acetylene reduction assay

Nitrogenase reduces acetylene (C_2H_2) to ethylene (C_2H_4), and the ethylene formed can be very sensitively detected by gas chromatography. The acetylene reduction assay (ARA) is the most sensitive method to elucidate biological N_2 fixation activity. It could be used to compare nitrogenase activities among treatments in laboratory experiments as well as in field experiments. However, the ARA cannot be quantitatively converted to the amount of N_2 fixed. It should be noted that the ARA does not relate directly to N_2 fixation, but instead measures electron flux through nitrogenase. Total electron allocation for both N_2 fixation and H_2 evolution will be measured by ARA. In addition, ARA is difficult to be applied in field experiment mainly due to difficulty of full recovery of nodulated root system.

The standard ARA method involves enclosed detached nodules or nodulated root systems in air tight containers and exposing them to an atmosphere containing C_2H_2 (Peoples and Herridge 1990). However, detached nodules are ready to desiccate and decrease N_2 fixation activity.

For hydroponically cultivated legume plants, the ARA can be measured in intact plants in a sealed pot (Fig. 1A). About 10 % of the air volume inside is replaced by pure acetylene. After incubation in a short period for 15 min or 30 min, 0.5 mL of the gas inside is sampled using a hypodermic syringe, and the gas is analyzed by GC (gas chromatography) equipped with FID detector. The column is Porapack N (GL Sciences). About 99.5 % standard ethylene was diluted to 1000 times and 0.5mL was injected.

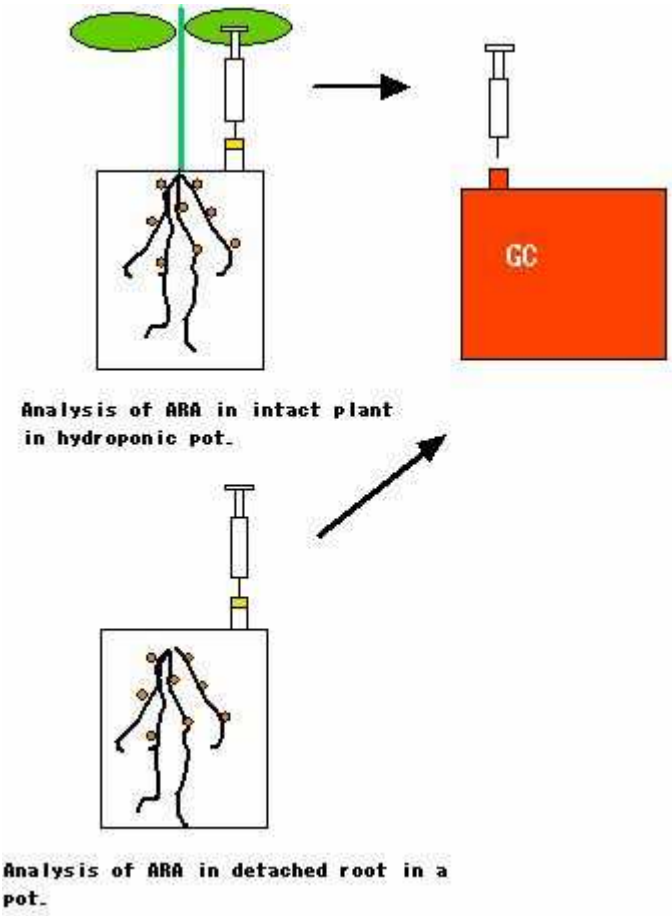


Fig.1A: ARA measurement of nodulated legumes in intact plants and detached roots

Detached nodulated roots are most frequently used for ARA analysis (Fig.1B). Detached root system is put in a glass jar (700 mL) and 10 % of the gas inside is replaced by acetylene. After 20 min incubation at 25℃, 0.5 mL of gas inside the jar was taken and concentration of ethylene is analyzed. After ARA

measurement, roots are dried and nodule DW is measured. ARA is expressed as μmole ethylene formed per hr per plant or μmole ethylene formed per hr per g DW of nodules (specific ARA).

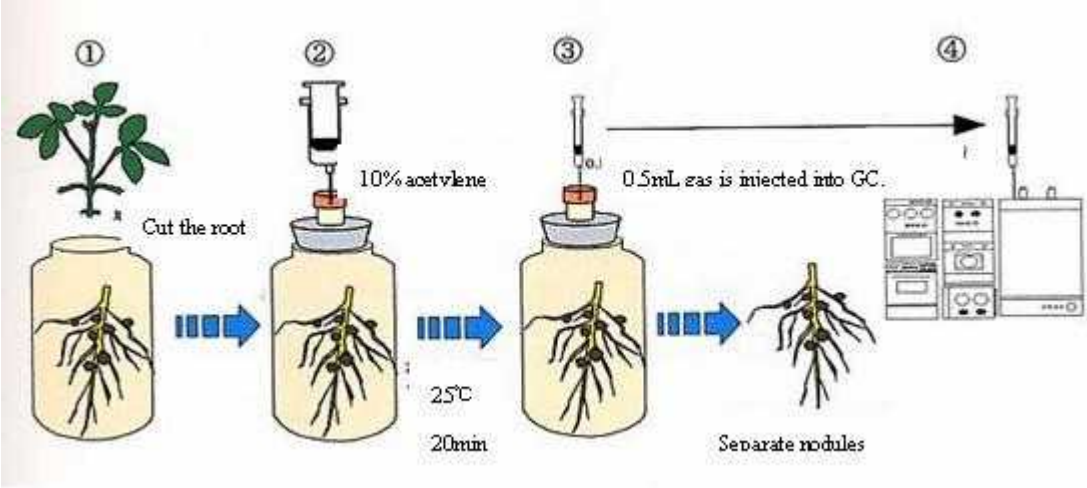


Fig.1B: ARA measurement of nodulated legumes in intact plants and detached roots

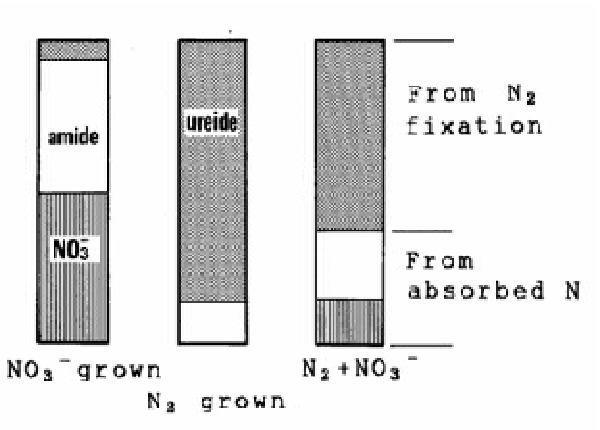
2.1.2. H_2 production assay

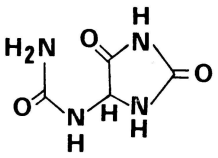
When air is replaced by inert gas such as Ar, nitrogenase catalyzes the evolution of H_2 gas instead of N_2 fixation. This H_2 evolution can be used for the index of nitrogen fixation activity like ARA (Witty and Minchin 1998). This method is used for physiological researches instead of ARA, but not applied to the field evaluation so far.

2.1.3. Relative ureide method

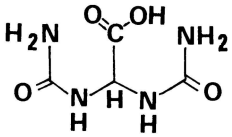
Kushizaki et al. (1964) discovered that nodulated soybean plants accumulate ureide compounds, allantoin and allantoic acids in the shoots. Comparing $^{15}N_2$ fixed in nodules and $^{15}NO_3^-$ absorbed in the roots, it was confirmed that most of the ureides in the shoots derived from nodules (Ohyama and Kumazawa 1979). Similar $^{15}N_2$ fixation studies revealed that ammonia a primary N_2 fixation product in bacteroids of a symbiotic form of rhizobia in nodules is rapidly excreted to the plant cytosol and assimilated into glutamine via the GS/GOGAT system, and synthesized to ureide via *de novo* synthesis of purine base (Ohyama and Kumazawa 1978, 1980a, 1980b, 1980c, 1981a, 1981b).

Many tropical grain legumes, such as soybean, common bean, cowpea, pigeon pea, and mung bean that have spherical determinate type of nodules transport the bulk of fixed N as ureide (allantoin and allantoic acid). On the other hand, nitrate and amino acids (especially amide, asparagine) are the major forms of N derived from soil and fertilizer N transported by roots (Ohyama and Kumazawa 1979, Ohtake et al. 1995).



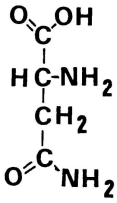


Allantoin



Allantoic acid

Nitrate



Asparagine

Fig. 2: Concept of relative ureide method and chemical structures of major nitrogen compounds in soybean xylem sap

Dr. Herridge (1990) developed the relative ureide method for evaluation of % Ndfa by analyzing the nitrogen composition of xylem sap obtained from bleeding sap from a cut stump, or vacuum collection from shoot (Fig.2, 3, 4). The concentration of ureide-N, nitrate-N and amino-N can be easily determined by colorimetry.

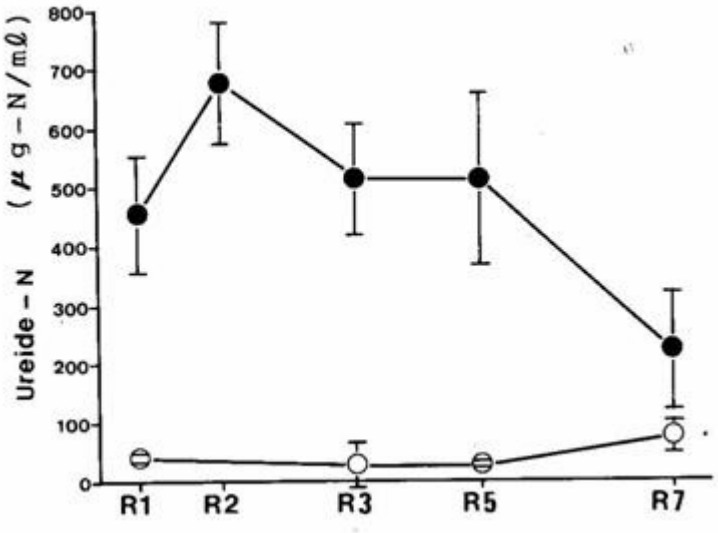


Fig. 3: Ureide-N concentrations in xylem sap of nodulating (T202) and non-nodulating (T201) isolines of soybeans

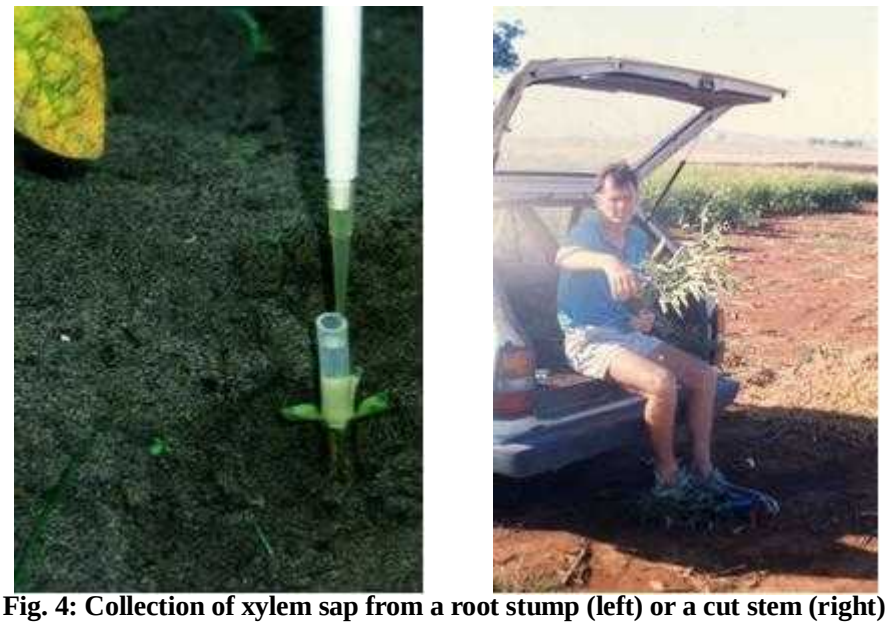


Fig. 4: Collection of xylem sap from a root stump (left) or a cut stem (right)

A study on a range of food legume species (soybean, green gram, black gram, cowpea, pigeon pea and common bean) indicated that the differences in the relationship between ureide-N and N_2 fixation are likely to be minor (Peoples and Herridge 1990).

This method is reliable in the field experiment of soybean, without any requirement of reference plants. It is the easiest way to measure %Ndfa in farmers field, because no preparation is necessary before sampling. This method is also applicable for experiments with variable N fertilizer application. In field conditions, the simple equation can be adapted for the estimation of % Ndfa.

$$\%\ \text{Ndfa} = \frac{\text{Ureide-N}}{\text{Ureide-N} + \text{Nitrate-N} + 2 \times \alpha\text{-Amino-N}} \times 100$$

The original equation proposed by Herridge was "α-Amino-N" instead of "2 x α-Amino-N" in the above equation. Based on the analysis of root bleeding xylem sap amino acids compositions, 2N amide Asparagine was the major amino acid thoughout the stages and the average N number in amino acids was 1.7, so we use "2 x α-amino-N" for the estimation.

By periodical sampling of legume plants and xylem sap, quantitative estimation of the seasonal changes in N_2 fixation activity and N absorption rate is possible（Takahashi et al. 1993). We usually sample soybean plants four or five times, at R1 (initial flowering), R3 (maximum shoot growth), R5 (pod filling) and R7 (yellow leaf) stages for xylem sap and plant N analyses.

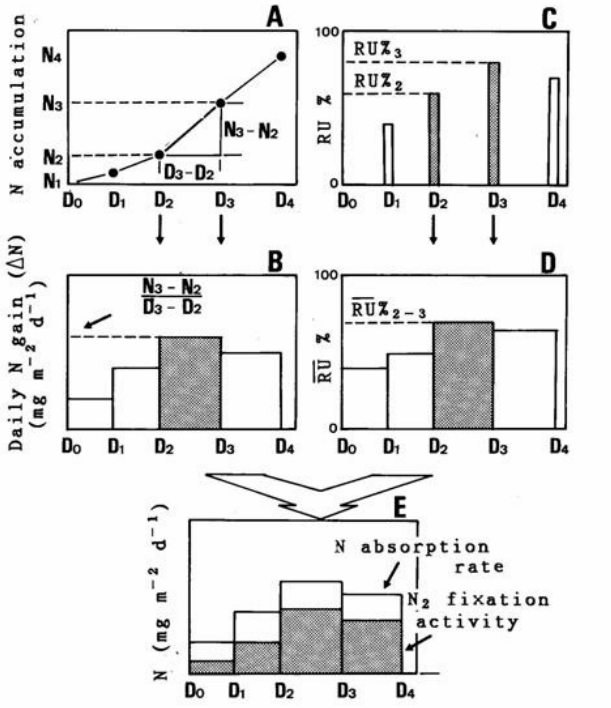


Fig. 5 shows the outline of the estimation of N_2 fixation activity and N absorption rate based on the data obtained by relative ureide method and total N analysis (Takahashi et al. 1993).

Soybean plants were harvested at D_1, D_2, D_3 and D_4. We usually harvest at R1, R3, R5 and R7 stages for D_1, D_2, D_3 and D_4. D_0 means the day of sowing. The N content of soybean shoot or whole plant including roots is determined by Kjeldahl digestion method. Average daily N increase in plant is calculated between successive sampling.

Daily N gain $\Delta N_{2\text{-}3} = (N_3 - N_2)/(D_3 - D_2)$

Fig. 5: Outline of the estimation of N_2 fixation activity and N absorption rate based on the data obtained by relative ureide method and total N analysis

The root bleeding xylem sap is collected from a cut stump for 30 min, and the percentage of relative

ureide N value (RU %) is calculated at each sampling time. The average RU % is calculated as follows:

$RU\%_{2\text{-}3} = (RU\%_2 + RU\%_3)/2$

Exceptionally, the first phase between D_0 and D_1, $RU\%_{0\text{-}1} = RU\%_1$

Combining the data of daily N gain and average RU %, the daily N_2 fixation activity and daily N absorption rate can be calculated as follows:

Daily N_2 fixation activity = $\Delta N_{2\text{-}3} \times RU\%_{2\text{-}3} /100/D_{2\text{-}3}$

Daily N absorption rate = $\Delta N_{2\text{-}3} \times (100 - RU\%_{2\text{-}3})/100/D_{2\text{-}3}$

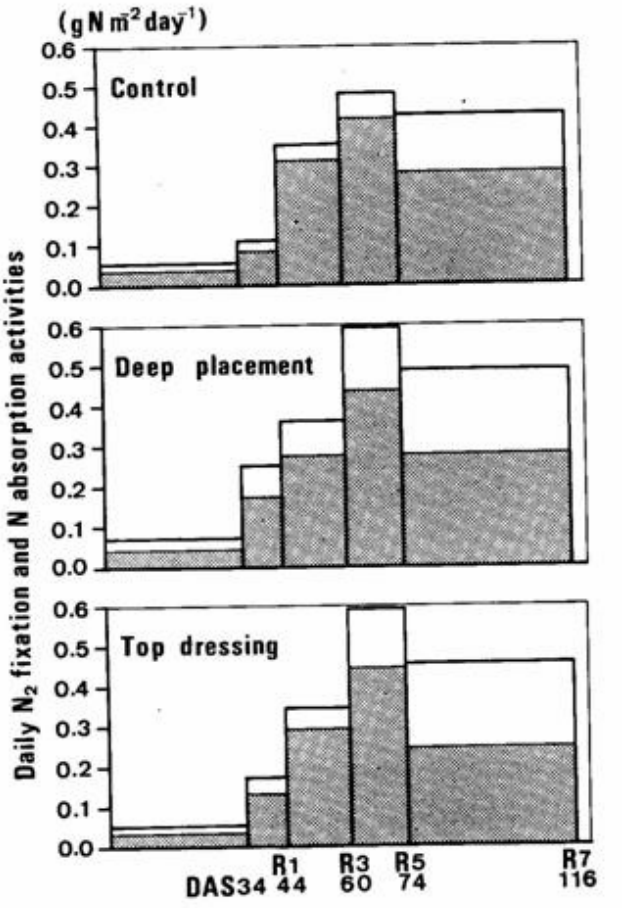


Fig. 6: Seasonal changes in daily N_2 fixation activityand N absorption rate estimated by relative ureide method (Takahashi et al.1992)

The examples of the evaluation of Ndfa by relative ureide method are shown in Fig. 6 (Takahashi et al. 1992) and Fig. 7 (Suganuma et al. 2001). A high dose of N fertilizer application generally depresses nodulation and N_2 fixation, and plant growth and seed yield are not improved in soybean cultivation. However, a deep placement of coated urea (slow release N fertilizer) did not depress N fixation and supplement N from the lower roots; plant growth and the seed yield exceeded (5.9 t/ha) the control cultivation (4.8 t/ha) (Takahashi et al 1992, 1999).

Fig. 7 shows the rate of N_2 fixation and N absorption by Williams and the hypernodulation mutants NOD1-3 that is partially tolerant to nitrate. The % Ndfa was higher in NOD1-3 (65 %) than Williams (58 %), the rate of N_2 fixation and N absorption was much lower in NOD1-3 than in Williams.

