ANALYSIS OF NITROGEN FIXATION AND TRANSPORT IN SOYBEAN (*Glycine max* (L.) Merr.) USING NITROGEN ISOTOPES AS TRACER

By

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Contents

Abbreviations

ATP: Adenosine triphosphate

ADP: Adenosine phosphate

BAS: Bio imaging analyzer system

BNF: Biological nitrogen fixation

DAP: Day's after planting

DW: Dried weight

FOV: Field of view

GC: Gas Chromatography

GOGAT: Glutamate synthase

GS: Glutamine synthetase

Lb: Leghemoglobin

N_2: Di-nitrogen

N_{dfa}: Nitrogen derived from air

N_{dff}: Nitrogen derived from fertilizer

N_{dfs}: Nitrogen derived from soil

NF: Nitrogen fixation

PETIS: Positron-emitting Tracer Imaging System

ROI: Region of interest

TAC: Time-activity curve

CHAPTER 1

INTRODUCTION

1.1. Background of research

Legume is a large group with about 18,000 species (Ohyama et al., 2008a), of which soybean plant takes the important position because soybean seed has one of the most important protein sources for human and livestock in the world (Ohyama et al., 2013).

Nitrogen element is one of the most necessary nutrient elements that is required for growth and development of every organism. This element also plays an important role in plant life. Because soybean seed contains a high concentration of protein, about 35-40% based on dry weight, so that soybean plants need a large amount of nitrogen. It is estimated that one ton of soybean seed requires 70-90 kg of nitrogen (Ohyama et al., 2008a). In soybean, nitrogen usually derived from three sources; air, soil, and fertilizers, of which nitrogen derived from atmosphere via symbiotic nitrogen fixation makes up from 60-75% in converted paddy fields in Niigata (Takahashi et al., 1993a). Although N_2 is rich in air and accounts for about 78%, it cannot be utilized by eukaryotes, such as plants, fungi and animals. Only some species of prokaryotic microorganisms can use it directly from atmosphere via biological nitrogen fixation (BNF) process. However, some legume species form root nodules and they can use atmospheric

nitrogen by symbiosis with nitrogen fixing microorganisms.

It is clear that BNF plays a major role in the plant life especially in legume species, and under N deficiency conditions. Through a symbiotic nitrogen fixation process, legume plants can use atmospheric nitrogen as a nutritional source for their growth and development. It was estimated that 39×10^6 tons of nitrogen is fixed by legume species every year (The Nature and Properties of Soils, 2002). Soybean plant has also the ability to fix dinitrogen (N_2) from the atmosphere in the root nodules and absorb nitrogen nutrition from either fertilizer or soil. Soybean plants need a large amount of nitrogen nutrient to synthesize seed storage protein especially in the pod filling stage, but the nitrogen nutrient obtained from atmosphere is sometimes not enough at specific stages (Ohyama, 1983). Therefore, in order to get the highest yield and quality of soybean seeds it is necessary to provide a large amount of nitrogen nutrient depending on the requirement in various growth stages. The understanding of physiological process of BNF and the transport of fixed-N are very important for improving legume cultivation in order to increase crop productivity, promote the contribution of BNF in soybean crop in each stage and provide enough nutrition for growth and seed yield. Furthermore we can save the chemical fertilizers and protect environment problem of N pollution.

Until now, there are many methods to be used to investigate nitrogen fixation and transportation in plants such as the total nitrogen different method

(Gauthier et al., 1985), acetylene reduction assay method, ureide assay method (Herridge et al., 1990). However, all these methods are indirect methods so that they don't provide the real rate of nitrogen fixation and the information about transport of fixed-N in living plants.

The methods using nitrogen isotope are considered to be the best tool for studying of nitrogen fixation in plants. By using ^{15}N stable isotope, researchers have found the pathways of nitrogen compounds assimilating and transporting in plants. The results indicated that N_2 is reduced into ammonia in nodules and then assimilated through different pathways in legume species (Ohyama and Kumazawa, 1978a). In soybean plant, it was found that ureides (allantoic acid and allantoin) are synthesized in nodules and transferred to the shoots via xylem system (Matsumoto 1977), while the main transport forms of nitrogen absorbed from roots were nitrate and asparagine (Ohyama and Kumazawa, 1978 and 1979). All of N forms are considered to be transported to shoots via xylem vessels, but major part of N forms from the roots was first translocated in leaves and then re-distributed to pod and seed, while some parts of the fixed N originating from nodules were directly moved to the pods and seeds in addition to the leaves (Ohyama, 1980).

In addition, the positron-emitting tracer imaging system (PETIS), which has been developed in recent decades, gave the outstanding analytical method in the field of plant nutrition. PETIS system detects γ-ray generated by positron-

emitting nuclides and we can observe the movement of positron-emitting radioisotopes in a living plant at real-time (Kume et al, 1997). This new technique provided the visualization of the dynamic transport and allocation of metabolites at large distance scales and consequently gave information for understanding whole-plant physiological response to environmental change in real time (Kiser et al., 2008). In the past decades, PETIS was used to study of nutrient in plant such as carbon (C) in broad bean (Matsuhashi et al., 2005), sorghum (Keutgen et al., 2005), soybean (Kawachi et al., 2011), eggplant (Kikuchi et al., 2008), nitrogen (N) in soybean (Ishii et al., 2009, Ohtake et al., 2001, Keutgen et al., 2002, Sato et al., 1999), in rice (Kiyomiya et al., 2001), *Orobanche sp.* (Kawachi et al., 2008), cadmium (Cd) (Fujimaki et al., 2010; Ishikawa et al., 2011), manganese (Mn) in barley (Tsukamoto et al., 2006), iron (Fe) in barley (Tsukamoto et al., 2009), and Zinc (Zn) in barley (Suzuki et al., 2006).

There are many studies in the field of nitrogen fixation and the transport of fixed nitrogen in soybean plant, but the results are not much clear about the rate of fixed-N from nodules and the transport of fixed-N to various organs of soybean plant.

To elucidate the turnover rate of fixed-N in soybean nodules and transport mechanism of fixed-N, this study used $^{15}N_2$ and $^{13}N_2$ isotopes as tracers.

1.2. Literature review

1.2.1. Biological nitrogen fixation and nitrogen nutrition demand in soybean plant

Although nitrogen is dominant element on the Earth, but most of organisms cannot use gaseous nitrogen (N_2) directly from atmosphere except some prokaryotic microorganisms. Some plant species can use atmospheric nitrogen indirectly through BNF in a symbiotic process with these microorganisms. Therefore, BNF is not only important for the growth and development of legume plants, but also for nitrogen cycle in the global scale.

Legume plants can fix atmospheric di-nitrogen via symbiosis with soil bacteria, rhizobia. Because of its importance, the process of BNF has been studied intensively for a long time. The studies of BNF may promote crop production to improve the yield of grain crops for food and livestock when the population of the world is increasing rapidly. Furthermore, the use of chemical nitrogen fertilizer for crop cultivation is very large, estimated about 100×10^6 ton in 2009 (Ohyama et al., 2010), an excess or improper use of the chemical fertilizers sometimes resulted in pollution of soil and underground water. Research efforts to improve the nitrogen fixation activity in legume crops not only increase the crop production and the income for famer, but also decrease the environmental pollution.

Soybean plant is one of the most important legume crops and is the fourth largest grain crop after rice, wheat and maize. Soybean seeds contain a high concentration of storage protein (approximate 40% of dry weight), therefore providing a large amount of nitrogen is necessary to get high yield and high quality seeds. One of the most important characteristics of soybean plant is that it can also use nitrogen source indirectly from atmosphere in the symbiotic process with bacteria, rhizobia, N_2 fixing soil as well as soybean can absorb combined nitrogen such as mineralized N from soil or fertilizer N (Ohyama et al., 2010).

In the process of BNF, rhizobia obtain carbohydrate from a host plant and they fixe atmospheric N_2 to NH_4^+ in the root nodules, and then they give fixed NH_4^+ to the plant cells, and ammonia is assimilated into N compounds such as amino acids and ureides (Russelle, 2008). In soybean nodules, the major fixed ammonia is excreted to cytosol in infected cells, and then it is assimilated into amino acids via glutamine synthetase/glutamate synthase (GS/GOGAT) (Ohyama and Kumazawa, 1978b). The previous results indicated that a major part of fixed nitrogen is assimilated into ureides, allantoin and allantoate by de novo synthesis and transported from nodules to shoots via xylem system (Ohyama, 1981).

The symbiotic nitrogen fixation activity is influenced by many biotic and abiotic factors (Sprent and Minchin, 1983). The nitrogen fixation rate is highest at the end of flowering and during the pod filling (Harper, 1974). It has been determined that the increase of soybean yield was related to the increase of

the amount of fixed nitrogen and total N derived from atmosphere (Herridge and Bergersen, 1988). It is estimated that the average of fixed nitrogen in soybean was 75 kgN ha^{-1} (LaRue and Patterson, 1981), but in good condition it could reach to 300 kgN ha^{-1} (Keyser and Li, 1992), and the BNF can supply more than 50% of the total N requirement for the whole life. It is clear that BNF is very important for agricultural system, especially at nowadays when pollution and climate change are increasing day by day then limited use of chemical fertilizers and increased use of organic fertilizers, especially increasing the ability of BNF of tree crops are one of the best ways to protect our planet.

1.2.2. Mechanism of biological nitrogen fixation

The process of BNF in legume plant is taken place in root nodules. First, the host plant roots excrete the phenolic compounds such as daidzein and genistein in soybean plant (Charrier *et al.* 1995, Shirley 1996, Bladergroen and Spaink 1998) signal molecules to attract rhizobia and stimulate the expression of nodulation genes (Nod-genes). When rhizobia habitat in root system they will induce the formation of nodules after trapped by hair root curling. During the root curling process, rhizobia are entrapped in the loop of root hair and they proliferate, and move through the infection thread toward the inner cortex of root to form a nodule in the inner root cortex (**Figure 1.1**)
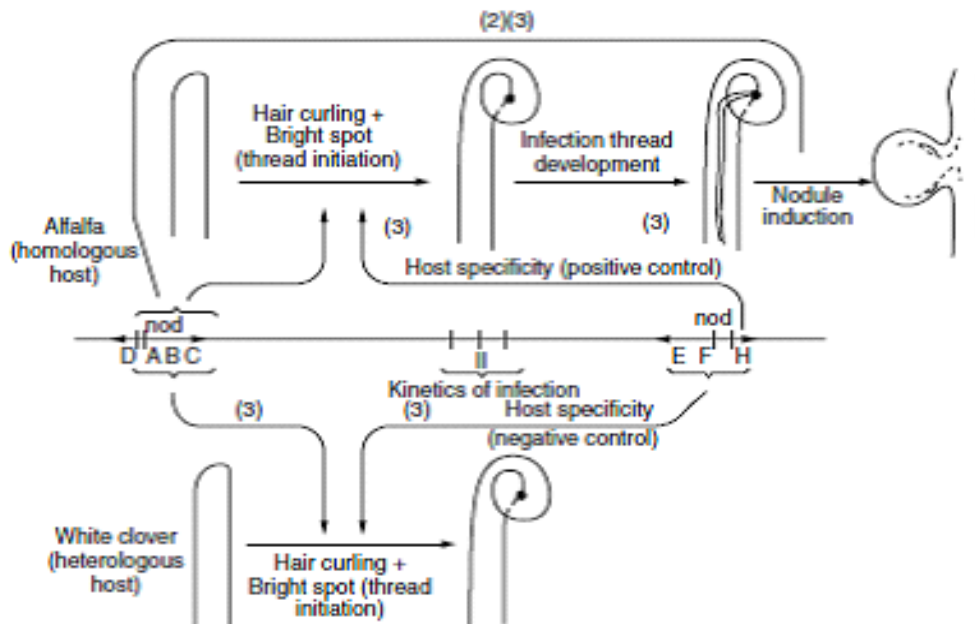
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Figure 1.1: The infection process through the root hairs and the simultaneous

formation of the nodule (Debell´e, F., et al., 1986).

Soybean nodules appear about 10 days after sowing when the seeds are inoculated with a compatible rhizobia, and they reach 3 mm in diameter after 20 days cultivating (Ohyama et al., 2010). The soybean nodule is structured by many layers (**Figure 1.2**), there is the symbiotic region in the center, which consists of the mosaic of small uninfected cells and large infected cells. The infected cells contain O_2 binding protein "leghemoglobin (Lb)" that keep an important role in in protection of nitrogenase and maintenance of respiration to

provide ATP in nodules (Ohyama et al., 2008b). Nodule cortex layer is surrounded the internal symbiotic region that has a function in regulating O_2 permeability for nitrogen fixation process in order to adapt high or low O_2 concentrations.
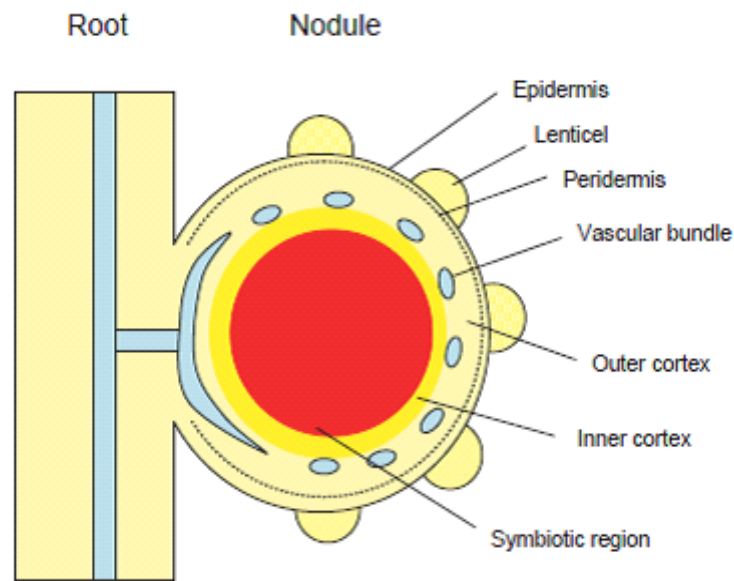

Figure: 1.2 Model structure of soybean root nodule (Ohyama et al., 2008).

The atmospheric nitrogen fixation is conducted based on the catalysis of nitrogenase enzyme in three steps (Rees et al., 2005). First, the Fe-protein of nitrogenase reduced by electron carriers such as flavodoxin and ferredoxin and then, single electron is transferred from Fe-protein to Mo-Fe protein in Mg-ATP dependent process. Finally, the electron is transferred to the substrate, which already bound to the active site of Mo-Fe protein complex and the cycle is

repeated until sufficient electrons and protons form to reduce the substrate.

$$N_2 + 16ATP + 8H^+ + 8e^- \rightarrow 2NH_3 + H_2 + 16ADP + 16P_i$$
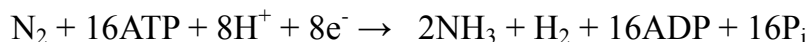
The energy used in this process is obtained from the host plant. The initial product of nitrogen fixation process is known as ammonia (Ohyama and Kumazawa, 1978b). It has been found that more than 90% of fixed nitrogen in soluble fraction is ammonium only after soybean nodules were exposed to $^{15}N_2$ gas to soybean nodules (Bergersen, 1965). A high concentration of ammonium is poisonous to plant cells, so that it should be immediately converted to amino acids (Day et al., 2001).

Two enzyme glutamine synthetase (GS) and glutamate synthase (GOGAT) operate in tandem to form glutamate synthase cycle of ammonium assimilation (**Figure 1.3**) (Lea, 1997).

In the glutamine synthase pathway, glutamate is converted into glutamine with ATP-dependent catalyzed by GS:

Glutamate + Ammonia + ATP $\rightarrow$ Glutamine + AMP + P_i

This reaction requires divalent cation, such as Mg^{2+}, Mn^{2+} or Co^{2+} as a cofactor. Next, the GOGAT catalyzes the reaction between glutamine and 2-oxoglutarate to convert glutamine into glutamate:
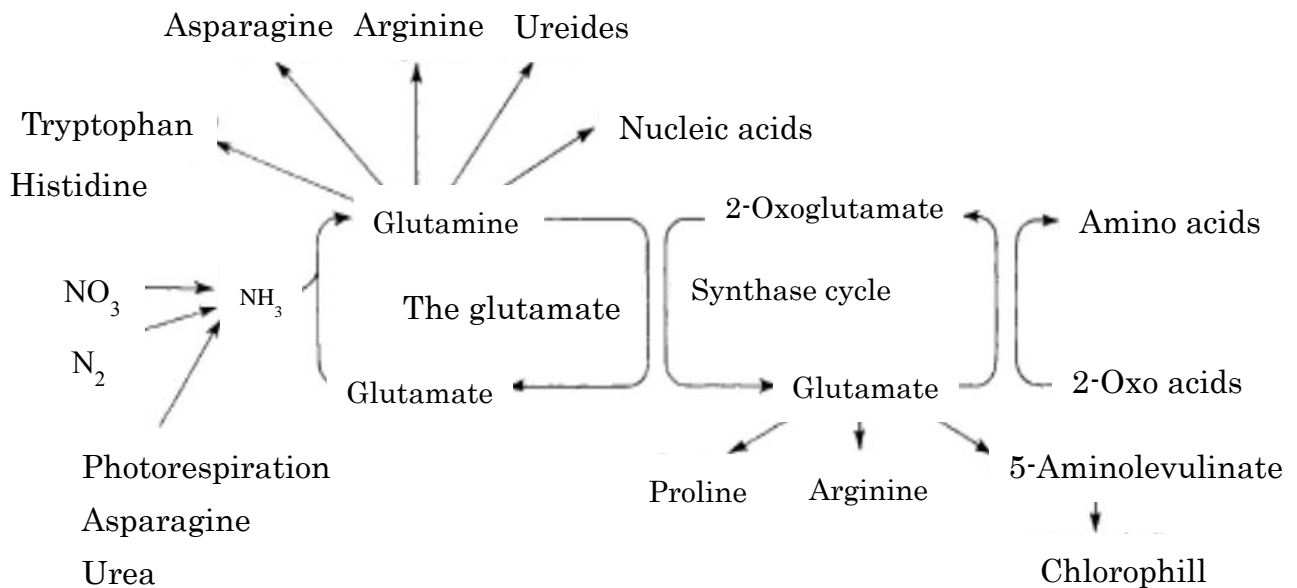
Glutamine + 2-oxoglutarate + $2e^- \rightarrow$ 2 glutamate

Asparagine Arginine Ureides

Tryptophan

Histidine

Nucleic acids

Glutamine

2-Oxoglutamate

Amino acids

NO_3 → NH_3

The glutamate

Synthase cycle

N_2

Glutamate

Glutamate

2-Oxo acids

Photorespiration

Asparagine

Proline   Arginine

5-Aminolevulinate

Urea

Chlorophill

Figure 1.3: The assimilation of ammonia in higher plants via the glutamine synthetase/glutamate synthase cycle (Lea, 1997).

