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Analysis of Nitrate Absorption and Transport in Non-nodulated and Nodulated Soybean Plants with $^{13}NO_3^-$ and $^{15}NO_3^-$

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Nodulating (T202) and non-nodulating (T201) soybean isolines were hydroponically cultivated, then nitrate labeled with ^{13}N or ^{15}N, was added to the culture solution in order to investigate the nitrate absorption and transport in soybean. The accumulation pattern of the absorbed ^{13}N in the first trifoliate was observed by positron emitting tracer imaging system (PETIS) as well as bioimaging analyzer system (BAS). The ^{15}N abundance of each part was determined by emission spectrometry.

Real time changes in two dimensional image of the radioactivity could be monitored by PETIS, besides the distribution ^{13}N in whole plant could be observed by BAS. However quantitative data were hardly obtained by the ^{13}N analysis. Stable isotope ^{15}N is more reliable in the quantitative analysis in each part. Combing the data obtained by ^{15}N and ^{13}N tracer experiments, the absorption and translocation of N *in plant should be more clearly figured out.*

Key Words: soybean, nodule, positron emitting tracer, radioisotope, $^{13}NO_3^-$, stable isotope, $^{15}NO_3^-$

1. Introduction

Nitrogen is one of the most important, as well as an essential element of plants. The availability and utilization is strictly related to the growth, yield and the quality of crops. The studies of nitrogen absorption, transport and metabolism are leading topics in the field of plant nutrition. Soybean (*Glycine max* (L.) Merr.) forms root nodules with soil bacteria, *Bradyrhizobium japonicum*. Bacteroids, the symbiotic state of rhizobium in nodules can convert atmospheric nitrogen to ammonia. In return for receiving the fixed nitrogen from the bacteroids, the host plant provides photosynthates to the bacteroids as a carbon source. Soybean can also use the combined nitrogen, such as nitrate, absorbed from the medium. However, it is well known that the nodulation and N_2 fixation are depressed by exogenous combined nitrogen, especially by nitrate[1]. The precise mechanism of the inhibition by nitrate for nodule formation and N_2 fixation, have not been elucidated yet. Therefore, it is important to investigate the absorption manner and the transportation of nitrate both in nodulated and non-nodulated soybeans.

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Radioactive ^{13}N (half life time of 9.97 min) is available by cyclotron for the tracer studies of plant nitrogen nutrition. Recently, positron emitting tracer imaging system (PETIS)[2] has been devised for observing two dimensional image of positron emittion in plant supplied with positron emitting nuclide, such as ^{13}N[3], ^{11}C[4] and ^{18}F[2),5] produced by cyclotron. The stable isotope, ^{15}N, has been used for the investigation of nitrogen absorption and assimilation[6)−13)] in soybean.

In this report, nitrate labeled with ^{13}N was supplied to the culture medium of a soybean plant, and the distribution of radioactivity from ^{13}N in the petiole and first trifoliate leaf was observed by PETIS. In addition, the absorption and distribution of ^{15}N labeled nitrate in roots, nodules, stems, and leaves of soybean were quantitatively determined.

2. Experimentals

Nodulating (T202) and non-nodulating (T201) isolines of soybean were used in this study. The seeds were sterilized with 0.7 L L^{-1} ethanol for a minute, then 5 g L^{-1} sodium hypochrolite solution for 5 min, after that, thoroughly washed with a tap water. The seeds of T202 were inoculated with *Bradyrhizobium japonicum* strain USDA110 (10^8 cells mL^{-1}). All the seeds were sown in a tray filled with vermiculite, and grown in a green house under natural light conditions. Two weeks after sowing, the plants were transplanted to the hydroponic culture in a glass bottle (800 mL). The composition of the nitrate free medium was as follows; K_2SO_4 109, K_2HPO_4 8.5, KCl 0.935, $CaCl_2 \cdot H_2O$ 183.0, $MgSO_4 \cdot 7H_2O$ 123, H_3BO_4 0.367, $CuSO_4 \cdot 5H_2O$ 0.032, $MnSO_4$ 0.189, $ZnSO_4 \cdot 7H_2O$ 0.144, $(NH_4)_6Mo_7O_{24}$ 0.004, $CoSO_4$ 0.028, $NiSO_4 \cdot 6H_2O$ 0.0035, EDTA·Na_2 18.6, $FeSO_4 \cdot 7H_2O$ 13.9 (mg L^{-1}). The initial pH of the solution was about 6.2. The T202 plants were cultivated in the culture solution, and the T201 plants were cultivated in the culture solution containing 1 mM $NaNO_3$. Thirty days after sowing, the plants were treated for tracer studies.

