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.

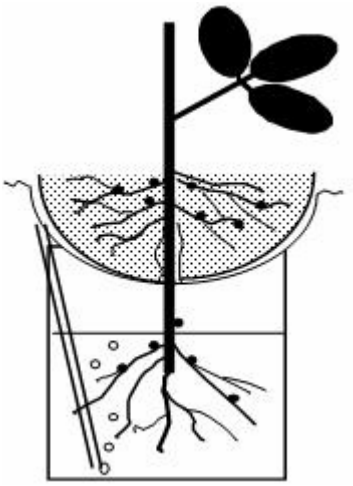

Fig. 1 A: Tow-layered pot for soybean culture (Fujikake et al. 2002)

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.

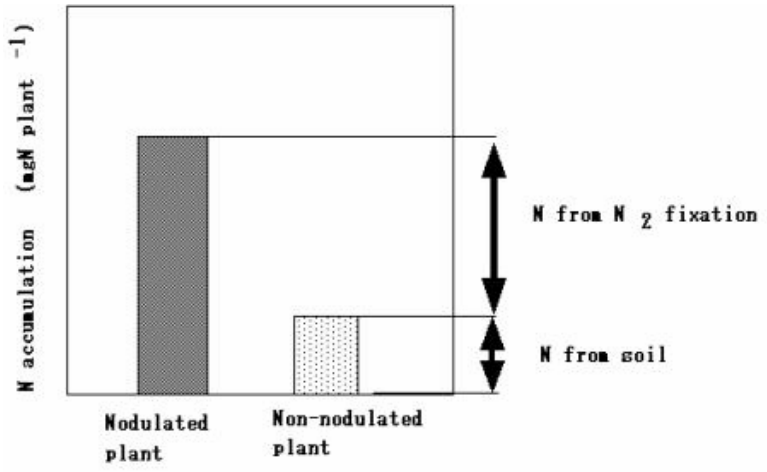

Fig. 2: Concept of N difference method

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).

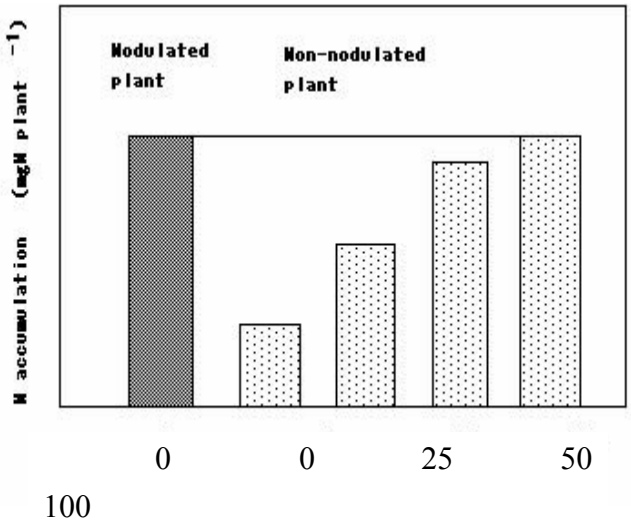

Fig. 4: Concept of N fertilizer equivalence

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.