By using $^{15}N_2$ experiments, it has been demonstrated that the ^{15}N abundance of ammonia initially increased and reached the maximum value rapidly after a few minutes of $^{15}N_2$ exposure. This suggested that there were more than one compartment of ammonia in nodules and the ammonia pool may be one of which directly derived from nitrogen fixation (Ohyama and Kumazawa, 1978b, 1980). The glutamine increased highest until 10 minutes of $^{15}N_2$ exposure but not continue afterward and it seem to be synthesized near the site of N_2

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fixation (Ohyama and Kumazawa, 1978b). All ^{15}N incorporated compounds are transported immediately to organs of soybean plant via xylem system.

1.2.3. Transport of fixed nitrogen in soybean plant

As mentioned early, after N_2 is fixed, the fixed-N is exported immediately from bacteroid to cytosol and assimilated by GS/GOGAT pathway to glutamate and metabolized to various amino acids (Ohyama et al., 2008b). In soybean plant, a major part of fixed-N is metabolized to ureides (including allantoin and allantoic acid) in nodules through de novo synthesis, so that they are considered as the main nitrogen compounds transported from soybean nodules to other parts (McClure and Israel, 1979; Ohyama et al., 1989b).

The main transport route of fixed N from the nodule to the shoot is considered via xylem system, as well as the transport of absorbed nitrate (NO_3^-) in the roots. Previous ^{15}N tracer experiments comparing $^{15}N_2$ fixation and $^{15}NO_3^-$ absorption indicated that some portions of N, which originated from N_2 fixation, were translocated directly to the pods and seeds in addition to transportation to the leaves (Ohyama, 1983). On the other hand, nitrate nitrogen (NO_3^-) was primarily transported to the leaves then re-transported to the pods and seeds. Pate et al. (1979) reported that, in white lupin (*Lupinus albus* L.), 68% of the N received by leaves from xylem was re-exported from the leaves with

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photosynthates via phloem system, and that 48% of N incorporated into the growing nodule was supplied from the shoot downward via phloem, this recycling N is very important for roots and nodules growth for their N nutrition. However, the rate of recycling N from the shoot to the roots has not been investigated in nodulated soybean plants.

Based on the obtained results, Ohyama et al., (2008a) assumed conclusion for the fixed nitrogen transport described by the model following:
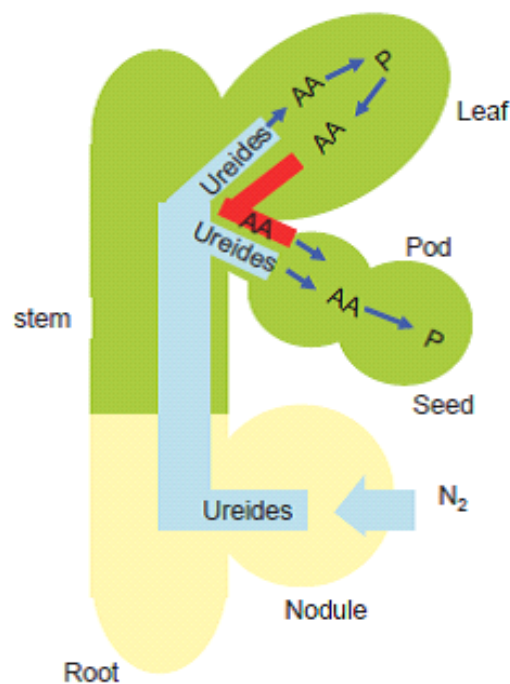

Figure 1.4: Model of transport of N from N fixation in soybean plant (Ohyama et al., 2008).

The **Figure 1.4** shows the transportation of fixed nitrogen in soybean plant, the fixed N from nodules as the forms of allantoin and allantoic acid is translocated to the upper parts including shoots, leaves and pods via xylem system and re-distributed to pods, roots via phloem system (Ohyama et al., 2008b). First, fixed ammonia is incorporated into amide group of glutamine and then into glutamic acid and allantoin and allantoic acid. There are two main pathways of fixed N transport to be distributed. One, the fixed N is carried from root nodules up to shoot in the xylem. The other major flow path is that fixed N moves from mature leaves to growth and storage organs in the phloem system.

1.2.4. Measurement of biological nitrogen fixation

Up to today, there are several methods used to measure NF rate in crops, but no one can provide an accurate measure of NF for all legume species under diversely environmental conditions. Each method has its own advantages and disadvantages (Peoples et al., 1989). In general, there are several approaches for estimating NF in legume plants. The first is to estimate the NF activity based on the increase in total N of plant and soil system (N balance method). The second is to separate plant nitrogen into the fraction derived from soil and atmosphere such as N difference, ^{15}N abundance, ^{15}N isotope dilution and relative ureide methods. The last is to measure the activity of nitrogenase that is responsible for N_2

fixation such as acetylene reduction and hydrogen evolution methods (Unkovich et al., 2008). Some of which will be described briefly in the following sections.

1.2.4.1. ^{15}N stable isotope method

The ^{15}N isotope method is one of the most reliable methods used to investigate the contribution of BNF to legume crops using ^{15}N-labeled gas or nitrogen ^{15}N labeled fertilizers (Pauferro et al., 2010). It based on the principle that the ^{15}N/ (^{14}N + ^{15}N) molar ratio of the atmosphere is 0.364%, thus the contribution of biological nitrogen fixation is ^{15}N/^{14}N ratio in plant tissue supplied either calculated from with the ^{15}N$_2$ or ^{15}N$_2$ fertilizer or soil (Danso, 1995). Depending on each technique and condition to be applied, the ^{15}N method may be classified into:

1) ^{15}N$_2$ isotope gas method

2) ^{15}N isotope dilution method

3) The A-value method

^{15}N$_2$ labeled gas method was applied to study nitrogen fixation long time ago. In this method, nodulated plants were incubated in ^{15}N$_2$ labeled gas, the ^{15}N abundance in the plant tissue of fixing plants will be significantly higher than that of the natural abundance in air or soil. This method uses ^{15}N isotope gas directly to treat legume plants in laboratory condition in order to detect BNF.

However, the environment within the chamber is different compared that of in the field and plants were treated with $^{15}N_2$ only a short time. Therefore, the results obtained from such experiments usually applied for short-term $^{15}N_2$ feeding experiment and it is difficult to use for the long-term experiment during entire growing season (Knowles, 1980). Although it is very sensitive and precise, this method cannot be applied in the field because plant roots need to be enclosed in an air-tight system.

1.2.4.2. ^{13}N radioisotope method

Nitrogen-13 is a radioisotope of nitrogen with short half-life only 9.97 minutes. It has been applied in positron emission tomography (PET). One of the advanced methods employed ^{13}N in the field of plant nutritional study developed recently was positron-emitting tracer imaging system (PETIS). This method can overcome the obstacle that previous methods could not perform. PETIS method was used relatively wide to study of nutrients in plants such as wheat (Matsuhashi et al., 2006), barley (Suzuki et al., 2006, Tsukamoto et al., 2006), eggplant (Kikuchi et al., 2008), soybean plant (Ishii et al., 2009; Ohtake et al., 2001). Besides, PETIS is also used to investigate the pollutant metal accumulated in food crops such as rice (Fujimaki et al., 2010; Ishikawa et al., 2011). Recently, by applying mathematical models in quantifying of radioisotope activity in time course, the rate export and import of labeled elements were calculated broad bean (Matsuhashi et al., 2005), soybean (Ishii et al., 2009; Keutgen et al., 2002).

The PETIS apparatus (Hamamatsu Photonics, Hamamatsu, Japan) used in studying of plant nutrition has two head detectors that are opposite with each other (**Figure 1.5**). The detectors consist of arrays of scintillators and photomultipliers, which detect γ-rays and export spatial information of the incident points on the head surface. The test plant will be placed in the center between two head detectors at a distance of 10 cm to each detector head. PETIS system can detect γ-rays created by positron-emitting nuclide and can observe the movement of labeled elements in living plant in real time (Kume et al., 1997), and this technique provides the capacity to visualize the dynamic transport or allocation of metabolites at scales of centimeters and consequently gives information for understanding whole-plant physiological response to environmental change in real time (Fujimaki 2007; Fujimaki et al., 2010; Kawachi et al., 2011). The detection of γ-rays from the annihilation of positron is possible to be tracked the transport and distribution of radiotracers in test plant as a function of time.
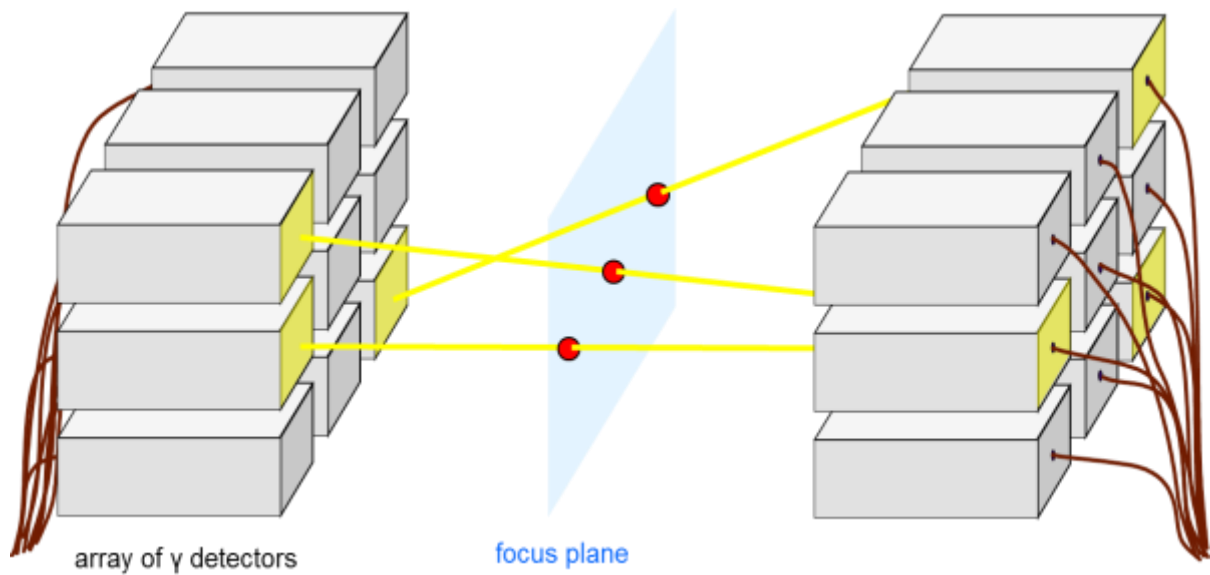
array of γ detectors   focus plane

Figure 1.5: Diagram of two head detectors (Courtesy of Dr. Shu Fujimaki, JAEA).

PETIS method is a non-invasive technique, which allows the visualization and assessment of radioactive tracers in sample plants without destroying. Therefore, the sample plant can be used repeatedly many times under the same environmental condition. The repetitive measurement on the same plant makes it possible assessing of biological variances of an individual plant.

To investigate the BNF activity and N assimilation in PETIS experiment, plants are treated with ^{13}N-labeled tracers (^{13}N$_2$, ^{13}NH$_4^+$ or ^{13}NO$_3^-$). The radioactive nucleus decays with emitting a positron (e+) and neutrino (ν). The positron will travel through the material, losing energy by collisions with

electrons (e−). Once the positron reaches thermal energies it annihilates with an electron, which results in two 511 keV gamma rays (γ) emitted in opposite directions (**Figure 1.6**).

Gamma rays are attenuated very little by test plant tissue and detected by two head detectors. PETIS system will reconstruct an image of the two-dimensional distribution of the tracer and the image will be used for estimating accumulation and transport of the N tracer in plant.
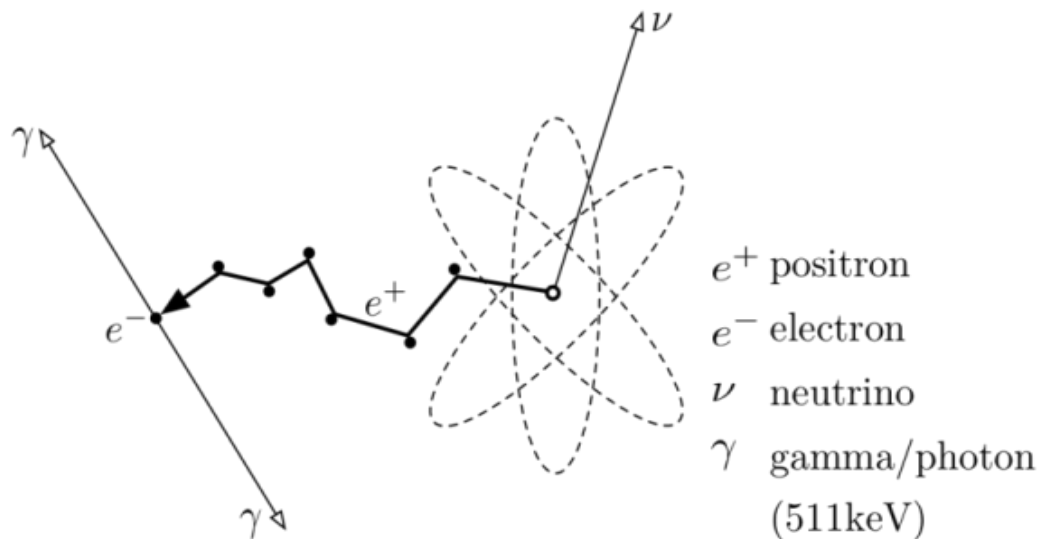
γ

ν

e^-

e^+

γ

e^+ positron
e^- electron
ν neutrino
γ gamma/photon
(511keV)

Figure 1.6: Diagram of positron annihilation (Jens Langer, 2007).

1.3. Objectives

The objectives of my research were as follows:

1) Quantitative analysis of the initial transport of fixed nitrogen from nodules and transport and distribution of fixed-N in various organs by pulse-labeled $^{15}N_2$ experiment.

2) Visualization of the initial transport of fixed nitrogen in nodulated soybean plant using $^{13}N_2$ tracer gas and PETIS in real time.

3) Studying of the effects of O_2 partial pressure in rhizosphere on nitrogen fixation activity and transport of fixed $^{13}N_2$ in soybean using PETIS.

Chapter 2

MATERIALS AND METHODS

In this chapter, the general methods will be described and the detail used in specific experiment will be addressed in the corresponding chapters.

2.1. Plant cultivation

2.1.1. Seed germination

Soybean (*Glycine max* [L.]Merr. cv. Williams) seeds were sterilized with 70% ethanol for 30 second and sodium hypochlorite solution with 5g L^{-1} of available Cl for 5 min and then thoroughly washed with deionized water. The seeds were inoculated with the suspension of *Bradyrhizobium japonicum* (strain USDA 110) and sown on a vermiculite tray. Ten days after sowing, the seedlings with primary leaves expansion were transferred to 1 L glass bottle containing 800 mL of nitrogen-free nutrient solution or plastic containers containing 20 L of nitrogen-free nutrient solution (Fujikake et al., 2002). The nutrient solution was changed periodically depending on each experiment, usually three times a week. The solution was continuously aerated by an air-pump.

2.1.2. Growth conditions.

Soybean plants were cultivated hydroponically in 1 L glass bottle greenhouse for the N stable isotope experiment and in 20 L plastic container in growth room for radioisotope experiment under the following conditions: temperature: 16 h light at 28^oC and 8 h in darkness at 20^oC; humidity: 65%; and irradiance: 400 μE m^{-1} x s^{-1} under florescence light-tubes.

2.1.3. Composition of nutrient solution.

All experiments the nutrient solution was used without N element based on the recipe of nutrient solution (Fujikake et al. 2002): (K_2SO_4:109 mg L^{-1}, K_2HPO_4: 8.5 mg L^{-1}, KCl: 0.935 mg L^{-1}, $CaCl_2 \cdot H_2O$: 183.0 mg L^{-1}, $MgSO_4 \cdot 7H_2O$: 123 mg L^{-1}, H_3BO_4: 0.367 mg L^{-1}, $CuSO_4 \cdot 5H_2O$: 0.032 mg L^{-1}, $MnSO_4$ 0.189 mg L^{-1}, $ZnSO_4.7H_2O$: 0.144 mg L^{-1}, $NiSO_4 \cdot 6H_2O$: 0.0035 mg L^{-1}, ethylenediamine-tetraacetic acid$\cdot$2Na: 18.6 mg L^{-1}, $FeSO_4 \cdot 7H_2O$: 13.9 mg L^{-1}; pH: 6.0).

