# 大豆のチッソ代謝に関する研究 (6)


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# Studies on Nitrogen Metabolism of Soybean Plants

## VI. Utilization and distribution of nitrogen derived from nitrate and symbiotic fixation

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Field-grown soybeans, like other crop plants, can utilize N from the soil, but normally they also fix atmospheric N through the symbiotic relationship with $N_2$ fixing bacteria. In nodulated soybeans, therefore, the metabolic sources of N transported from roots to shoots should consist of two components: one deriving from root-absorbed N, and one from symbiotically fixed N. There is accumulating evidence that the predomonant forms of N compounds supplied from these two N assimilatory systems may be qualitatively different. The results from xylem sap analysis[15] have revealed that the predominant forms of N transport in the nodulated soybeans grown in N-free nutrient solution are allantoin and allantoic acid, whereas those in the nonnodulated ones supplied with nitrate are amino acids and nitrate. When both nitrate absorption and $N_2$ fixation are simultaneously occurring, all of these N compounds appear in the stem exudate[8,15,17]. However, little is known as to whether these internal N sources would serve equally well for the growth of soybeans.

In the experiment described below, nodulated soybeans were grown on $^{15}N$-nitrate throughout their growth and the relative contributions of nitrate and atmospheric N to the vegetative and reproductive growth have been investigated. An attempt was also made to isolate allantoin to determine the origin of allantoin-N.

### Materials and Methods

Inoculated soybeans (*Glycine max* Merr. c.v. Kogane daizu) were grown in green house hydroponic systems using cloth as a water sustaining agency as well as a root supporting medium as described earlier[9]. Throughout the experiment (from May 25 to September 15, 1977) each plant root was grown in a nylon bag (20 cm wide and 30 cm deep) in order to avoid mutual tangling. These nylon bags were inserted between the two-folded sheet of cloth which had been fixed in the hydroponic apparatus.

For the first 10 days after germination only water was supplied to ensure good nodulation. Subsequently, all the plants were supplied with a Hoagland's solution of quarter-strength (50 ppm N) containing $^{15}N$-labeled nitrate (3.32 atom %). The concentration was maintained by frequently checking the solution with a horticultural E.C. meter.

At full bloom stage half of the plants were transferred to another set of hydroponic systems supplied with the same nutrient solution except that $^{14}N$-nitrate replaced $^{15}N$-nitrate. This latter group of plants was used to examine the subsequent distribution of previously acquired $^{15}N$. After 76 days (the end of pod developing stage) the concentration of nutrient solution of both series was increased to half-strength (100 ppm N) until maturity.

Samplings were made at irregular intervals at 9 selected growth stages. The harvested plants were separated into different plant parts, oven-dried at 70°C, and weighed. The dried tissues were ground in a Willey mill and used for the determination of total N and isotopic enrichments. The isotopic enrichments of allantoin-, amino-, and protein-N in the stems (plus petioles), pods, roots, and nodules were also

measured.

Allantoin was isolated according to the methods of Brown *et al.*[1] with some modifications. Tissue dry powderes (1–5 g) were extracted with boiling water and then centrifuged at 5,000 rpm for 15 minutes. The residue was used for analysis of insoluble-N (protein-N). The supernate was concentrated to 10 ml in a vacuum rotary evaporator at 50°C, and centrifuged. The resulting supernate was passed through a column of Dowex 50W-8X, $H^+$ form, and the column was washed with water. The pH of the solution passed through this column was taken to 6.0, and then 5% basic lead acetate was added to this solution. The precipitate was removed by centrifugation and the excess lead in the supernate was precipitated by adding 5% sulfuric acid. After removing precipitate by centrifugation, the pH of the solution was brought back to 6.0 using 5% sodium hydroxide, and the solution was treated with Darco and filtered. The volume of the filtrate was reduced in vacuo to 30 ml and filtered again. Allantoin was precipitated by the addition of 20 ml of mercuric acetate solution (0.2 g mercuric acetate and 2 g sodium acetate in 20 ml water, adjusted to pH 6.0 with acetic acid). Most of the pod and nodule extracts reacted with this reagent at pH 6.0 to form mercury allantoin, while stem and root extracts hardly reacted at pH 6.0 but precipitation occurred at pH 7.0. Although the latter precipitates are more likely to be mercury allantoate on the basis of their precipitation behavior, identification was not made. After standing overnight, the precipitates were collected by centrifugation, washed with water, and allantoin-N in the precipitates was converted to ammonia by the Kjeldahl procedure for the determination of $^{15}N$ abundance. For pod and nodule extracts the supernate solutions after collecting precipitates at pH 6.0 were saved for further precipitation at pH 7.0.