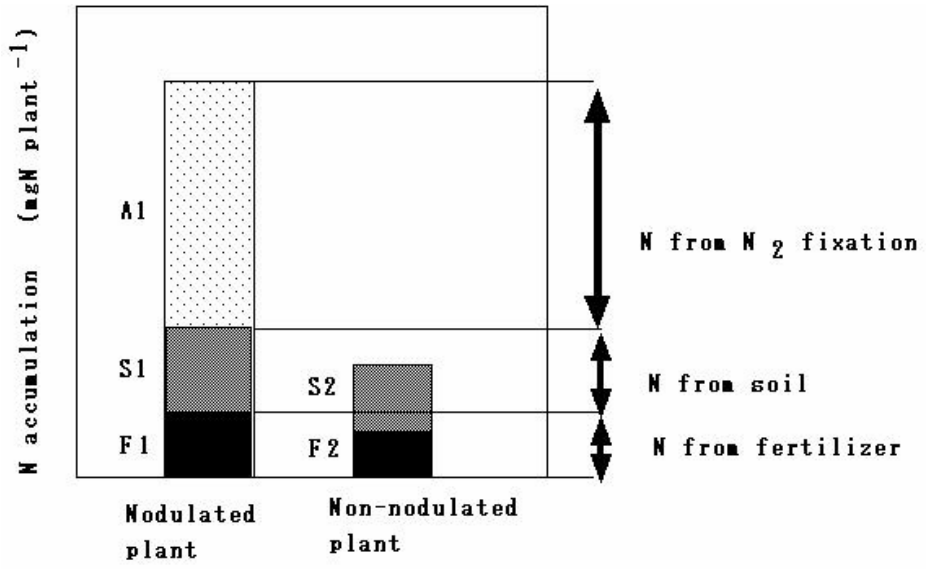

Fig. 5: Concept of ^{15}N dilution method for the evaluation of Ndfa, Ndfs and Ndff

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.