2.2. ^{15}N stable isotope experiment

2.2.1. Experiment process

In this experiment, soybean plants were treated with ^{15}N labeled gas at two stages: 36 DAP (young vegetative stage before flowering) and 91 DAP (pod-filling stage). The ^{15}N labeled gas war prepared by mixing 80% ^{15}N labeled gas (^{15}N$_2$: Ar = 3:7, 99.7 atom% Shoko Co. Ltd., Tokyo, Japan) with O$_2$. The test soybean plants were inserted in a 1 L cylinder and then exposed to mixed gas in the feeding apparatus (**Figure 2.1A**) through inlet tube and displacement of nutrient solution was drained by outlet tubes. Nutrient solution was remained in the cylinder about 300 mL. One hour after introducing ^{15}N labeled gas, all gas remained was flushed out and treated soybean plants were exposed under the natural air conditions. Treated soybean plants were sampled at 1, 2, 4, and 8 hours after starting the ^{15}N$_2$ exposure with four replications. At each time, soybean plants were divided into six sections (S1, S2, S3 for the upper parts and R1, R2, R3 for lower parts); each section was separated into stem, leaf, pod shell and seed for the upper parts; and root and nodule parts for the lower part (**Figure 2.1B**). All samples were frozen in liquid nitrogen quickly, and freeze-dried in vacuum machine.
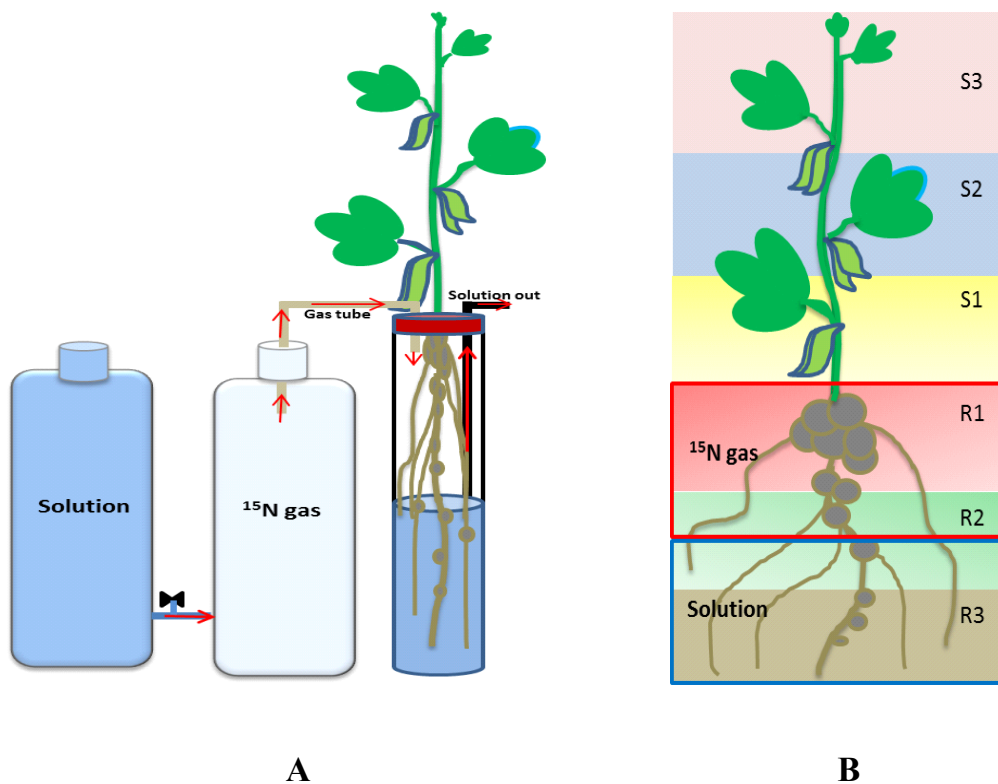
A

B

Figure 2.1: Illustration of setup for ^{15}N experiment.

A: Model of the ^{15}N$_2$ gas feeding apparatus; the ^{15}N$_2$ (^{15}N$_2$:Ar:O$_2$=24:56:20) feeding cylinder on the right hand-side, the ^{15}N$_2$ reserve bottle in the middle, and the solution reserve bottle on the left hand-side. Soybean plants were fixed in the center hole of the rubber stopper sealed with plastic clay.

B: Soybean samples were divided to 6 segments (S1, S2, and S3 for the shoot and R1, R2, and R3 for the underground part) with an equal length of the stem or primary roots.

2.2.2. Measurement of ^{15}N content

Freeze-dried samples of soybean plants were ground in a vibrating machine (CMT, Tokyo, Japan) into a fine powder. Samples were analyzed for total N and ^{15}N using an automated N and C analyzer by Mass Spectrometry machine (Thermo Finnigan EA 1112). The percentage of N derived from ^{15}N$_2$ (%^{15}N$_{dfa}$) in N total was calculated by the following equation:

%^{15}N$_{dfa}$=100 x ^{15}N atom% excess of sample / ^{15}N atom% excess of labeled ^{15}N$_2$ gas

2.3. ^{13}N radioisotope experiment

2.3.1. Synthesis of [^{13}N]N$_2$

Depending upon previous studies (Ishii et al., 2009), [^{13}N]N$_2$ was produced at the cyclotron facilities in TIARA (Japan Atomic Energy Agency, Takasaki, Gunma, Japan) by bombarding CO_2 in ten minutes with 0.5μA of 18.3 MeV proton beam delivered from a cyclotron. The production of [^{13}N]N$_2$ can be described by following scheme (**Figure 2.2**).

The rapid production method for the [^{13}N]N$_2$ tracer based on previous study (Ishii et al., 2009) and some modification was described as follows: 38 mL of pure CO_2 gas was filled into a target chamber with 5×10^5 Pa and then it was irradiated with a proton beam delivered from a cyclotron. After irradiating, 15

mL of non-radioactive nitrogen gas was added to the target chamber as the carrier gas in order to carry the N radioactive from target chamber to the receiver. The mixed gases after irradiating (including CO_2, $[^{13}N]N_2$, $[^{13}N]N_2O$ and N_2) were purified by passing through a glass column containing soda lime powder (Soda lime No.1; Wako Pure Chemical Industries, Osaka, Japan) to absorb all CO_2, and then mixed gas went through a glass column containing pure granular copper (LUDISWISS, Switzerland) placed in a furnace at $600^{o}C$ in order to deoxidize $[^{13}N]N_2O$ to $[^{13}N]N_2$. The purified gas was collected in a syringe for checking contamination by gas chromatography. After purifying, twenty-five mL of the $[^{13}N]N_2$ radioactive gas was collected in a syringe and then the obtained gas was mixed with 15 mL of non-radioactive N_2 gas and proper volume of O_2 or He gas depending on each experiment to make the finally desire composition of the tracer gas for treatment.
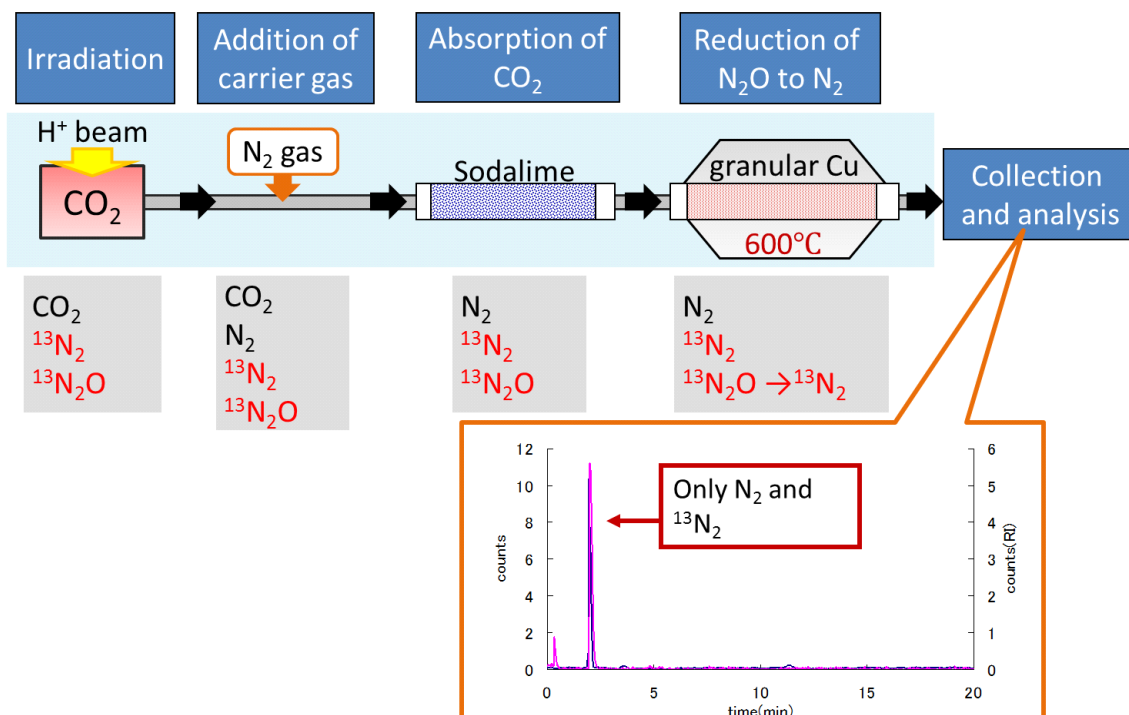
Figure 2.2: Schematic diagram of production of ^{13}N tracer gas (Courtesy of Dr. Satomi Ishii, JAEA).

2.3.2. ^{13}N tracer gas treatment and imaging with PETIS

The PETIS system for imaging experiment was set up as shown in **figure 2.3**. The root system of soybean plants was inserted into an acrylic box and the base stem of the plant at the top of acrylic box was sealed by plastic clay to prevent gas leakage. The inlet and outlet of the gases and solution were connected with silicon tubes and controlled by valves (**Figure 2.3A**). The acrylic box was placed in the middle between the two detector heads of PETIS (Modified type of PPIS-

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4800; Hamamatsu Photonics, Hamamatsu, Japan) in a growth chamber (**Figure 2.3B**) with relative humidity of 65% at 28°C, so that the main observation area was located at the center of the field of view (FOV). The light was maintained at a photon flux density of approximately 150 µmol photon m^{-2}s^{-1}.

First, root system was adapted to a non-radioactive gas for 30 min. Then, the culture solution in the acrylic box was raised to the inner top of the acrylic box to flush out the initial gas. Subsequently**,** the 50 mL of solution was drained off and 50 mL of the tracer gas containing [^{13}N]N$_2$ was introduced to the box at the same time. The ^{13}N tracer gas was kept for 10 min in the acrylic box, and flushed out by raising the solution in the acrylic box.

PETIS imaging was started when [^{13}N]N$_2$ tracer was filled in the acrylic box. Each frame (image) was obtained in every 10 seconds for 1 hour.
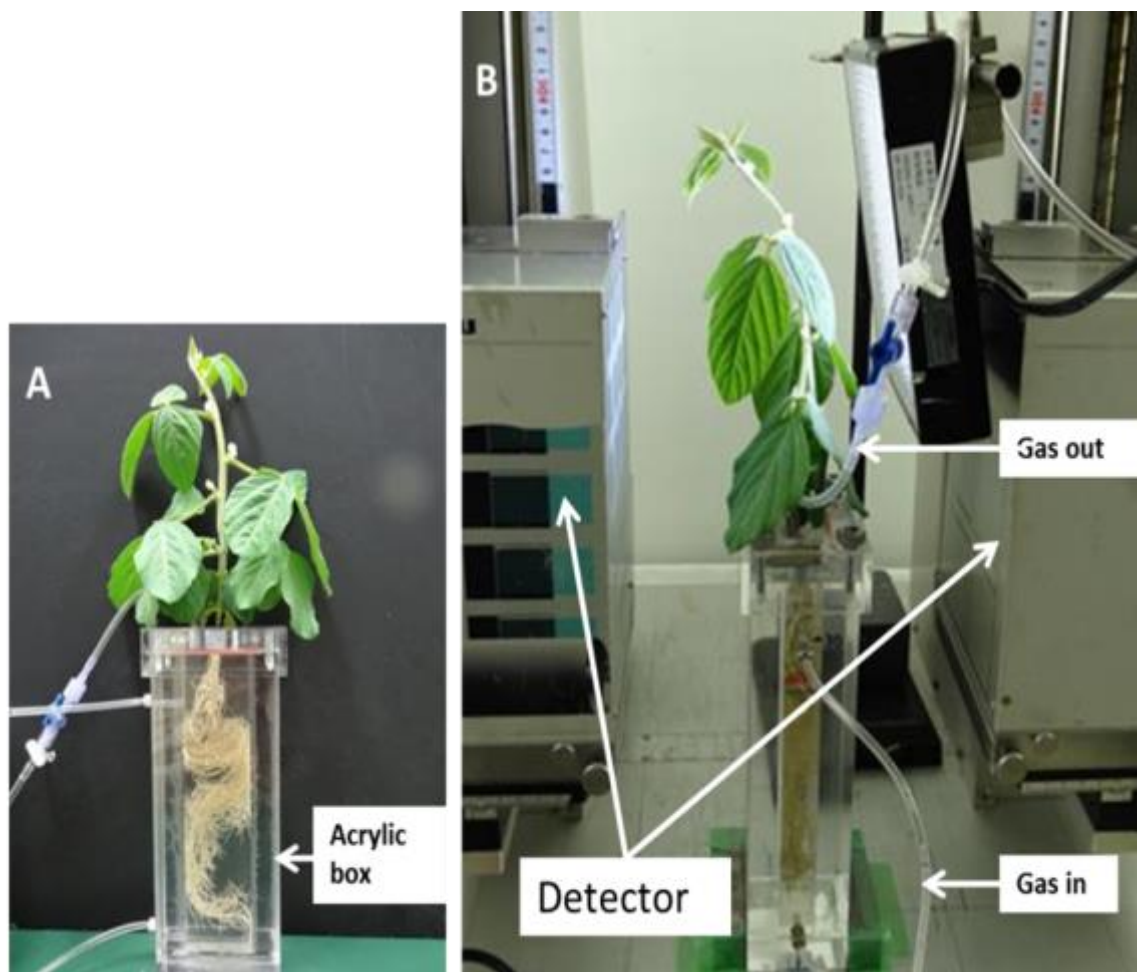
Figure 2.3: Soybean plant was set up for [^{13}N]N$_2$ experiment.

A: The test soybean plant in acrylic box.

B: PETIS imaging

2.3.3. Analysis of [^{13}N]N$_2$ fixation and transport in soybean plant

All PETIS image data were reconstructed and analyzed by using NIH image J 1.45 software. To estimate the dynamic of [^{13}N]N$_2$ accumulated in

nodules and the translocation of fixed $[^{13}N]N_2$ from nodules to the upper stem, the regions of interest (ROIs) on the integrated PETIS images (**Figure 2.4**) were drawn and extracted at clump of nodules and on the stem. The time-activity curves (TACs) were generated from ROIs of a series PETIS images in 60 minutes, these curves were corrected for physical decay of $[^{13}N]N_2$. The data of TACs will be used for estimating the rate import and export of $[^{13}N]N_2$ at ROIs.
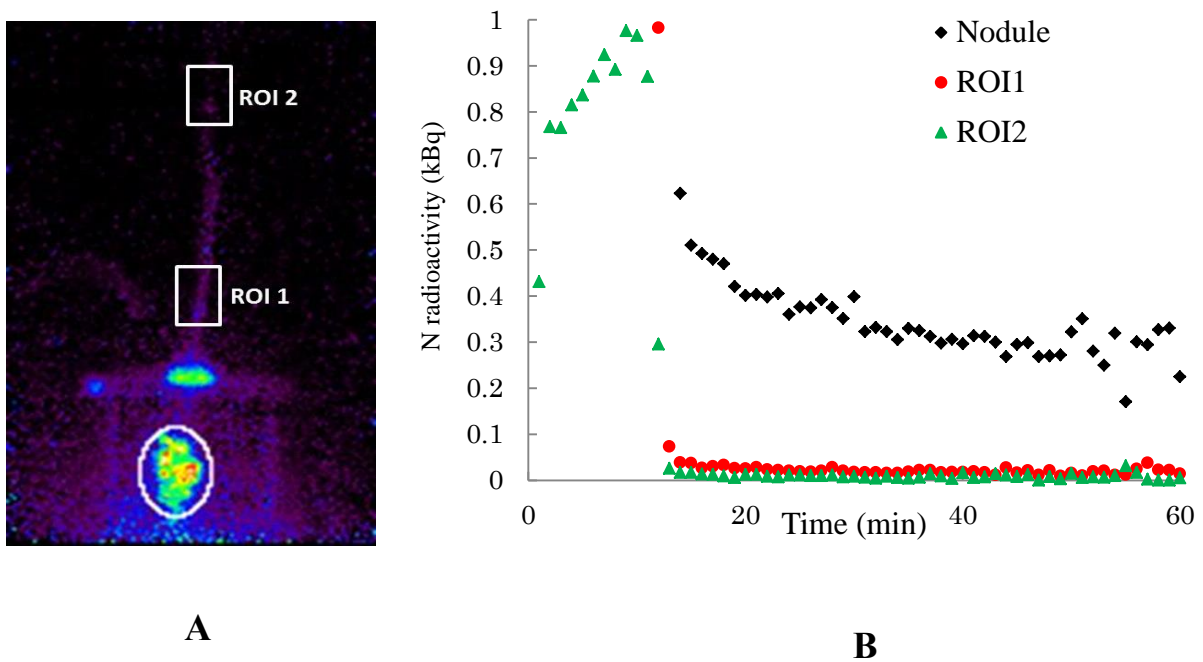

A

B

Figure 2.4: The integrated PETIS images of ^{13}N activity and the time-activity curves (TAC) in soybean plant and ROIs.

A: The integrated PETIS image

B: The time-activity curves

Chapter 3

Quantitative Analysis of the Initial Transport of Fixed Nitrogen in Nodulated Soybean Plants using ^{15}N as a Tracer

3.1. Summary

Quantitative analysis of the initial transport of fixed ^{15}N in intact nodulated soybean plants was investigated by a ^{15}N pulse-chase experiment. Soybean seeds (*Glycine max* [L.] Merr. cv. Williams) were inoculated with *Bradyrhizobium japonicum* (USDA110) and cultivated in hydroponics. A pulse-chase experiment with ^{15}N$_2$ exposure to nodulated roots for 1 hour, followed by 0, 1, 3, and 7 hours of non-labeled conditions (chase-period) was carried out at the vegetative stages (36 days after planting; DAP) and pod-filling stage (91DAP). Plant roots and shoots were separated into three sections (basal, middle, and distal parts) with the same length of the main stem or primary root. As a result, about 77% (36 DAP) and 80% (91 DAP) of fixed N was distributed in the basal part of the nodulated roots at the end of 1 hour of ^{15}N$_2$ exposure. A similar amount of ^{15}N was distributed in the basal, middle, and distal parts of the shoots just after 1 hour of ^{15}N$_2$ exposure. About 90% of fixed ^{15}N was retained in the nodules after 1 hour of ^{15}N$_2$ exposure at 91 DAP and ^{15}N distribution was higher in the basal nodules (78%) than in the middle (12%) and distal nodules

(0.1%). The percentage distribution of ^{15}N in the nodules at 91 DAP, decreased from 90% to 7% during the 7 hours of the chase-period, and increased in the roots (14%), stems (54%) leaves (12%), pods (10%), and seeds (4%) during this period. ^{15}N distribution was negligible in the distal root segment, suggesting that nitrogen fixation activity was negligible and recycling fixed N from the shoot to the roots was very low for the 7 hours of the chase-period.