The nitrogenous compounds adsorbed by the column were eluted with 2 N HCl and directly subjected to the Kjeldahl digestion. This fraction, largely consisted of amino acids, will be referred to as amino acid fraction.

The determination of $^{15}N$ abundance was carried out by the emission spectroscopic technique described previously[9]. The amount of symbiotically fixed N in each organ was estimated by subtracting the amount of $^{15}N$ (calculated for 100% in nitrate administered) from total N of the organ. Similarly, the amount of $^{15}N$ taken up after the full bloom stage was calculated by subtracting the amount of prebloom-$^{15}N$ from total N.

## Results

Accumulation of N derived from nitrate and symbiotic fixation proceeded nearly parallel with each other over the period from 28 to 84 days (early pod filling stage), both showing rapid increases during the flowering (Fig. 1). With the development of seeds, accumulation of N from fixation began to level off while N from nitrate ($^{15}N$) continued to accumulate at maximal rate throughout the period of most rapid

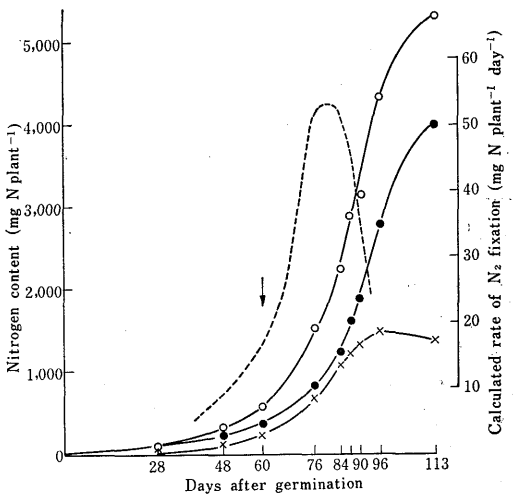


Fig. 1. Changes in nitrogen content and daily rate of $N_2$ fixation of soybean plants.
(○) : total N; (●) : N derived from nitrate; (×) : N derived from symbiotic fixation; dotted line, calculated rate of daily $N_2$ fixation; an arrow indicates the time of full bloom.

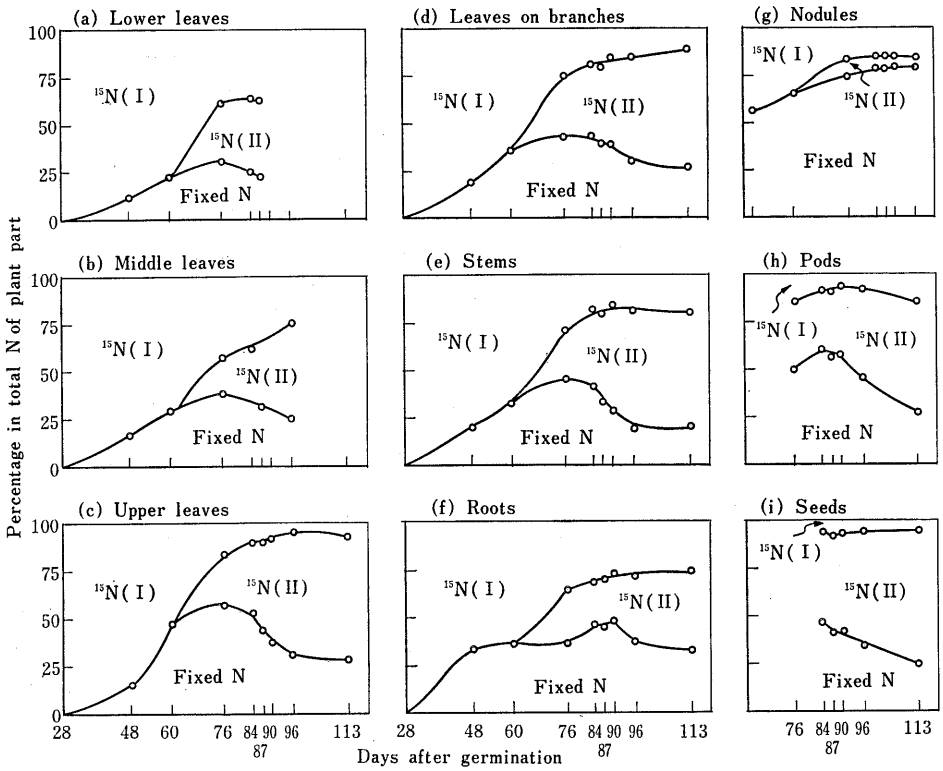


Fig. 2. Seasonal changes in the relative proportion of nitrate-derived N and symbiotic N in the total N of different plant parts.
Fixed N: N from symbiotic fixation; $^{15}N$ (I): N derived from nitrate applied before full bloom; $^{15}N$ (II): N derived from nitrate applied after full bloom.

pod-fill stage. As a result, the contribution from symbiotic fixation to total plant N was reduced from 47% at early pod-fill stage to 26% at maturity.