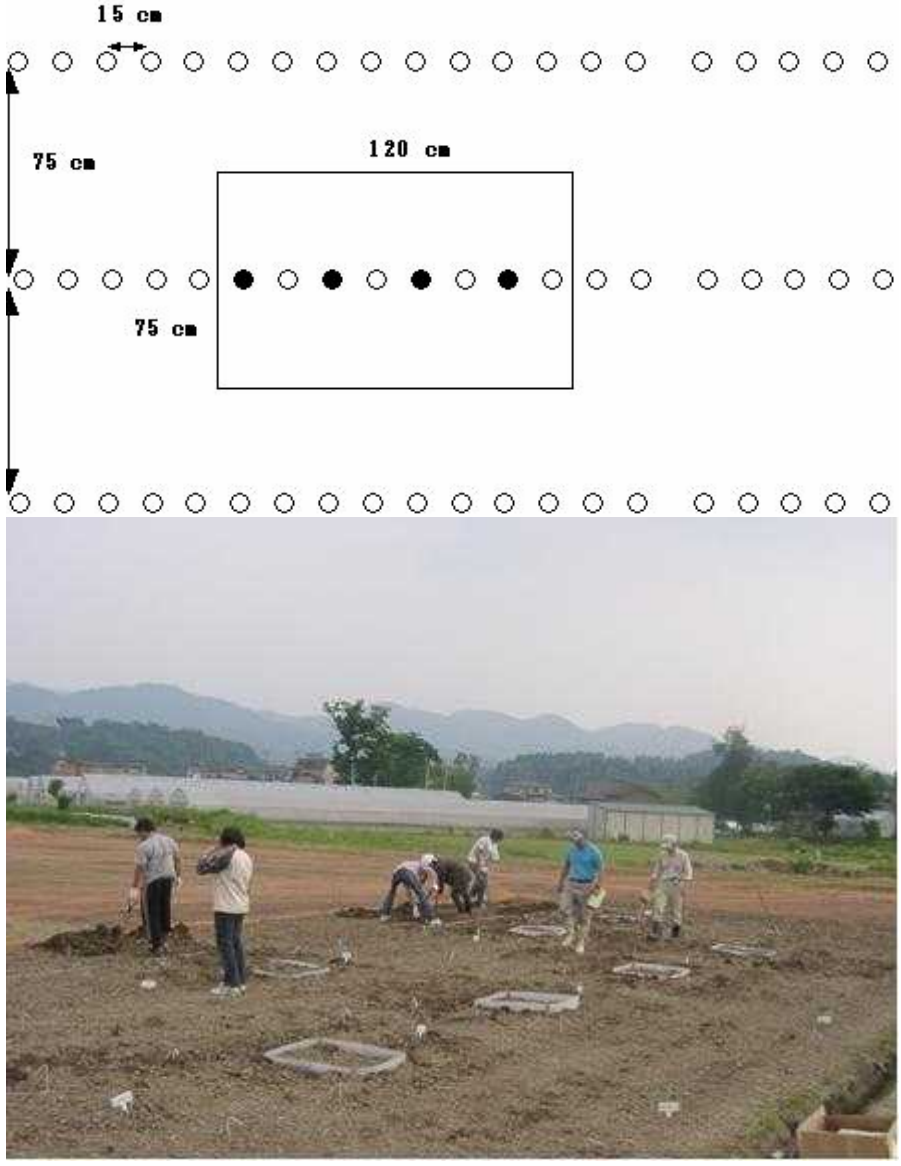

Fig. 6: An example of ^{15}N treatment for soybean experiment in the field

^{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)

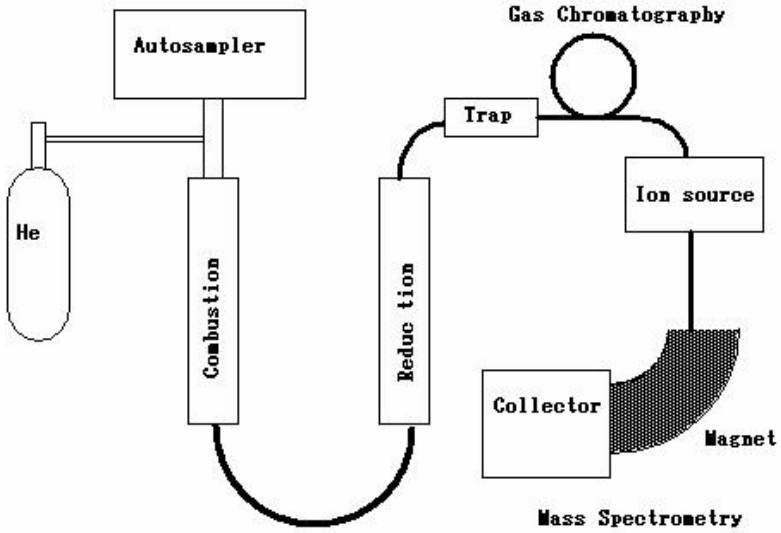

Fig. 8: Schematic diagram of an isotope ratio mass spectrometer combined with sample combustion system (FAO/IAEA)

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.