3.2. Background of this study

Soybean plants require a large amount of N because the seeds contain a high concentration of protein. They assimilate a large amount of nitrogen during both the vegetative and reproductive stages, and the total amount of N assimilated in a plant has been significantly correlated with the soybean seed yield (Ohyama et al., 2012). Soybean plants can fix dinitrogen (N_2) in the air by the root nodule, which is a symbiotic organ with the soil bacteria, bradyrhizobia. Soybean roots also absorb inorganic nitrogen, usually nitrate (NO_3^-), from the soil. Sole N_2 fixation is known to be not enough for the maximum seed yield of soybeans, and it is necessary to use both N_2 fixation and N absorbed from roots (Harper, 1974, 1987, Tajima et al., 2004; Tewari et al., 2006). When soybean plants depend only on N_2 fixation, vigorous vegetative growth does not occur, which results in a reduced seed yield. On the other hand, a heavy supply of N fertilizer often depresses nodule development and N_2 fixation activity, and

induces nodule senescence, which also results in a reduced seed yield. In addition, an excess N supply causes luxuriant shoot growth, which results in lodging and poor pod formation. Therefore, no nitrogen fertilizer or only a small amount of N fertilizer is applied for soybean cultivation as "starter N" to promote initial growth after germination.

Nitrogen fixation is a symbiotic process with a legume and rhizobia association, in which legume roots incorporate the soil bacteria, rhizobia and form root nodules. Rhizobia turn to bacteroids, the symbiotic state of rhizobia, in the infected cells in nodules, and start to fix atmospheric dinitrogen (N_2). Ammonia is known to be an initial product of nitrogen fixation catalyzed by the enzyme "nitrogenase" (EC 1.18.6.1), and more than 90% of fixed N in the soluble fraction of the soybean nodule was detected as ammonia after the exposure of $^{15}N_2$ to soybean nodules for 1 minute (Bergersen, 1965). Fixed ammonia was also shown to be rapidly excreted from bacteroids to the plant cytosol of nodule cells and initially assimilated by the GS/GOGAT system (Ohyama and Kumazawa, 1978, 1979, 1980), which was then metabolized into ureides (allantoin and allantoic acid) through *de novo* synthesis of purine bases.

The N element including fixed-N and N absorbed from soil and fertilizers was consider transporting in xylem system, of which N forms originated from N_2 fixation were translocated directly to the pods and seeds in addition to transportation to the leaves (Ohyama, 1983). On the other hand,

nitrate nitrogen (NO_3^-) was primarily transported to the leaves then re-transported to the pods and seeds. The initial transport of nitrate absorbed by the roots, a part of ^{15}N derived from labeled $^{15}NO_3^-$ was quickly detected in the first 15 minutes in xylem sap collected from the cut end of the stump (Ohyama et al., 1989a). The transport form of N from absorbed NO_3^- in the roots was shown to be mainly nitrate and asparagine with a small amount of ureides (Ohyama and Kumazawa 1979, Ohtake et al. 1995). From the petiole girdling experiment, transported N compounds, such as ureides, nitrate, and asparagine, from N_2 fixation and NO_3^- absorption were not directly re-exported from the leaves, were metabolized once in the leaves then re-exported to the growing parts via phloem (Ohyama and Kawai 1982). The sink activity of pods and seeds may be related to the N re-transportation rate from the leaves to the pods because N-deficient soybean plants accumulated N in their pods from $^{13}NO_3^-$ or $^{15}NO_3^-$ much faster than those in N-sufficient soybean plants (Ohtake et al. 2001).

Regarding the relationship between nitrogen fixation and nitrate absorption, nitrate is known to inhibit nodule growth and nitrogen fixation activity (Streeter 1988, Fujikake et al., 2003, Ohyama et al. 2011). Nitrate absorbed from the lower part of the roots is not readily transported to the soybean nodules attached to the upper part of the roots (Yashima et al., 2005), while nitrate can be directly absorbed from the nodule surface (Mizukoshi et al., 1995). Nitrate absorption and its transport pattern were not affected by nodulation

relative to the nodulated and non-nodulated isolines of soybean, when a short-term distribution pattern was observed using a positron-emitting tracer imaging system (Sato et al., 1999).

Until now, little evidence has been obtained for quantitative analysis of the initial transport and distribution of fixed N in nodulated soybean plants. Ishii et al. (2009) conducted positron-imaging of ^{13}N in the nodulated roots of intact soybean plant exposed to $^{13}N_2$, and estimated the average nitrogen fixation rate to be 0.17 μmol N_2 h^{-1}, with a decreased rate of assimilated nitrogen in the nodule of 0.012 μmol N_2 h^{-1}. This result indicates that the translocation rate of fixed N is relatively low just after N_2 fixation. In the case of soybean nodules, the major part of fixed ammonia should be assimilated by the GS/GOGAT pathway, and synthesized into ureides, allantoin, and allantoic acid through purine base biosynthesis and degradation (Tajima et al. 2004, Ohyama et al. 1978, 1979, 2009); therefore, a relatively long time may be required for the assimilation and transport of fixed N from the nodules.

In this study, we conducted a pulse-chase experiment after a 1-hour exposure of $^{15}N_2$ labeled air to the nodulated roots to analyze the quantity of the initial transport of fixed-N in soybean.

3.3. Materials and Methods

3.3.1. Plant materials and culture

Soybean (*Glycine max* [L.] Merr., cv. Williams) seeds were sown on a plastic tray filled with vermiculite on 10 June, 2011. On 20 June, 2011, when the primary leaves developed, each plant was transferred into a 1 L glass bottle containing 800 ml of nitrogen-free nutrient solution, the concentration was described in section 2.1.3 of chapter 2. The pH of the solution was adjusted at 5.5-6.0 and renewed at intervals of three days. The solution in the bottle was continuously aerated by an air pump. The plant was changed from a 1 L bottle to a larger bottle (2 L) 36 DAP. Plants were cultivated in a greenhouse under natural conditions and transferred to the plant growth chamber (SANYO, Growth Cabinet MLR-350) under controlled conditions with a day length of 16 h at $28^{o}C$ and night length of 8 h at $18^{o}C$ one day before the $^{15}N_2$ treatment.

3.3.2. $^{15}N_2$ pulse-chase experiment

Pulse-chase experiments were conducted twice at the vegetative stage before the flowering (36 DAP) and pod-filling stage (91 DAP). Experiments were conducted during daytime conditions and the details were described in section **2.2.1** of chapter 2.

3.3.3. Measurement of N concentration and ^{15}N abundance

Freeze-dried samples were ground into a fine powder with a vibrating mill (CMT, Tokyo, Japan). The N concentration (mgN/gDW) and ^{15}N abundance (atom % ^{15}N) of each sample was analyzed using a Stable Isotope Mass Spectrometry (Thermo Finnigan EA 1112). The percentage of N derived from ^{15}N$_2$ (%^{15}N) and total N content were calculated by these data and DW, and the percentage distribution of fixed N in each section (36DAP) or each part (91DAP) was presented.

3.4. Results

3.4.1. Plant growth and nitrogen fixation activity at two stages

Soybean plants were cultivated in a nitrogen-free culture solution without supplying combined nitrogen. The plants were well-nodulated and uniformly grown, and reached the pod-filling stage at the second treatment stage (91 DAPs). Table 3.1 shows the dry weight of each section of the plants at 36 DAPs and 91 DAPs, respectively. The average total dry weight of the soybean plant was 1.62 g at the vegetative stage (36 DAPs) and 28.3 g at the pod-filling stage (91 DAPs). The average total nitrogen content of the soybean plant was 53.7 mg at the vegetative stage (36 DAPs) and 1,178 mg at the pod-filling stage (91 DAPs) (data not shown). Although the length was the same among the three

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sections of the shoot and roots, the biomass (DW) and N content of each section were different. In both stages, the middle part of the shoot (S2) showed the highest dry weight, followed by S3 (distal section) and S1 (basal section) in the shoot. The dry weight of the roots was markedly high in R1 (basal section) than in R2 (middle section) and R3 (distal section) at both 36 and 91 DAPs. The top/root DW ratio increased slightly from 2.66 at 36 DAPs to 3.11 at 91 DAPs.

Table 3.1: Dry weight of each segment of shoot and root at 36 DAP and   91 DAP (g part^{-1}).

	36 DAP (g)	91 DAP (g)
S3	0.300 (0.029)	7.67 (0.78)
S2	0.505 (0.024)	8.57 (0.56)
S1	0.372 (0.032)	5.13 (0.37)
Shoot total	1.177 (0.075)	21.37 (0.79)
R1	0.294 (0.016)	5.03 (0.34)
R2	0.080 (0.010)	1.41 (0.10)
R3	0.069 (0.026)	0.44(0.03)
Root total	0.443 (0.034)	6.88 (0.79)
Total/plant	1.620 (0.080)	28.25 (0.92)

DAP: Days after plating, S1, S2 and S3 shows the basal to terminal part of shoot with equal length, and R1, R2 and R3 shows the basal to terminal part of root with equal length, Number in parenthesis is standard error (n=16).

The total nitrogen content derived from nitrogen fixation per plant was 133 µgN at 36 DAPs, and 497 µgN at 91 DAPs (**Figure 3.1A**). However, the concentration of ^{15}N per total gDW of the soybean at the vegetative stage (86 µgN/gDW) was higher than that of the soybean at the pod-filling stage (19µg ^{15}N/gDW) (Figure 4.1B). From the results of field estimations of nitrogen fixation activity by the relative ureide method, nitrogen fixation activity from R5 to R7 was about 10 mgN per day per plant (Tewari et al., 2007). Therefore, the average nitrogen fixation activity in this experiment (about 500 µgN/h =12 mgN/day) at 91 DAPs in our system was equivalent to the nitrogen fixation activity measured in field estimations.
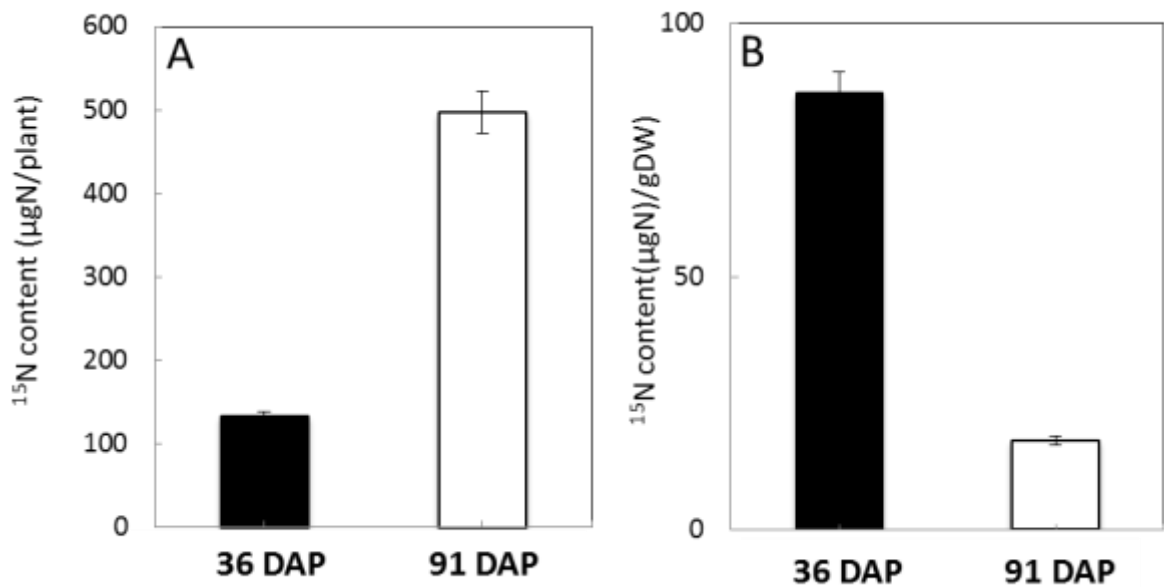
Figure 3.1: Average amount of nitrogen fixation per plant or per gDW for 1 h of

^{15}N$_2$ **gas feeding**

A: The amount of total fixed ^{15}N per plant.

B: The amount of fixed ^{15}N per gram DW.

Bar on the column shows the standard error (SE).

3.4.2. Translocation of fixed nitrogen among the shoot and root sections

In order to analyze the movement of fixed nitrogen in soybean plants, shoot and roots were divided into three sections with an equal length of the main stem (S1, S2, and S3) or primary root (R1, R2, and R3) as shown in **Figure 3.1B**. Changes in percentage distribution of ^{15}N (%^{15}N) in soybean plants are shown in **Figure 3.2.**

In both stages, about 80% of the fixed ^{15}N remained in the underground parts, mainly in the R1 section, 1 h after ^{15}N$_2$ air exposure, and the proportion of ^{15}N distributed in R1 then gradually decreased thereafter. The rate of the decrease in %^{15}N in the basal section of the roots (R1) was faster until 4 h in 36 DAPs plants than in 91 DAPs plants; however, almost the same percentage remained in the underground parts at 8 h 36 DAPs (25%) and 91 DAPs (21%). The distribution of ^{15}N in R2 was very low at about 1-2% of total fixed ^{15}N at 36 DAPs, but was over 10% at 91 DAPs. The distribution of ^{15}N in R3 was negligible at any sampling time, although some nodules were formed in this section **(Table 3.2).** This may be due to the nodules in the lower roots being

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inactive, or respiration in nodules soaked in solution being restricted and nitrogen fixation activity being depressed. Furthermore, $\%^{15}N$ did not increase during the chase-period in R3 at both 36 DAPs and 91 DAPs, suggesting that recycling fixed N from leaves to the distal part of the roots and nodules may need longer than 8 h.

Interestingly, the $\%^{15}N$ was almost the same among the S1 (6%), S2 (8%), and S3 (8%) segments in the shoot 1 h after $^{15}N_2$ exposure at 36 DAPs, suggesting that the same amounts of fixed N were transported to each section and reached the upper (S3) part of the shoot through xylem via a transpiration stream. At 36 DAPs, the $\%^{15}N$ in S1 increased from 1 h to 4 h and then decreased at 8 h, which was the equivalent part transported to S2 and S3. On the other hand, the $\%^{15}N$ in each part of the shoot (S1, S2, and S3) consistently increased at 91 DAP. These differences may be related to the different growth stages, the vegetative stage at 36 DAPs and reproductive stage at 91 DAPs, at which pods and seeds developed.
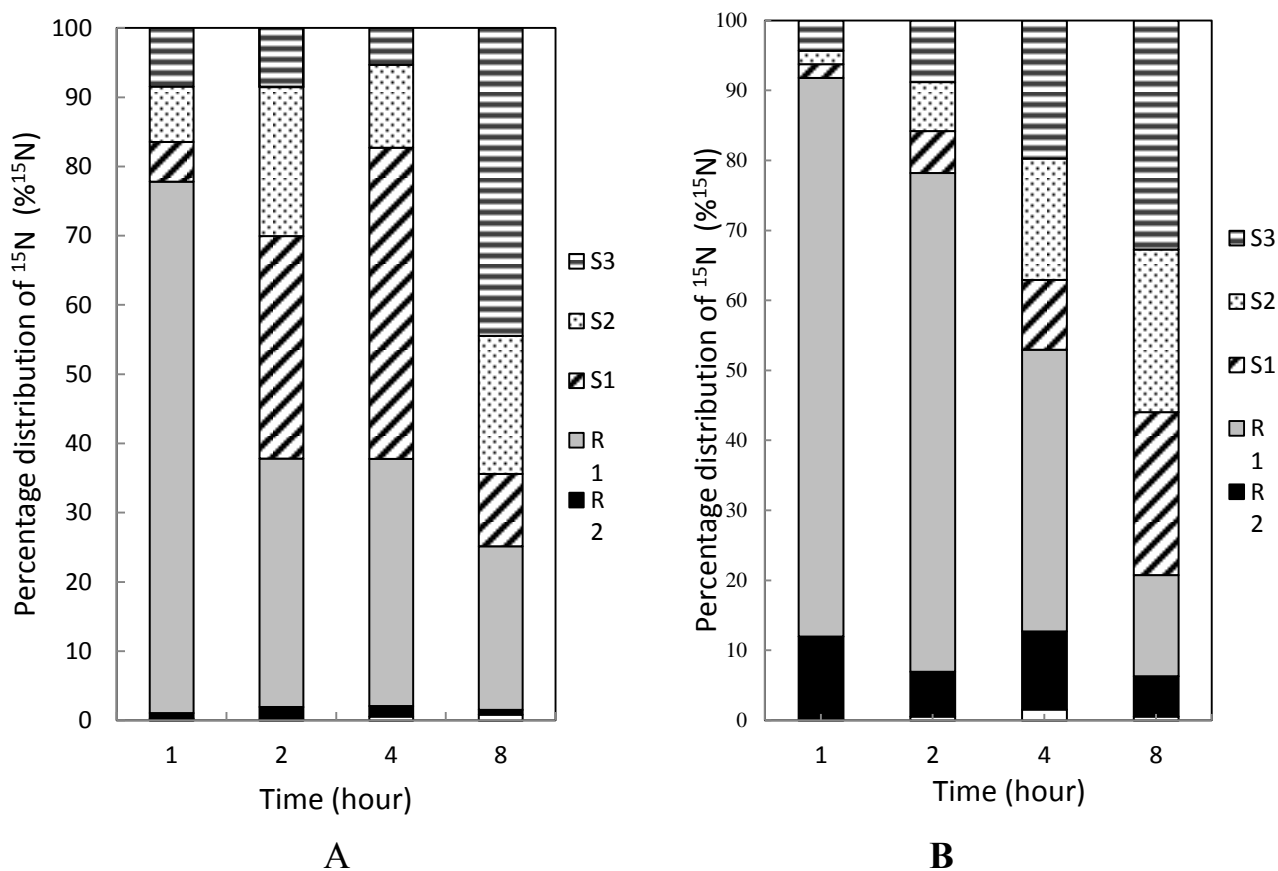
Figure 3.2: Changes in the percentage distribution of fixed N in each section of the shoot (S1, S2, and S3) and roots (R1, R2, and R3) of the soybean plant at 36 DAP and 91 DAP.

A: Soybean plants at 36 DAPs.

B: Soybean plants at 91 DAPs.

