

We are IntechOpen, the world's leading publisher of Open Access books Built by scientists, for scientists

6,900

Open access books available

186,000

International authors and editors

200M

Downloads

Our authors are among the

154

Countries delivered to

TOP 1%

most cited scientists

12.2%

Contributors from top 500 universities



WEB OF SCIENCE™

Selection of our books indexed in the Book Citation Index
in Web of Science™ Core Collection (BKCI)

Interested in publishing with us?
Contact book.department@intechopen.com

Numbers displayed above are based on latest data collected.
For more information visit www.intechopen.com



Amino Acid Metabolism and Transport in Soybean Plants

Takuji Ohyama, Norikuni Ohtake, Kuni Sueyoshi,
Yuki Ono, Kotaro Tsutsumi, Manabu Ueno,
Sayuri Tanabata, Takashi Sato and
Yoshihiko Takahashi

Additional information is available at the end of the chapter

<http://dx.doi.org/10.5772/intechopen.68992>

Abstract

The ammonium produced by nitrogen fixation in the bacteroid is rapidly excreted to cytosol of infected cell of soybean nodules and then assimilated into glutamine and glutamic acid, by glutamine synthetase/glutamate synthase pathway. Most of the nitrogen is further assimilated into ureides, allantoin, and allantoic acid, via purine synthesis, and they are transported through xylem to the shoots. Nitrate absorbed in the roots is reduced by nitrate reductase and nitrite reductase to ammonia either in the roots or leaves. The ammonia is also assimilated by glutamine synthetase/glutamate synthase pathway, and mainly transported by asparagine, and not ureides. The nitrogen transported into leaves is readily utilized for protein synthesis, and then, some of them are decomposed and retransported to roots, apical shoots, and pods via phloem mainly in the form of asparagine.

Keywords: soybean, amino acid, nitrogen fixation, nitrate absorption, nodule, root, leaf

1. Introduction

1.1. Role of amino acids in plants

Plants are photoautotrophs, and they can synthesize all organic compounds from inorganic materials such as carbon dioxide (CO₂), water (H₂O), and minerals using light energy. Amino acids are the key metabolites in nitrogen (N) metabolism of higher plants. First, the inorganic

N, such as ammonium absorbed in the roots or produced from nitrate reduction, nitrogen fixation, and photorespiration, is initially assimilated into glutamine (Gln) and glutamate (Glu) by the glutamine synthetase (GS)/glutamate synthase (GOGAT) pathway. Second, amino acids are the essential components of proteins. Third, amino acids are used for long-distance transport of nitrogen among organs (roots, nodules, stems, leaves, pod, seeds, and apical buds) through xylem or phloem. Fourth, nonprotein amino acids may play a role in protecting plants from feeding damages by animals, insects, or infection by fungi. In this chapter, we would like to review the amino acid metabolism in soybean nodules, roots, leaves, pods, and seeds. In addition, we will introduce the amino acids transport via xylem and phloem among organs.

Soybean plants absorb inorganic N from the roots, and they can fix atmospheric N_2 in the nodules associated with soil bacteria rhizobia. **Figure 1** shows a model of nutrients and water flow via xylem and phloem in soybean plants. Soybean roots absorb water and nutrients in soil solution, and they are transported to the shoots via xylem vessels by the transpiration and root pressure. The fixed N in nodule is also transported to the shoots via xylem. On the other hand, photoassimilates (mainly sucrose), amino acids (Asn, etc.), and minerals (potassium, etc.) are transported from leaves to the apical buds, roots, nodules, and pods via the phloem by osmotic pressure or protoplasmic streaming.