Nitrate labeled with ^{13}N was produced from ^{16}O (p, α)^{13}N reaction by AVF cyclotron of Takasaki ion accelerators for advanced radiation application (TIARA). About 6 g of ice (H_2O) was used as a target, and irradiated by proton beam with 1 pμA for 20 min, which was accelerated by AVF cyclotron. The $^{13}NO_3^-$ was purified by passing through a cation exchange column and a basic alumina column. The plants were transferred to a 50 mL of culture solution with aeration. Nitrate labeled with ^{13}N was added to the culture solution, after the radioactivity was measured. The distribution of the absorbed ^{13}N was observed in the first trifoliate leaf by PETIS (Fig. 1). Data were collected for every minute until 40 min after $^{13}NO_3^-$ addition. The detection area was 48 mm × 50 mm rectangle, which are composed a $Bi_4Ge_3O_{12}$ scintillator array coupled to a position sensitive photomultiplier (Hamamatsu Photonics Co., Japan). Two γ-ray originated from annihilation of positron were detected simultaneously. The middle point of a line between the two detect position is a radioactive point where the plant is set. The radioactivity was calibrated automatically for half life time of supplied ^{13}N. The sensitivity of activity in the detection area is different, so the calibration is automatically done for the positional effect[14]. After PETIS observation, the radioactivity in the whole plant was observed by a bioimaging analyzer system (BAS1500, Fujifilm Co., Japan).

In the experiment using ^{15}N, the nodulated T202 plants were pretreated with or without 1 mM NO_3^- for 1 day before the addition of $^{15}NO_3^-$. A half of group of T201 plants (4 plants) were cultivated in nitrate free culture solution for 1 day

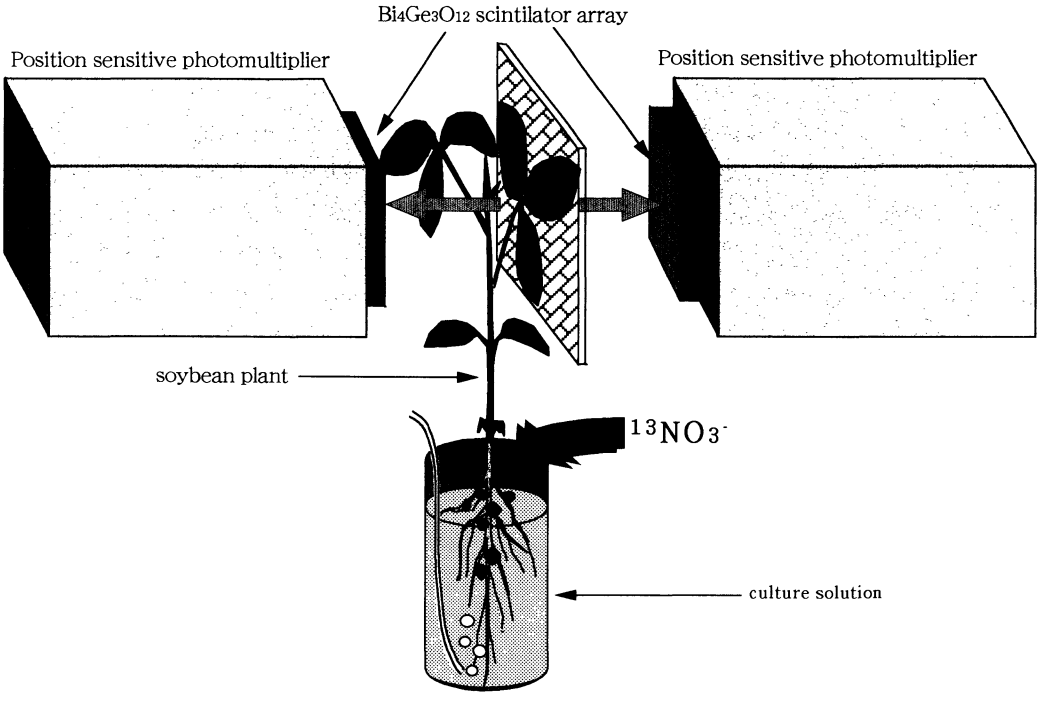

Fig. 1 Real time observation of radioactivity in the first trifoliate of soybean plant by PETIS (positron-emitting tracer imaging system).