3.4.3. Translocation of fixed nitrogen in each organ at the pod-filling stage

The nodules and roots in the root sections, and the stems, leaves, pods and seeds in the shoot sections were separated for plant samples on 91 DAP, and the dry weight (**Table 3.2**) and total nitrogen content in each part (**Table 3.3**) were measured. The DW and N contents of stems were higher in S1 (basal part) than in S2 and S3 because the S1 stem was thicker than the others. In contrast, the DW and N contents were lower in the leaves, pods, and seeds in S1 than in the S2 and S3 sections (**Table 3.2, Table 3.3A**). The DW and N contents in the nodules and roots were higher in R1 than in the R2 and R3 sections (**Table 3.2, Table 3.3B**). The DW of the nodules in R1, R2, and R3 at 91 DAPs were 1.13 g, 0.24 g, and 0.13 g, respectively.

Table 3.2: Dry weight of each segment at 91DAPs (gDW part^{-1}).

	Stems (g)	Leaves (g)	Pods (g)	Seeds (g)	Nodules (g)	Roots (g)
S3	1.63 (0.51)	3.50 (0.40)	1.72 (0.17)	0.82 (0.11)		
S2	1.53 (0.09)	4.36 (0.32)	1.73 (0.17)	0.95 (0.12)		
S1	2.74 (0.14)	1.47 (0.22)	0.63 (0.14)	0.29 (0.05)		
R1					1.13 (0.08)	3.90 (0.29)
R2					0.24 (0.03)	1.17 (0.10)
R3					0.13 (0.02)	0.31 (0.03)
Total	5.90 (0.55)	9.33 (0.60)	4.08 (0.23)	2.06 (0.21)	1.50 (0.89)	5.38 (0.44)

DAP: Day after plating, DW: Dry weight, S1, S2 and S3 shows the basal to terminal part of shoot with equal length, and R1, R2 and R3 shows the basal to terminal part of root with equal length, Number in parenthesis is standard error (n=16).

Table 3.3A: Nitrogen content in stems leaves, pods and seeds in shoot segments S1, S2, and S3 at 91 DAPs.

	Stems (mgN part^{-1})	Leaves (mgN part^{-1})	Pods (mgN part^{-1})	Seeds (mgN part^{-1})	Total (mgN part^{-1})
S3	41.8 (2.9)	168 (19)	62.6 (6.5)	46.6 (6.5)	319 (30)
S2	46.7 (2.9)	182 (13)	65.7 (6.3)	69.7 (8.5)	364 (26)
S1	74.6 (3.8)	62 (9.3)	18.3 (4.0)	23.1 (3.1)	178 (13)
Total	163 (8)	412 (27)	147 (9)	139 (14)	861 (48)

S1, S2 and S3 shows the basal to terminal part of shoot with equal length, Number in parenthesis is standard error (n=4).

Table 3.3B: Nitrogen content in nodules and roots in root segments R1, R2, and R3 at 91 DAPs.

	Nodules	Roots	Total
	(mgN part^{-1})	(mgN part^{-1})	(mgN part^{-1})
R1	71.3 (5.1)	160 (12)	231 (15)
R2	14.8 (1.7)	48.8 (4.3)	63.6 (4.5)
R3	6.1 (1.2)	18.0 (1.7)	24.1 (1.6)
Total	92.2 (5.5)	227 (14)	319 (17)

R1, R2 and R3 shows the basal to terminal part of root with equal length, Number in parenthesis is standard error (n=4).

Figure 3.4A shows changes in the amount of labeled N (µgN/gDW) in the nodules and roots of each root section. The labeled N concentration in the nodules in the R1 section was very high (246 µgN/gDW) at the end of 1 h of $^{15}N_2$ exposure. The labeled N concentration in the nodules decreased 2 h (185 µgN/gDW), 4 h (85 µgN/gDW), and 8 h (56 µgN/gDW) after starting the $^{15}N_2$ treatment. The half time of fixed N release from the nodules was about 2 h. The

labeled N concentration in the roots in the R1 section increased at 4 h (44 μgN/gDW), then decreased at 8 h (17 μgN/gDW), suggesting the passage of fixed N through the root to the shoot. The labeled N concentration in the nodules in the R2 section at 1 h after $^{15}N_2$ exposure was about 55μgN/gDW and was much lower than those in the nodules in the R1 section. The labeled N concentration in the nodules and roots in the R3 section was negligible at any time, suggesting that nitrogen fixation did not occur in this section although some nodules were visible. In addition, recycling fixed N from the shoots to the distal part of the roots was not detected after 8 h of the treatment.

Figure 3.4B shows changes in the labeled N concentration in the shoot organs after feeding $^{15}N_2$ for 1 h. In the S1, S2, and S3 sections, the labeled N concentration in the stems increased rapidly until 2 h, then became constant or slightly increased from 4 h to 8 h. The labeled N concentration in the leaves increased until 4 h in all sections, and then gradually increased until 8 h. The labeled N concentration in the pods and seeds was negligible 1 h after $^{15}N_2$ feeding, and then consistently increased from 2 to 8 h. When these sections were compared the labeled N concentration was slightly higher in the upper part of the shoot section than in the basal section (S3>S2>S1).
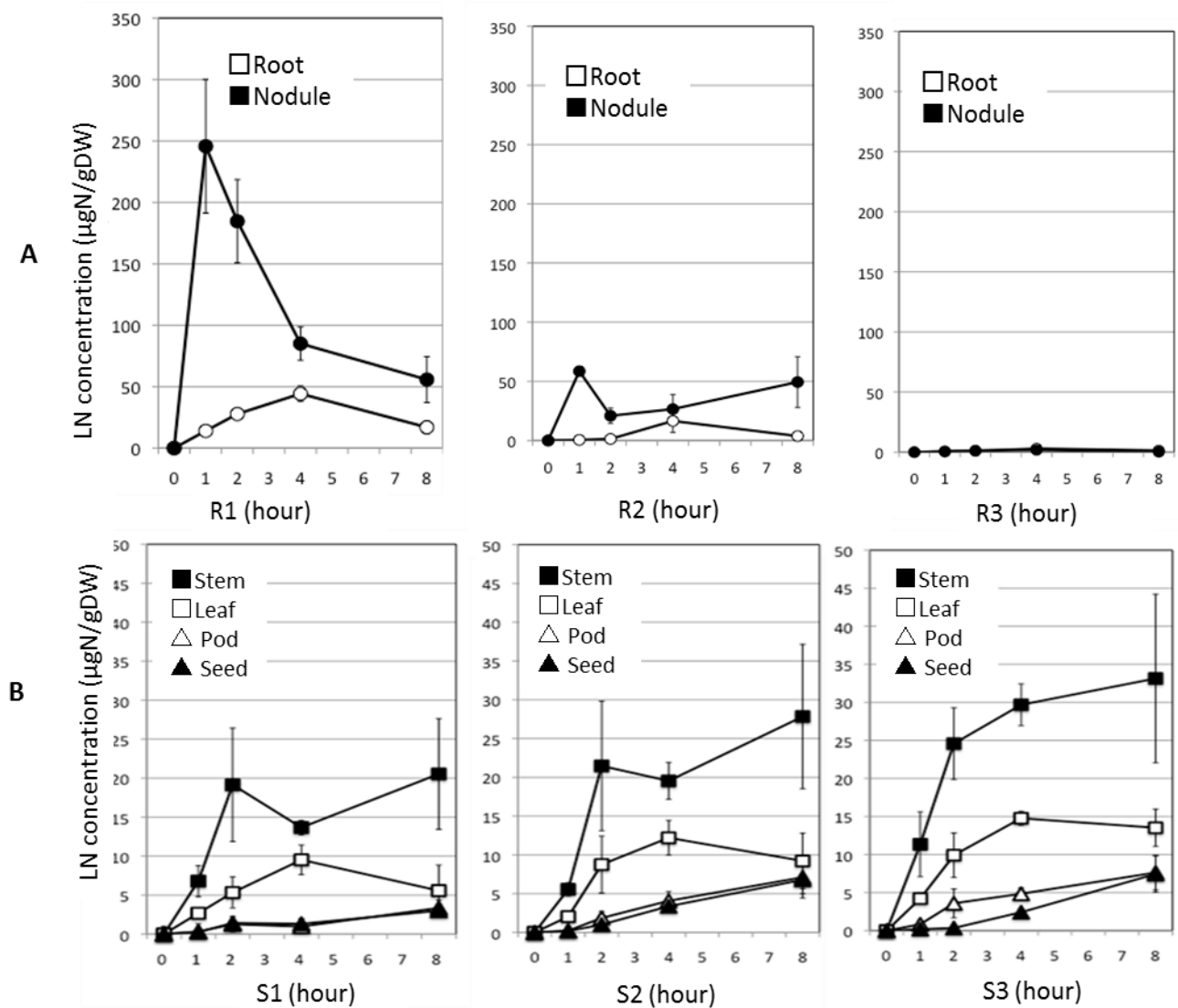
Figure 3.3: Changes in the amount of fixed N in the nodules, roots, stems, leaves, pods, and seeds of the soybean plant at pod-filling stage (91 DAP).

A: Underground part R1, R2, and R3.

B: Shoot part, S1, S2 and S3

Bar on the column shows the standard error (SE).

Figure 3.4 shows changes in the percentage distribution of labeled N ($\%^{15}N$) in each part of the soybean plants 91 DAPs. In Figure 4.4, the percentage of labeled N in each organ shows the sum of the $\%^{15}N$ in S1+S2+S3 for the shoot and R1+R2+R3 for the roots. At the end of feeding $^{15}N_2$ for 1 h, 90% of fixed N was distributed in the nodules, 2% was in the roots, 5% in the stems, 2% in the leaves, and 1% in the pods. The percentage of labeled N in the nodules decreased to 74% at 2 h, 32% at 4 h and 7% at 8 h. The $\%^{15}N$ in the roots increased from 2 h (3%) to 4 h (20%), and then decreased at 8 h (14%). The $\%^{15}N$ in the stems increased steadily from 1 h (5%), 2 h (13%), 4 h (29%) and 8 h (54%). The $\%^{15}N$ in the leaves also increased from 1 h (2%), 2 h (6%), 4 h (10%), and 8 h (12%). The $\%^{15}N$ in the pods and seeds was negligible at 1 h, but steadily increased from 2 h, and reached 10% in the pods and 4% in the seeds at 8 h.
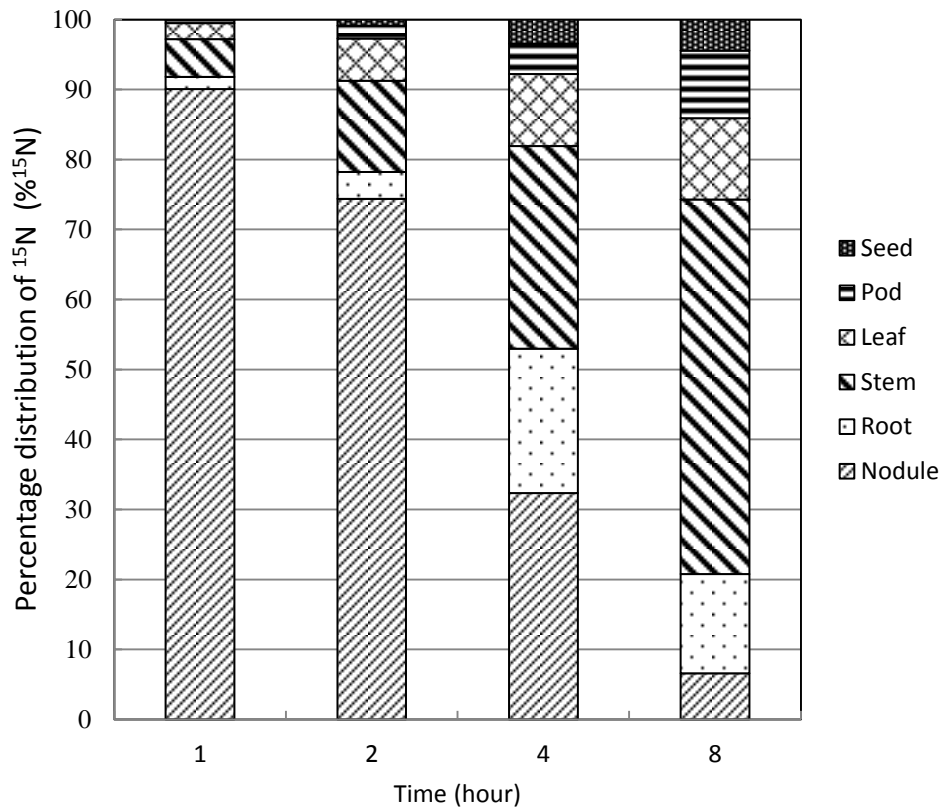
Figure 3.4: The percentage ^{15}N distribution in the nodules, roots, stems, leaves, pods, and seeds of the soybean plant at the pod-filling stage (91 DAP).

3.5. Discussion

3.5.1. Export rate of fixed nitrogen from the nodules

At the end of 1 h of exposure of ^{15}N$_2$, about 80% and 92% of labeled N remained in the underground part at 36 DAP and 91DAP, respectively (**Figure 4.3**). In addition, about 90% of fixed N remained in the nodules just after 1 h of exposure to ^{15}N$_2$ at 91 DAP, and 10% of the fixed ^{15}N was exported to the roots

and shoot (**Figure 3.4**). These results indicate that a large percentage of fixed N was retained in the nodules for initial 1 h at both the vegetative (36 DAPs) and reproductive (91 DAPs) stages, and only a small percentage of fixed nitrogen in the nodules was rapidly transported to the shoot after being exported from the nodules within 1 h.

The soybean nodule is a symbiotic organ, and has an infected region in the center and nodule cortex around the infected region (Mizukoshi et al., 1995). Vascular bundles in the nodule outer cortex around the infected region are connected to root vascular bundles, which supply photosynthates from the shoot to the nodules via phloem and transport fixed N from the nodule to the shoots via xylem vessels. In addition, the infected region has complex compartments, which are separated by several membranes, such as the bacteroid membrane, symbiosome membrane, infected cell membrane, and uninfected cell membrane. A major form of fixed N (about 80-90%) has been shown to be transported from soybean nodules as ureides (allantoin and allantoic acid) (Ohyama and Kumazawa 1978, 1979, Ohyama et al., 2009). The metabolic process of ureide synthesis is via *de novo* purine synthesis in the infected cells of nodules, and further degradation occur in uninfected cells in soybean nodules (Tajima et al. 2004). It was confirmed from short time $^{15}N_2$ gas exposure to soybean nodules that the incorporation of ^{15}N into ureides was relatively rapid, and was detected 10 min after $^{15}N_2$ gas exposure (Ohyama and Kumazawa 1978, 2009). Although

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the rapid synthesis of ureides has been previously reported, this is the first quantitative evidence to show that the transport of 90% of fixed nitrogen is retained in the nodules for longer than 1 h, and then most of it is transported from the roots and shoot during the subsequent 7 hours.

3.5.2. Comparison of nodulation and nitrogen fixation among the root segments.

A comparison of the nodule dry weight (Table 4.2) and amount of fixed ^{15}N (**Figure 3.4A**) in the nodules in the basal section (R1), middle section (R2), and apical section (R3) of the roots at 91 DAPs, revealed that the concentration and percentage distribution of ^{15}N in the R1 nodules were significantly higher than those in the R2 and R3 sections. The amount of fixed ^{15}N in nodules attached to R3 was especially negligible (**Figure 3.4A**). This was partly because the nodule mass was much lower in R3 than in R1. However, the amount of ^{15}N based on the gDW of nodules was also lower in R2 and R3 nodules than in R1 nodules (**Figure 3.4A**). This may be due to the fact that the nodules in these sections were inactive.

3.5.3. Transport of fixed nitrogen in the shoot segments

The transportation of nitrogen in the plant was reflected by changes in the ^{15}N content (**Figure 3.4**) and percentage distribution of labeled N among the

organs (**Figure 3.5**) at 91 DAPs. At both stages, fixed N was almost simultaneously transported to the basal section of the shoots (S1) as well as the middle (S2) and distal sections (S3). Therefore, the amount of ^{15}N fixed in the stem initially increased, followed by the leaves (**Figure 3.4B**). On the other hand, the incorporation of fixed ^{15}N in the pods and seeds was later than the stems and leaves.

Previous studies subjected to nodulated soybean plants to ^{15}N$_2$ or ^{15}NO$_3^-$ pulse-chase experiments at the pod-filling stage with 10 h of ^{15}N feedings and subsequent 5 days of non-labeled chase-period (Ohyama 1983). Immediately after 10 hours of ^{15}N$_2$ exposure, 36% of the fixed ^{15}N remained in the nodules and the rest was located in the roots (9%), stems (17%), leaves (18%), pods (10%), and seeds (10%). Within 5 days of the chase-period, the percentage of fixed ^{15}N decreased in the nodules (21%), roots (6%), and stems (9%), and increased in the seeds (36%), leaves (17%), and pods (13%). Meanwhile, the distribution of absorbed ^{15}N derived from ^{15}NO$_3^-$ under the same conditions was high in the roots (36%), stems (17%), and leaves (36%) and very low in the nodules (0.4%), pods (5%), and seeds (5%) 10 h after ^{15}NO$_3^-$ feeding period. At the end of 5 days of the pulse-chase period, ^{15}N originating from ^{15}NO$_3^-$ decreased in the roots, stems, and leaves, but increased in the pods (8%) and seeds (44%).

3.5.4. Recycling fixed nitrogen from the shoot to the roots.

Recycling N from the shoots to the apical growing part of the roots or young nodules via phloem may be essential to support their initial growth. In this study, the amount of ^{15}N in the R3 section was negligible until 8 h after the $^{15}N_2$ treatment. This indicates that recycling fixed N may be a long-term process.

3.6. Conclusion

The nitrogen fixation ability and fixed-N transport among plant parts were determined on soybean plants cultivated hydroponically in N free nutrient solution. The results showed that young soybean plants have higher activity in nitrogen fixation than mature soybean. The fixed-N remained in nodules relatively long time before transported to the other organs. The initial transport of fixed-N was not significant between two growth stages and fixed-N was translocated in priority to vegetative organs rather than to reproductive organs.