Recently, we developed the analysis of ureide-N, nitrate-N and asparagine by capillary electrophoresis (Figs. 8, 9 Sato et al. 1998).

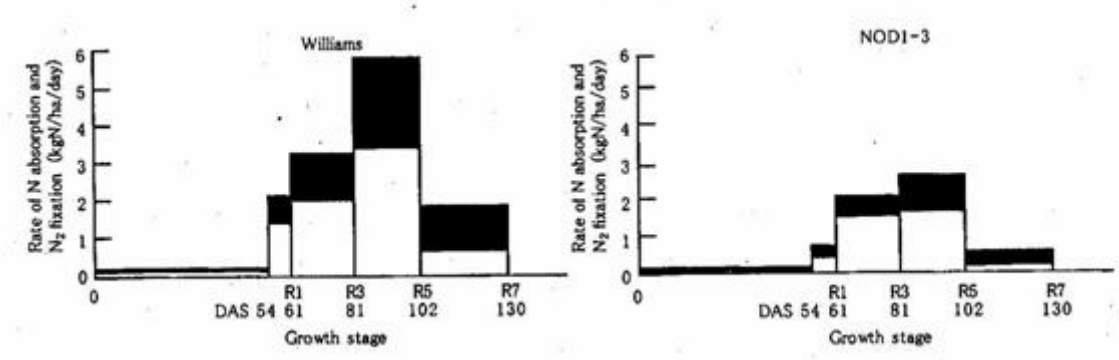


Fig. 7: Seasonal changes in daily N_2 fixation activity and N absorption rate in Willams and its hypernodulation mutant NOD1-3 cultivated in the field estimated by relative ureide method

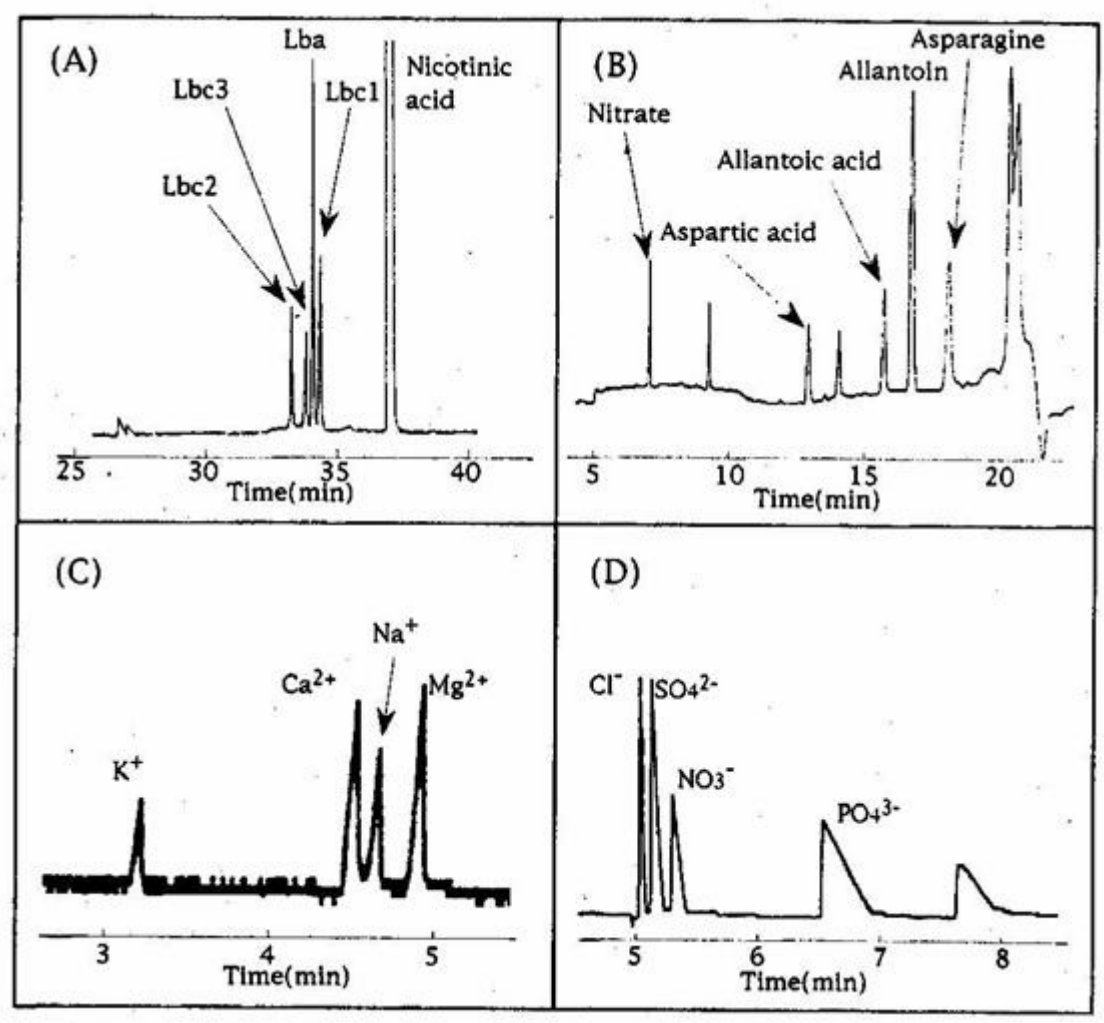


Fig. 8: Analysis of xylem solutes including N constituents by capillary electorophoresis

Fig. 9: Apparatus of Capillary Electrophoresis

Procedures for colorimetric analysis of ureide-N, amino-N and nitrate-N

The procedure below is based on the laboratory methods used in the laboratory of Prof. Dr. Harper (Univerisity of Illinois) and introduced by Takahashi and Ohyama (1993).

Ureide-N

Ureide-N concentration is determined by Young-Conway method (1942).

1) 50 μL of xylem sap is taken into a test tube
2) 3 mL of 0.083M NaOH solution is added.
3) Heat the test tubes in boiling water bath for 8 min.
   All the allantoin is degraded into allantoic acid by alkali hydrolysis.
4) Cool the test tubes in cool-water.
5) 1 mL of ice-cooled phenylhydrazinium solution is added.
   Phenylhydrazinium solution: Dissolve 0.33 g of phenylhydrazinium chloride in 100 mL of water, and add 100 mL of 0.65M HCl solution. Cool in ice water bath before use.
6) Heat the test tubes in boiling water bath for 2 min.
   All the allantoic acid is degraded into urea and glyoxilic acid.
7) Tubes are immediately cooled in ice-water bath for 15 min.
8) Add 2.5 mL of ferricyanide solution and wait for 30 min in ice-water bath.
   Ferricyanide solution: Dissolve 1.67 g of potassium ferricyanide in 100 mL of water. Add 400 mL of 10 M HCl solution. Cool in ice-water before use.
9) Measure the absorbance at 520 nm by spectrometry.

Standard 5 mM allantoin solution (280 μgN mL): Dissolve 197.7mg of allantoin in 250 mL of water.

Amino-N

α-Amino-N concentration is determined by the ninhydrin method (Herridge 1984).

1) 50 μL of xylem sap is taken into a test tube.
2) Add 1.5 mL of citrate buffer into the tube.
   Citrate buffer: Dissolve 56 g of citrate and 21.3 g of NaOH in 1 L of water.
3) Add 1.2 mL of ninhydrin solution.
   Ninhydrin solution: Dissolve 0.958 g of ninhydrin and 33.4 mg of ascorbic acid in 3.2 mL of water. Then solution is mixed with methoxyethanol (methylcellosolve) up to total of 100mL.
4) Boil the test tube in boiling water bath for 20 min with aluminum foil lid.
5) Add 3 mL of 60 % ethanol (60mL of ethanol plus 40 ml of water), and cool until room temperature.
6) Measure the absorbance at 570 nm by spectrometry.

Standard 10 mM amino acid solution (140 μg α-amino-N mL^{-1}): Dissolve 132 mg of asparagine (150mg of asparagine monohydrate) plus 146 mg glutamine in 200 mL of water. This solution contains 5mM asparagine

plus 5 mM glutamine. Total N concentration is 280 μg N mL^{-1}. Be careful to use α-amino-N concentration for the calculation of simple relative ureide equation with "2 x α-amino-N".

Nitrate-N

Nitrate-N concentration is measured by Cataldo's method (Cataldo et al. 1974).

1) 50 μL of xylem sap is taken into a test tube.
2) Add 200 μL of salicylic acid-sulfate solution into the tube, mix well and wait for 20 min.
   Salicylic acid-sulfate solution: Dissolve 5 g of salicylic acid in 100 mL of concentrated sulfric acid.
3) Add 5 mL of 2M NaOH solution into tube. Mix well and wait for 20 min.
4) Measure the absorbance at 410 nm.
   Standard nitrate solution (70 μgN mL^{-1}) : Dissolve 425 mg of sodium nitrate in 1 L of water.

2.2. P- solubilizing Activity

To determine the phosphate solubilizing activity of microorganism, the Pikovskaya's medium without agar is prepared. (see chapter 4.1.2. Isolation of mineral phosphate solubilizer)

If solubilization of rock phosphate or other insoluble phosphate is to be studied, $Ca_3 (PO_4)_2$ can be replaced by rock phosphate or other substrates containing phosphorus. The medium is sterilized in the autoclave and then inoculated with P-solubilizing microorganism. The microbe is allowed to grow in the appropiate conditions for 3 to 7 days. The clear solution is then collected by the filteration or centrifugation. The water soluble phosphorus in the clear solution is than determinated by spectrophotometric method.

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III. Carriers for Biofertilizers

1. Carrier Materials

1.1. Introduction

Biofertilizers are usually prepared as carrier-based inoculants containing effective microorganisms. Incorporation of microorganisms in carrier material enables easy-handling, long-term storage and high effectiveness of biofertilizers. Among various types of biofertilizers, bacterial inoculant is one major group which includes rhizobia, nitrogen-fixing rhizobacteria, plant growth-promoting rhizobacteria, phosphate-solubilizing bacteria, and so on. Basically, the carrier-based inoculant of these bacteria can be prepared by a common procedure. In this chapter, type of carrier materials available for biofertilizers, and preparation in general of carrier-based inoculants will be described.

Most of the bacteria included in biofertilizer have close relationship with plant roots. *Rhizobium* has symbiotic interaction with legume roots, and rhizobacteria inhabit on root surface or in rhizosphere soil. To achieve the successful inoculation of *Rhizobium* or rhizobacteria, large population of the bacterial strain must be placed close to the emerging root, so that the majority of nodules are formed by the inoculated rhizobial strain, and that the inoculated rhizobacterial strain occupies the rhizosphere as major member of rhizobacteria. If the population is not large enough, the native rhizobia / rhizobacteria will occupy most of the root nodules / rhizosphere, leading to unsatisfactory effect of inoculation.

The most common way of inoculation is "seed inoculation", in which the inoculant (bacteria-carrier mixture) is mixed with water to make slurry-form, and then mixed with seeds. In this case, the carrier must be a form of fine powder. To achieve the tight coating of inoculant on seed surface, use of adhesive, such as gum arabic, methylethylcellulose, sucrose solutions, and vegetable oils, is recommended. Any locally available sticky material, which is non-toxic to bacteria and seeds, can be used as adhesive.

Seed inoculation may not always be successful, i.e. the inoculation resulted in low nodule occupancy of the inoculated rhizobial strain, or low establishment of the inoculated rhizobacterial strain. This might be due to low population and/or low survival of the inoculated bacterial strain on the seed surface and in the soil. In such instance, "soil inoculation" will be adopted, whereby a large population of a bacterial strain can be introduced into the soil. For soil inoculation in general, granular inoculant is placed into the furrow under or alongside the seed. This enhances the chance for the inoculated strain to be in contact with plant roots.

1.2. Carrier material

Various types of material are used as carrier for seed or soil inoculation. For preparation of seed inoculant, the carrier material is milled to fine powder with particle size of 10 -40 μm. According to the "Handbook for Rhizobia" (Somasegaran and Hoben, Springer, 1994), the properties of a good carrier material for seed inoculation are: (1) non-toxic to inoculant bacterial strain, (2) good moisture absorption capacity, (3) easy to process and free of lump-forming materials, (4) easy to sterilize by autoclaving or gamma-irradiation, (5) available in adequate amounts, (6) inexpensive, (7) good adhesion to seeds, and (8) good pH buffering capacity. Needless to say, (9) non-toxic to plant, is another important property.

Peat is the most frequently used carrier material for seed inoculation. Peat-based rhizobial inoculant is already used in many countries and a number of information is available on the properties and effect of the inoculant.

For soil inoculation, carrier material with granular form (0.5 – 1.5 mm) is generally used. Granular forms of peat, perlite, charcoal or soil aggregates are suitable for soil inoculation. Various types of material used or tested as carrier for bacterial inoculant (mostly *Rhizobia*) is listed in Table 1.

Other essential criteria for carrier selection relating to survival of the inoculant bacteria should be considered. (1) Survival of the inoculant bacteria on seed. Seeds are not always sown immediately after seed coating with the inoculant bacteria. The bacteria have to survive on seed surface against drying condition until placed into soil. (2) Survival of the inoculant bacteria during the storage period. (3) Survival of the inoculant bacteria in soil. After being introduced into the soil, the inoculant bacteria have to compete with native soil microorganisms for the nutrient and habitable niche, and have to survive against grazing protozoa. Such carrier materials that offer the available nutrient and/or habitable micro-pore to the inoculant bacteria will be desirable. In this sense, materials with micro-porous structure, such as soil aggregate and charcoal, will be good carrier for soil inoculant.

1.3. Sterilization

Sterilization of carrier material is essential to keep high number of inoculant bacteria on carrier for long storage period.

Gamma-irradiation is the most suitable way of carrier sterilization, because the sterilization process makes almost no change in physical and chemical properties of the material. Detail of gamma-irradiation will be described in another chapter. In brief, carrier material is packed in thin-walled polyethylene bag, and then gamma-irradiated at 50 kGy (5 Mrads).

Another way of carrier sterilization is autoclaving. Carrier material is packed in partially opened, thin-walled polypropylene bags and autoclaved for 60 min at 121 ºC. It should be noted that during autoclaving, some materials changes their properties and produce toxic substance to some bacterial strains.

1.4. Appendix

As an example of the manipulation of rhizobial inoculant, our paper entitled "Enhanced Growth and Nodule Occupancy of Red Kidney Bean and Soybean Inoculated with Soil Aggregate-Based Inoculant" printed in *Soil Science and Plant Nutrition* (48 (2), 251-259, 2002) will be useful. Abstract of the paper is as follows. For the reprint request, please mail to asenoo@mail.ecc.u-tokyo.ac.jp.

ABSTRACT

Volcanic ash soil, which is widely distributed in Japan, contains a large amount of well-structured soil aggregates. By using these aggregates as carrier materials, we prepared (brady)rhizobial inoculants for red kidney bean (*Phaseolus vulgaris*) and soybean (*Glycine max*). Autoclaved soil aggregates were inoculated with *Rhizobium tropici* CIAT899R or *Bradyrhizobium japonicum* USDA110R, incubated for 15 or 21 days at 30℃, slowly air-dried at 20℃ to prepare the aggregate-based inoculants, and stored at various temperatures. The populations of CIAT899R and USDA110R in the aggregate-based inoculants were maintained during several months of storage at 20℃. When the aggregate-based inoculants were mixed with soil, CIAT899R and USDA110R cells showed a remarkably improved survival in soils compared with those mixed with soil without carrier material. The effect of the aggregate-based inoculants on the growth of red kidney bean and soybean was examined in pot experiments. By placing a small amount of the inoculant just beneath the seeds at the time of sowing, plant growth was significantly enhanced compared with the use of traditional peat-based inoculant. In addition, nodule formation on the upper part of soybean roots and nodule occupancy by the inoculated strain were remarkably enhanced by the aggregate-based inoculant. It is suggested that soil aggregates might be suitable carrier materials for preparing cheap and effective (brady)rhizobial inoculants.

Table 1 Carriers materials used for biofertilizers

Carrier material	Inoculant bacterium	Characteristics
Sterilized oxalic acid industrial waste[1]	*Rhizobium*	- seed inoculation - *Rhizobium* multiplication in carrier in ambient temperature up to 90 days. - Carrier sterilization contributed significant increase in grain yield, nodule number and nitrogen content.
Alginate-perlite dry ganule[2]	*Rhizobium*	- soil inoculation - *Rhizobium* strains survived in dry granules beyond 180 days. - The inoculant can be stored in a dry state without losing much viability.
Composted sawdust[3]	*Bradyrhizobium* , *Rhizobium* and *Azospirillum*	- seed inoculation - Good growth and survival of the inoculant strains.
Agriperlite, Expanded clay, Kaolin, Celite, Diatom, Porosil MP, Micro-cel, Vermiculite[4]	*Agrobacterium radiobacter* K84	- Crown gall control - Screening was performed to find improved formulation of K84 cells. - Effect of carrier storage temperature and carrier water content on survival of K84 was examined.
Cheese whey grown cells in peat[5]	*Rhizobium meliloti*	- seed inoculation - Better survival at various temperature during storage, even under desiccation
Mineral soils[6]	*Rhizobium*	- seed inoculant - *Rhizobium* survived better at 4 C than at higher temperature.
Coal[7]	*Rhizobium*	- seed inoculant - Seven among eight tested coals supported the growth and survival of *R. phaseoli* strains. Most contained more than 10^7 rhizobia per g after 12months.
Granular inoculants amended with nutrients[8]	*Bradyrhizobium japonicum*	- soil inoculant - Betonite granules, illite and smectite granules, or silica granules amended with glycerol, Na glutamate and inoculated with either peat or liquid *Bradyrhizobium japonicum* inoculants. - enhanced early nodulation of soybean and increased N content of grain
Soybean oil or peanut oil added with lyophilized cells[9]	*Rhizobium*	- seed inoculant - Provide more protection than peat-based inoculant when rhizobia are inoculated on seeds and exposed to condition of drought and high temperature.
Perlite[10]	*Rhizobium, Bradyrhizobium, Bacillus*	- seed inoculant - Combination of a sucrose adhesive with the perlite carrier gave better survival of bacteria on seeds - Produced similar number of nodules, nodule dry

		weight, crop yield and nitrogen content as peat-based inoculants
Wastewater sludge[11]	*Sinorhizobium meliloti*	- seed inoculant - Result showed the suitability of using sludge as a carrier because it had the same or a higher potential than peat to support survival of *S. meliloti.*
Wheat bran, sugarcane baggas[12]	*Rhizobium*/ *Bradyrhizobium* and rock-phosphate-solubilizing fungus *Aspergillus niger*	- soil inoculant - The number of codoultured microorganisms was the highest with peat, followed by bran and sugarcane baggas.
Nutrient-supplemented pumice[13]	*Rhizobium*	- seed inoculant - Good storage and handling properties and could be mixed directly with the seeds during the sowing process

2. Carrier Sterilization using γ –irradiation

2.1. Introduction

About 100 years ago, an England scientist Ernest Rutherford designated three kinds of radiation release from uranium α –ray, β –ray and γ –ray. The entities of α –ray, β –ray and γ –rays are helium ions (positively charged particles), electrons (negatively charged particles) and photons (ionizing electromagnetic waves), respectively. These are collectively called "ionizing radiation". For radiation sterilization purpose, γ –irradiation is the most suitable because of its high penetrating activity. In this chapter, the properties of ionizing radiation, the effects of radiation on microorganisms, the necessity of radiation sterilization as well as the practical example for carrier sterilization will be described.