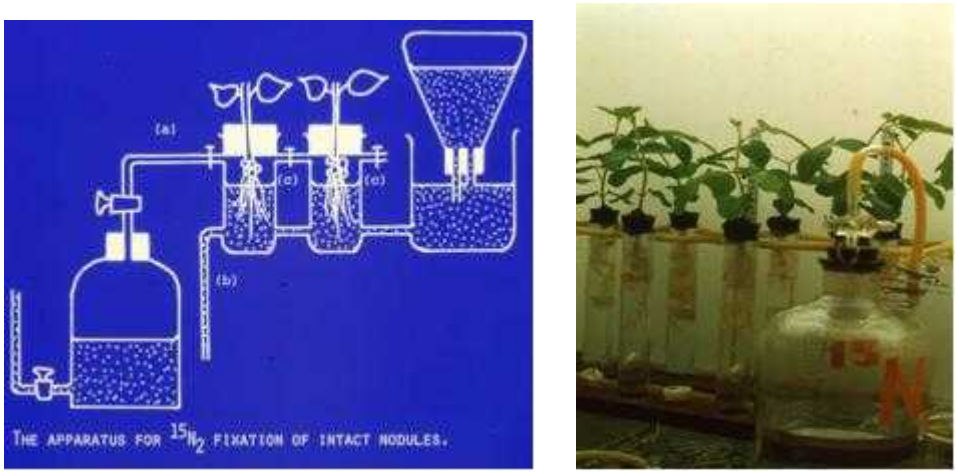

Fig. 9: Apparatus for $^{15}N_2$ fixation of intact soybean plants

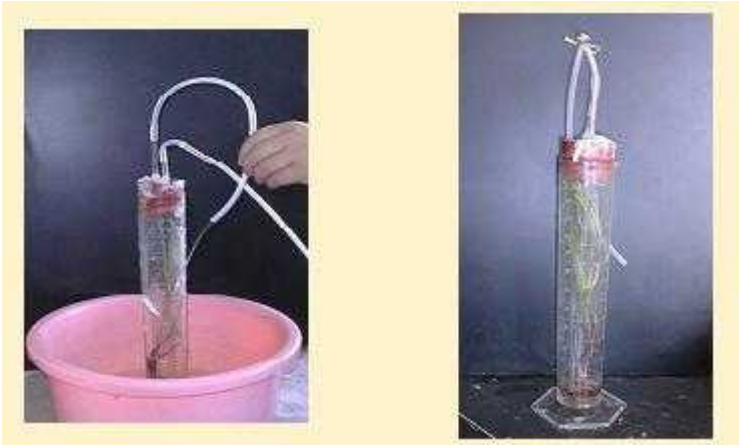

Fig. 10: Apparatus for $^{15}N_2$ fixation for sugarcane plants