Chapter 4

Visualization of initial transport of fixed nitrogen in nodulated soybean plant using $^{13}N_2$ tracer gas in real-time

4.1. Summary

The observation of initial transport of fixed nitrogen in intact soybean plants at real-time was conducted by using the positron-emitting tracer imaging system (PETIS). Soybean root nodules were fed with $[^{13}N]N_2$ for 10 minutes, and the radioactivity of $[^{13}N]N$ tracer was recorded for 60 minutes. The serial images of nitrogen fixation activity and translocation of fixed nitrogen in the plant were reconstructed to estimate the fixed-N transport to the upper shoot. As a result, the signal of nitrogen radiotracer moving upward through the intact stem was successfully observed. This is the first report that the translocation of fixed-N is visualized at real-time in soybean plant by a moving image. The signal of nitrogen radiotracer was found at the base stem at about 20 minutes after the feeding of tracer gas and it took 40 minute to reach the upper stem. The velocity of fixed nitrogen translocation was estimated about 1.63 cm min^{-1}. The autoradiography taken after PETIS experiment showed a clear picture of transport of fixed ^{13}N in the whole plant that the fixed-N moves not only via xylem system but also via the phloem system to the shoot after transferring from xylem to phloem in the stem although it has been generally considered that the

fixed-N in nodule is transported dominantly via xylem by transpiration stream toward mature leaves. This also suggests that the initial transport of fixed-N was mainly into the stem and subsequently translocated to young leaves and buds via phloem system. These new findings in the initial transport of fixed nitrogen of soybean will become the basis for the future study with the whole legume plants.

4.2. Background

Since the understanding of biological nitrogen fixation and fixed-N transport is very important for applying to legume cultivation in order to increase crop productivity, so that the dynamic of BNF and fixed-N transport have been concerning many researchers. In soybean plant, after fixation a major part of fixed-N is metabolized to ureides in nodules and then transported to the upper parts including shoots, leaves and pods via xylem system and redistributed to pods, seeds and roots via phloem system (Ohyama et al., 2009).

There are two main routes that fixed-N is transported in legume plant. One, fixed-N is moved from root nodules via the xylem system to shoot. The other one, fixed-N after incorporating into various N compounds in mature leaves moved from the mature leaves to growth organs or the storage organs by phloem system (Oghoghorie and Page, 1972).

Up to now, various methods have been used in the field of BNF, of

which the positron emitting tracer imaging system (PETIS) developed in recent decades for researching in the field of plant nutrition is considered one of the most advanced methods. This method can overcome the obstacle that previous methods could not perform. Especially, this method could visualize the moving of some radioisotopes in intact plant with. PETIS was used successfully in the first real-time images of nitrogen fixation activity in intact soybean plant (Ishii et al., 2009), and in the analysis of nitrate transport in soybean (Sato et al., 1999)

In this study, PETIS was used to elucidate more clearly the pathway and the velocity that the fixed nitrogen was transported and translocated in soybean plant in the initial time.

4.3. Materials and Methods

4.3.1. Plant material and cultures

The cultivation of soybean plants (*Glycine max* [L.] Merr. cv. Williams) was described in section **2.1** of chapter 2. The soybean plants at 26-30 day-old were used for the experiments.

4.3.2. Synthesis of the [^{13}N]N$_2$ tracer

The detail of ^{13}N]N$_2$ production was described in section **2.3.1** of the chapter 2. In this experiment, 25 mL of the pure [^{13}N]N$_2$ gas after producing was

mixed 15 mL non-radioactive nitrogen and 10 mL O_2 gas to make the final composition of O_2:N_2 = 2:8 for the experimental treatment.

4.3.3. [^{13}N]N_2 tracer gas treatment and imaging with PETIS

Soybean plants were fed with ^{13}N tracer gas in a growth chamber (**Figure 2.3B**). The detail process was described in section **2.3.2** of the chapter 2.

4.3.4. Estimation of nitrogen fixation rates and exporting rates of assimilated nitrogen in the root nodules

The general analysis processing of PETIS data was described in section **2.3.3** of the chapter 2. In order to estimate the fixed-N activity, the average radioactivity (Bq) of the first 10 frames after the flushing out of [^{13}N]N_2 tracer gas was calculated and then converted into the amount of total nitrogen (μmol N_2). This value indicates the amount of total nitrogen fixed by the nodules during 10 minutes of ^{13}N exposure and was used to estimate the rate of nitrogen fixation (μmol N_2 h^{-1}).

To analyze the export of fixed nitrogen from the nodules, a linear regression was made on the data points of the time-activity curve of each sample for 20 minutes from the end of flushing out of the tracer gas, and the slope of the line was converted to the decreasing rate of fixed N in nodule (μmol N_2 h^{-1}).

4.3.5. BAS imaging

To obtain autoradiography images, the plants were exposed to the imaging plates of a bio-imaging analyzer (BAS GUGE 2040, Fujifilm, Tokyo, Japan) for 30 minutes. After exposure, the plates were scanned with a bio-imaging analyzer system (GE Healthcare, Typhoon FLA 7000). The autoradiograph image was reconstructed by using NIH image J1.45.

4.4. Results

In the $[^{13}N]N_2$ tracer experiment using PETIS system, the nitrogen fixation activity and the transport of fixed nitrogen are reflected by the ^{13}N radioactivity accumulated at detected area of the plant. The **figure 4.2** shows the test plant and a serial images recorded by PETIS after flushing out of $[^{13}N]N_2$ gas from the acrylics box. Due to the small field of view, the detectors were only concentrated around the upper nodules and lower shoot (**Figure 4.1A**) to observe the dynamics of nitrogen fixation in the nodules and transport of the fixed nitrogen to upper parts. The PETIS images were taken in every ten second, and **figure 4.1B** shows the restacked images in a sequence of 5 minutes (equal to 30 frames) of all frames. It was demonstrated that just after five minutes exposing to $[^{13}N]N_2$ gas, the fixed nitrogen has already been at the base stem and then gradually moved up to shoot (**Figure 4.1B**).
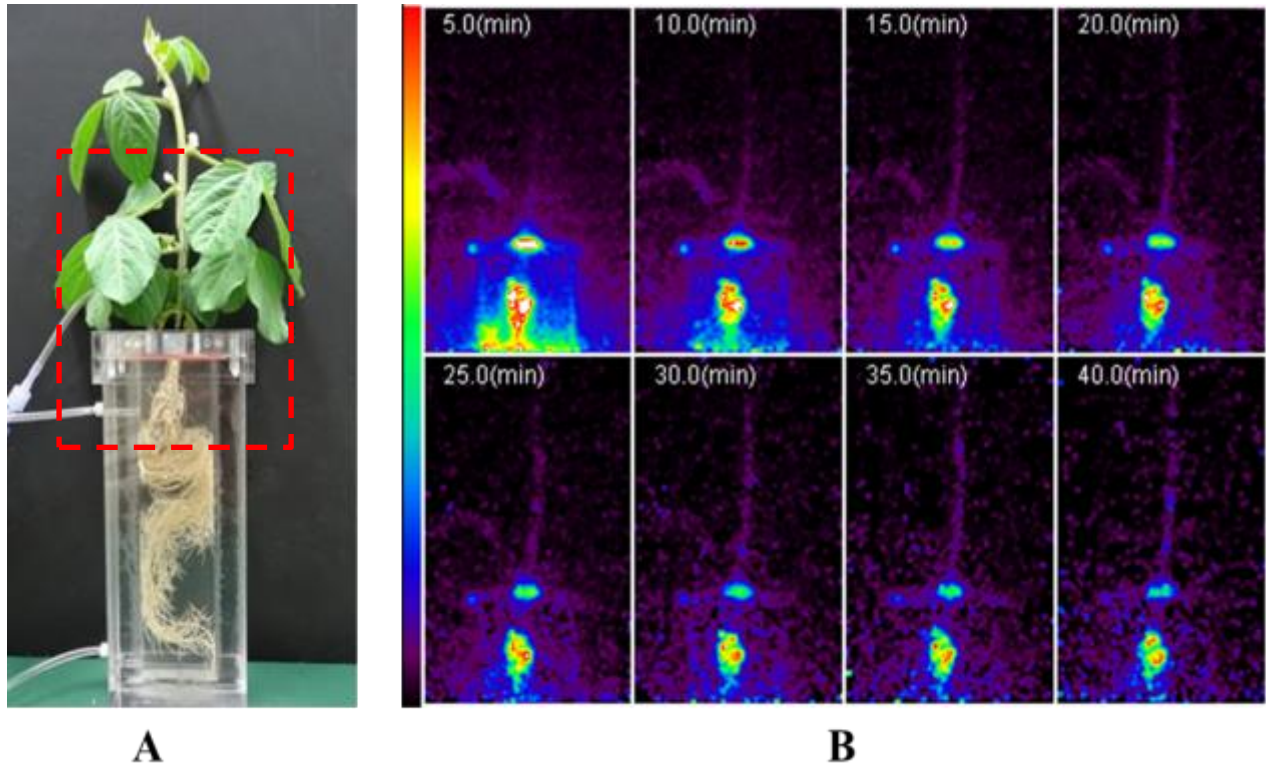
Figure 4.1: Test soybean plant and PETIS images.

A: Test plant at 26 DAPs

B. Serial PETIS images of ^{13}N movement in soybean shoot. Each image was generated by restacking of 30 original frames.
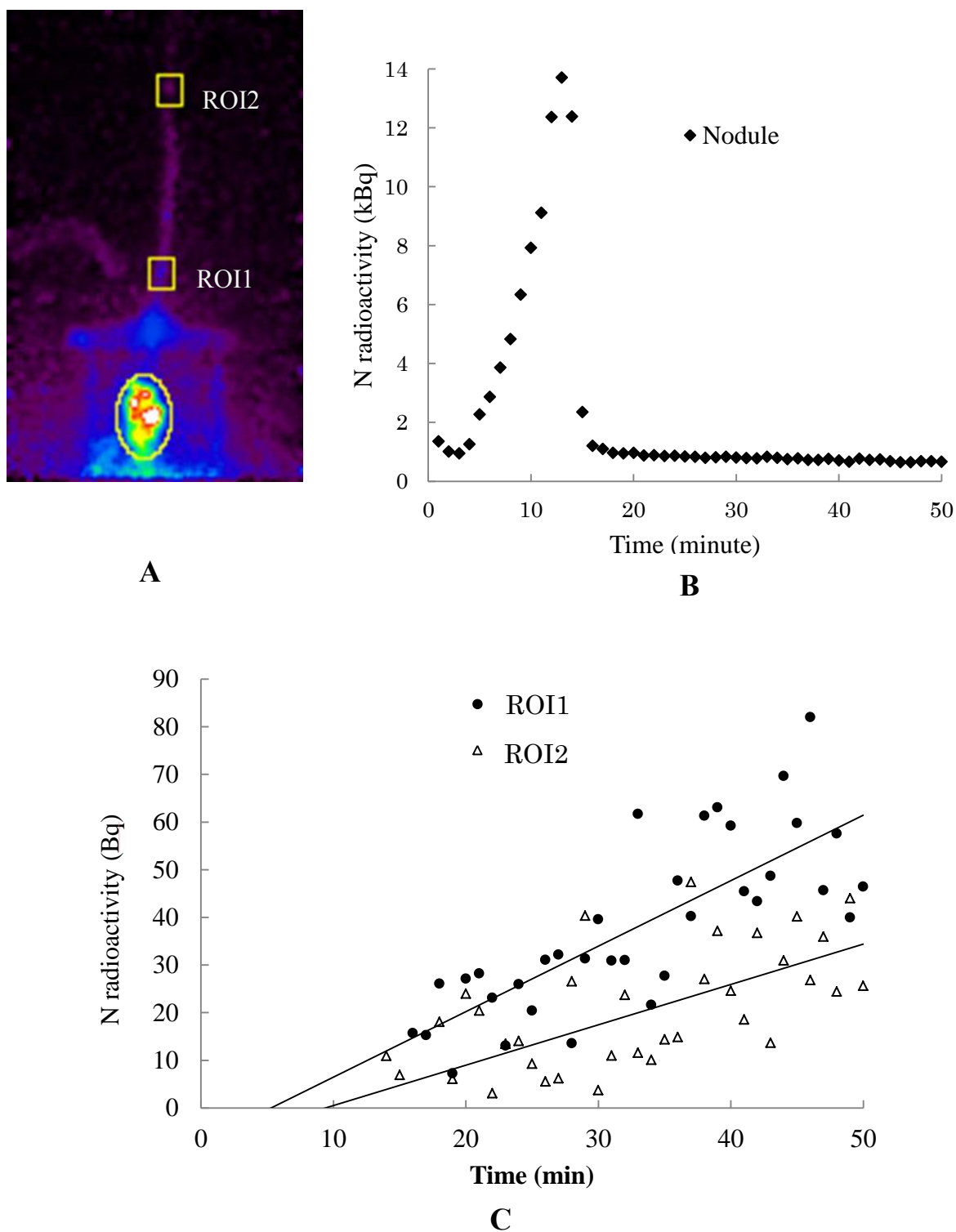
Figure 4.2: Analysis of time-activity curves generated from PETIS data.

A: Two selected ROIs on PETIS image

 B: The time-activity curves showing the fixed N accumulated at nodules

C: The time-activity curves showing the fixed N accumulated at two ROIs

The PETIS data was used to analyze the dynamic of nitrogen fixation activity and the translocation of fixed nitrogen from nodules to shoot. To estimate the nitrogen fixation rate and transport velocity of fixed nitrogen, the regions of interest (ROIs) were set on the nodule zone, base stem (ROI1), and upper stem (ROI2) along the stem (**Figure 4.2A**). **Figure 4.2B** shows the time activity curve from the nodule zone. It was estimated from the value just after flushing out of the tracer and the subsequent slope that the average rate of nitrogen fixation was about 0.538 µmol N_2 h^{-1} and the export rate of fixed nitrogen from nodules to the other parts was evaluated to be 0.017 µmol N_2 h^{-1} (**Table 4.1**). The distribution rate of fixed nitrogen form nodules to base stem (ROI1) and upper stem (ROI2) in the initial time was low, it was estimated about 0.0169µmol N_2 h^{-1} and 0.0101µmol N_2 h^{-1} at ROI1 (equal to 3.14% at base stem) and ROI2, respectively (**Table 4.1**).

Table 4.1: Evaluation of nitrogen fixation rate and export rate of fixed-N.

Plant	N_2 fixation rate (μmol N_2 h^{-1})	Fixed-N export rate (μmol N_2 h^{-1})	Fixed-N distribution (μmol N_2 h^{-1})	
			ROI1	ROI2
1	0.6054	0.0148	0.0147	0.0064
2	0.5278	0.0173	0.0211	0.0146
3	0.5560	0.0194	0.0118	0.0061
4	0.4626	0.0166	0.02018	0.0163
Mean(SE)	0.538±0.0298	0.0170±0.001	0.0169±0.0022	0.0106±0.0025

To estimate the velocity of the initial transport of fixed-N movement in the stem from root to the shoot, we used the data from TACs at two ROIs to calculate the arrival time of fixed-N at the base stem (ROI1) and the upper stem (ROI2). **Figure 4.3** shows the time activity curves (TACs) from the stem zone (ROI1 and ROI2). From the time-lag between the two TACs, the velocity of movement of fixed nitrogen in the stem was estimated about 1.63 cm min^{-1}.

In order to determine more clearly the initial transport of fixed nitrogen ([^{13}N]N_2 tracer) in soybean plant we subjected the test plant to the autoradiograph

after PETIS investigating. The photograph and BAS image of test plant (**Figure 4.3**) showed the accumulation of fixed-N in nodules and translocation to the shoot. In particular, in BAS image the ^{13}N radioactivity signal was observed in young leaves and new shoot (apical bud) but not in mature leaves and roots.

A   **B**
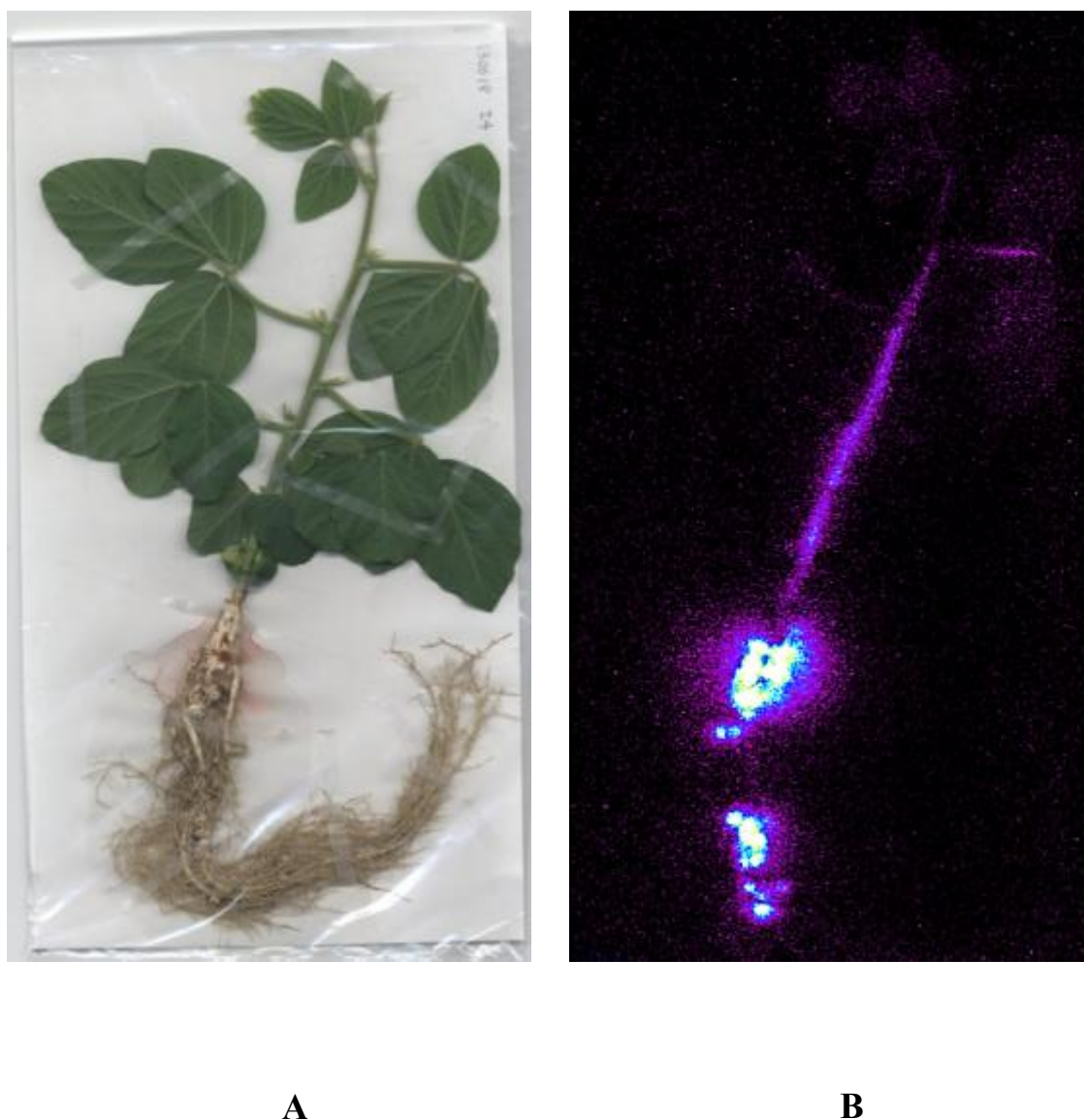
Figure 4.3: The photograph and autoradiograph of the test soybean plant.