The time course of the rate of daily $N_2$ fixation is also shown in Fig. 1. The rate of daily $N_2$ fixation reached its peak (53 mg N per day) at early pod-fill stage and then decreased drastically as seeds developed.

Fig. 2 shows the seasonal changes in the relative contribution of symbiotic and nitrate-derived N in different plant parts. In the lower leaves which include the primary leaves and trifoliate leaves 1 to 4 (numbered from bottom of the main stem), the percentage of N from symbiotic fixation may be more or less overestimated because these leaves perhaps contained an appreciable amount of cotyledon-derived N which, with the method employed in this study, cannot be distinguished from symbiotically fixed N. For other plant parts, however, cotyledon-derived N, if present, may well be negligible.

The percentage contribution of symbiotic N in the leaves of all groups (Fig. 2 a, b, c and d) increased until 76 days and decreased thereafter as leaves approached senescence. The maximum percentage values of symbiotic N for middle leaves (trifoliate 5 to 8), upper leaves (trifoliate 9 to 12), and for leaves on the branches were approximately 40, 55 and 45%, respectively. The nitrate N applied after the full bloom stage ($^{15}N$(II) in Fig. 2) was easily incorporated into the leaves of all groups and contributed the major portion of the leaf N in the late growing season.

The stems showed the same general pattern as that found in the leaves, with the symbiotic N reaching its highest percent-

age (45%) when the rate of $N_2$ fixation was at its maximum (Fig. 2e). On the other hand, somewhat different patterns was obtained with the roots (Fig. 2f). The percentage of symbiotic N in the roots increased rather sharply up to day 48 and formed a plateau at this level (36%), though a small peak appeared later at the pod filling stage. Another characteristic feature of roots may be that they contained postbloom-$^{15}$N ($^{15}$N absorbed after the full bloom) in relatively small proportion as compared with leaves and stems.

As expected, the percentage of symbiotic N in the nodule total N was exceptionally high (70 to 80%), although the nodules at early stages of their development derived appreciable portion of N (40%) from nitrate (Fig. 2g). In the nodules postbloom-$^{15}$N constituted only a fraction of their total N in the later stages of growth.

The total N of the pods at early pod-fill stage (84 days) contained symbiotic N, prebloom-$^{15}$N, and postbloom-$^{15}$N in the ratio 59:8:33 (Fig. 2h). Since the input rates of N from $N_2$ fixation and nitrate uptake were nearly equal during the period of pod development (Fig. 1), the ratio symbiotic N: postbloom-$^{15}$N (59:33 or 1.8:1) obtained is significantly higher than would be expected from the over-all input ratio (1:1). It is, therefore, conceivable that symbiotic N might have been preferentially taken up by the pods. The percentage of symbiotic N in the pod total N, however, decreased to 28% during the seed development, with a corresponding rise in the percentage of postbloom-$^{15}$N.

The relative proportion of symbiotic N to total N in the seeds was consistently lower than that in the comparable pods (Fig. 2i). Over the period of seed development, the percentage of symbiotic N decreased from 46 to 25%, and that of prebloom-$^{15}$N from 8 to 5%, whereas that of postbloom-$^{15}$N increased from 46 to 70%.

The time course of N accumulation in the seeds is shown in Fig. 3. Postbloom-$^{15}$N continued to accumulate over the entire period of seed development, while the accumulation of symbiotic N and prebloom-$^{15}$N took place only during the first 2 weeks of seed growth. The final amounts of symbiotic N, prebloom-$^{15}$N, and postbloom-$^{15}$N in the seeds were 991, 212 and 2789 mg, respectively, which represented 72, 56 and 77% of the respective N components accumulated in the whole plant at maturity.

A marked difference in the extent of $^{15}$N enrichment was observed between allantoin and amino acids or proteins (Fig. 4). Although N content of these compounds varied with the time of sampling and with plant parts from which they were isolated, allantoin had considerably lower $^{15}$N content than either amino acids or protein (Fig. 4).

Stem allantoin (precipitated at pH 7.0) collected before the flowering stage showed the relatively high $^{15}$N content (approximately 35% of the allantoin-N) which was followed by a marked decrease until pod-fill stage, and then increased rapidly as seeds developed. Almost strictly inverse relationship was found between the changes in $^{15}$N content of stem allantoin and those in the daily rate of $N_2$ fixation (Fig. 4a).