before $^{15}NO_3^-$ supply. The other group of T201 (4 plants) was cultivated with 1 mM $NaNO_3$ continuously. The plants were transplanted to a plastic container (50 mL) with aeration, then 50 μL of 1 M $Na^{15}NO_3$ solution (70.7 atom% ^{15}N) were added to the culture solution to be a 1 mM $NaNO_3$ solution as a final concentration. All the plants were incubated under light condition for an hour. Plants were frozen with liquid nitrogen and freeze-dried, then separated into leaves, stems, roots and nodules. Plant samples were weighed and ground into fine powder by a vibrating sample mill (TI-100, C.M.T. Co., Japan). The ^{15}N abundance in each organ was determined by an emission spectrometer[7] (N150, Jasco Co., Japan).

3. Results and Discussion

The imaging picture was monitored for every minute, and the integration of 5 min images are shown in Fig. 2. The ^{13}N image began to be observed in the petiole during 6 and 10 min. The radioactivity in the petiole was gradually increased until the end of the treatment. About 10 min after addition of $^{13}NO_3^-$, the radioactivity was also detected in leaf blade.

The radioactivity in detected area in Fig. 2 were plotted for T202 and T201 (Fig. 3 A). There was a significant difference in radioactivity increasing manner even within the same plant species under the same condition. In order to compare the relative patterns of the time lag and the increase of ^{13}N transport, the relative radioactivity was calculated as in Fig. 3 B, where the radioactivity at 40 min after start was normalized as 100%. In the figure, ^{13}N radioactivity was increased linearly in leaves after about 5 – 10 min time lag, indifferent to nodule formation.

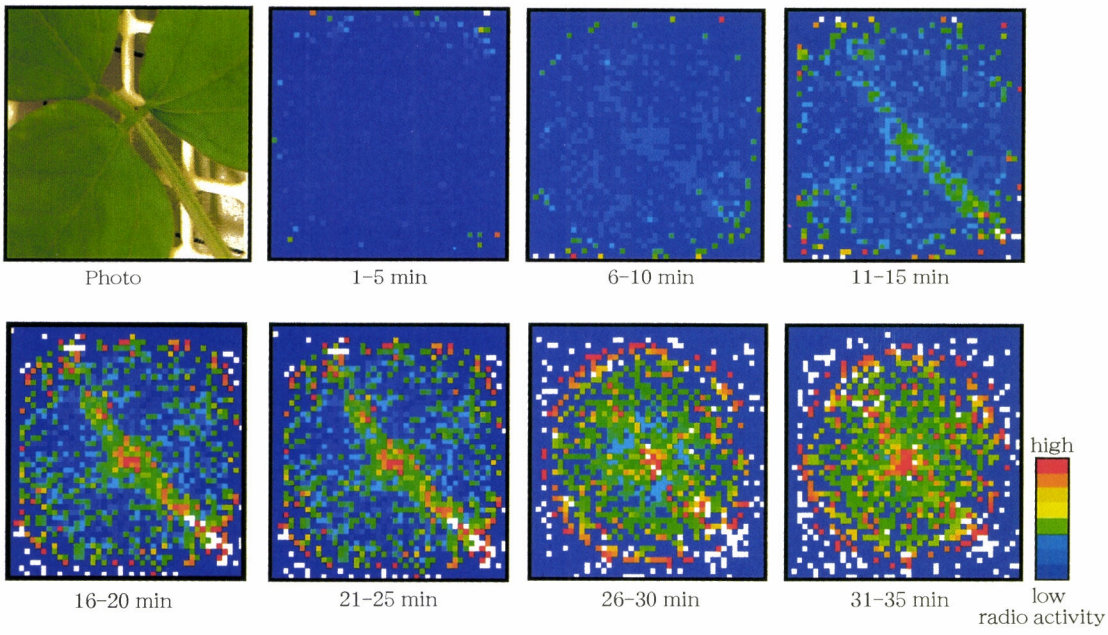

Fig. 2 Imaging of soybean leaf supplied with $^{13}NO_3^-$ by PETIS.