2.2. The properties of ionizing radiation

Atoms are electrically neutral in that the number of negatively charged electrons is exactly equal to the number of positively charged protons. However, when there are energy sources available, atoms can gain or loss electrons and acquire a net electrical charge. This process is called "ionization". In a simple term, ionization is the gain or loss of electrons. Ionization of atoms by γ –rays mainly proceeds through Compton effect. In this process, γ –ray collides with and transfers part of its energy to a loosely bound electron in an atom. The γ –ray with reduced energy is scattered in a new direction and involved in the ionization of other atoms until it loses energy enough for ionization reaction. As a result of the γ –ray collision with an atom, an electron is ejected from its atom, and acts as β –ray (negatively charged particle) to create a new ionization that is mainly occurred by inelastic collision.

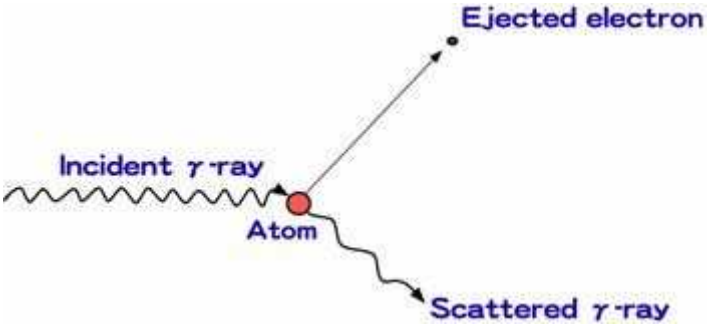

Fig. 1: Compton effect

2.3. The effects of radiation on microorganisms

If γ –rays collide with atoms of biological materials such as protein, lipid, carbohydrates and nucleic acid, the atoms are ionized and receive damages (direct effect of radiation). Among these biological materials, nucleic acid is the most sensitive material to ionizing radiation despite the proportion of nucleic acid in the total cellular components is only 1%.

From the aspect of biological effect of radiation, another important property of γ –rays is water radiolysis. The water molecular occupies 80% of the total cellular components. When a water molecule is ionized by γ –irradiation, many radical species including hydroxy radical, hydrogen radical, hydroperoxy radical and superoxide are produced. These radical species are highly reactive to biological materials, especially to nucleic acids. The effect of radiation on biological materials via water radiolysis is called "indirect effect of radiation".

In general, there exist two types of DNA damage induced by the direct and indirect effects of radiation; DNA strand breaks and base oxidative damages. DNA strand break is the dissociation of the phosphodiester bond of the main chain in DNA. DNA strand break causes loss of the continuity of genetic information and arrest of replication process, thereby results in cell death. Base oxidative damage is occurred at the nucleotide base in DNA, and at the base moiety of nucleotide pool in cytosol. This type of DNA damage causes replication errors and the accumulation of genetic mutation, thereby results in cell death.

However, the radiation resistance of living organisms differs widely in individual species. In general, microorganisms exhibit more radiation resistance than animals and plants. One of the explainable reasons for this resistance is that the cell nucleus (target of radiation) of microorganisms is much smaller than those of animals and plants. Another reason can be explained by DNA protection and repair capacity. DNA protection includes spore formation (the resting stage of cell) and radical scavengers such as catalase, superoxide dismutase and carothenoids. Microorganisms have a great variety of DNA repair capacity with different effectiveness by which the difference in radiation resistance arises. It has been known that there exist non-sporing but extremely radiation resistant bacteria that inhabit in many natural places such as soil and environmental waste including animal dung and plant chip. However, all the radiation resistant bacteria isolated so far are non-pathogenic. The most problematic issue for the sterilization of microorganisms is the presence of soil-born spore-forming bacteria, which are highly resistant to radiation, desiccation and heat. Some spore-forming bacteria are infectious and highly pathogenic for human and farm animals; e.g. *Bacillus anthracis* (anthrax), *Clostridium tetani* (tetanus), *Clostridium botulinum* (gas gangrenous). Some other spore-forming bacteria with lesser toxicity can cause food poisoning and opportunistic infections.

As mentioned above, bacterial spore is highly resistant to radiation. However, when there are nutrients available, the spore germinates to produce a vegetative cell that is much more sensitive to radiation (Fig. 2). This process (germination) is occurred in minutes. The difference in the moisture condition can affect the radiation resistance. As shown in Fig. 3, wet cells of *Escherichia coli*, that are nonsporing bacterium, are more sensitive to radiation compared to desiccated cells. This difference in survival rate is due to indirect effect of radiation by water radiolysis. Therefore, controlling the moisture conditions is very important for effective sterilization of carrier materials.

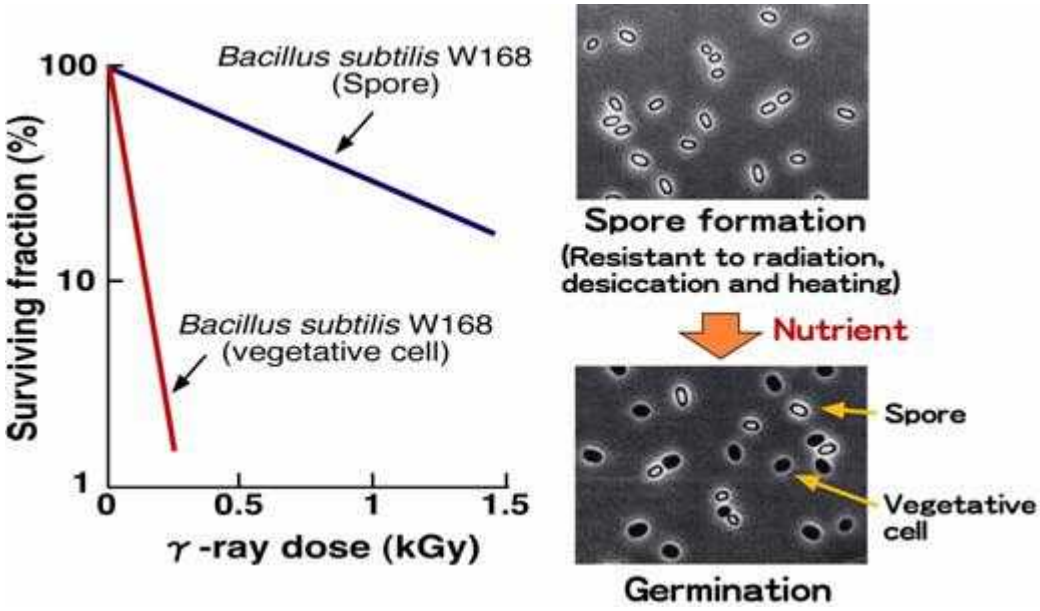

Fig. 2: Bacterial spore and vegetative cell

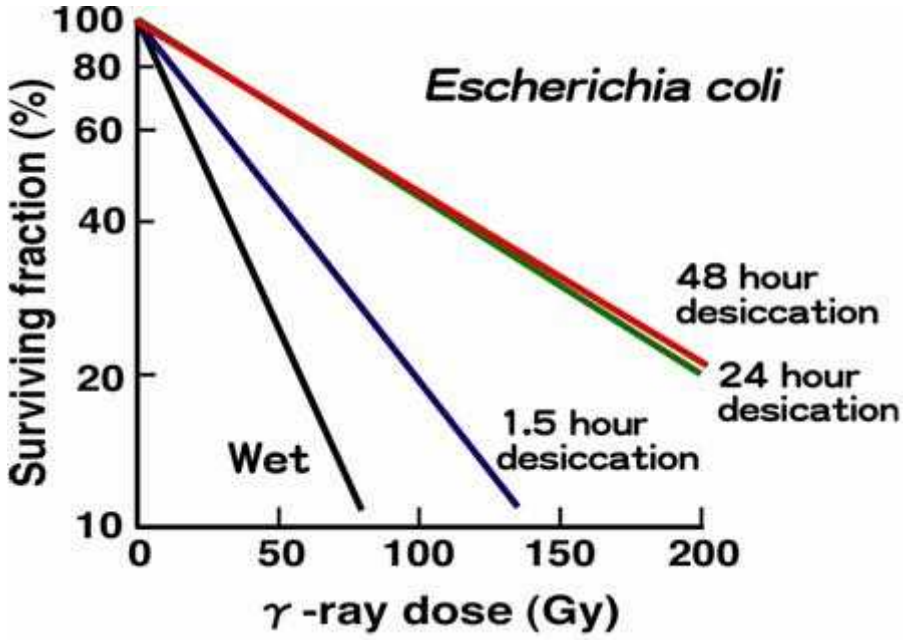

Fig. 3: Desiccation and hydration

2.4. The necessity of radiation sterilization

The purpose of sterilization of carrier materials for biofertilizer can be divided into two categories. 1) To offer nutrient and place to the inoculant bacteria against the occupation by the contaminated and/or native bacteria. This is important to keep the number of inoculant bacteria on carrier during the storage period before use. 2) To prevent undesirable dispersion of pathogenic bacteria to agricultural field. In other words, radiation sterilization is essential to reduce the risk of field contamination and infection.

2.5. The practical example for carrier sterilization

A proposed outline of sterilization process is described below.

a. Preparation of materials

a-1. Prepare the appropriate amount of carrier material (10 kg is recommended).

a-2. Divide into 10 polyethylene packages (Thickness: approx. 0.1 mm, Size: approx. 20 cm x 30 cm)

with 1 kg carrier.

a-3. Seal the packages using a heat sealer.

a-4. (Option 1) If the carrier is a highly dry material, wet with an appropriate amount of water (to increase the indirect effect of radiation).

a-5. (Option 2) If the presence of spore-forming bacteria is suspected in the carrier, add an appropriate amount of nutrient liquid medium (to promote the germination of spore).

b. Irradiation

b-1. Divide the carrier packages into 2 dose groups.

b-2. Irradiate each group by 25 kGy or 50 kGy of γ –rays at room temperature in the atmosphere. In the almost all cases, radiation sources are cobalt-60 or cesium-137. Irradiation dose can be controlled by changing the distance from the radiation source. The total irradiation time is dependent on the source activity. (Option: Instead of γ –rays, electron-beams can be used for radiation sterilization). A margin of error of plus or minus 10% is allowed for irradiation dose. No limit for dose rate. A short interruption of irradiation during the total time for required dose can be allowed. Follow the requirements for each irradiation facility. A practical example of irradiation is illustrated in Fig. 4.

b-3. After irradiation, preserve the irradiated packages at room temperature under the sealed condition until the inoculation of microorganisms.

Fig. 4: A practical example of irradiation

d. Confirmation of sterilization effect.

d-1. Prepare 1 g of carrier samples (non-irradiated, 25 kGy and 50 kGy irradiated samples).

d-2. Mix with 9 ml of sterile water to make suspension.

d-3. Dilute the suspension by serial 10-fold dilutions using sterile water and spread on nutrient agar plates.

d-4. Incubate (at 30 °C in general) and count bacterial colony number.

(Note: For this experiment, some experimental equipment is required; autoclave, clean bench, temperature-controlled incubator, etc. The same protocol can be used for monitoring survival of the inoculant microorganisms in carrier during the storage period.)

e. Inoculation of microorganisms to carrier.

e-1. Prepare starter culture for inoculation. Optionally, appropriately dilute with sterile water for moisture and cell number adjustment.

e-2. Inject the culture to the carrier package using a sterile disposable plastic syringe with a needle.

e-3. Seal the needle hole with a waterproof tape.

e-4. Keep the package at appropriate temperatures for maturation and storage. Although the temperatures suitable for maturation and storage are dependent on the inoculant microorganisms, 30 °C for maturation and 20 °C-30 °C for storage will be suited for inoculants in most cases.

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2.6. Appendix:

γ –ray irradiation facilities and electron-beam irradiation facilities potentially available for carrier sterilization in Asia are listed below.

γ-ray Irradiation Facilities for Commercial Use

Name	Year established	Shielding capacity	Source Activity	Purpose of irradiation	Remarks
INDONESIA					
1. Panoramic Irradiator	1979	125 kCi	75 kCi	Polymerization, Sterilization, Food Preservation	PATIR-BATAN
2. Latex irradiation	1984	400 kCi	215 kCi	Latex Vulcanization, Sterilization, Food Preservation	PATIR-BATAN
3. Indo Gamma	1991		4 MCi	Sterilization, Food Preservation	
4. Gamma Chamber	1998		10 kCi	Tissue Bank	Jamil Hospital
KOREA					
KAERI	1975 (1998)	176 cm	0.13 MCi	Research	Co60
Greenpia Tech	1986	~ 180 cm	1 MCi	Commercial	Co60
MALAYSIA					
Ansell	1977	4 MCi	2.0 MCi	sterilization	medical products
MINT	1989	2 MCi	1.5 MCi	sterilization	medical products/spices
Sterilgamma	1993	6 MCi	2.0 MCi	sterilization	medical products
ISOTRON	2001	4 MCi	1.0 MCi	sterilization	Medical products
PHILIPPINES					
Multipurpose irradiation facility, PNRI	1989	250,000 Ci	70,000 Ci	radiation sterilization; food irradiation	semi-commercial (pilot scale) service
THAILAND					
Kendal Gammatron Co.Ltd.	1984	500 kCi	150 kCi	Sterilization of medical supplies	Nakorn Prathom
Thai Irradiation Center	1993	3 MCi	450 kCi	R&D on radiation processing	Government own (OAP) Pratumthani
IBA S&I (Thailand) Ltd.	1999	3 MCi	1 MCi	Sterilization and others	Rayong
GAMMA-STER (Thailand) Ltd.	2000	6 MCi	1 MCi	Sterilization and others	Chonburi
Name	Year established	Shielding capacity	Source Activity	Purpose of irradiation	Remarks
VIETNAM					
SVST-Co-60	1999	2 MCi	400 kCi	- Sterilization of Medical products - Food pasteurization -Polysaccharides degradation	Hungarian type
RPP-150	1991	1 MCi	107 kCi	Food preservation	Russian type
Gamma Cell	1983 1987	16.5 kCi + 9 kCi		R&D (Present activity: 3 kCi)	Russian type

	Year established	Shielding capacity	Source Activity	Purpose of irradiation	Remarks
JAPAN					
Radia Ind. Co. Ltd.	1972		1.5 + 2 + 3 MCi	Sterilization	
Shihoro Agriculture Coop.	1973		1 MCi	Potato Irradiation	
Terumo Co. Ltd.	1983		3 × 2 MCi	Sterilization	
Koka Isotope Co. Ltd.	1987		2 MCi	Sterilization	
JMS Co. Ltd.	1987		3 × 2 MCi	Sterilization	
Nissho Co. Ltd.	1988		3 MCi	Sterilization	
Asahi-Med-ical Co. Ltd.	1988		1.5 MCi	Sterilization	
Japan Radiat. Serv. Co. Ltd.	1996		3 MCi	Sterilization	
CHINA					
Chengdu	1978			Spices, sausage, garlic	
Shanghai	1986			Apples, Potatoes, Onions, Garlic	
Zhengzhou	1986			Tomatoes	
Nanjing	1987				
Jinan	1987				
Lanzhou	1988				
Beijing	1988				
Tienjin	1988				
Daqing	1988				
Jianou	1991			Rice, Garlic, Spices	
Beijing	1995				
Dalian	1998				
Zhongshan	1999				
Inner Mongolia	1999				
Shuanglin	2000				

Installation of Electron Accelerators for Industrial Purpose

No.	Application	Location	Machine Ratings	Years
China				
1	Heat shrinkable	Jilin Radiation	3.0MV 40mA	1984
2	Heat shrinkable	Engineering Physics	3.0MV 10mA	1987
3	Wire & cable	Tianshui Cable	2.2MV 25mA	1989
4	Wire & cable	Yantai Cable	2.0MV 20mA	1991
5	Wire & cable	Xian Wire	2.0MV 30mA	1993
6	Heat shrinkable/cable	Taiyuan	2.5MV 30mA	1993
7	Wire & cable	Sichuan Cable	2.0MV 10mA	1993
8	Heat shrinkable	Chengdu Shuangliu	2.0MV 10mA	1993
9	Wire & cable	Changshou Cable	2.5MV 20mA	1994
10	Wire & cable	Liyang Cable	2.5MV 20mA	1994
11	Heat shrinkable	Changchun Chemistry	2.5MV 30mA	1994
12	Wire & cable	Xinhua Cable	1.5MV 40mA	1995
13	Wire & cable	Xinhua Cable	1.5MV 30mA	1995
14	Wire & cable	Guangdong cable	2.5MV 40mA	1995
15	Heat shrinkable	Nuclear Technology	2.0MV 20mA	1995
16	Wire & cable	Kunming Cable	2.5MV 30mA	1995
17	Wire & cable	Shanghai Cable	2.5MV 33mA	1995
18	Wire & cable	Huangshi Cable	2.5MV 40mA	1995
19	Wire & cable	Shenyang Cable	2.0MV 10mA	1995
20	Heat shrinkable	Dayu Shrink-tube	2.0MV 10mA	1995
21	Heat shrinkable	Dayu Shrink-tube	2.0MV 10mA	1995
22	Heat shrinkable	Tianjin Tech-Physics	2.0MV 10mA	1995
23	Wire & cable	Yangzhong Cable	2.0MV 10mA	1995
24	Wire & cable	Jiangxi Cable	2.0MV 10mA	1995
25	Wire & cable	Shanghai-minhang Cable	2.5MV 30mA	1996
26	Heat shrinkable	Changchun Chemistry	1.5MV 40mA	1997
27	Heat shrinkable	Changchun Chemistry	1.5MV 40mA	1997
28	Heat shrinkable	Changchun Chemistry	1.5MV 40mA	1997
29	Heat shrinkable	Changchun Chemistry	1.5MV 40mA	1997
30	Wire & cable	Tianjin Cable	2.5MV 20mA	1997
31	Wire & cable	Lanxi Cable	2.5MV 40mA	1997
32	Wire & cable	Huaian Cable	2.5MV 25mA	1997
33	Flue gas	Chengdu Power	800kV 400mAX2	1997
34	Wire & cable	Zhengzhou Cable	2.5MV 30mA	1998
35	Wire & cable	Zhunhua Cable	2.5MV 25mA	1999
36	Heat shrinkable	Chengdu Shuangliu	3.0MV 30mA	2000
37	Heat shrinkable	Shenzhen Plastic	2.5MV 30mA	2000
38	Wire & cable	Sijiazhuang Cable	2.5MV 20mA	2001
Indonesia				
1	R&D(Curing)	PATIR *1	300kV 50mA	1984
2	R&D(Cross-linking)	PATIR *1	2.0MV 10mA	1993
3	Tire	GT *2	500kV 150mA	1998

*1: Center for the Application of Isotopes and Radiation Technology

*2:PT Gajah Tunggal

No.	Application	Location	Machine Ratings	Years
Korea				
1	Wire & cable	LG Cable	750kV 65mA	1984
2	Wire & cable	LG Cable	1.5MV 65mA	1987
3	Wire & cable	LG Cable	1.0MV 100mA	1988
4	Wire & cable	LG Cable	2.0MV 50mA?	2000
5	Wire & cable	LG Cable	1.0MV 100mA?	2000
6	Wire & cable	Taihan Electric Wire	1.5MV 65mA	1988
7	Wire & cable	Dongyang Cable	1.0MV 50mA	1996
8	Heat shrinkable	Daewon Cable	1.0MV	1991
9	Tube	Daeryak Industry	1.0MV	1998
10	Wire & cable	Hankok KDK	1.0MV?	1997
11	Wire & cable	KyuangShin Co.	1.0MV 65mA	1990
12	Tire	Hankok Tire	500kV 150mA	1993
13	Tire	Hankok Tire	500kV 150mAX2sets	1996
14	Tire	Kumho & Co.	800kV 100mAX2sets	1990
15	Foarmed polymer	Youngbo Chemical	500kV 100mA	1990
16	Foarmed polymer	Youngbo Chemical	1.0MV 100mA	1998
17	Foarmed polymer	Tongil Ind.	800kV 65mA	1992
18	Curing	Tetrapack	175kV 300mA	
19	Cross-linking	Ceratech Co.	1.0MV 50mA?	
20	Waste water	Dyeing Complex	1.0MV 40mA?	1998
21	R & D	KAERI	300kV 25mA	1975
22	R & D	KAERI	2.0MV 45mA	2000
23	R & D/Service	EB Tech	1.0MV 40mA	
24	R & D/Service	EB Tech	1.0MV 40mA	
25	R & D	Youngnamu Univ.	800kV 35mA	1998
Malaysia				
1	R&D(Curing)	MINT *1	200kV 20mA	1991
2	R&D(Cross-linking)	MINT *1	3.0MV 30mA	1991
3	Wire & cable	Sumitomo *2	800kV 100mA	1995
4	Wire & cable	Sumitomo *2	2.0MV 50mA	2001
5	Packaging film	W.R.Grace	550kV 60mAX2	1996
6	Packaging film	S.K.Ploymer	150kV 460mA	1997
	*1:Malaysian Institute for Nuclear Technology Research (MINT)			
	*2:Sumitomo Electric Interconnect products, Johor			
The Philippines				
1	Sterilization	Terumo	10.0MV 28kW	2000
2	Tire	Yokohama	500kV 100mA	1998
Thailand				
1	Sterilization	Thai Klinipro	2.4MV 10kW	1997
2	Gem stones	IBA S&I	15MV 8.5kW	2000
3	Foarmed polymer	Sekisui Thai	800kV 100mA	1997
Vietnam				
	No installation of electron accelerator			

Installation of Electron Accelerators in Japan

Application	Low Energy E ≦300keV	Medium Energy 300keV＜E ≦ 3MeV	High Energy 3MeV＜E ≦ 10MeV	Total
Wire & cable	1	51	0	52
Foamed polymer	4	12	0	16
Heat shrinkable	15	17	1	33
Tire	3	20	0	23
Radiation curing	46	2	0	48
Flue gas & waste water	1	7	0	8
Sterilization	3	2	6	11
Irradiation service	7	11	4	22
Research and development	120	2	1	123
Total	200	124	12	336

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.