Pod allantoin (precipitated at pH 6.0) isolated at early pod-fill stage exhibited the $^{15}$N content of 14%, a value which was

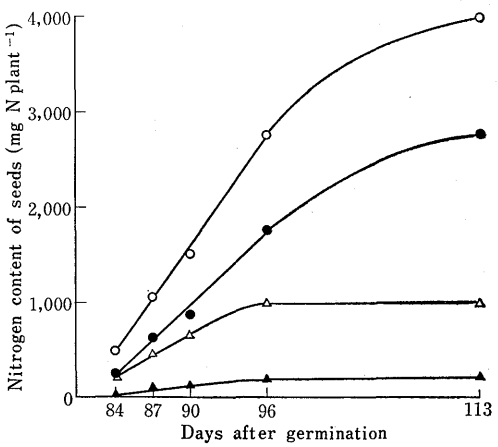


Fig. 3. Time course of nitrogen accumulation by soybean seeds.
(○) : total N; (△) : N derived from symbiotic fixation; (●) : N derived from nitrate applied after full bloom; (▲) : N derived from nitrate applied before full bloom.

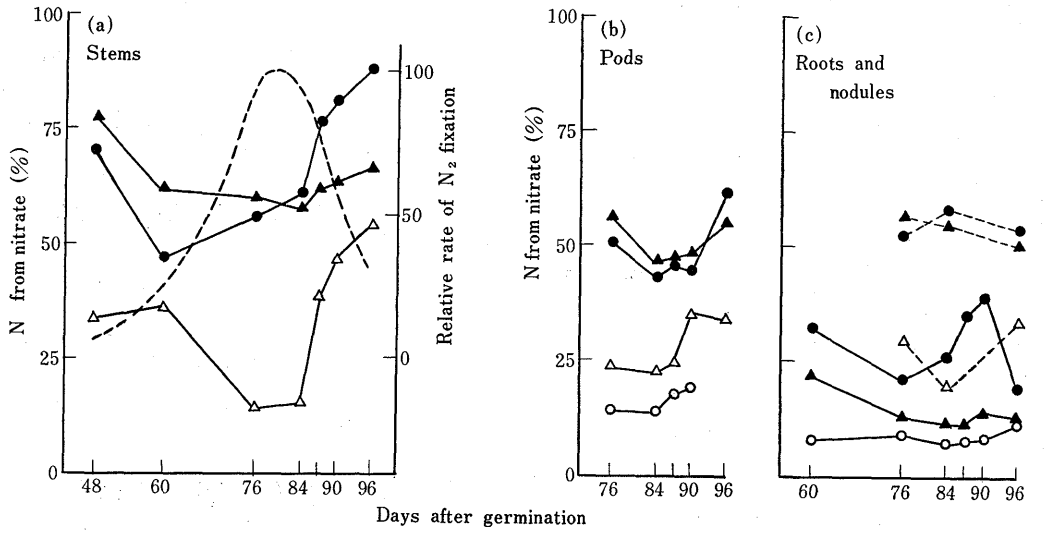


Fig. 4. Percentage content of nitrate-derived N in allantoin-N, amino-N, and protein-N isolated from (a) stems, (b) pods, (c) roots (dotted lines), and nodules (solid lines). (○) : allantoin precipitated at pH 6.0; (△) : allantoin precipitated at pH 7.0; (●) : amino acids; (▲) : proteins. Nodule allantoin is a mixture of allnatoin precipitated at pH 6.0 and 7.0.
Dashed line in (a) shows the relative rate of daily $N_2$ fixation.

nearly one-forth as low as the $^{15}N$ content of the pod amino acids and protein (Fig. 4b). The pod allantoin precipitated at pH 7.0, presumably containing allantoic acid, had consistently higher $^{15}N$ content than that precipitated at pH 6.0.

The root allantoin (precipitated at pH 7.0) also showed its minimum $^{15}N$ content (19%) at early pod-fill stage when $N_2$ fixation was most active (Fig. 4c). Root allantoin isolated either before or after this period yielded $^{15}N$ content of approximately 30%.

Nodule allantoin recorded the lowest $^{15}N$ content (7–8%) of all examined (Fig. 4c). In contrast, nodule amino acids had the $^{15}N$ content fourfold greater than nodule allantoin and two to threefold greater than nodule protein.