A: Photograph of soybean plant at 26 DAPs   **B:** Autoradiograph

4.5. Discussions

The transportation of fixed nitrogen in legume plants has been studied for a long time, but this mechanism still needs to be elucidated more precisely. By using PETIS method, the movement of fixed nitrogen in soybean plant imaged clearly in this study for the first time. The translocation of fixed nitrogen was observed at base stem at about five minutes after feeding of ^{13}N radiotracer and at full stem (only in the FOV) at 40 minutes (**Figure 4.2**). This evidence suggested that fixed-^{13}N after synthesized in the nodules move to the shoot in a short time, which is similar to a previous result that ^{13}N was detected first at the trifoliate of soybean plant at 5-10 minutes after introducing ^{13}NO$_3^-$ (Sato et al., 1999), and ^{13}N reached at base stem of rice in 2 minute and at newest leaf in 6 minutes after supplying ^{13}NH$_4^+$ (Kiyomiya et al., 2001). It implies that the timing of movement of the fixed nitrogen from soybean nodules was not delayed even if it is compared to that of absorbed nitrogen from the culture solution. In other word, it does not need much time more than a few minutes to convert N$_2$ into new nitrogen compounds before starting transport of them to other. The velocity of the initial transport of fixed-^{13}N was estimated as about 1.6 cm min^{-1} at vegetative stage. This result was similar to previous result found that the speed of ^{13}N compound moving in rice plant at vegetative stage was 8.6 cm min^{-1} (Kiyomiya et al., 2001), and the movement of ^{13}N fixation compounds, ^{13}NO$_3^-$ and ^{13}NH$_4^+$ at rate of 6-12 cm min^{-1} in alfalfa root and shoot (Cadwell et al.,

1984). Therefore, it was suggested that most of the fixed-^{13}N is transported smoothly on the transpiration stream in the xylem from root to shoot as well as nitrate and ammonium.

Although the signal intensity of ^{13}N radioactivity was observed early at the base stem, it was still weak at the end of PETIS experiment, and the translocation of nitrogen radioactivity could not be seen in the whole plant because of the limitation of the field of view (FOV) by PETIS experiment. However, this phenomenon observed more clearly by the BAS image (**Figure 4.3**) performed after the end of PETIS measurement. In BAS image, the signal of N radiotracer was presented only in young leaves and bud (**Figure 4.3**). This result suggested that fixed nitrogen was transported in priority to upper organs (young leaves and bud) to create new compounds for plant growth rather than transported to mature leaves. This is consistent with my results found that fixed-^{13}N was exported to young upper parts of shoot especially stem more than lower parts of shoot (results in chapter 3). When observing the transport of $[^{13}N]NO_3$ in soybean plant by autoradiography, Sato et al. (1999) also found that the radioactivity of ^{13}N tracer was high in young and mature trifoliate leaves compared to primary leaves, while the $[^{13}N]N$ radiotracer derived from fixation presented here was only observed in young leaves. This suggesting that the fixed nitrogen was translocated to young leaves and buds, while nitrogen that absorbed from fertilizer and soil was transported to all shoots especially mature leaves. It

should be also noted in the BAS image that no signal was detected in the nodules and root of distal region nevertheless many nodules attached there. These nodules were immersed in the culture solution so that they could not contact to $[^{13}N]N_2$ tracer gas and could not directly fix it. Therefore, this result suggests that the recycling of fixed nitrogen from the shoots to distant parts of roots and nodules via phloem needs longer than 60 minutes.

No signal of ^{13}N radiotracer was found in the old leaves although they were close to the source of fixed nitrogen (nodules). This evidence may change the previous concept that the initial translocation of fixed-N through the shoot not only moves in xylem system by transpiration stream toward mature leaves (Ohyama et al., 2008a; Pate et al., 1979a; Pate et al., 1979c). It suggests that the fixed-N is transferred from xylem to phloem in the stem, and then translocated to young leaves and buds via phloem system. The new finding in this study on the initial transport of fixed nitrogen of soybean will become the basis for future study in nitrogen transport in the whole legume plants.

Chapter 5

Evaluation of the effects of low partial pressure of O_2 on nitrogen fixation in soybean using a positron-emitting tracer imaging system

5.1. Summary

Nitrogen fixation activity changes under various environmental conditions, of which partial pressure of oxygen is one of the most effective factors in legume plant. The effects of low partial pressure of oxygen in rhizosphere on the symbiotic nitrogen fixation activity and translocation to various organs were evaluated in real-time analysis by a PETIS. Soybean nodules were treated with mixed gas containing ^{13}N labeled N_2 with a various proportions of O_2, and the nitrogen fixation in the nodules was visualized by the PETIS. The results showed that under normal condition (pO_2: 0.20 atm) the nitrogen fixation ability of soybean plant was highest compared to that of lower O_2 conditions (pO_2: 0.00 and 0.10 atm). The nitrogen fixation activity of soybean nodules was strongly depressed with low O_2 concentrations, although it was not inhibited completely even at 0.00 atm pO_2. In contrast to the nitrogen fixation, the export of fixed-N from the nodules was enhanced by lower O_2 concentration.

5.2. Background

Oxygen plays dilemmatic roles in biological N_2 fixation processes in legume nodules. It is necessary for the respiration of bacteroids in nodules to supply energy for nitrogen fixation and assimilation, but it is well known that nitrogenase, the enzyme catalyzing synthesis of ammonia from atmospheric N_2, is very sensitive to molecular O_2 and irreversibly destroy nitrogenase (Criswell et al., 1976). To sustain the symbiotic nitrogen fixation process, legume nodules have to regulate the internal O_2 concentration in the central infected region at an optimal level. Such regulation is operated by a barrier with variable permeability to O_2 under various environmental conditions (Sheehy et al., 1983).

Soybean root nodules express a nodule specific protein leghemoglobin (Lb) in infected cells of the central region. This protein shows red color like hemoglobin, and O_2 binds to Lb forming LbO_2. The presence of Lb protects O_2 damage on nitrogenase, with providing sufficient O_2 for the respiration of bacteroids. Lb is essential for maintaining N_2 fixation in legume nodules and the mutants lacking Lb cannot fix N_2.

The previous studies found that when oxygen tension increased in root medium, it favoured the nitrogen fixation and oxygen tensions increased up to 50% it stimulates the fixation but if it excesses that level then the nitrogen fixation was inhibited (Ferguson and Bond, 1954). Bergersen (1962) pointed that the nitrogen fixation was inhibited by increased external pO_2 at 80%. The effect

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of rhizosphere pO_2 on the assimilation of $^{15}N_2$ in the nodules attached to the intact plants was investigated (Ohyama and Kumazawa, 1980). The incorporation of ^{15}N into 80% ethanol soluble fraction was depressed with decreasing pO_2 (0.20, 0.10, 0.05, 0.00 atm). The nodules under at pO_2=0.00 atm, the ^{15}N incorporation into all nitrogen compounds was nearly zero except ammonia. At pO_2=0.10 atm the incorporation of ^{15}N into allantoin was most strongly depressed.

So far, the studies of effect of O_2 partial pressure on nitrogen fixation were mostly based on the acetylene reduction activity. This is a simple, inexpensive and high sensitive method for studying of nitrogen fixation activity in legume plants, but the results only show the change of acetylene to ethylene and do not provide the real rate of nitrogen fixation (People et al., 1989). Besides, nitrogen stable isotope (^{15}N) was also used to estimate the effect of various pO_2 concentrations in intact soybean nodules (Ohyama and Kumazawa, 1980, and F. J. Bergerson, 1961), but this method also didn't explain the effects of O_2 under various O_2 concentrations at the actual time.

The PETIS, an advanced method, is very sensitive and accurate method. It can observe the changes of nitrogen fixation activity with very low concentration at real time. The aims of this study were to visualize and investigate the nitrogen fixation activity under the effects of low pO_2 in intact soybean root nodules in real time.

5.3. Materials and Methods

5.3.1. Plant culture

Soybean (*Glycine max* [L.]Merr. cv. Williams) plant cultivation was described in section **2.1** of chapter 2. The soybean plants at 26-30 day-old were used for the experiments.

5.3.2. [^{13}N]N$_2$ production

The detail of [^{13}N]N$_2$ production was described in section **2.3.1** of chapter 2. In this experiment in order to make various composition of O$_2$, the purified [^{13}N]N$_2$ radioactive gas was mixed with the non-radioactive N$_2$ and oxygen to make the final desire component for treatment described as follows:

- First, 25 mL of the pure [^{13}N]N$_2$ gas after purification was mixed with 15 mL of non-radiotracer N

- Next: The mixed N$_2$ gas (including [^{13}N]N$_2$ and N$_2$) was mixed with 10 mL of O$_2$ gas to make final composition of 20% O$_2$, or 5 mL of O$_2$ gas + 5 mL of He gas to make final composition of 10% O$_2$, or 10 mL of He gas to make the final composition of gas to make final composition of 0% O$_2$.

5.3.3. [^{13}N]N$_2$ tracer gas treatment and imaging with PETIS

The PETIS system for imaging experiment was set up shown in **figure**

2.3B, and described in section **2.3.2** of chapter 2. The procedure of ^{13}N tracer feeding was executed as follows: Before treating nitrogen radiotracer gases with different pO$_2$, the soybean root system was adapted to a non-radioactive gases with the same compositions except for [^{13}N]N$_2$ for 30 min. Then, the culture solution in the acrylic box was raised to the inner top of the acrylic box to flush out the initial gas. Subsequently, the solution was drained off 50 mL and filled in with 50ml of the tracer gas of [^{13}N]N$_2$ at the same time. The ^{13}N tracer gas was kept for 10 min in the acrylic box, and flushed out by raising the solution in the acrylic box. PETIS imaging was performed when [^{13}N]N$_2$ tracer starting filled in the acrylic box. Each frame (image) was obtained in every 10 seconds for 1 hour.

5.3.4. Estimation of nitrogen fixation rates and decreasing rates of assimilated nitrogen in the root nodules

The analysis processing of PETIS data was described in section **2.3.3** of the chapter 2 and section 4.3.4 of the chapter 4.

5.4. Results

To observe the effects of O$_2$ partial pressure on BNF in soybean nodules, the soybean plants were treated with nitrogen tracer gas ([^{13}N]N$_2$) containing various oxygen concentrations (pO$_2$= 0.00, 0.10 and 0.20 atm). Ten minutes after exposing to [^{13}N]N$_2$ tracer gas, the signal of N radioactivity was observed in the nodules by

PETIS images. These images were restacked in a sequence of 5 minutes (equal to 30 frames) of all frames after [^{13}N]N tracer gas flushing out (**Figure 5.1**), and used to estimate nitrogen fixation activity.
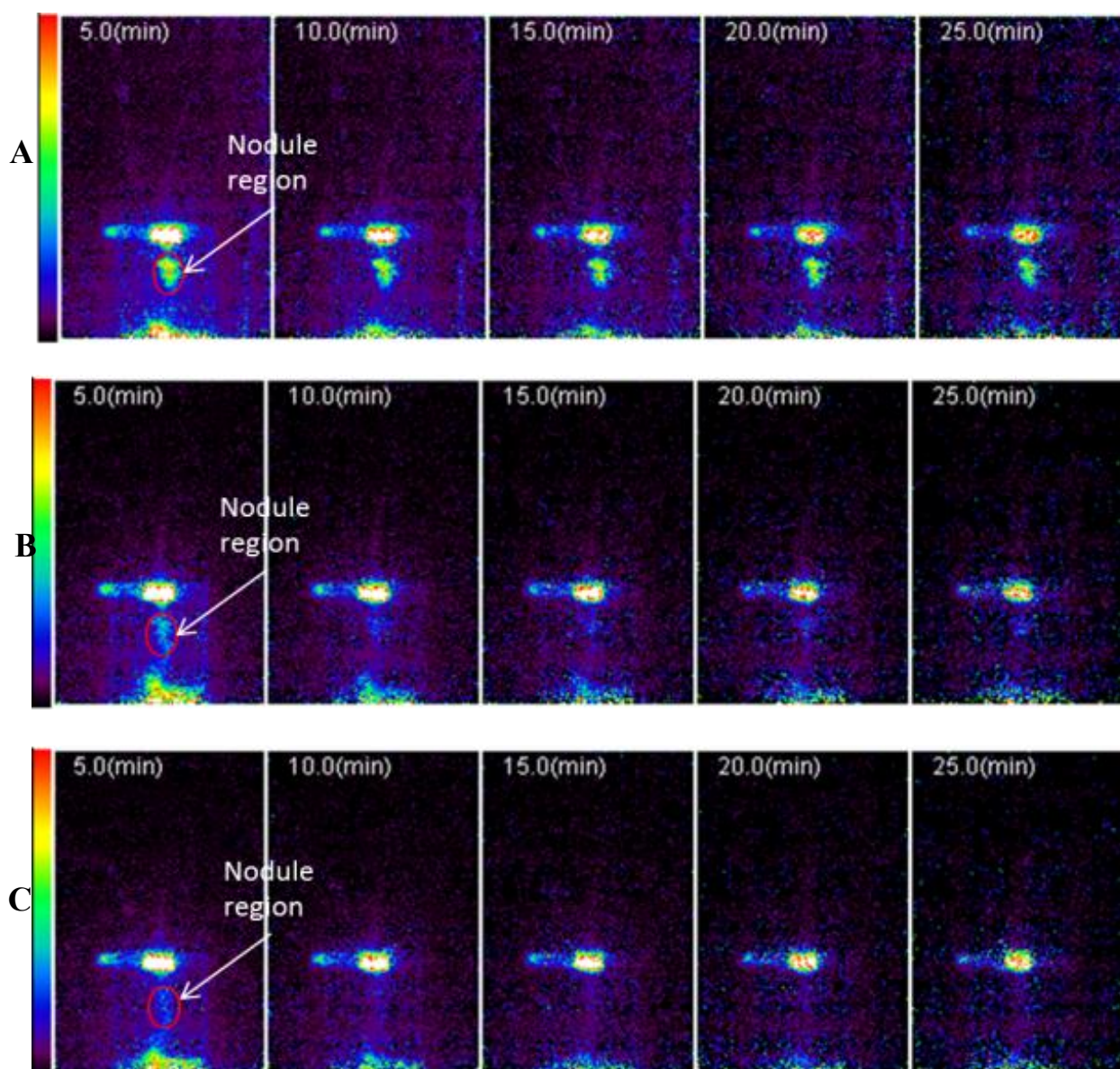
Figure 5.1: The PETIS images of ^{13}N activity in soybean nodules at different O$_2$ components.

- Soybean plant was used at 26 day-old

- **A,** pO_2=0.20 atm. **B,** pO_2=0.10 atm. **C,** pO_2=0.00 atm.

The signal intensity of $[^{13}N]N_2$ tracer was significantly different under the conditions with different oxygen concentration. The ^{13}N tracer accumulated in root nodules was strongest at pO_2 0.20 atm and weakest at without O_2.

The differences in nitrogen fixation activity among O_2 concentrations were seen more clearly when the time-activity curves (TACs) were generated from regions of interest (ROIs) on root nodules of a single soybean plant depending on each pO_2 partial pressure (**Figure 5.2**). The time-activity curves showed the different intensity of nitrogen radioactivity among various O_2 partial pressures. The radioactivity fixed in nodules at pO_2 0.00 atm was very low, but not zero.

The difference of nitrogen fixation rate and export rate of fixed-N among various O_2 concentrations were calculated as shown in the **figure 5.3**. The average rate of nitrogen fixation in normal condition (pO_2=0.20 atm) was highest (0.272 $\mu mol\ N_2\ h^{-1}$) in comparison to the other O_2 conditions (0.181 and 0.060 $\mu mol\ N_2\ h^{-1}$ in pO_2=0.20 and 0 atm, respectively). However, the average rate of export of fixed nitrogen from the nodules was opposite to the fixation rate, it was highest at pO_2=0 atm and lowest at pO_2=20 atm.