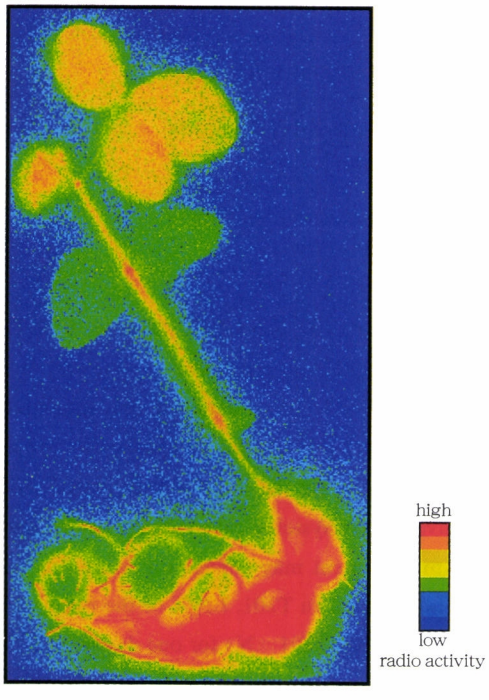

Fig. 4 Imaging of ^{13}N in T202 plant by Bioimaging analyzer.

The relative patterns of ^{13}N radioactivity were almost identical between nodulated (T202) and non-nodulated (T201) plants. It was suggested that nodulation may not affect the NO_3^- absorption in roots and transport to the leaves.

Figure 4 shows the BAS image of nodulated T202 treated with $^{13}NO_3^-$ for 1 h. The distribution pattern of radioactivity was basically the same in T201 and T202 irrespective of N treatment. Radioactivity of ^{13}N was the highest in roots followed by stems and leaves. Radioactivity of ^{13}N at the primary leaves was lower than those of first trifoliate leaves in spite of high activity at the stem node of primary leaves. The result suggests that nitrate absorbed from medium is transported to the first trifoliate leaves and terminal buds before the primary leaves. It is interesting that there was little ^{13}N activity in the nodules, while radioactivity of ^{13}N in roots was high, indicating that the nitrate absorbed from the roots was not transported to the nodules within a short

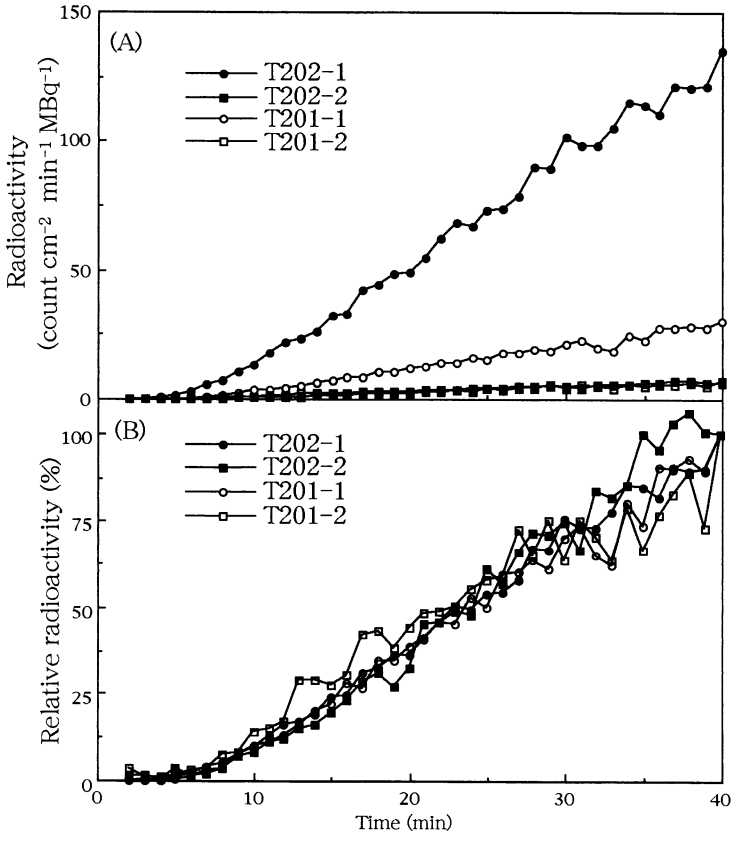

Fig. 3 Radioactivity per area (A) and relative radioactivity (B) of the detected area for T202 and T 201.
(A) After half life correction, the count was devided by initial radioactivity (MBq) of added NO_3^- at 0 time.
(B) The relative radioactivity to that at 40 min were plotted, which mean the radioactivity at 40 min is normalized as 100%.
Number in the figure shows experimental replication.

period (1 h).