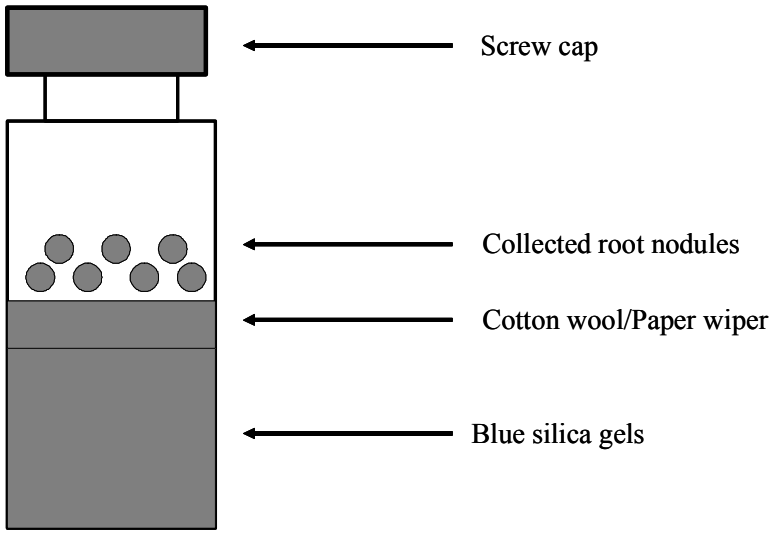

Fig.1: A preservation vial for root nodules

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.

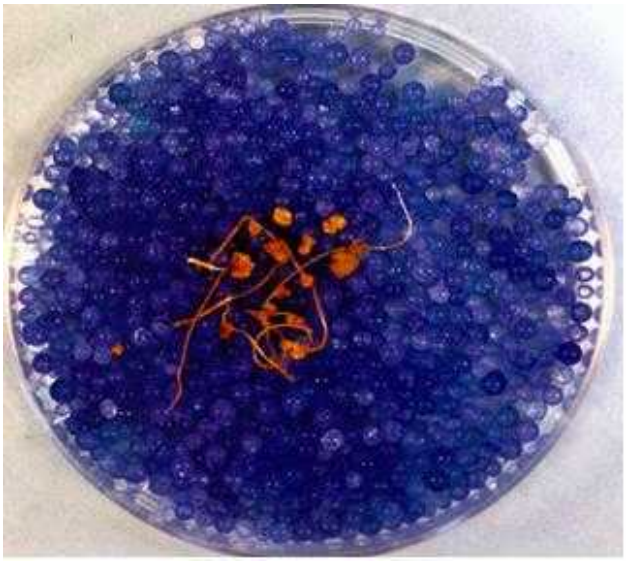

Fig. 2: Dried root nodule in blue silica gels

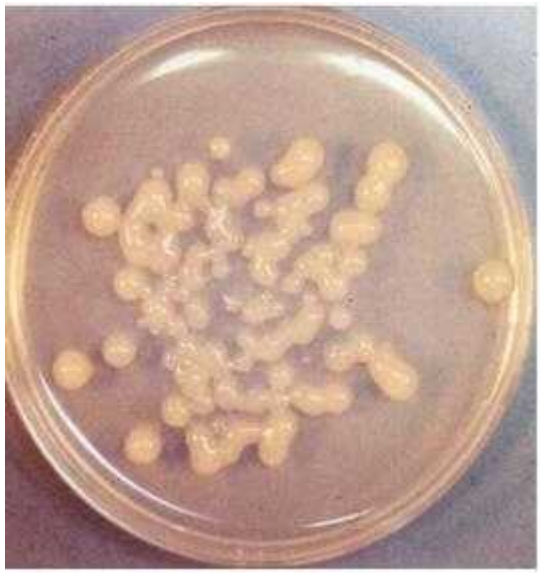

Fig.3: Well separated *Bradyrhizobium* colonies isolated from a dried root nodule originated in Wild *Vigna* plant

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-

Poster with the information of "Mamezo" products.

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.

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.

Factory building

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.

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.

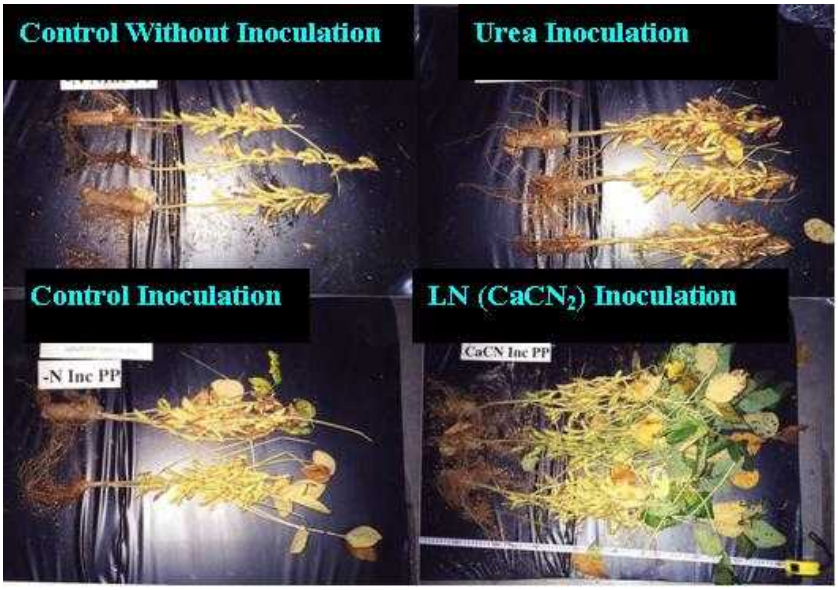

Fig. 3: Comparison of inoculation and urea or calcium cyanamide deep placement in reclaimed Yamakita Field where no indigenous *Rhizobium* was detected. Upper left (Non-inoculated without N deep placement), Lower left (Inoculated paper pot without N deep placement), Upper right (Inoculated paper pot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

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

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

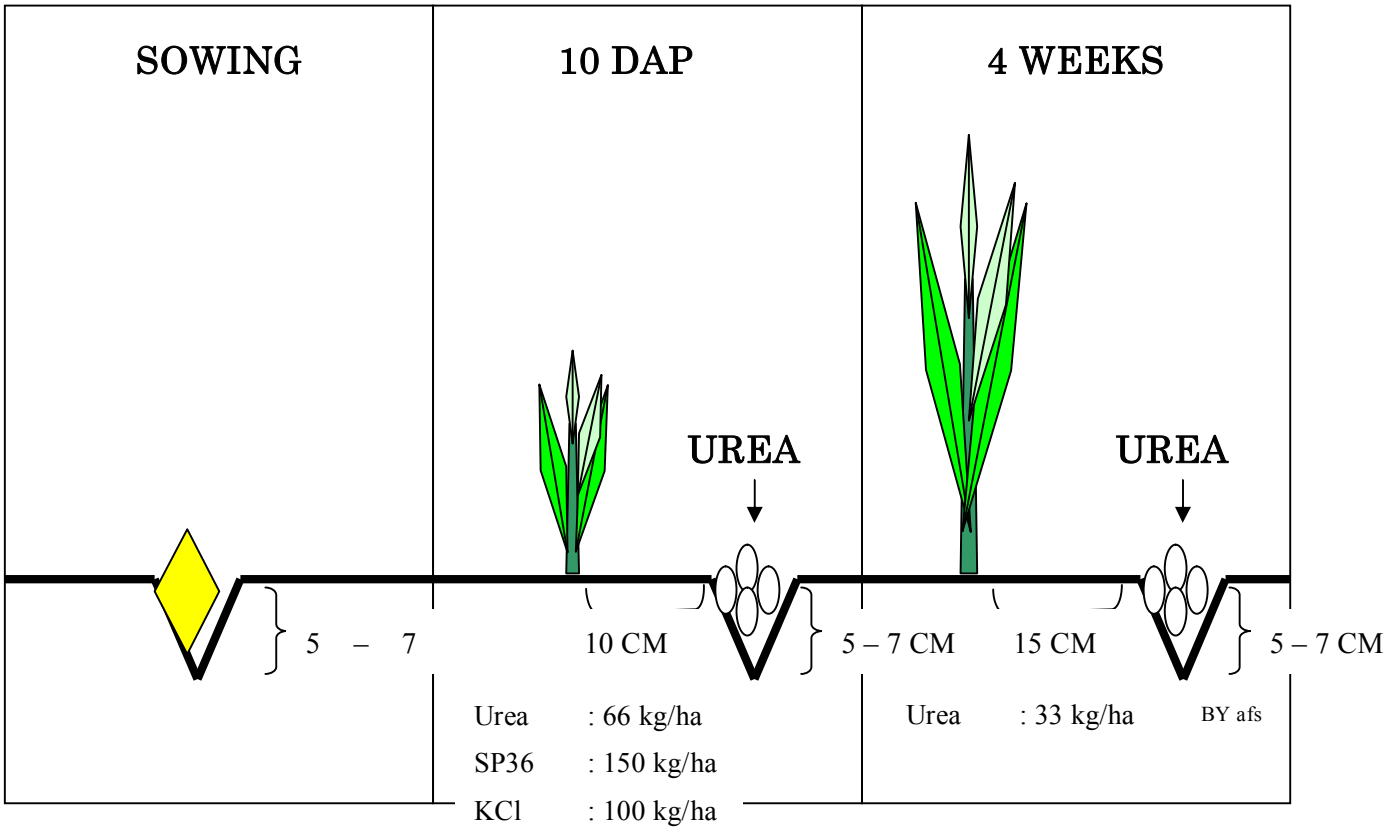

Fig. 1: Schematic diagram of fertilizer application (*)

(*). 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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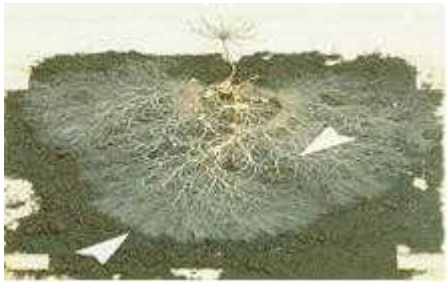
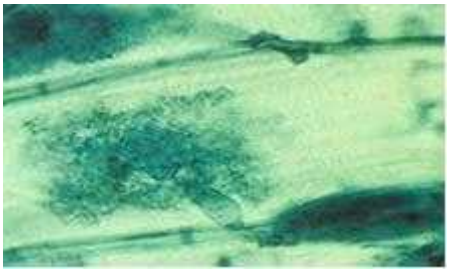
3. Mycorrhiza

3.1. Introduction



Mycorrhizal fungi are species of fungi that intimately associate with plant roots forming a symbiotic relationship, with the plant providing sugars for the fungi and the fungi providing nutrients such as phosphorus, to the plants. Mycorrhizal fungi can absorb, accumulate and transport large quantities of phosphate within their hyphae and release to plant cells in root tissue.

A mycorrhiza ("fungus – root") is a type of endophitic, biotrophic, mutualistic symbiosis prevalent in many cultivated and natural ecosystems. There are three major groups of mycorrhiza: Ectomycorrhiza, Ectendomycorrhiza and Endomycorrhiza. Ectomycorrhiza and endomycorrhiza are important in agriculture and forestry. In Thailand, endomycorrhiza biofertilizer has been investigated for ten years. Initially the mycorrhizal biofertilizer production is for economic crops such as fruit trees (durian, longan, sweet tamarind, mangosteen, papaya). Now the biofertilizer can be used for vegetables and rubber.



Endomycorrhiza (vesiclular arbuscular mycorrhiza; VA mycorrhiza; now known as arbuscular mycorrhiza,AM) play a very important role on enhancing the plant growth and yield due to an increase supply of phosphorus to the host plant. Mycorrhizal plants can absorb and accumulate several times more phosphate from the soil or solution than non–mycorrhizal plants. Plants inoculated with endomycorrhiza have been shown to be more resistant to some root diseases.

Arbuscular Mycorrhizal (AM) fungi (or Vesicular-Arbuscular Mycorrhizal, VAM fungi), belonging to the Phylum Glomeromycota are symbionts with terrestrial plant roots. It is now generally recognized that they improve not only the phosphorus nutrition of the host plant but also its growth, which may result in an increase in resistance to drought stress and some diseases. Therefore, AM fungi offer a great potential for sustainable agriculture, and the application of AM fungi to agriculture has been developed. In fact, in some countries the AM fungal inocula have been commercialized. Since it is laborious and cost-consuming for production of AM fungal inocula because of their obligate biotrophic nature, the ways to increase the function of the indigenous AM fungi in soil have also been developed. In this manual, the introductory techniques with AM fungi are presented.

General procedure has been documented in various text books and web sites. In this manual some additional information is described together with the outline of procedure.

3.2. Benefits of Mycorrhizal Biofertilizer

Mycorrhiza plays a very important role on enhancing the plant growth and yield due to an increase supply of phosphorus to the host plant. Mycorrhizal plants can absorb and accumulate several times more phosphate from the soil or solution than non–mycorrhizal plants. Plants inoculated with endomycorrhiza have been shown to be more resistant to some root diseases.

Mycorrhiza increase root surface area for water and nutrients uptake. The use of mycorrhizal biofertilizer helps to improve higher branching of plant roots, and the mycorrhizal hyphae grow from the root to soil enabling the plant roots to contact with wider area of soil surface, hence, increasing the absorbing area for water and nutrients absorption of the plant root system. Therefore, plants with mycorrhizal association will have higher efficiency for nutrients absorption, such as nitrogen, phosphorus, potassium, calcium, magnesium, zinc, and copper; and also increase plant resistance to drought. Benefits of mycorrhizal biofertilize can be seemed as follows:

1. Allow plants to take up nutrients in unavailable forms or nutrients that are fixed to the soil. Some plant nutrients, especially phosphorus, are elements that dissolve were in water in neutral soil. In the extreme acidic or basic soil, phosphorus is usually bound to iron, aluminum, calcium, or magnesium, leading to water insolubility, which is not useful for plants. Mycorrhiza plays an important role in phosphorus absorption for plant via cell wall of mycorrhiza to the cell wall of plant root. In addition, mycorrhiza help to absorb other organic substances that are not fully soluble for plants to use, and also help to absorb and dissolve other nutrients for plants by storage in the root it is associated with.
2. Enhance plant growth, improve crop yield, and increase income for the farmers. Arising from improved water and essential nutrients absorption for plant growth by mycorrhiza, it leads to improvement in plant photosynthesis, nutrients translocation, and plant metabolism processes. Therefore, the plant has better growth and yield, reduce the use of chemical fertilizer, sometimes up to half of the suggested amount, which in turn increases income for the farmers. As in the trial involving mycorrhizal biofertilizer on asparagus it was observed that, when the farmers used suggested amount of chemical fertilizer together with mycorrhizal biofertilizer, it was found that the crop yield improved by more than 50%, and the farmers' income increased 61% higher than when chemical fertilizer alone was used.
3. Improve plant resistance to root rot and collar rot diseases. Mycorrhizal association in plant roots will help plant to resist root rot and collar rot diseases caused by other fungi.
4. It can be used together with other agricultural chemicals. Mycorrhiza are endurable to several chemical substances; for example; pesticide such as endrin, chlordane, methyl parathion, methomyl carbofuran; herbicide such as glyphosate, fuazifopbutyl; chemical agents for plant disease elimination such as captan, benomyl, maneb triforine, mancozed and zineb.



3.3. Isolation of Arbuscular Mycorrhizal Fungi

3.3.1. Taxonomy of AM fungi

AM fungi show the peculiar characteristics in morphology and physiology. Spores of AM fungi are generally formed in soil and their sizes (50-500 μm in diameter) are much larger than those of other fungi. There is no septum in their hyphae. No sexual growth-phase has been observed. Spores germinate when they are under favorable conditions, extend their hyphae and colonized plant roots. The fungi penetrate the hyphae into cortex layer of roots and form the hyphal organs, "vesicles" and "arbuscules" which are characteristics to AM fungi (Fig. 1). AM fungi belonging to Gigasporaceae are known not to form vesicles. Colonization on plant roots is essential for proliferation of AM fungi. AM fungi are thus recognized as obligate symbiotic fungi. The interaction between AM fungi and plants is generally mutualism based upon nutrient exchange.