These results would indicate a remarkable accessibility of symbiotic N to the processes of allantoin synthesis and provide further evidence in support of the idea that allantoin synthesis occurs primarily in the nodules. However the more or less constant occurrence of $^{15}N$ in allantoin-N fraction would also suggest that at least small amount of nitrate-N is utilized, directly or indirectly, for the synthesis of tissue allantoin.

## Discussion

Symbiotic fixation contributed 26% of the total N assimilated throughout the plant life. This is about the same order as that appearing in the literature[2]. The time curve for the calculated daily $N_2$ fixation rate also followed the same general pattern as those reported by other workers[5,6,7,11,16,18,19], but in this experiment fixation activity collapsed somewhat earlier. This rapid decline in $N_2$ fixing activity may have been partially due to the increased concentration of nitrate (as well as other nutrients) in the nutrient solution immediately prior to seed development, and partially to a failure to detect $N_2$ fixation at the later stages of growth because of insensitive method employed.

The efficiency of transfer of symbiotic N to the seeds was 72%. Similarly 56 and 77% were obtained for prebloom-$^{15}N$ and postbloom-$^{15}N$, respectively. When the

over-all efficiency for nitrate N ($^{15}N$) was calculated, a value of 75% was obtained. It seems, therefore, that both nitrate and atmospheric N are equally well utilized by the soybean plant for the production of seed yield. However, a high transfer efficiency (77%) of postbloom-$^{15}N$ must be emphasized. Although late N application to soybeans under field conditions is generally ineffective, Harper[7] has indicated that hydroponically grown soybeans are responsive to application of fertilizer N during later growth stages. The present results suggest that nitrate N applied after full bloom could play an important role in seed production of soybeans if, as Harper[7] has pointed out, moisture conditions are favorable to facilitate nutrient uptake.

The analysis of tissue allantoin revealed that nitrate-derived N and symbiotic N differ significantly in their capabilities of being incorporated into allantoin. A ready incorporation of symbiotic N into allantoin shows the close relationship between the processes of $N_2$ fixation and allantoin synthesis. Furthermore, the fact that allantoin from nodules, among other plant parts, was most heavily enriched with symbiotic N gives further evidence that nodules are a probable site of allantoin formation.

On the other hand, the stem allantoin collected at 96 days contained $^{15}N$ in such a high concentration that more than half the allantoin-N was derived from nitrate (Fig. 4a). It seems clear, therefore, that symbiotic N is not the sole source for allantoin-N.

## Summary

Nodulated soybeans were grown on $^{15}N$-nitrate throughout the life cycle to investigate the relative contribution of nitrate and atmospheric N to the vegetative and reproductive growth of soybeans. The calculated daily rate of $N_2$ fixation increased with vegetative growth of the plant and reached a maximum at early pod-fill stage. By this time, the amount of N derived from symbiotic fixation became nearly equal to that from nitrate uptake. Thereafter, $N_2$ fixing activity declined sharply while nitrate uptake continued at an almost maximal rate until late seed development stage. As a result, the contribution of symbiotic N to total plant N at maturity was reduced to 26%.

The efficiency of transfer of N to the seeds was calculated to be 72% for symbiotic N and 75% for nitrate-derived N, indicating that both sources of N are equally well utilized by soybeans for the production of seeds. However, symbiotic N and nitrate differ significantly in their capabilities of being incorporated into allantoin. Symbiotic N is much more readily incorporated into allantoin, indicating a close relationship between the processes of $N_2$ fixation and allantoin synthesis.

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〔和 文 摘 要〕

## 大豆のチッソ代謝に関する研究

### 第6報 培地および根粒に由来するチッソの体内における分布と利用について

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根粒菌を接種した大豆（品種コガネダイズ）を生育全期間にわたって $^{15}N$ 硝酸塩を含む培養液で水耕栽培し，培地から吸収した N と根粒の N 固定活動に由来する N が，大豆の生長にそれぞれどのように貢献しているかを調べた．根粒の N 固定活動は栄養生長とともに活発となり，1日当たりの N 固定量は稔実初期に最高に達した．この時期までに植物体内に蓄積した N についてみると，培地由来のものと N 固定に由来するものがほぼ等しい割合で存在していたが，その後 N 固定作用は稔実の進行とともに急激に衰えたのに対し，培地からの N 吸収は引きつづき活発に行われ，その結果成熟期の全 N に占める固定 N の割合は 26% に低下した．

N の全蓄積量中種子に移行した割合を，固定 N と培地由来 N について個別的にみると，前者72%，後者75% となり，双方とも種子生産に効率よく用いられていることがわかった．しかし両者の間にはアラントインへのとりこみに大きな差がみられ，アラントイン態 N の源泉は主として固定 N であると考えられた．