Figure 5 shows the percentage of nitrogen derived from $^{15}NO_3^-$ in each organ. After 1 h treatment with 1 mM $^{15}NO_3^-$, ^{15}N abundance was able to be measured in every part including nodules. The percentage of labeled N in total N (LN%) of roots was the highest, followed by stems, petioles and leaves. The ^{15}N incorporation was the lowest in nodules in nodulated T202 plants irrespective of pretreatment. In nodulated T202 with nitrate pretreatment (T202+N), LN% were higher than those of T202 without pretreatment (T202−N) in most of organs. The result indicates that the nitrate absorption is increased by one day pretreatment of NO_3^-. It is assumed that the inducible nitrate transporter may be induced by exogenous nitrate during the pretreatment period. Alternatively, the physiological condition of the plant with pretreatment may change on account of nitrate absorption. In T201, LN% of the plant with N free solution (T201−N) for one day before ^{15}N treatment was lower than those of the plant with continuous nitrate supply (T201+N). The result indicates that soybean plant rapidly responds to

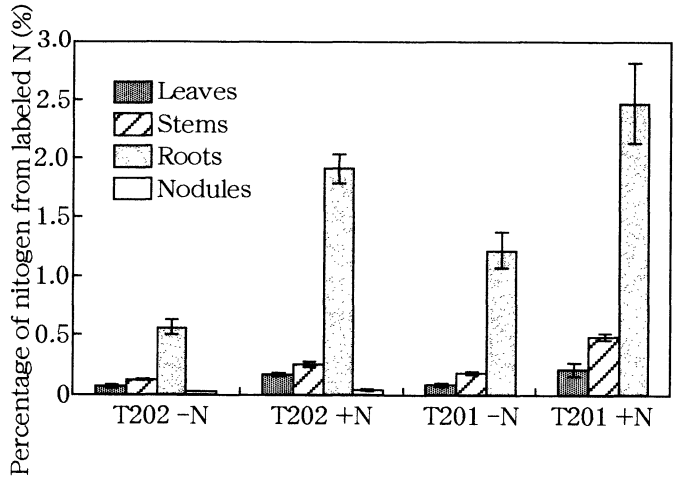

Fig. 5 Percentage of nitrogen derived from labeled N in each part of soybean plants.
Bar indicates standard error.

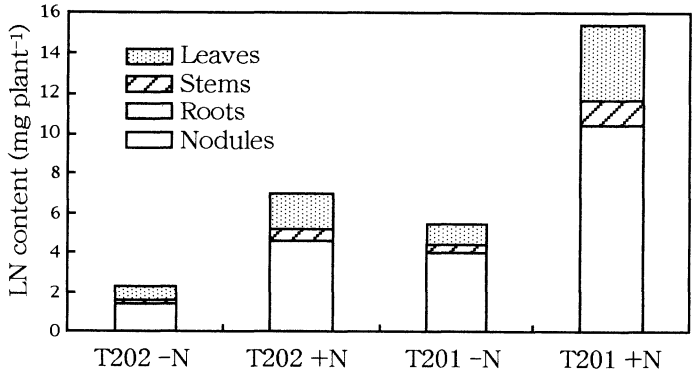

Fig. 6 Content of labeled N in each part of soybean plants.

the removal of exogenous nitrate, although the reason is not clear why nitrate absorption rate is decreased in the plant with the pretreatment without nitrate. In this experiment, LN% of T201 plants were higher than those of T202 plants both in −N and +N treatment.

Figure 6 shows LN content ^{15}N in various organs. LN content in T201 was higher than that of T202 both in −N and +N treatment. But it is difficult to conclude that nitrate absorption rate in non-nodulation soybean is higher than that of nodulation soybean, because the cultivation conditions before ^{15}N treatment were different between non-nodulation soybean (T201) and nodulation soybean (T202), consequently growth rates were different between T201 and T202.