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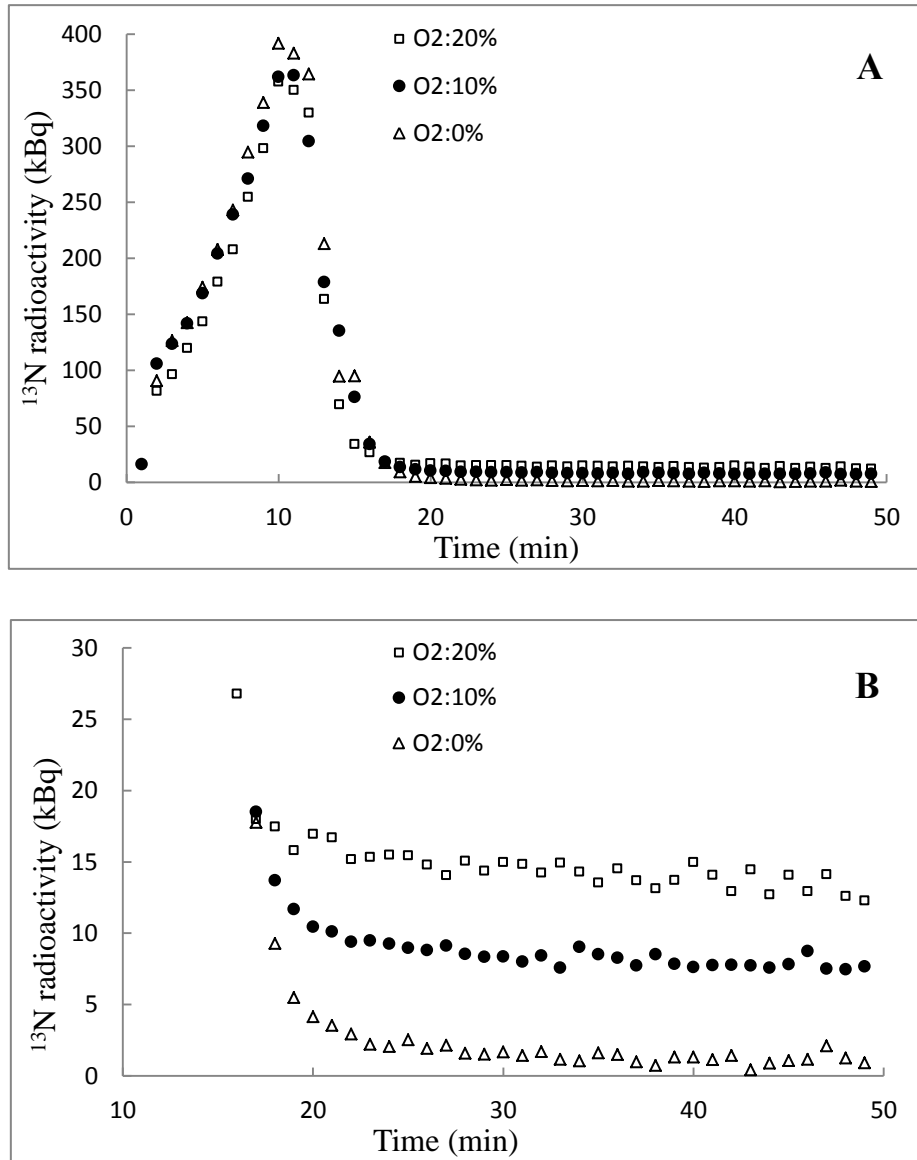
Figure 5.2: The time-activity curve of ^{13}N radioactivity accumulated in soybean nodules after feeding of [^{13}N]N$_2$ tracer gas at difference O$_2$ proportion.

A: Time-course of ^{13}N radioactivity at nodule region;

B: The magnification of time-course of ^{13}N radioactivity after ^{13}N$_2$ gas tracer flushing
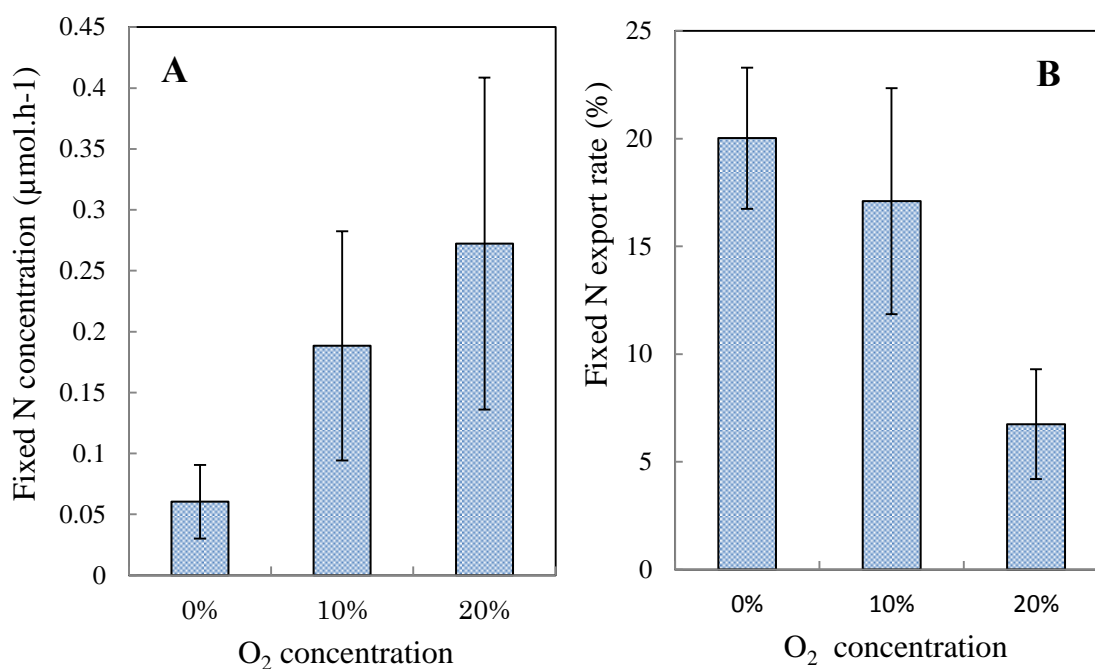
Figure 5.3: The N fixation rate and export rate of fixed -N in soybean at difference

   O₂ proportion.

   A: Nitrogen fixation rate

   B: The export rate of Fixed- N

   Bar on the column shows the standard error (SE means standard error, n=4).

5.5. Discussion

In the PETIS imaging experiment, the BNF of soybean plant is reflected by the ^{13}N radiotracer accumulated in the nodules. The signal intensity of

radiotracer accumulated in nodules was significantly different among O_2 concentrations. The results showed that nitrogen fixation activity of soybean nodules was depressed strongly by the decline of rhizosphere pO_2 even in short time affected. At normal condition, the NF activity was higher than that of the other lower O_2 concentration, but it was not inhibited completely at without O_2 (**Figure 5.2B**). Accordingly, BNF was very susceptible to pO_2 changes in short time although BNF continued at decreasing pO_2 concentration. The nitrogen fixation activity can be affected within short time (30 minutes) by lowered partial pressure of O_2 in the rhizosphere.

The export rates of fixed N were estimated from decreasing rates of the ^{13}N signal in the nodule region and reflect the physiological movement of fixed N from nodules to other parts of plant. The results were opposite to that of rate in which the export rate of fixed N in nodules at pO_2 0.20 atm was lowest (**Figure 5.4**). It seems likely that the enhancement of fixed-N export acts to complement the inhibited fixation and promote relative function cells to get more O_2 from upper ground through the soft tissues of epidermis. This mechanism might be important to maintain the nitrogen fixation activity in legume plant.

In conclusion, the PETIS data provided clear evidences of the rapid effect of lowered O_2 concentrations on nitrogen fixation activity and export of the fixed nitrogen. By using PETIS, a single plant was tested repeatedly in various O_2 compositions, so that the individual variation can be limited at the lowest

level. Therefore, the results, using intact soybean plants, reflected the real nature of symbiotic nitrogen fixation in soybean plant. Our research was only carried out under lower O_2 proportion conditions than normal O_2 proportion condition, it should be conducted in the future also with higher O_2 proportion to make the complete evidence.

CHAPTER 6

GENERAL DISCUSSION

The understanding of physiology of nitrogen fixation and transport mechanism in plant is very important for improving legume crop production in the yield as well as the quality. The overall goal of this study was to get better understanding in nitrogen fixation activity at two growth stages of soybean plant, the initial transport of fixed nitrogen, and the effects of partial low O2 concentrations on biological nitrogen fixation activity and transport in soybean plant by using nitrogen isotopes, ^{15}N and ^{13}N.

6.1. The analyzing methods

There are many methods used in the field of BNF and the transport of fixed-N in legume plant. In this study, I used nitrogen isotopes (including ^{15}N stable isotope and ^{13}N radioisotope) as tracers to measure the activity of nitrogen fixation and the initial transport rate of fixed-N from soybean nodules. These methods were considered to be one of the best ways for the analysis of assimilation and transport of nutrient elements in plants. The ^{15}N stable isotope method has advantages in quantitative analysis, it can be used at any place such as in laboratory, greenhouse and on the fields as well. While the ^{13}N radioisotope method is very sensitive and precise, it can be used for the analysis of kinetics and visualization in plant at real-time. Using two methods in this study has

complimented each other and the combination of the results successfully figured out more clearly the phenomenon that appears in plant life. Indeed, in this study by using nitrogen stable isotope I calculated the amounts of fixed-N at the first hours and the distribution proportion of fixed-N from nodules to the other organs during the chase-period experiment. On the other hand, by using radioisotope I estimated the timing and velocity of the initial transport of fixed-N. Moreover with BAS image applied nitrogen radioisotope, I found a new pathway of the initial transport of fixed-N in soybean plant, in which fixed-N may be transferred from xylem to phloem system in the stem

6.2. Nitrogen fixation activity at growth stages

It seems natural that the NF activity of plant depends on the growth stages. The precise measurement of nitrogen fixation activity in each growth stage can help to determine the N nutrient requirement during plant life in order to supplement the nutrition timely for plant growth and development. In this study, it was found that young soybean plant have higher activity in nitrogen fixation per total DW than soybean plant at pod-feeling stage, but the total amount of fixed-N per plant at pod-feeling stage was higher than that of vegetative stage. This indicates that nitrogen fixation activity was still high to support soybean growth, although specific nitrogen fixation decreased. Thus, in cultivation of legume crops we can adjust the amount of nitrogen fertilizer with lower dosage depending on the young growth stage decrease the input without

any effect on seed productivity and quality, and save the agricultural environment.

6.3. Fixed nitrogen transport in soybean plant

The dynamics of fixed-N in a short time after ^{15}N fixation was investigated intensively. The results showed that 80-90% of fixed-N remained in 1 hour in root nodules before being transported to the other organs and it was translocated in priority to young leaves and shoots rather than to mature leaves and root. The PETIS experiments revealed that it takes only less than 15 minutes to start exporting the fixed N from the nodules after feeding of ^{13}N$_2$. The fixed ^{13}N goes up to the upper shoot region around 40 minutes at a speed of 1.6 cm min^{-1}. One of the most important finding in this study is that the fixed-N was translocated only to young leaves and bud, but not to the mature leaves. This evidence confirmed that fixed-N was transported in stem via not only in xylem system but also in phloem system. Depending on previous studies and combining with our study, we designed a new model for the initial transport of fixed-N in soybean plant following (**Figure 7.1**).
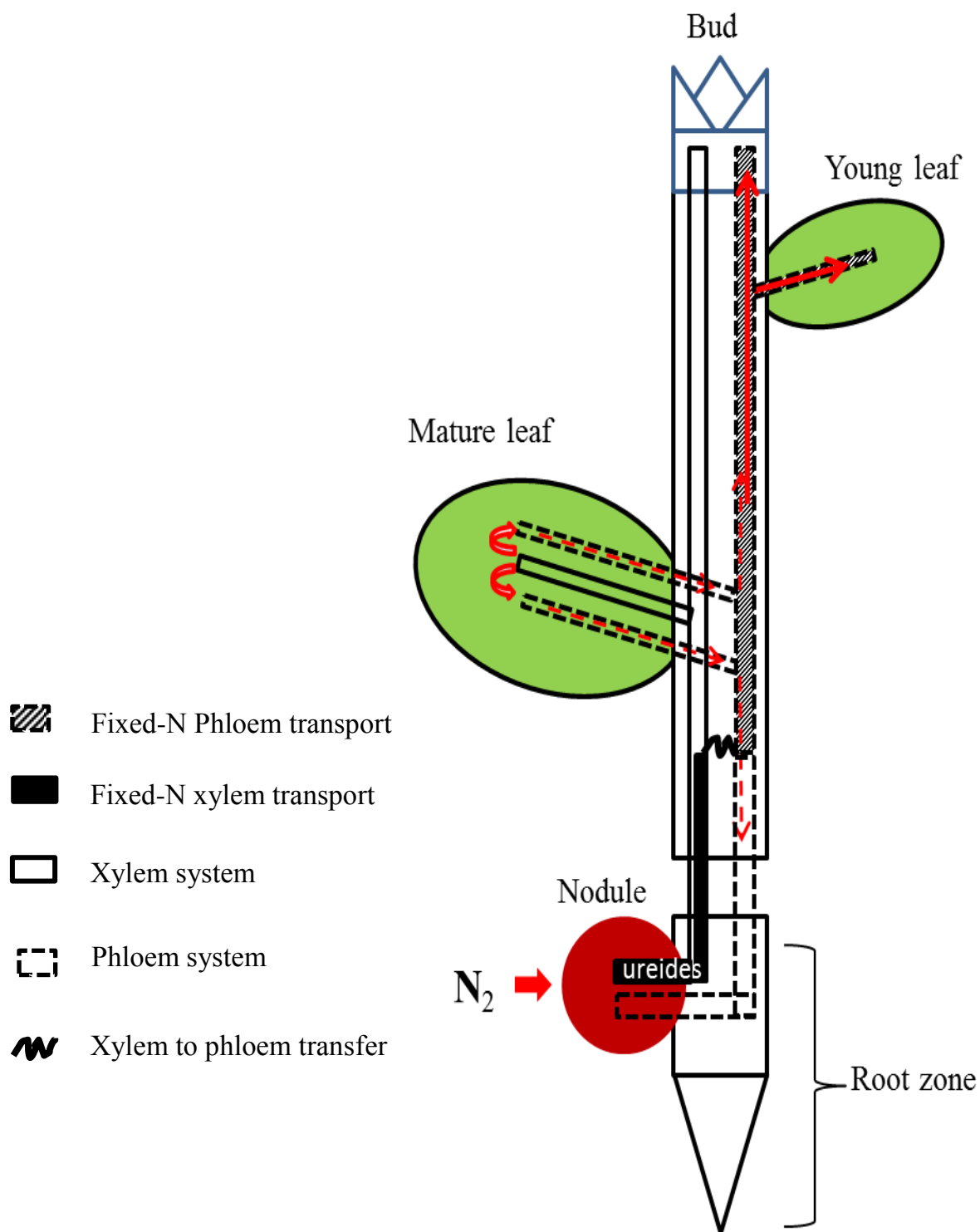
Figure 6.1: The model of transport of fixed-N in soybean plant.

The major feature of the model describes the pathway of fixed-N moving in soybean plant. Nitrogen from the atmosphere is fixed in nodules and then the fixed-N is rapidly assimilated into ureides (allantoin and allantoate). First, it is loaded to xylem system within the root zone and then transported to the stem. In the stem of shoots, it is transfer from xylem system to phloem system and translocated in priority to young leaves and buds. In the stem fixed-N is mainly transported in the phloem vessel and very little fixed-N is transported in xylem vessel. The transformation of fixed-N between xylem and phloem is very flexible, may be they can change to each other in the certain condition. This explains that in case of analysis the transport of fixed-N by bleeding sap from stump (Layzell and Larue, 1982; McClure and Israel, 1979; Pate et al., 1980; Pate et al., 1979c) the fixed-N was still presented in excrete sap in the basal pave of main stem.

The new finding in the initial transport of fixed nitrogen of soybean will become the basis for future study on transport of fixed N in legume plants. As the next study, it is necessary to determine where and how the fixed nitrogen is transferred from the xylem system to phloem system and transported in stem.

6.4. The effects of O_2 on nitrogen fixation activity in soybean plant

Oxygen is considered one of the essential factors which affects most strongly on nitrogen fixation activity. In this study, the nitrogen fixation activity of soybean plant was found to be decreased strongly along with reduced oxygen concentration 0 and 0.1 atm pO_2 around the rhizosphere even in a short period of treatment. In contract, the export of fixed-N was found to be increased under the lowered concentrations of O_2.

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ABSTRACT

The quantitative analysis of nitrogen fixation and the initial transport of fixed nitrogen in intact nodulated soybean plants (*Glycine max* [L.] Merr. cv. Williams) during relatively short time (8 h) was conducted at the vegetative stage (36 DAP) and pod-filling stage (91 DAP) by the ^{15}N pulse-chase experiment. The nodulated roots of intact soybean plants were exposed to N_2 gas labeled with a stable isotope ^{15}N for 1 hour, followed by 0, 1, 3 and 7 hours of exposure with normal air. The results showed that young soybean plants at 36 DAP showed higher N_2 fixation activity based on the dry weight (86µg/g DW) compared with pod filling soybean plants at 91 DAP (19µg/g DW). In both stages, approximately 90% of the fixed ^{15}N was retained in the nodules and the ^{15}N distribution in the basal nodules (78%) was higher than that of in the middle (12%) and distal nodules (0.1%) after 1 hour of stable isotope $^{15}N_2$ exposure. The distribution of fixed ^{15}N in the nodules decreased from 90% to 7% and increased in the roots (14%), stems (54%), leaves (12%), pods (10%), and seeds (4%) during the initial 7 hours of the chase-period at 91 DAP. The distribution of fixed ^{15}N was negligible in the distal root segment, suggesting that the recycling of fixed N from the shoot to the roots was very low within 7 hours after fixation.

The observation of fixed nitrogen transported in soybean plant by using ^{13}N-labeled gas tracer and a positron-emitting tracer imaging system (PETIS) showed that the signals of N radioactivity could be observed in the stem at 20

minutes after feeding with $^{13}N_2$ tracer gas. This is the first observation that the transport of fixed nitrogen in the stem could be observed at real-time in soybean plant. However, due to the short half-life of ^{13}N (9.97 minutes) and short exposing time, the signal intensity of the fixed N translocation in the upper stem observed by PETIS was weak, but the autoradiography taken after PETIS experiment showed a clear picture of transport of fixed ^{13}N in intact soybean plant. The result suggested that the fixed ^{13}N translocation through the shoot may not move only in xylem system as the previous concept that the fixed N in nodule is transported through xylem by transpiration stream by mature leaves, but the fixed N may be transferred from xylem to phloem in the stem. This result indicates that the initial transport of fixed N was mainly in the stem and translocated to young leaves and buds via phloem system. The new finding in the initial transport of fixed nitrogen of soybean will become the basis for the future study of fixed-N transport with the whole legume plants.

The effects of oxygen concentration in rhizosphere on the symbiotic nitrogen fixation in real-time was evaluated under various O_2 partial pressure conditions. Soybean nodules were treated with mixed gas containing ^{13}N-labeled N_2 with various O_2 concentrations, and the nitrogen fixation activity in the nodules was analyzed by PETIS. The results showed that under normal condition (20% O_2) the nitrogen fixation activity of soybean plant was higher compared to

that of under the other conditions (0% O_2 and 10% O_2). The nitrogen fixation activity of soybean nodules was strongly depressed with low O_2 concentrations, although it was not inhibited completely even at 0%. On the other hand, the export rate of fixed nitrogen from nodules was not affected by the changes of oxygen conditions.

Tables

Figures

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