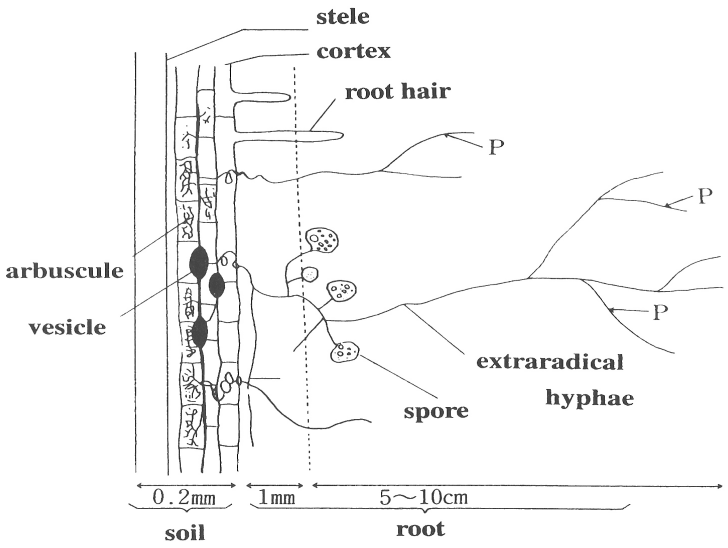


Fig. 1: Schematic picture of arbusucular mycorrhizal fungi colonizing roots and their hyphal extension into soil.

Because of morphological characteristics such as no hyphal septum, AM fungi had long been recognized as a member of Zygomycota. Recent molecular phylogenetic studies showed that Zygomycota is poly-phyletic and that AM fungi should be separated from other Zygomycota. A new Phylum Glomeromycota has been proposed for AM fungi. Current classification system is summarized in Fig. 2. This classification is mainly based upon the sequence data of rRNA gene. However, some new genera have been raised with relatively small numbers of isolates, so further study may revise the present classification system.

In this manual, the morphological characteristics of representative genera are shown in the following sections. Although there is a recent trend that the sequence data of AM fungi is over-emphasized for the identification, the conventional morphological observation is still important and should not be neglected for identification.

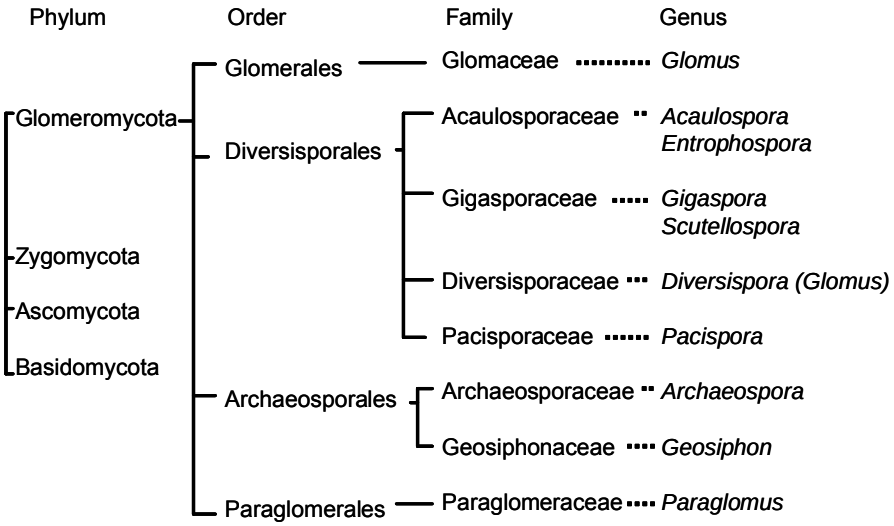


Fig. 2: Taxonomy of arbuscular mycorrhizal fungi.
http://www.tu-darmstadt.de/fb/bio/bot/schuessler/amphylo/amphylogeny.html

3.3.2. Observation of arbuscular mycorrhizal fungi in roots

Arbuscular mycorrhizal fungal structure in roots is usually not observed without appropriate staining. Freshly collected root samples should be washed gently and be free from soil particles. Ultrasonic treatment is effective to disperse soil particles closely adhered to roots.

Roots are treated with 10 % KOH solution for 30 min to 1-2 hours in a hot bath, depending on thickness of root structure. Treated roots are washed with water and treated with 2 % HCl solution. Acidified root samples are stained with 0.05 % trypan blue (or acid fuchsin) in lactic acid for 10-15 min in a hot bath or for a few hours without heating. The roots are destained with lactic acid or lacto-glycerol and are now ready for microscopic observation. The stained roots may be observed first under a dissecting microscope with transmitted illumination and then observed under a compound microscope. Fungal structures are stained and can be easily recognized.

3.3.3. Isolation of spores from soils and their observation for identification

Spores of AM fungi in soil can be collected by the wet sieving method. The gravity of spores is a little lighter than that of soil particles. Successive decantation of soil suspension followed by sieving with fine mesh can concentrate the spores from soil. Since the spores are globular or sub–globular in 50–500 µm in diameter, they, in sievings can be recognized under a dissecting microscope.

Equipments:

1) Sieve: Sieves with various mesh size. At least the following mesh size are required; 1 mm, 100 µm and 50 µm. Other sizes such as 500µm and 250µm are preferable. Stainless steel sieves are commercially available. However, it is possible to make a plastic sieve with PVP tubes and nylon mesh by yourself.
2) Fine glass pipettes: Tip of disposable glass Pasteur pipette (1 ml) is softened with flame of gas burner and is sharpened. Various sizes of tips fitting to sizes of spores can be prepared.
3) Forceps: Light fine tweezers is preferable. Tweezers Model 113SA (OHM-Werkzeuge, Germany) is the

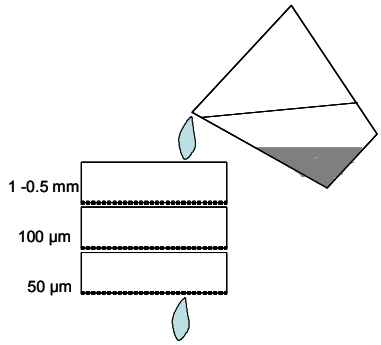
most convenient with good handling. Contact address: OHM-Werkzeuge, Flamerscheid 3a, P.O. Box 42791-41, D-42799, Leichlingen, Germany. Tips of the tweezers should be sharpened with a fine file or sandpaper and polished with abrasives.

4) Dissecting microscope: Stereoscopic zoom microscope with bifurcated illuminator of fiber arm is advisable. Transmitted illumination system is also needed.
5) Compound microscope: Biological compound microscope is needed. Nomarsky's DIC illumination system is advisable.

Procedure:

Ten to 50 g of freshly collected soil sample is put into 1 to 2 litters of plastic beakers. Usually rhizosphere soils are rich in AM fungal spores. Beaker size can be changed depending on the soil sample size. Soil is suspended with about 500 ml to 1 litter of tap water. Soil macro-aggregates should be crushed with hand. After 10-30 seconds* of settling down of soil particles, the upper layer of soil suspension is poured into the sieving (Fig. 3). The procedure should be repeated until the upper layer of soil suspension is transparent. The sievings on the fine mesh is collected into a small beaker and dispersed with ultra sonication. Weak sonication (i.e. 30W 30 sec) is enough, and strong sonication may destroy fungal spores. Then the dispersed sample is again passed through the sieve. Depending on toughness of soil aggregate, the sonication process can be repeated. Usually AM fungal spores are collected on 100 μm. Some small spores are on 50 μm. To collect large spores such as *Gigaspora margarita*, 250 μm sieve is efficient.

Fig. 3: Sieving of soil suspension.



Spores of AM fungi have characteristic shapes and colours, and so it is not difficult to discriminate the spores in organic debris collected on the sieves. However, it is recommended that those who have not yet observed the spores before should learn from the experts how the spores look like. Pictures of spores in the textbooks and the websites may be helpful to recognized AM fungal spores.

In the soil such as grassland soil rich in organic debris, it may be hard to find the spores hidden by the debris. In such a case, sucrose density centrifugation technique is often used to separate spores from the organic debris.

3.3.4. Morphological observation of spores for identification

Morphology of spores is a basis for identification of AM fungi, because the hyphae and the organs such as arbuscules and vesicles are not specific to species. Spores collected from soil often deteriorate so that they may be used only for tentative identification at genus level. For detailed observation, culturing the target AM fungus is required, and the spores from pot culture should be used. At least 30-50 spores from the same morphological spore type should be observed, and more observation is

recommended.

1) Observation of intact spores under dissecting microscope:

Spores collected from soil or culturing medium are put in a watchglass or a small Petri dish, and their shape, colour and the attachment to spores are observed. Spores should be classified into each spore type based upon morphology. For each spore type, detailed observation is conducted.

For color description, standard colour chart such as soil colour chart or "colour chart of glomalean fungi" (see INVAM web site) should be used. The colour chart should be under the same illumination as used for spore observation, because the colour itself is greatly affected by the characteristics of illumination.

It should be reminded that hyphal attachments such as sporiferous saccule for Acaulosporaceae and subtenting hyphae for Gigasporaceae are often lost during collection of spores from soil.

2) Observation of spores mounted on slide glass under a compound microscope:

Spores are mounted with polyvinyl lactoglycerol (PVLG) on a slide glass. Several slides should be made. These are for intact spores mounted with PVLG, for crushed spores mounted with PVLG, for spores mounted with PVLG containing Meltzer's reagent.

The morphological characteristic as indicated in Table 1 should be recorded. These characteristics may be helpful to identify genus of the target fungus (Fig. 4). However, some genera such as *Archaeospora* needs not only these morphological characteristics but also sequence data. For species identification, the characteristics should be compared with those in the species description in the original reference. Many species description and pictures are also available in INVAM website. Detailed information for morphological observation is available in review by Morton and his website. Species identification without enough expertise may cause trouble in scientific society, so those who wish to identify species of AM fungi should consult the experts in AM fungal taxonomy.

For spore size, at least 40-50 spores should be examined, and more examination is preferable.

PVLG (poly-vinyl lacto-glycerol):

Polyvinyl alcohol (polymerization 1000-1500), 1.66 g, is dissolved in 10ml of deionized water. Complete dissolution may need 6 hours at 80 ºC. The dissolved polyvinyl alcohol is mixed with 10 ml of lactic acid and 1ml of glycerol. It can be used more than a day after preparation.

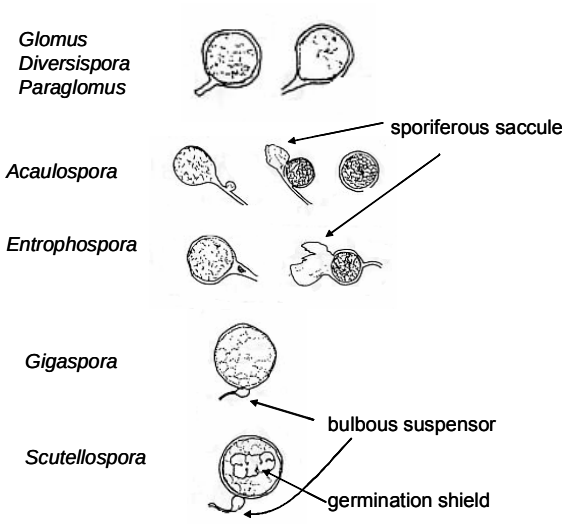


Fig. 4: Morphology of representative genera of arbuscular mycorrhizal fungi.

Table 1 Morphological character of spores of AM fungi

Shape:	(i.e. globular, spherical, irregular etc)
Size:	Globular: diameter (minimum – average – maximum) Irregular shape: length x width (minimum – average – maximum)
Colour:	(compare with Standard Colour Chart)
Hyphal attachment:	(i.e. sporiferrous saccule, bulbous suspensor etc) sporiferrous saccule = *Acaulospora, Entrophospora, Archaeospora* bulbous suspensor = *Gigaspora, Scutellospora*
Auxiliary cell:	(presence = *Gigaspora, Scutellospora*, none)
Sporocarp:	(presence, none)
Germination shield:	(presence =*Scutellospora*, absence)
Surface ornamentation:	(i.e. smooth, rough, reticulate etc)
Vesicle:	(presence or absence in mycorrhizal roots)

* These characters should be recorded with careful observation of many spores.

3.3.5. Culturing AM fungi

AM fungi need the symbiotic association with plants for proliferation. Therefore, culturing AM fungi is to inoculate AM fungi to host plant and to grow the inoculated plant. For the AM fungal inoculum, spores collected form soil can be used. However, spores in soil are not always active in colonizing plants. Therefore, trapping culture is often employed. Soil or sieving of soil is used as inoculum (Soil Trap Culture). To isolate AM fungi colonizing roots, mycorrhizal plants collected from field can be transplanted to potting medium as Plant Trap Culture (Murakoshi et al. 1998) (Fig. 5).

Potting medium: Sterile soil or soil-sand mixture is usually used. We prefer to use commercially available "Akadama-tsuchi" which is collected from subsoil of volcanic ash soil and is prepared for horticulture use (ref Saito NP or picture). Various potting materials for horticulture can be also used. However, the materials for potting medium should be low in available phosphate and preferably not rich in organic matter. In some cases the fungi isolated from some specific soils may need the specific soil properties for their growth.

Host plant: Various mycotrophic plants can be used: leguminous species (i.e. *Trifolium* spp., *Medicago* spp., *Lotus japonicus*) and grass species (i.e. *Lolium* spp., *Paspalum notatum*), and other herbaceous species (i.e. *Plantago* spp.). Onion and leek (*Allium* spp.) are also good hosts. AM fungi generally do not show host specificity but some species show host preference. Therefore, the plant species from which the target AM fungus is isolated can be used as a host plant.

Growth conditions**:** Any conditions, which support good growth of host plants, are acceptable. To avoid contamination, a growth chamber is preferable. If greenhouse is used, it should be kept clean. It should be reminded that cross-contamination or contamination from dust is inevitable under open-air conditions, even in growth chamber. To prevent cross-contamination from other pot culture in the same chamber, use of

plastic bag (SUNBAG, Sigma Co.) is advisable (Walker & Vestberg 1994).

Single spore isolation**:** To purify an isolated fungus, single spore isolation is needed. Even if the spores are morphologically identical, it often contains contaminants whose morphology is very similar. Successive pot culture of such multispore isolates would cause unexpected outbreak of the contaminant. Furthermore, even if the culture contains only one species, it may be composed of genetically diverse populations. For such a genetic studies or population genetics, the purification through single spore isolation is essential.

For single spore isolation, no specific equipment is needed. For efficient handing, two sets of dissecting microscopes are place side by side. One microscope is for picking up single spore from spores in a dish. Another is for inoculation of a spore on roots. Seedling placed in the pot is placed under another microscope. Under the first microscope, single spore is picked up and transferred under the second microscope. Under the second microscope, the spore is placed on fine roots or root tip of the seedling (Fig. 6). If culture is successful, the detailed morphological observation is required. Potting medium can be dried by stopping watering to the pot. After the host plant wilt, the dried soil containing spores can be stored for a year at 4-5°C. It is advisable that the isolated fungi are re-cultured every year. Flow of isolation and culture of AM fungi is summarized in Fig. 7.

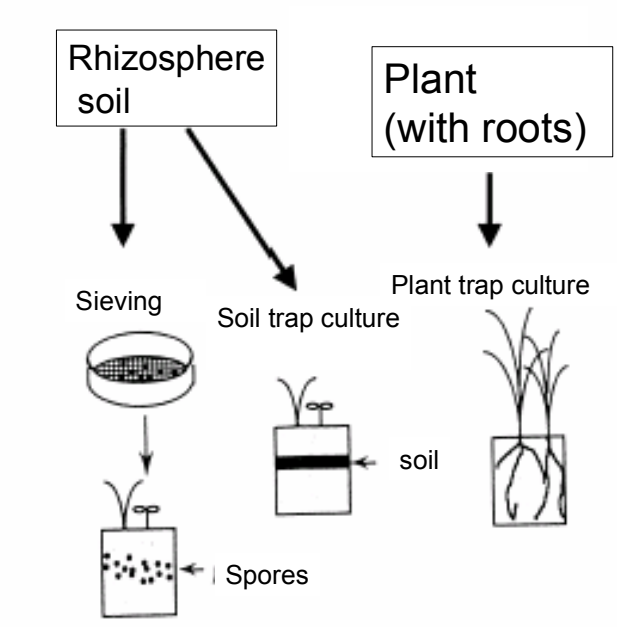


Fig. 5: Methods for trapping AM fungi.

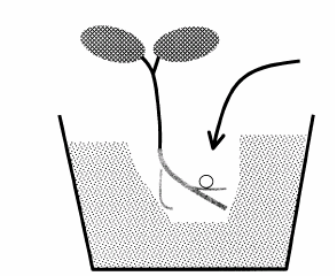


Fig. 6: Single spore isolation.

Throughout this procedure, the followings should be reminded.

1) Origin information of the isolated fungi should be recoded in detail as much as possible. (i.e. site description (latitude, elevation, vegetation, soil type, cropping history etc.), soil properties etc.)
2) At each culture step, voucher specimen of spores should be prepared and stored.

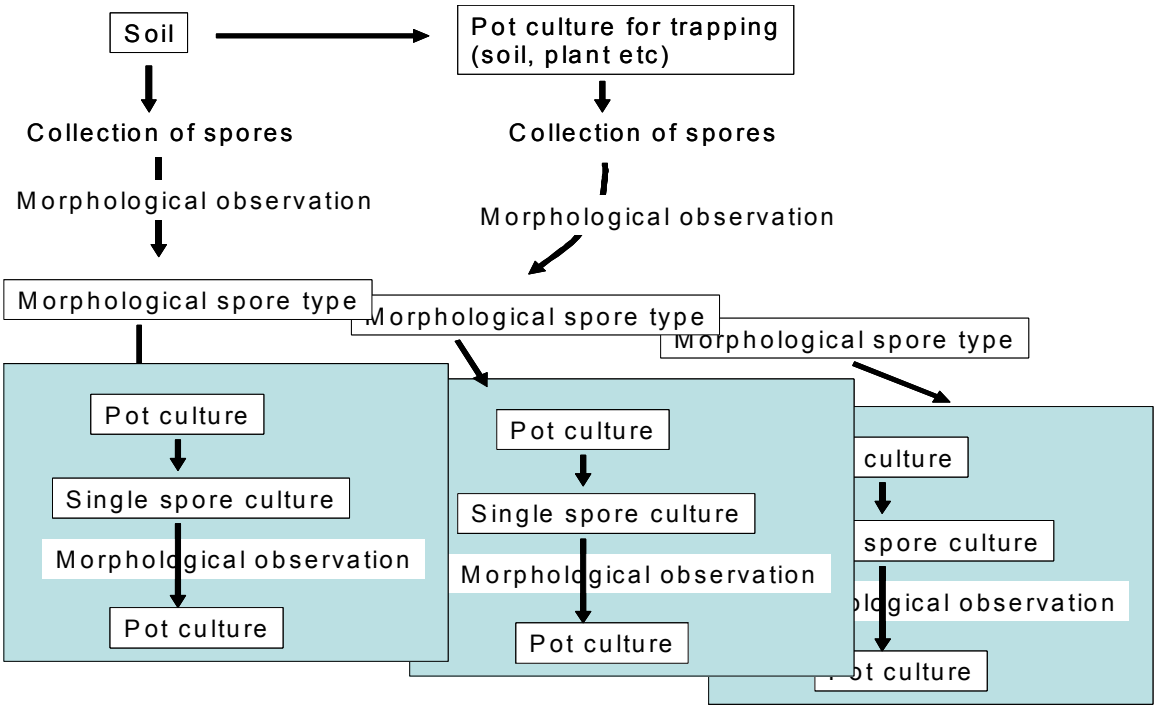


Fig. 7: Flow of isolation and culture of AM fungi.