The % distribution patterns of LN among organs were slightly changed by nitrate pretreatment both in T202 and T201. While the % distribution of LN in roots increased from 56% to 64% in T202 plant by nitrate pretreatment, although the % distribution of LN decreased from 72% to

67% in T201 plant by nitrate pretreatment. The stems share similar percentage of LN among treatments (about 8%), and the nodules share only 2% of absorbed $^{15}NO_3^-$.

It has been proved by ^{15}N experiments that the major transport forms of N from root nodules are ureides, allantoic acid and allantoin, on the other hands, the main transport forms of N absorbed from roots are nitrate and asparagine[8),9)]. Although both N forms are transported to the shoots via xylem vessels, the utilization of N sources is suggested to be different. A major part of the absorbed N is transported and assimilated in leaves at first then re-distributed to the pods and seeds, some part of the ureides originating from nodules are directly transported to pods as well as leaves[9),10),13)].

So far the fate of N absorbed from roots has been investigated after relatively long-term feeding period of $^{15}NO_3^-$ for several hours to months. By continuous supply of $^{15}NO_3^-$ for 28 days after transplanting to hydroponics, the percentage of N from nitrate (labeled-N) and from N_2 (non-labeled N) was determined in each organ. The results shows that about 20% of N was from nitrate in nodules and 40 – 50% of N was derived from nitrate in roots, stems and leaves[13)]. The result means that N from nitrate is used in the nodules and N from N_2 fixation is also used in roots, possibly by recycling of N from shoots via phloem. By tracer experiments using tungstate and $^{15}NO_3^-$, it was suggested that nitrate per se can be absorbed from the nodule surface into cortical region of nodules via symplastic transport[6)].

In the experiment using ^{13}N and ^{15}N for a short term within 1 h, it was clearly figured out as follows. While the ^{13}N was heavily and uniformly accumulated in the roots (Fig. 4), NO_3^- is rapidly absorbed from the roots, then some part can be detected in petioles and leaf blades with in 5 – 10 min after administration (Fig. 2). However, the nodules were hardly labeled by ^{13}N as well as ^{15}N within 1 h, although they are soaked in the labeled solution.

From the results obtained by ^{13}N and ^{15}N tracer studies, the merits and demerits of using ^{13}N and ^{15}N is considered as follows. Radioactive tracer ^{13}N has made it possible to monitor two dimensional real time changes of the activity by PETIS, although the detectable area is relatively small (48 × 50 mm) at present. By BAS image, the distribution image of whole plant can be observed. The disadvantage of ^{13}N tracer experiment is that the quantitative studies are difficult, while sensitivity of ^{13}N is higher than that of ^{15}N. Furthermore the experiment should be done in a hot laboratory near the cyclotron only in a short period about an hour, because ^{13}N is radioisotope with short half life (9.97 min).

We can use stable isotope ^{15}N at any place including fields. Using ^{15}N, quantitative analysis can be done, so we can see how much N derived from the labeled source. The disadvantage of ^{15}N utilization as a tracer is low sensitivity, and the analytical procedures are tedious and time consuming compared with ^{13}N tracer studies. Combing the data obtained by ^{15}N and ^{13}N tracer experiment, the absorption and translocation of N in plant can be more clearly figured out.

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要　旨

根粒非着生と根粒着生ダイズにおける $^{13}NO_3^-$ と $^{15}NO_3^-$ を用いた硝酸吸収と移行の解析

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ダイズにおける硝酸吸収と移行を調べるために，同質遺伝子系統の根粒着生ダイズ（T202）と根粒非着生ダイズ（T201）を水耕栽培し，^{13}N と ^{15}N トレーサ実験を行った。TIARA AVF サイクロトロンで作製した $^{13}NO_3^-$ を水耕培地へ投与した。第一本葉における吸収された ^{13}N の分布を，ポジトロンイメージング装置（PETIS）とバイオイメージングアナライザ（BAS）を用いて観測した。

また，別の実験では $^{15}NO_3^-$ を1時間供給し，各器官の ^{15}N 増加量を発光分光法により定量した。放射能活性の二次元画像の経時的変化を，PETIS により観測でき，植物全体における ^{13}N の分布を BAS により観察できた。しかし，^{13}N の実験では定量的なデータを得るのは困難であった。安定同位体の ^{15}N による分析は，各々の器官における標識窒素の割合や量の定量的解析に有効であった。^{15}N と ^{13}N トレーサ実験のデータを組み合わせることにより，植物における窒素の吸収と移行がより明確になると期待される。

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