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.

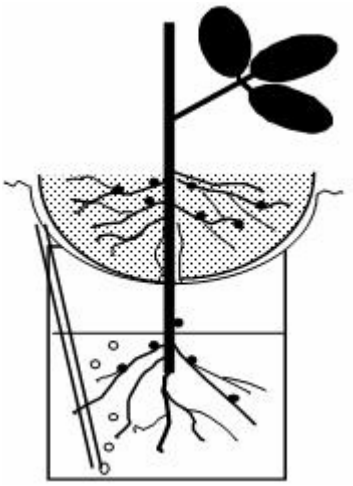

Fig. 1 A: Tow-layered pot for soybean culture (Fujikake et al. 2002)

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.

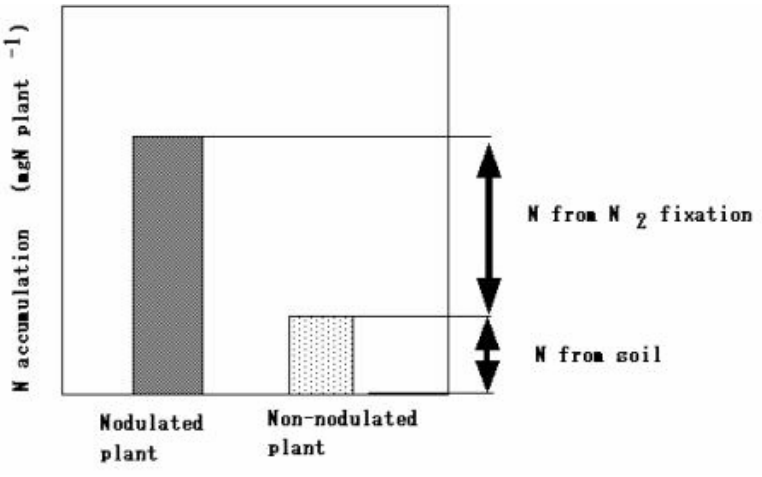

Fig. 2: Concept of N difference method

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).