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

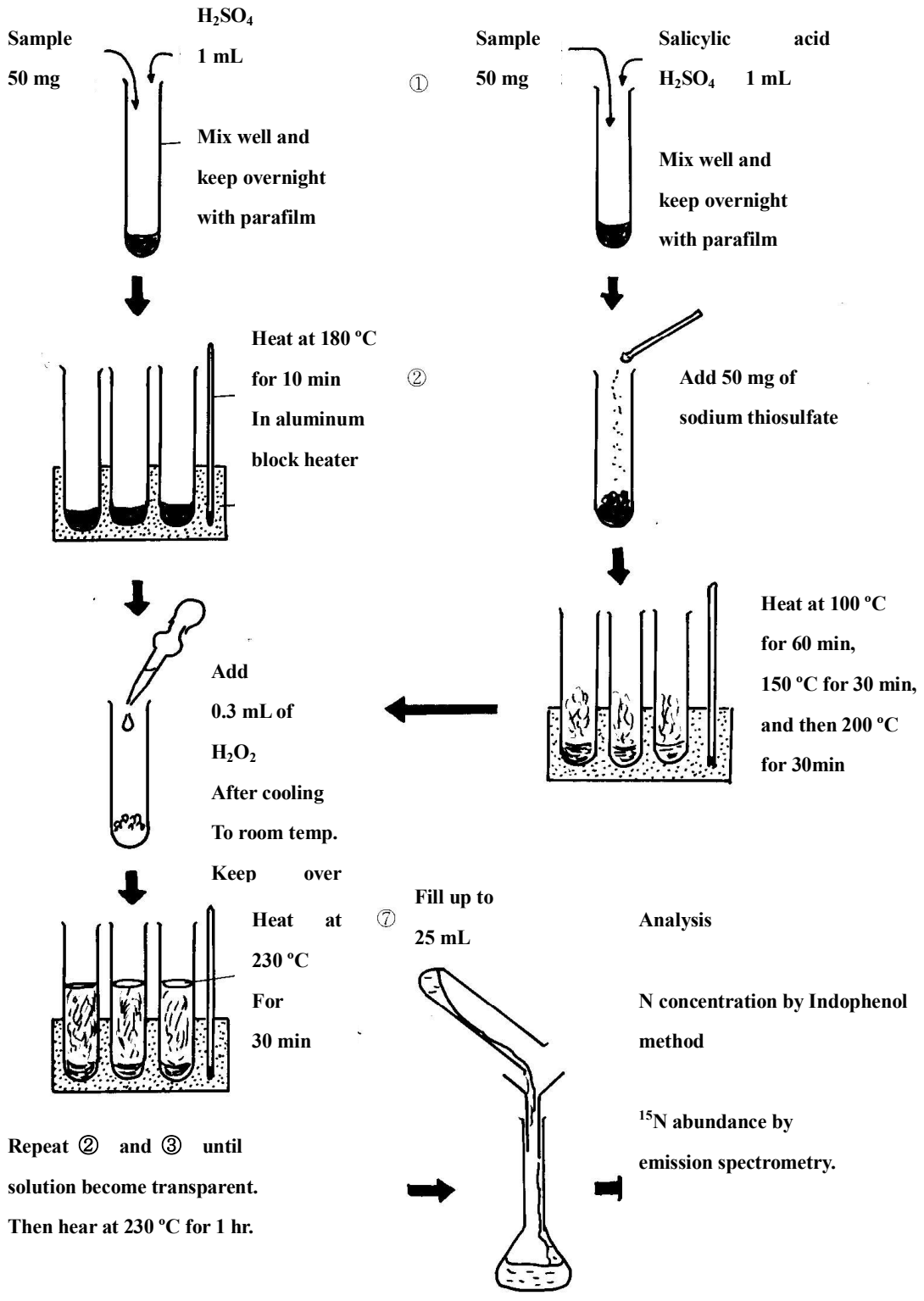


Fig. 12: Kjeldahl digestion of plant samples with low concentration of nitrate (left) and high concentration of nitrate (right)