3.3.6. DNA extraction from spores

Molecular phylogenetic information is essential for taxonomy of AM fungi. Sequence data for conserved genes such as rRNA is obtained by PCR amplification followed by sequencing with DNA extracted from spores. Many protocols for DNA extraction are reported. One of the protocols which we have used is described.

Cleaning of spores:

Clean spores are collected with tweezers or fine Pasture pipette. Spores should wash several times with sterile water with sonication for 10-20 seconds. If spores show water repellency, surfactant such as Tween 80 can be used. Microplate with 6 or 12 wells is convenient for successive washing.

Crushing spores:

We use a cap of Eppendorf tube. The cap is cut from the tube and placed upside down on the stage of a dissecting microscope. Twenty µl of Instagene (Biorad) is added to the cap. A spore or spores are put into the cap and crushed thoroughly with a micro-pestle or any fine rod. The tube itself is put on the cap with crushed spores. Then the tube is centrifuged for while for spinning down the reagent with crushed spores from the cap into the tube.

Extraction of DNA:

Follow the instruction provided by manufacture. Further purification of DNA with ethanol/chloroform precipitation is sometimes needed to remove inhibitor for PCR amplification.

PCR amplification and DNA sequencing:

Conditions of PCR amplification depend on the primers you will use. AM fungi are multi-nuclear organisms and often show polymorphisms in their sequence. It is advisable to sequence several clones from

the target fungus and to check the phylogenetic position of the sequence by constructing phylogentic tree if the target genes are located within a reasonable clade of the tree (Sawaki et al. 1998).

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3. Saito, M. & Marumoto, T. (2002) : Inoculation with arbuscular mycorrhizal fungi: the status quo in Japan and the future prospects. *Plant Soil* 244: 273-279.
4. Sawaki, H., Sugawara K. & Saito M. (1998) Phylogenetic position of an arbuscular mycorrhizal fungus, *Acaulospora gerdemannii*, and its synanamorph *Glomus leptotichum*, based upon 18S rRNA gene sequence. *Mycoscience* 39: 477-480.
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3.4. Inoculant Production

3.4.1. Preparation of trap culture inoculum of arbuscular mycorrhizal fungal spores

The following text is adapted from INVAM publication. For the establishment of inoculum from monospecific cultures trapping of healthy arbuscular mycorrhizal fungi (AMF) spores is often a necessity. Spore trap cultures can also aid in AMF identification. AMF spores sampled directly from a field plot may appear healthy but are not viable. The spores may appear differently, due to weathering and intrinsic soil environment effect, either physical, chemical or biological. Trap cultures are important in the following situations:

- When AMF colonization is high in roots of a plant community, but with little or no spores produced, especially in arid and hydric soil conditions.
- Where the soil has high microbial activity, especially in tropical environments, with relatively high temperature and humidity moisture). Organic matter content at these sites can be high. Under these environments, AMF spores may physically transform resulting in difficulty in species identification.
- To gather abundant healthy spores of different species and establish monospecific cultures for specific purposes.

Procedures

i. Rhizosphere soil is collected, with shoots of trap plant cut at the crown, and roots are finely chopped and mixed with the soils using a sharp chopper.
ii. The chopped roots and soil are mixed 1:1 (v/v) with autoclaved coarse sand in a mechanical mixer, or massaged well in a durable plastic bag.
iii. The soil mix is then transferred to a 15 cm plastic pot.
iv. Plant seeds of suitable trap plant such as tropical signal grass, *Brachiaria decumbens*, into the pot.
v. The pot cultures are maintained in a greenhouse for at least 3 months, and check sporulation from time to time. By the fourth month AMF sporulation may be at the peak. Sanitary tests may also be carried out to ensure no contamination from parasitic fungi occurs.
vi. Keep fertilizer application to a minimum, to encourage AMF proliferation.
vii. Trap culture pots are later left to dry under shade for up to 2 weeks.
viii. Harvest the spores using the sieving and decanting techniques or the density-gradient centrifugation technique.
ix. The monospecific spores are ready for inoculation onto seedlings of the desired crops.

3.4.2. Inoculation of AMF

Two weeks before spore inoculation, the desired seedlings (e.g. oil palm, vegetable, pasture grass) are prepared in suitable containers filled with sandy loam soil.

i. The seedlings are gently uprooted singly on in a small bunch, and have a gentle stream of water sprayed onto the roots so that they stick together.
ii. Spores collected from 3.3.1 are suspended in water and about 200 µl of the spore suspension are pipetted onto the moist roots.
iii. The inoculated seedlings are immediately transplanted into containers of suitable size, containing sterilized soil.
iv. The soil is topped with a sterile growth medium, watered gently under shade, before transferring into the greenhouse.
v. To encourage colonization of AMF onto seedling, fertilizers are not given during the early growth stage of the seedlings.

3.4.3. Problems and potential for AMF inoculum production and utilization

i. Situations where effective indigenous AMF population is low.
ii. Inoculation is best for transplanted crops, where soil disturbances has reduces AMF inoculum potential.

3.5. Inoculant Application

1. Application rate of VA mycorrhiza biofertilizer is 10 g or 1 spoonful per plant.
2. VA mycorrhiza biofertilizer can be used at any stage of plant growth. However, for maximum benefits it should be applied during seedling stage or placed at the base of plant hole before planting. After two weeks of application, other suitable fertilizers can be applied.
3. For planting by stem cutting, the growing media are mixed with VA mycorrhiza biofertilizer prior to planting. The cutting stocks can be transferred to field one month after roots have developed.
4. For transplanting, simply sprinkle VA mycorrhiza biofertilizer adjacent to the plant roots and cover with soil.
5. For grown trees, soil under the plant canopy is trenched or the leaf litter under the tree is removed. About 10 g (1 spoonful) per plant of VA mycorrhizal biofertilizer is applied to the root hair system and then covered with soil.
6. VA mycorrhizal biofertilizer can be used in combination with several types of biofertilizers (e.g.*Rhizobium* biofertilizer, or PGPR).

3.6. Preservation and Precautions

1. Mycorrhizal biofertilizer can be kept under shade at room temperature. Normally AM fungi can live for 1-5 years, depending on the species.
2. Avoid using VA mycorrhizal biofertilizer on plants with root rot or stem rot. Mycorrhizal biofertilizer is more useful when applied prior the infection.
3. Avoid using VA mycorrhiza biofertilizer with some chemical products such as fosetyl, metalazyl and metalaxyl mancozeb since these substances can inhibit growth of VA mycorrhizal fungi.

References

Text books and other useful web-sites:

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2. Sieverding, E. *Vesicular-Arbuscular Mycorrhizal Management in Tropical Ecosystems,* Deutche Gesllshaft für TEchnische Zusammnearbeit (GTZ) GmbH (1991)
3. Norris, J.R., Read, D.J. and Varma, A.K. (ed.) *Methods in Microbiology, Vol. 24, Techniques for the Study of Mycorrhiza,* Academic Press (1992)
4. Brundrett, M. et al. *Working with Mycorrhizas in Forestry and Agriculture,* Australian Centre for International Agricultural Research (1996).
5. *Working with Mycorrhizas in Forestry and Agriculture* http://www.ffp.csiro.au/research/mycorrhiza/
   Various methodologies and many beautiful pictures are available.
6. International Culture Collection of Arbuscular and Vesicular-Arbuscular Mycorrhizal Fungi
   http://invam.caf.wvu.edu/
7. Taxonomy and species description are available. Representative isolates are distributed upon request.
   The International Bank for the Glomeromycota
   http://www.kent.ac.uk/bio/beg/englishhomepage.htm
8. GINCO (Glomeromycota in vitro collection)
   http://res2.agr.ca/ecorc/ginco-can/index_e.htm
9. Microorganisms Section of the NIAS Genebank,
   National Institute of Agrobiological Scineces (NIAS), Japan
   http://www.gene.affrc.go.jp/micro/index.html
10. These sites provide various information and their isolates are distributed upon request.
    Mycorrhiza Information Exchange
    http://mycorrhiza.ag.utk.edu/
11. The Mycological Society of Japan
    http://wwwsoc.nii.ac.jp/msj7/english/index_e.html

4. Phosphate Solubilizers

4.1. Isolation of Microbial Strains

A considerable number of bacterial species are able to exert a beneficial effect upon plant growth. Mostly they are associated with the plant rhizosphere, so they are called as rhizobacteria. This group of bacteria has been termed plant growth promoting rhizobacteria, and among them are strains from genera such as *Alcaligenes, Acinetobacter, Arthrobacter, Azospirillum, Bacillus, Burkholderia, Enterobacter, Erwinia, Flavobacterium, Paenibacillus, Pseudomonas, Rhizobium, and Serratia*. They are used as biofertilizers or control agents for agriculture improvement, and there are numerous researchers for the area with the agricultural environment conservation.

Phosphorus is second only to nitrogen in mineral nutrients most commonly limiting the growth of crops. Phosphorus is an essential element for plant development and growth making up about 0.2 % of plant dry weight. Plants acquire P from soil solution as phosphate anions. However, phosphate anions are extremely reactive and may be immobilized through precipitation with cations such as Ca^{2+}, Mg^{2+}, Fe^{3+} and Al^{3+}, depending on the particular properties of a soil. In these forms, P is highly insoluble and unavailable to plants. As the results, the amount available to plants is usually a small proportion of this total.

Several scientists have reported the ability of different bacterial species to solubilize insoluble inorganic phosphate compounds, such as tricalcium phosphate, dicalcium phosphate, hydroxyapatite, and rock phosphate.

4.1.1. Mechanisms of phosphate solubilization

The principal mechanism for mineral phosphate solubilization is the production of organic acids, and acid phosphatases play a major role in the mineralization of organic phosphorus in soil. It is generally accepted that the major mechanism of mineral phosphate solubilization is the action of organic acids synthesized by soil microorganisms. Production of organic acids results in acidification of the microbial cell and its surroundings.

The production of organic acids by phosphate solubilizing bacteria has been well documented. Gluconic acid seems to be the most frequent agent of mineral phosphate solubilization. Also, 2-ketogluconic acid is another organic acid identified in strains with phosphate solubilizing ability (Table 1).

Table 1. Microbial strains producing organic acid

Organic acid	Strains
Gluconic acid	*Pseudomonas* sp., *Erwinia herbicola, Pseudomonas cepacia, Burkholderia cepacia*
2-Ketogluconic acid	*Rhizobium leguminosarum, Rhizobium meliloti, Bacillus firmus*

Strains of *Bacillus* were found to produce mixtures of lactic, isovaleric, isobutyric and acetic acids. Other organic acids, such as glycolic, oxalic, malonic, and succinic acid, have also

been identified among phosphate solubilizers. Strains from the genera *Pseudomonas*, *Bacillus* and *Rhizobium* are among the most powerful phosphate solubilizers (Rodriguez et al. 1999. Biotechnology Advances 17: 319-339).

Chelating substances and inorganic acids such as sulphideric, nitric, and carbonic acid are considered as other mechanisms for phosphate solubilization. However the effectiveness and their contribution to P release in soils seems to be less than organic acid production.

4.1.2. Isolation of mineral phosphate solubilizer

Detection and estimation of the phosphate solublization ability of microorganisms have been possible using plate screening methods. Phosphate solubilizers produce clearing zones around the microbial colonies in media. Insoluble mineral phosphates such as tricalcium phosphate or hydroxyapatite are contained in the media.

Also the bromophenol blue method that produce yellow halos following pH drop through the release of organic acids is more reproducible and has greater correlation in comparison with the simple halo method. However, clearing zones on agar plate method is generally used (Fig. 1). Pikovskays's medium (Table 2) is a general medium for selection of phosphate solubilizer.

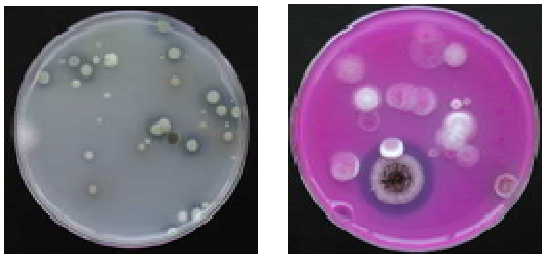

Fig. 1: Phosphate solubilizer forming clear zone.
Bacteria (left), Fungi (right)

Table 2. Composition of medium for phosphate solubilizers
(Pikovskays's medium)

Components	Amounts (g l^{-1})
Glucose	*10*
$Ca_3(PO_4)_2$	*5*
$(NH_4)_2SO_4$	*0.5*
NaCl	*0.2*
$MgSO_4$.7H2O	*0.1*
KCl	*0.2*
Yeast extract	*0.5*
$MnSO_4$. H_2O	*0.002*
$FeSO_4.7H_2O$	*0.002*
pH	*7.0*

4.2. Inoculant Production

Microorganisms with potential as plant growth promoters have been used to produce inoculants. Potential materials that are able to support good growth and survival of bacteria are needed in inoculant production. Many materials have been evaluated, including different types of coals, bentonite, corn oil, mineral soils, peat, peat moss, vermiculite, and perlite. Peat is commonly used material for inoculant carrier. Finely ground peat is most commonly used in conventional inoculant production. However, peat is not always available, because some peat may inhibit growth of some *Rhizobium* strains. There have been reports on the difficulty of obtaining autoclaved or gamma-irradiated peat as carriers. The high temperature during steam sterilization or the high dosage needed for irradiation might generate toxic substances for bacteria. However, perlite can be easily sterilized with no risk of producing toxic substances, because it is a volcanic stone, composed of a little-hydrated aluminium silicate.

The agronomic use of agro-wastes as substrates causes changes in the soil affecting its physico-chemical characteristics and microbial activity in the rhizosphere. The breakdown of such materials to simple sugars provides energy sources for heterotrophic microorganisms such as P-solubilizing and nitrogen fixing bacteria. Normally, the growth and metabolic activity of soil microorganisms are limited by the availability of nutrients. From this reasons, several kinds of agro wastes such as rice straw compost will be good carrier materials for the inoculants and improver of soil condition as following case study. However, the peat moss with phosphate solubilizer showed good survival of inoculants and effects on crops.

4.2.1. Cultivation

○ Media: proper media for inoculant (nutrient broth, yeast extract broth etc.)

○ Incubation condition: temperature, light, incubation period

In case of *Bacillus* sp.
-Nutrient broth for 3days at 27±1℃ on rotary shaker at 150 rpm.

4.2.2. Collection

○ After incubation period, collect microbial cells by centrifuge

○ Wash cells with distilled water or diluted saline solution

In case of *Bacillus* sp.
-Centrifuge 10 minutes at 4000-5000 rpm (Fig.2)
-Wash cultures with sterilized tap water
-Re-centrifuge washed cultures 10 minutes at 4000-5000 rpm

Fig. 2: Cultures centrifuged

4.2.3. Formulation of inoculants using carrier materials

If it is necessary, mix cells using carrier materials such as peat, vermiculite, perlite etc.

4.3. Inoculant Application

4.3.1. Inoculation method of phosphate solubilizer

Generally biofertilizers in powder form are applied as for organic matters onto the soil. This type is very convenient for users on the management of biofertilizer. Some biofertilizers are costly products for farmers, so their use would be restricted on the specific condition of agronomy.

Microorganisms are generally supplied by producers of biofertilizer, so it would only necessary that the users or farmers follow the application method prepared by manufacturers. However, the popular application method is regarded as next procedure (Fig. 3).

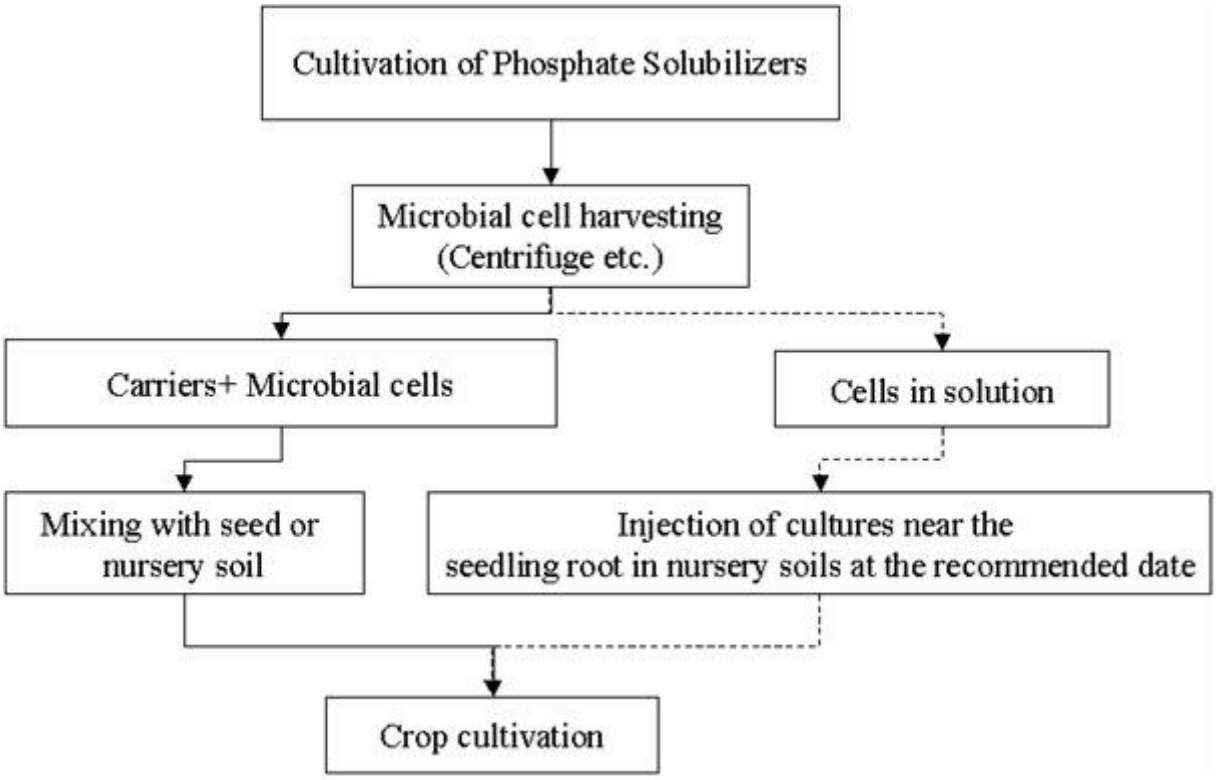

Fig. 3: Inoculation method of phosphate solubilizers.

4.3.2. Improvement of phosphate solubilizers

An alternative approach for the use of phosphate solubilizing bacteria as microbial inoculants is the use of mixed cultures or co-inoculation with other microorganisms. This evidence points to the advantage of the mixed inoculations of PGPR strains comprising phosphate solubilizing bacteria.

The effect of a combined inoculation of *Rhizobium*, a phosphate solubilizing *Bacillus megaterium* sub sp. *phospaticum* strain-PB and a biocontrol fungus *Trichoderma* spp. On growth, nutrient uptake and yield of chickpea were studied under glasshouse and field conditions. Combined inoculation of these three organisms showed increased germination, nutrient uptake, plant height, number of branches, nodulation, pea yield, and total biomass of chickpea compared to either individual inoculations or an uninoculated control (Rudresh et. al, 2004.).