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

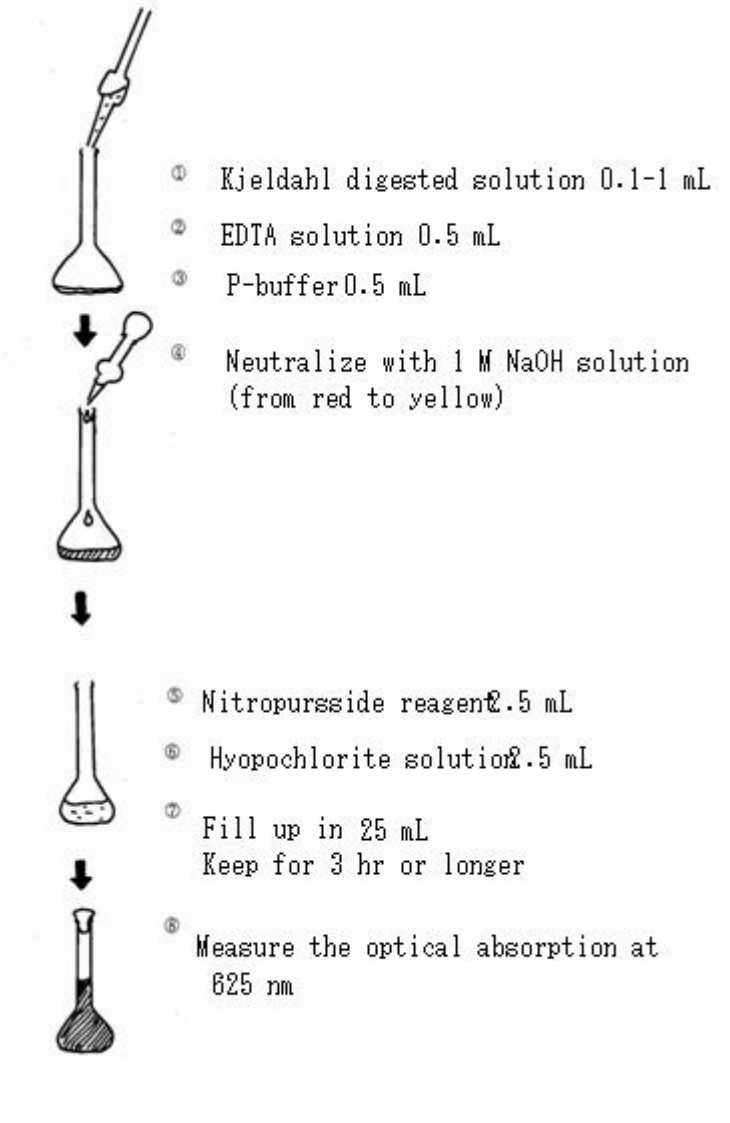


Fig. 13 shows the procedure for determination of the concentration of ammonium in Kjeldahl digested solution as mentioned above. 0.1-1 mL of digested solution is put into a 25 mL volumetric flask. The volume depends on the ammonium concentration estimated. 0.5 mL of EDTA solution and 0.5 mL of P-buffer 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.

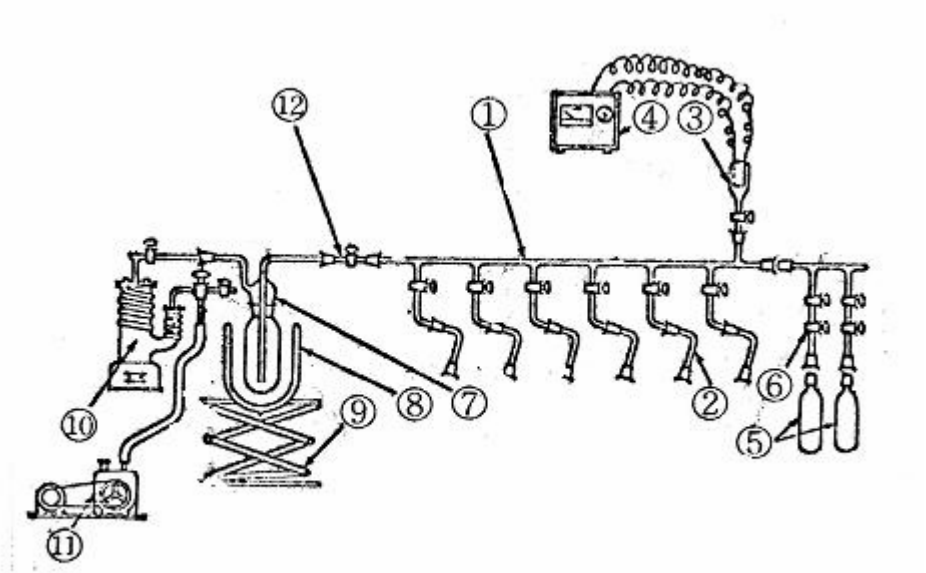
CaO reagent: Grind the calcium oxide blocks into a fine powder with mortar and pestle. Make a tablet (2 cm diameter and 1 mm thick) of CaO by pressing under the pressure at 4 ton cm^{-2}. Cut the tablet to 1 mm width sticks by a cutter knife. Heat the cut sticks in a crucible at 950℃ for 3hr in a muffle 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

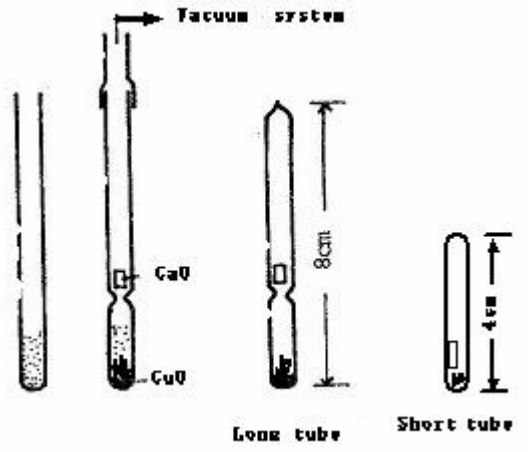


Procedure for making discharge tube

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).