On the other hand, it has been postulated that some phosphate solubilizing bacteria behave as mycorrhizal helper bacteria. It is likely that the phosphate solubilized by the bacteria could be more efficiently taken up by the plant through a mycorrhizal pipeline between roots and surrounding soil that allows nutrient translocation from soil to plant. Considerable evidence supports the specific role of phosphate solubilization in the enhancement of plant growth by phosphate solubilizing microorganisms. However, not all laboratory or field trials have offered positive results. Therefore, the efficiency of the inoculation varies with the soil type, specific cultivars, and other parameters.

4.3.3. Case study of improvement of soil condition for phosphate solubilizers on the fifty-years long-term experiment in rice paddy soils in Korea

A considerably higher concentration of phosphate solubilizing bacteria is commonly found in the rhizosphere soil. Also the fungal genera with this capacity are *Penicillium* and *Aspergillu* (Suh, et al., 1995; Whitelaw et al., 1999). A continued exploration of the natural biodiversity of soil microorganisms and the optimization and manipulation of microbial interactions in the rhizosphere of crops represents a prerequisite step to develop more efficient microbial inoculants. Also, it is desirable to find methods to manage indigenous microbes in soil for sustainable agriculture. The answer may be obtained from diverse field tests managed for improvement of soil condition. A case study for phosphate solubilizers has been conducted on a fifty-year long-term experiment in rice paddy soils in Korea.

4.3.3.1. Changes in physical properties of soils

An investigation was made on the effect of different soil amelioration on the physical and chemical properties of soil, the yield or quality of rice, and the activity of soil microbes in the plot where NPK, lime, and compost had been applied for fifty years.

The physical properties were most significantly affected by the application of compost, which reduced the bulk density and hardness of soil and increased the liquid and gas phase of soil

and cation exchange capacity of soil (Table 3).

Table 3. Effect of materials on physical properties of paddy soils

Treatment	Bulk density ($g\ cm^{-3}$)	Phase distribution (%)			CEC ($Cmol^{+}kg^{-1}$)	Hardness (㎜)
		Solid	Liquid	Gas		
Control	1.39	52.5	29.8	17.7	7.8	13.8
NPK	1.25	47.1	26.4	26.5	9.0	14.0
NPK+C	1.19	44.7	28.3	26.9	11.2	13.0
NPK+L	1.23	46.4	24.7	28.9	8.8	14.0
NPK+S	1.20	45.2	24.0	30.8	9.0	14.8
NPK+C+L+S	1.16	43.6	28.3	28.1	10.8	12.8

L; Lime, C; Compost, S; Silicate

4.3.3.2. Changes in chemical properties of soils

The application of compost tended to increase organic matter, available phosphorus and silicate, exchangeable cations in the soil (Table 4).

Table 4. Effect of materials on chemical properties of paddy soils

Treatment	pH (1:5)	OM ($g\ kg^{-1}$)	Av.P_2O_5 ($m\ kg^{-1}$)	Ex. ($cmol^{+}kg^{-1}$)			Av.SiO_2 ($㎎\ kg^{-1}$)
				K	Ca	Mg	
Control	6.0	18	40	0.10	5.0	0.9	89
NPK	5.7	21	194	0.12	4.7	0.9	44
NPK+C	5.7	32	241	0.11	5.7	1.1	47
NPK+L	6.6	23	166	0.12	5.9	1.5	51
NPK+S	6.6	20	167	0.09	7.7	1.2	256
NPK+C+L+S	7.0	33	270	0.12	10.0	1.6	386

L; Lime, C; Compost, S; Silicate

4.3.3.3. Changes in microbial diversity of soils

Diversity is an important concept in ecology, often in environmental monitoring and conservation management. But diversity is an intuitively obvious concept that is difficult to define and quantify. There are two parts to diversity: the number of species and the evenness of the distribution. A sample with more species is more diverse: while an evenly distributed community is more diverse than an unevenly distributed community with the same number of species. However, microbial communities are severely affected by agricultural managements such as tillage, and organic materials and chemicals for soil amendment.

A pattern was revealed from the long-term experiment showing different profiles of the ratio of functional species to bacteria. The ratios of fluorescent bacteria, Bacillus and Gram-negative bacteria to total aerobic bacteria in CLAPK (compost + lime + fertilizer) and CAPK (compost + fertilizer) plots were higher than other treatments. This result represents effect of amendments on soil microbial community (data not shown). Nevertheless microbial diversity was simplified, the functional groups were triggered by the application of substances to soil.

Apart from fertilization and enzymatic decomposition of organic compounds, microbial P-mobilization would be the only possible way to increase plant-available phosphorus. Many species of bacteria are able to solubilize phosphates in vitro and some of them can mobilize P in plants.

The phosphate solubilizing activity is significantly affected by material input. High positive correlation was observed between the phosphate solubilizing bacteria and organic matter contents in soils. The treatment of the agricultural materials is presented in Table 5. The available phosphorus contents ranged from 40 to 105 mg. FLC contained highest amount of available phosphorus than control. FLC showed high ratio of PB/TB between treatments. This means that lime and compost used as soil improver had positive effects on phosphate solubilizers.

Table 5. Effect of materials on available phosphorus and PB/TB ratio in paddy soils

Treatment	PH (1:5)	Av.P_2O_5 (mg kg^{-1})	PB/TB ratio (%)
Control	6.0	40	0.6
F	5.6	73	2.2
FL	6.3	73	5.0
FC	5.6	87	6.3
FLC	6.1	105	16.8

F; fertilizer (plot of ammonium sulfate and/or urea), FL; fertilizer + lime, FC; fertilizer + compost, FLC; fertilizer + lime + compost, PB/TB; phosphate bacteria/total aerobic bacteria

Modern agricultural practices largely rely on high inputs of mineral fertilizers to achieve high yield. These practices are now being reevaluated and are coming under increased scrutiny as our awareness of environmental conservation from the impact of chemical overuse. This research is very necessary to improve soil condition on environmentally friendly agriculture that is being practiced at present.

Also we should imagine that improvement of soil condition includes activation of functional microbes related to specific materials input. As shown in Table 6, diversity of phosphate solubilizers in soils was highly controlled by the agricultural management. Only three types of treatment are shown in the table. But as was commented earlier, compost directly affected microbial community in soils.

Table 6. Effects of amendment on phosphate solubilizer in soils

Treatment	Species of isolate	Number of isolate	
Chemical Fertilizer	*Bacillus simplex*	1	4
	Bacillus megaterium	2	
	Pseudomonas chlororaphis	1	
Lime + fertilizer	*Bacillus pumilis*	2	5
	Pseudomonas chlororaphis	1	
	Pseudomonas coronafaciens	1	
	Arthrobacter globiformis	1	
Compost + fertilizer	*Bacillus pumilis*	3	13
	Bacillus licheniformis	2	
	Pseudomonas fluorescens	4	
	Bacillus marinus	1	
	Bacillus subtilis	1	
	Bacillus lentimorbus	1	
	Pseudomonas chlororaphis	1	

4.3.3.4. Effects of organic matters on nutrient absorption of rice plants

Organic matter results in several benefits such as better soil structure that provides a more suitable medium for plant growth, supplies nutrients for the plant and also help to build up target microorganisms. The combined use of organic matter with inoculant in the build up of high bacterial populations that improve plant growth.

Effect of materials on amount of nutrient absorbed by rice is presented in Table 7. It is possible to suggest that the only driving force to dissolve phosphate is microbial activity, especially phosphate solubilizing bacteria, from the results shown in the table.

Table 7. Effect of materials on amount of nutrient absorbed by rice cultivated in paddy soils (unit: $kg\,ha^{-1}$)

Treatment	T-N	P_2O_5	K_2O	CaO	MgO
Control	46.4(50)	33.2(60)	76.4(63)	22.9	12.0
NPK	93.4(100)	54.9(100)	120.4(100)	38.0	23.4
NPK+C	107.5(115)	64.9(118)	161.9(134)	41.9	28.6
NPK+L	89.7(96)	52.5(96)	124.3(103)	36.7	22.9
NPK+S	94.0(104)	53.8(98)	137.1(114)	37.7	25.0
NPK+C+L+S	106.3(114)	63.1(115)	164.0(136)	35.4	26.0

L; Lime, C; Compost, S; Silicate

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V. Quality Control of Biofertilizers

1. General Concept of Quality Control

1.1. Definition of Biofertilizer

Biofertilizer is still an unclear term. It can be easily found that biofertilizers are identified as plant extract, composted urban wastes, and various microbial mixtures with unidentified constituents, and chemical fertilizer formulations supplemented with organic compounds. Likewise, the scientific literature has a very open interpretation of the term biofertilizer, representing everything from manures to plant extracts.

However biofertilizer is most commonly referred to the use of soil microorganisms to increase the availability and uptake of mineral nutrients for plants. So it is necessary to define the term "biofertilizer". There is a proposal that "biofertilizer" be defined as a substance which contains living microorganisms which colonizes the rhizosphere or the interior of the plant and promotes growth by increasing the supply or availability of primary nutrient and/or growth stimulus to the target crop, when applied to seed, plant surfaces, or soil.

Whether the existence of a microorganism increases the growth of plants by making nutrients more available or replacing soil nutrients or increasing plant access to nutrient, as long as the nutrient status of the plant has been enhanced by the microorganisms, the substance that was applied to the plant or soil containing the microorganisms, can be characterized as a biofertilizer. This definition separates biofertilizer from organic fertilizer containing organic matter.

1.2. Microbial Functions Newly Recommended as Biofertilizer

Numerous species of soil bacteria which flourish in the rhizosphere of plants, but which may grow in, on, or around plant tissues, stimulate plant growth. These bacteria are collectively known as plant growth promoting rhizobacteria (PGPR).

Some PGPR appear to promote growth by acting as both biofertilizer and biopesticides. The search for PGPR and investigation of their modes of actions are increasing at a rapid pace as efforts are made to exploit them commercially as biofertilizers.

Modes of PGPR action include fixing N_2, increasing the availability of nutrients in the rhizosphere, positively influencing root growth and morphology, and promoting other beneficial plant-microbe symbiosis. The combination of these modes of actions in PGPR is also addressed, as well as the challenges facing the more widespread utilization of PGPR as biofertilizers (Vessey. 2003. Plant and soil).

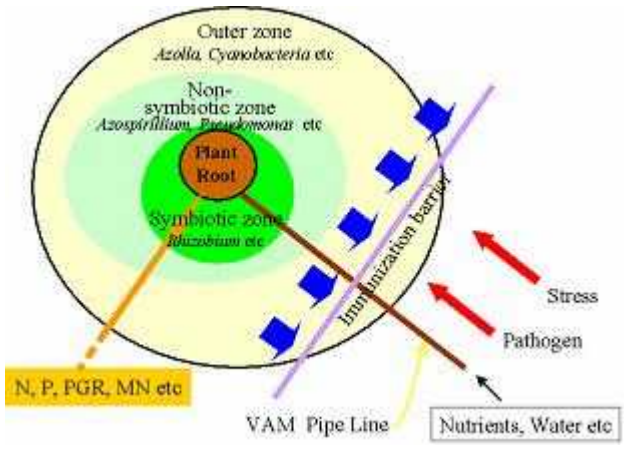

Fig. 1: Integrated microbial actions in soils.

There are two types of materials for agriculture, namely fertilizer or pesticide. It can be said that fertilizer is food, and pesticide is medicine for plants in conventional agriculture. On the other hand, biofertilizer and/or biopesticide are referred to each of them respectively in sustainable or environmentally friendly system (Fig. 1).

We have been interested that microorganisms mainly nitrogen fixer, phosphate solubilizer, and mycorrhizae are main sources for biofertilizer. As functional foods, restoratives and/or adjuvant are sometimes needed for human health care; plant growth promoting rhizobacteria may be one of interchangeable substances for crops.

There are several limitations to the use of biofertilizer for agricultural system. Primarily, efficacy is not reliable for most biofertilizer. This is because the mechanism of action of the biofertilizer in promoting growth is not well understood. However, research into biofertilizer is increasing, attempting to deal with these issues.

Research needs also to be conducted determining if and how variations in soil type, managements practices, and weather effect on biofertilizer efficacy. Furthermore, there is a block in biofertilizer development. It is difficult to test inoculant in field as routine experiments, as shown in Figure 2.

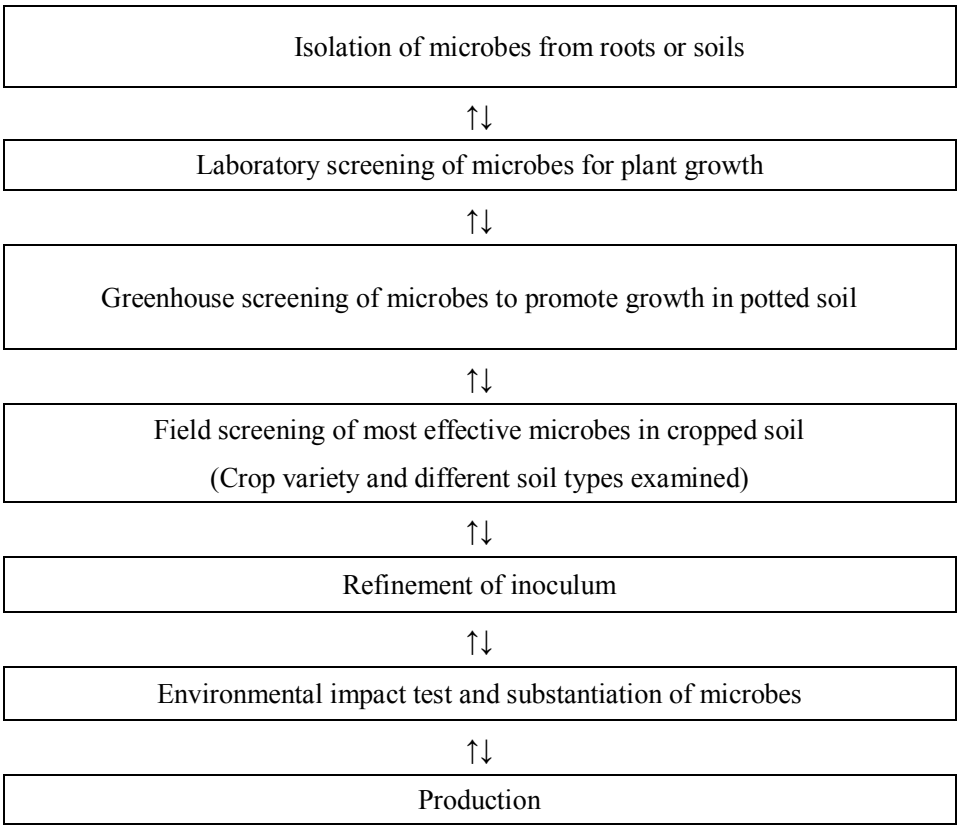

Fig. 2: Experimental process for biofertilizer.

1.3. Properties of Microbial Products

The microorganisms used for microbial products are bacteria of *Bacillus, Pseudomonas, Lactobacillus*, photosynthetic bacteria, nitrogen fixing bacteria, fungi of *Trichoderma* and yeast. Among the

microbes, the major species used for the inoculants is endospore-forming *Bacillus*. Usually, several species of microbes are used in microbial products with an available period of by- products of about 1~2 and/or 2~3 years.

Microbial products can be solid or liquid in form. Carriers used in solid type of microbial products are clay mineral, diatomaceous soil, and white carbon as mineral; rice, wheat bran, and discarded feed as organic matter. However, clay mineral and rice bran are most often used as carriers. Oftentimes, the effects of carriers and/or supplements are understood to represent the function of microbial products. It is important to seriously consider the control in the use of microbial products. In fact, farmers occasionally misunderstood this carrier effect as microbial action.

As manifested by producers, microbial products stimulate plant growth, decrease pest incidence, stimulate composting and ameliorate the soil. Among these functions, plant growth stimulation was often the main effect. However, there are instances when about 40% of the items are declared having multiple effects.

Quality of biofertilizer is one of the most important factors resulting in their success or failure and acceptance or rejection by end-user, the farmers. Basically, quality is meaning the number of selected microorganism in the active form per gram or milliliter biofertilizer. Quality standards are available only for *Rhizobium* in different countries. Specifications of biofertilizer are differ from country to country and maybe contain parameters like the microbial density at the time of manufacture, microbial density at the time of expiry, the expiry period, the permissible contamination, the pH, the moisture, the microbial strain, and the carrier. Quality has to be controlled at various stage of production (during mother culture stage, carrier selection, broth culture stage, mixing of broth and culture, packing and storage). Main parameters of biofertilizer in China are follows:

Forms	Liquid	Powder	Granular
Appearance	Without strange smell	Brown or black	Brown
Living target bacteria			
Fast-growing Rhizobium	$>0.5\times10^9$/ml	$>0.1\times10^9$/g	$>0.1\times10^9$/g
Slow-growing Rhizobium	$>1.0\times10^9$/ml	$>0.2\times10^9$/g	$>0.1\times10^9$/g
N fixation bacteria	$>0.5\times10^9$/ml	$>0.1\times10^9$/g	$>0.1\times10^9$/g
Si bacteria	$>1.0\times10^9$/ml	$>0.2\times10^9$/g	$>0.1\times10^9$/g
P bacteria			
Organic P	$>0.5\times10^9$/ml	$>0.1\times10^9$/g	$>0.1\times10^9$/g
Inorganic P	$>1.5\times10^9$/ml	$>0.3\times10^9$/g	$>0.2\times10^9$/g
Multi-strain bio-fertilizer	$>1.0\times10^9$/ml	$>0.2\times10^9$/g	$>0.1\times10^9$/g
Water content (%)		20-35	10
Size (ϕmm)		0.18	4.5
Organic matter (%C)		>20	>20

pH	5.5-7.0	6.0-7.5	6.0-7.5
Non-target bacteria Contamination (%)	<5	<15	<20
Valid period	>6 months	>6 months	>6 months

1.4. Quality Management

Quality management is very essential, and must be performed continually to control the microbial products in favor of the customers.

The guidelines used for evaluating quality are limited to the density of the available microorganisms and viability and preservation of the guaranteed microorganisms. It is important to set control plots that do not contain available microorganisms, but whose other compositions are the same as the final microbial products. Also it is highly desirable that the biofertilizer manifests the major effects for quality management of the final biofertilizer products. The major effects are used as indicators for the biofertilizer. Also, the effects are included as guaranteed activities of the biofertilizer.

It is an indispensable requirement to distinguish between the available microorganisms and the supplementary compositions on the effects of the biofertilizer guaranteed by the suppliers. If the final results of the two experimental plots are the same or cannot be confirmed statistically, then the product is only an organic matter.

This means that the effects of microbial products have to originate from the guaranteed microorganisms, and the target of the matters should be presented in details as a prescription. It is essential to evaluate precisely the functions under the given usage manifested by the applicant (fig. 3)

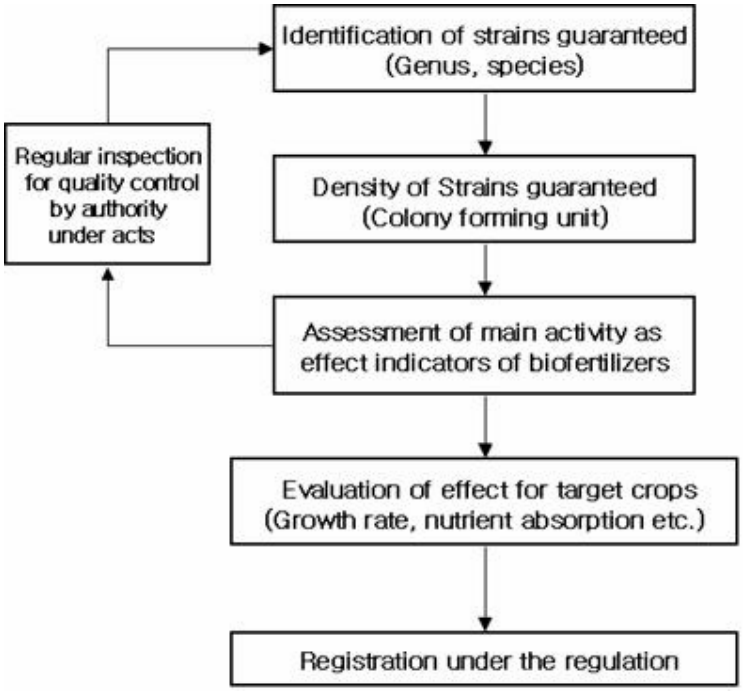

Fig. 3: Procedure of biofertilizer quality control.

Biofertilizers, known as microbial products, act as nutrient suppliers and soil conditioners that lower agricultural burden and conserve the environment. Good soil condition is imperative to increased crop production, as well as human and/or animal health welfare. Thus, the materials used to sustain good soil condition, are treated as environmental matters. However, as mentioned earlier, there are still some problems to be met on the use of microbial products. More precise quality control must be made in favour of the customers. With this in mind, we will do our best to develop better production techniques and to improve the management system for microbial products.

Although the effects of biofertilizers are different among nations due to variances in climate and soil conditions, the importance of biofertilizer on environmental conservation in the 21st century, must not be ignored. In the same manner, various biotechnologies should be accepted for increasing the biofertilizer effects with concern for the environment.

Biofertilizers lessen the environmental burden emanating from the chemical compounds. Our viewpoints on biofertilizers are the same for biocontrol and bioremediation, because we are members of an ecosystem related to the world wide web of foods.

2. Procedures for Quality Control of Biofertilizer

2.1. *Rhizobium*

Quality checks on *Rhizobium* biofertilizer can be divided into three parts:

1. Mother culture test
2. Broth test
3. Peat test

2.1.1. Mother culture test

Before producing *Rhizobium* biofertilizer, the mother culture should be checked on the following:

1.1 Growth

1.2 Purity

1.3 Gram stain

Growth

By streaking a mother culture on yeast mannitol + congo red agar (YMA) plates, checking the growth of rhizobia. Fast–growing rhizobia colonies will appear in 3-5 days, and a slow–growing rhizobia will appear in 5-7 days.

Purity

Check purity by streaking culture on glucose peptone agar plate, and incubate for 24 hours at 30 °C. No growth or poor growth should be obtained on GPA. Good growth and color changes can be expected from contaminants.

Gram stain

A loop of mother culture is checked by Gram staining. Rhizobial cell is Gram–negative, retains safranin color. Cells should appear red and not violet when observed under the microscope.

2.1.2. Broth test

The following qualities of the broth samples must be checked to make sure that the broths are in good condition:

2.1 pH

2.2 Staining

2.3 Optical density

2.4 Total count

2.5 Viable number

pH

Slow–growing rhizobia such as rhizobia for soybeans, mungbean and peanut produce a little basic compounds. After incubation, the pH will increase. (example, pH before growing = 6.0, after growing pH = 6.1 – 6.2). If broth pH decreases, it means some contaminants occur; lower pH indicates presence of contaminants.

Staining (Gram stain or Fuchsin stain)

Rhizobial cells are stained for observation of shape and size of the cells. Cells of rhizobia are rod–shaped, with one or two cells sticking together. They do not appear in long–chain. Long–chained cells are indicative of contaminants.

Gram-stained cells should appear red, not violet. Fuchsin staining is an easier and faster method. Rhizobial cells can be routinely checked using Fuchsin stain.

Gram stain

Solutions	Reaction and appearance of bacteria	
	Gram–positive	Gram-negative
I. Crystal violet (CV)	Cells stain violet	Cells stain violet
II. Iodine solution (I)	CV-I formed within cells; Cells remain violet	CV-I formed within cells; Cells remain violet
III. Alcohol	Cell walls dehydrated, Shrinkage of pores occurs, Permeability decreases, CV-I complex cannot pass Out of cells, cells remain Violet	Lipid extracted from cell walls, porosity increases, CV-I is removed from cell.
IV. Safranin	Cells not affected, remain violet	Cells take up this stain, become red.

Optical Density

Broth culture with active rhizobial growth will become turbid in 3-4 days. Broth turbidity, or optical density using spectrophotometer (at 540 nm) will show readings of 0 –to 1.0 O.D. The value of O.D. correlates to number of cells. If O.D. values are high then cells number are also high. We can measure cells

from $10^7 – 10^9$ per milliliter; if the number of cells are low this method is not accurate. This method has its limitations: (i). It gives a direct count (viable + dead cells), (ii). Polysaccharide production in different media gives different results, and (iii). Limitation from the instrument itself.

Total count

Total count includes viable cells and dead cells by using Petroft-Hausser counter At least 10 small squares all around the total area are counted, and not only in one large square.

Precautions:
1. Cells have to be homogeneous.
2. Clumping of cells (use non–ionic detergent).
3. It gives total count only.
4. Petroft, cover slip must be properly positioned to get uniform depth.

Viable count

The number of living cells is counted by spread plate or drop plate methods. Doing spread plate by making serial dilutions from $10^{-1} – 10^{-6}$ or 10^{-7} (depend on concentration) then three replicates of 0.1 milliliter of broth from 10^{-6} and 10^{-5} are spread over the YMA + CR plates. Plates are incubated in incubator (28 – 30°C) or at room temperature for 7 days. Colonies of rhizobial cells are round, opaque and have smooth margin. They are white and do not absorb red color as well as the other bacteria. Calculation of the number of rhizobia per ml;

$$\text{no. of cells/ml} = \frac{\text{no. colonies x dilution factor}}{\text{vol. of inoculum}}$$

$$\text{For example, no. of cells/ml} = \frac{32 \times 10^6}{0.1} = 32 \times 10^7$$

2.1.3. Peat test

For the peat inoculant, we check these qualities:

1. pH
2. Moisture content
3. Viable number
4. Plant infection method (MPN)

pH

Maintain neutral pH for the inoculant. Since peat is acidic the pH has to be increased with $CaCO_3$.. Weigh 10 g of inoculant, pour 20 ml of distilled water, mix well with glass rod, incubate at least 30 minutes, and then measure with pH meter.

Moisture content

The optimum moisture content of peat-inoculant is between 40 – 50 %. At low moisture rhizobia will die rapidly. If moisture is high, inoculant may stick to the plastic bag and, thus, not good for rhizobial growth.

Viable number

The number of viable rhizobia is counted by spread-plate method as in the broth test. It is more difficult when analyzing non-sterile peat. Colonies may sometimes be contaminated by other bacteria. The well trained staff is needed to conduct this microbiological analysis.

A Plant Infection Analysis using Most Probable Number Method (MPN)

This is an indirect method of assessing plant infection on nodulation. It is widely used when peat is not sterile. It takes more time than spread plate method (because we have to grow plants). We usually do MPN to compare the results with a spread plate method. Some laboratories conduct the MPN analysis and not by the spread plate method.

This method is based on the assumptions that:

1. If a viable rhizobia is inoculated on its specific host, nodules will develop on that roots.
2. Nodulation on that inoculated plant is a proof of the presence of infective rhizobia.
3. Absence of nodule is a proof of the absence of infective rhizobia.
4. Uninoculated plants are used as control, with absence of nodule.

Estimation of MPN

Plants within any given pouch are considered as a growth unit. Nodulation is recorded + for "nodulated growth unit" or – for absence of nodule. The actual number of nodules on each plant has no meaning on MPN count. If replications are in quadruplicated, the reading may be 4, 3, 2, 1 or o units. The highest dilution should show no nodulation. Refer to table, ten-fold dilutions (Table A.14.6 in Handbook for Rhizobia, Somasegaran and Hoben, 1994) the number of replications is indicated by "n" and "s" signifies the number of dilution steps.

The estimated number of rhizobia per g is calculated by the formula:

$$x = \frac{m \times d}{v}$$

m = number from MPN table A.14.6 (Vincent 1970)

d = lowest dilution (first unit)

v = volume of a aliquot inoculated

Contaminants have some effect on counting. In the presence of contaminants, count of MPN will give lower results than plate counts (R.J. Roughley 1967).

2.2. Non-symbiotic N_2-fixer

In the laboratory, microbial growth may be represented by the increment in cell mass, cell number or any cell constituent. Utilization of nutrients or accumulation of metabolic products can also be related to growth of the organism. Growth, therefore, can be determined by numerous techniques based on one of the following types of measurement: (a) cell count, directly by microscopy or by an electronic particle counter, or indirectly by colony count, (b) cell mass, directly by weighing or measurement of cell nitrogen, or indirectly by turbidity; and (c) cell activity, indirectly by relating the degree of biochemical activity to

the size of the population.

The multiplication of *Azospirillum* is expected to have reached its maximal at 3-5 days after inoculation. Inoculants in autoclaved carriers are not expected to contain many inoculants. The recommended counting technique for BIO-N inoculant utilizing known volume of serial dilutions is the drop-plate method (Miles & Mistra). Plate dilutions are ranging from 10^{-4} to 10^{-7}. If proper aseptic procedures are not fully observed, contaminants may be accidentally introduced during the injection of the broth culture and during serial dilution and plating. Such contaminants will usually be detectable on these indicator media and their number should be reported together with their number of viable cells as additional measure of the quality.

Procedure:

A. Dilution

1. Weigh 10 g of BIO-N inoculant and inoculate it on 95 ml of distilled water
2. Shake vigorously and set aside.
3. Make serial dilution of the 95 ml inoculated with diluted BIO-N. To achieve this, set out 7 tubes each containing 9 ml of sterile diluents.
4. Use a fresh pipette tips for each dilution.

B. Plating

1. Use sterile Enriched Nutrient Agar plates which are at least 3 days old or have dried at 37 ^{o}C for 2 hours.
2. Plate dilutions 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}
3. Allow the drops to dry by absorption into the agar; then invert and incubate at room temperature. Wrap the plates with sterile paper.
4. After 3-5 days of incubation with daily observations count the colonies of the respected organisms of the BIO-N inoculant.
5. Preferred counting range should be 10-30 colonies.

C. Computation

Example:

If the average number of colonies per drop is 30 at 10^{-5} dilution, the number of viable cells is:

$$1/0.03 \text{ X } 30 \text{ X } 10^{-5} = 1{,}000 \text{ X } 10^{-5} = 1 \text{ X } 10^{-8} \text{ ml}$$

ENRICHED NUTRIENT AGAR

Name of chemical	g/l
Peptone	10
NaCl	1
Beef Extract	5
Agar	18
Distilled water	1,000 ml

2.3. Mycorrhiza – the arbuscular mycorrhizal fungi, AMF

The article is an adaptation from INVAM publication. Quality control in the production of AMF inoculum is essential for product consistency, reliability and reproducibility. This is applied to the laboratory, preparation room, growth room, storage room and the greenhouses, taking care into the design, to achieve the most efficient control in inoculum production.

2.3.1. Laboratory quality control

i. Spores are extracted from selected batches of monospecific spore cultures in the preparation room.
ii. The spores are transported in petri dishes to the laboratory and placed in a refrigerator before examination.
iii. The petri dishes are examined under stereoscopic microscopes.
iv. Description of the spores from each petri dish are recorded.
v. Petri dishes are the cleaned and dried.

2.3.2. Preparation room quality control

i. This room has to be isolated from the greenhouse and growth room, and should not receive unsterilised soil or potting media samples..
ii. Stored materials (cultures; sterilized growth media) are clearly labeled and placed in specific containers.
iii. Floor should always be clean, avoiding sweeping, which encourages distribution of dust.
iv. Benches and other surfaces are cleaned with wet towels.
v. Containers are surface-sterilized with 10% sodium hypochlorite.

2.3.3. Growth room quality control

i. The growth room should be temperature controlled (22 ºC), and air is exhausted to the outside (no recycling of stale air)
ii. Bench tops should be painted with anti-microbial paint.
iii. All surfaces should be sterilized periodically e.g. monthly.
iv. All samples are checked for contaminants and pathogens.
v. Watering is done manually, with great care to avoid cross-contamination.

2.3.4. Storage room quality control

i. All sampled stored are placed in plastic bags, with proper labelling, and surface of bags should be wiped clean before storage.
ii. Floors and bench tops are wiped regularly, and dusting or sweeping should be avoided to prevent generation of dust.

References

INVAM home page.

2.4. Phosphate Solubilizers

As discussed in the definition of biofertilizer, phosphate solubilizers (PS) must contain phosphate solubilizing bacteria or fungi. Commercially produced PS biofertilizers (PSB) must be certificated with guaranteed components such as type of strains, microbial density, and biological activity. If possible the rate of phosphorus absorption of target crops is more valuable as fertilizer. It is suggested that the procedure shown in next figure would be used for the quality control of biofertilizer (Fig. 1).

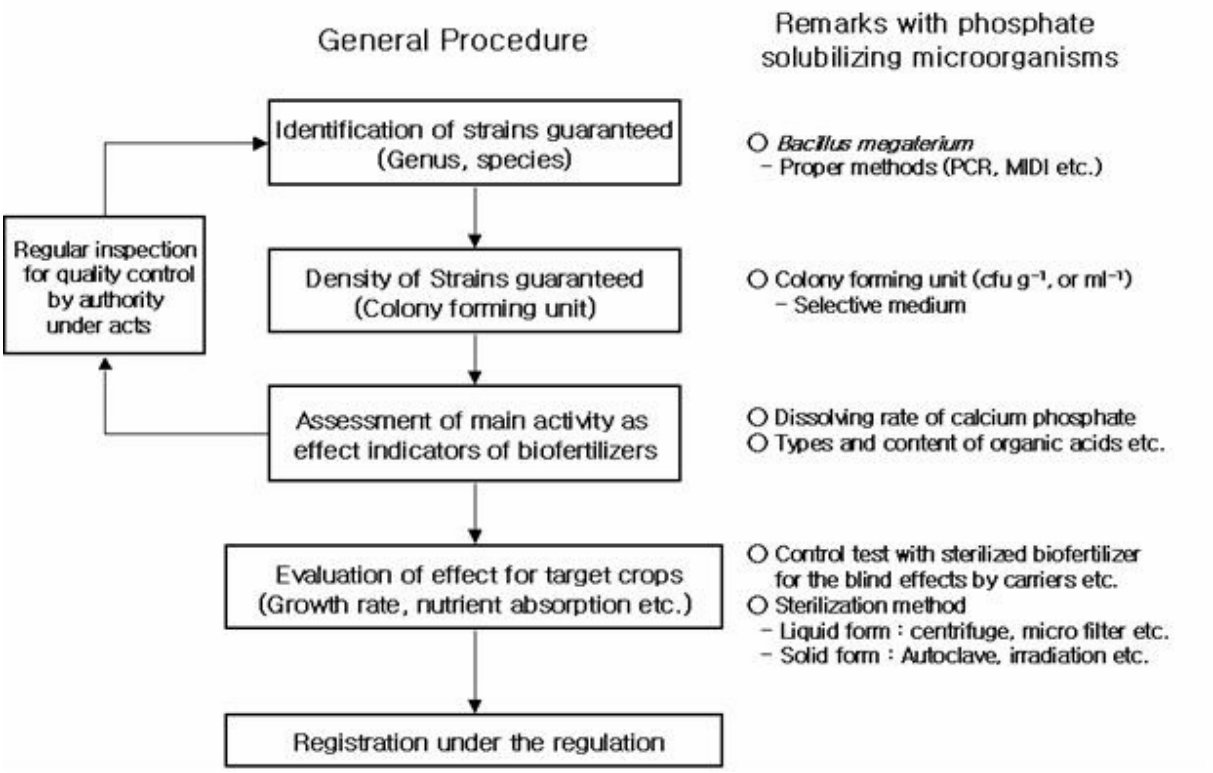

Fig. 1: General procedure for quality control of biofetilizers.

2.4.1. Inoculation on the Media

○ Dilute inoculants using diluents

○ Inoculate suitable diluted solution on selective agar medium

- Plate dilution method (Fig. 2)

Fig 2: Inoculation

2.4.2. Count of Colony Forming Units

Fig. 3: Colonies on agar medium

○ Count colonies forming clear zones on agar plate (Fig. 3)

○ Calculate colonies as per unit (g or ml)

[For example:

When average number of colonies is 45 on 10^{-5} series.

The total colony number is $45x10^{5}$ / g dry matter.]

2.4.3. Analysis of Organic Acid

Fig. 4: Solubilization of medium solution

○ Cultivation of isolate on solution medium contained unsoluble phosphate

○ Check the solubility on medium

- Transparence or clearance (Fig. 4)

○ Analysis of the organic acids in solution by the HPLC (Fig. 5)

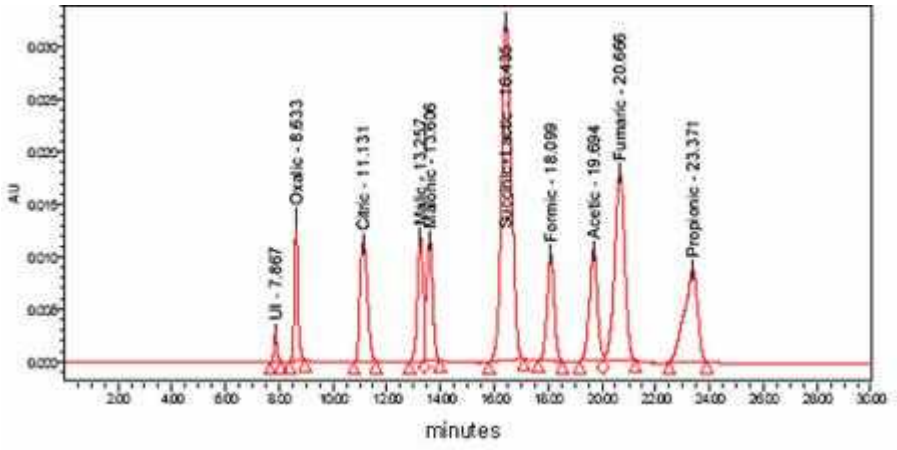

Fig. 5: Liquid chromatography of organic acids.

2.4.4. Test of Quality Certificated

○ Effects of inoculant which be proposed and certificated by manufacturers

- Plant growth rate, nutrient absorption of crops etc.

2.4.5. Prospects

Phosphate solubilizing microorganisms play an important role in plant nutrition through the increase in P uptake by the plant, and also plant growth promoting microbes are an important contributor to biofertilization of agricultural crops. Apart from fertilization, microbial P-mobilization would be the only possible way to increase available phosphate for plant. Accordingly, great attention should be paid to studies and application of new combinations of phosphate solubilizing bacteria and other plant growth promoting rhizomicrobes for improved results as mentioned early.

Concerns about the possible health and environmental consequences of using increasing amounts of mineral fertilizers and chemical pesticides have let to strong interest in alternative strategies to ensure yields and protection of crops. Use of microbial inoculants for biofertilizer in agriculture represents an attractive environmentally friendly alternative. This new approach to farming often referred to as sustainable agriculture.

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