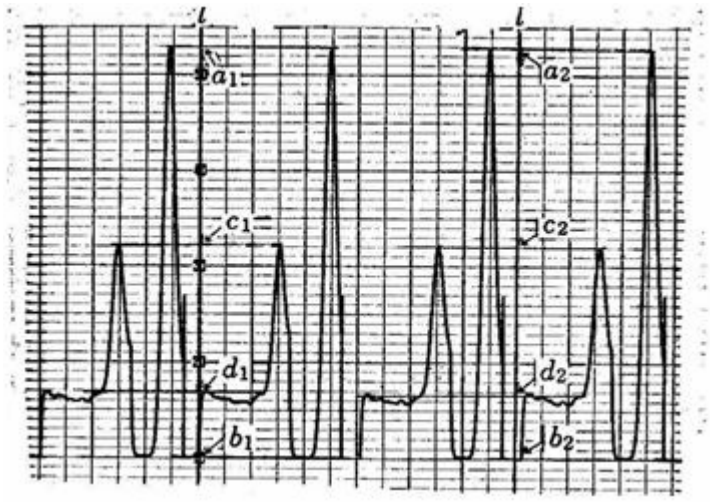
The tubes are wrapped in aluminum foil and heated at 560℃ for 30 min in a muffle furnace. In this step NH_4^+ in the tube is oxidized to N_2 and H_2O by CaO wire. After leaving overnight to ensure the absorption of water and impurities into CaO reagent, ^{15}N measurement can be done.

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

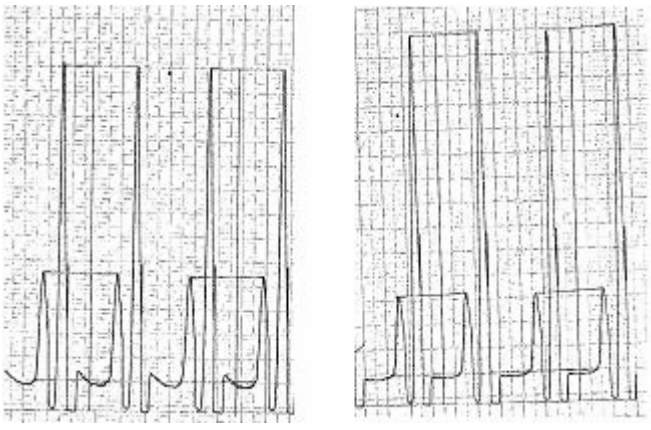


Four stable scans are recorded and the peak height of a pair of peaks are measured.

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:

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

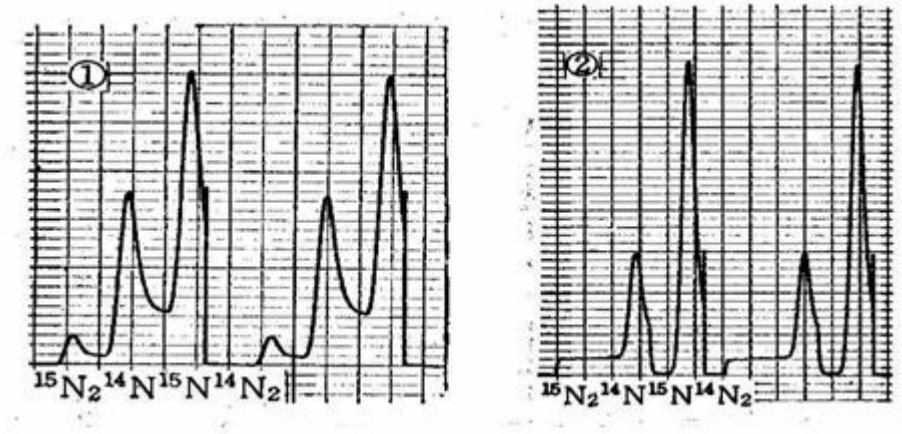
where, I_{28} and I_{29} are the peak height of $^{28}N_2$ and $^{29}N_2$ as shown in Fig. 7.

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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