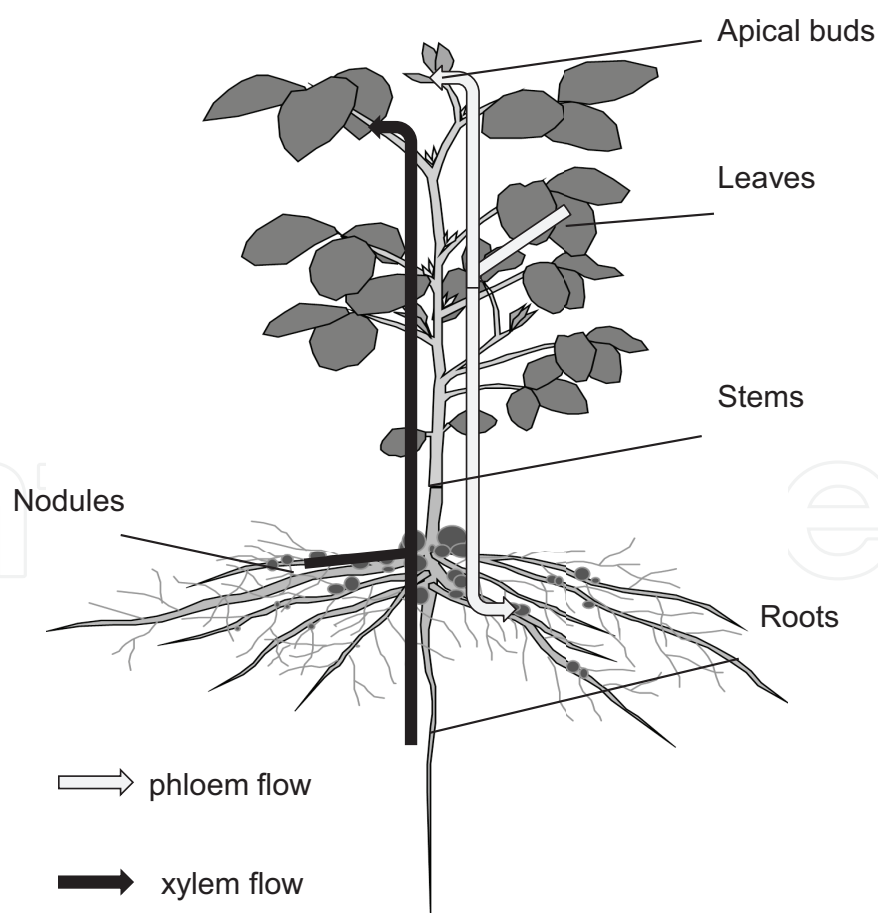


Figure 1. Transport pathways of nutrients and water by xylem and phloem in a soybean plant.

Figure 2 shows the distribution of radioactivity showing xylem flow (**Figure 2A**) [1] or phloem flow (**Figure 2B**) [2]. **Figure 2A** shows the positron imaging of the distribution of radioactivity in nodulated soybean (T202) after 1 hour of $^{13}\text{NO}_3^-$ supply to the root medium [1]. All parts of the roots exhibited the highest radioactivity (red), and stems and first trifoliolate leaf were relatively high (yellow). The radioactivity was not observed in the nodules, although they are attached in the roots. **Figure 2B** shows the positron imaging of distribution of radioactivity in nodulated soybean (cv. Williams). After ^{11}C -labeled CO_2 was exposed to the first trifoliolate leaf, and the radioactivity was monitored after 2 hours [2], the highest radioactivity was shown in the $^{11}\text{CO}_2$ -fed leaf (red) and stems (red) with apical bud (red) and root (yellow) and nodules (red). No radioactivity was observed in the primary leaf and other matured leaves. Nodules showed a higher radioactivity than that in the roots.

1.2. Role of amino acids on inorganic nitrogen assimilation

Ammonium ion (NH_4^+) is first assimilated into glutamine (Gln) combined with glutamic acid (Glu) by the enzyme glutamine synthetase (GS) consuming one molar of ATP (**Figure 3**). Then, the amide group of Gln is transferred to an organic acid, 2-oxoglutarate (2-OG), by glutamate synthase (GOGAT) in plastids using 2 molar of reduced Ferredoxin (Fd_{red}). Previously, NH_4^+ was considered to be initially assimilated into Glu by glutamate dehydrogenase (GDH). However, the enzyme GOGAT has been discovered in nitrogen-fixing bacteria *Aerobacter aerogenes* in 1970 [3], and GS/GOGAT cycle has been confirmed as the principal route of ammonium assimilation in plants [4–8].

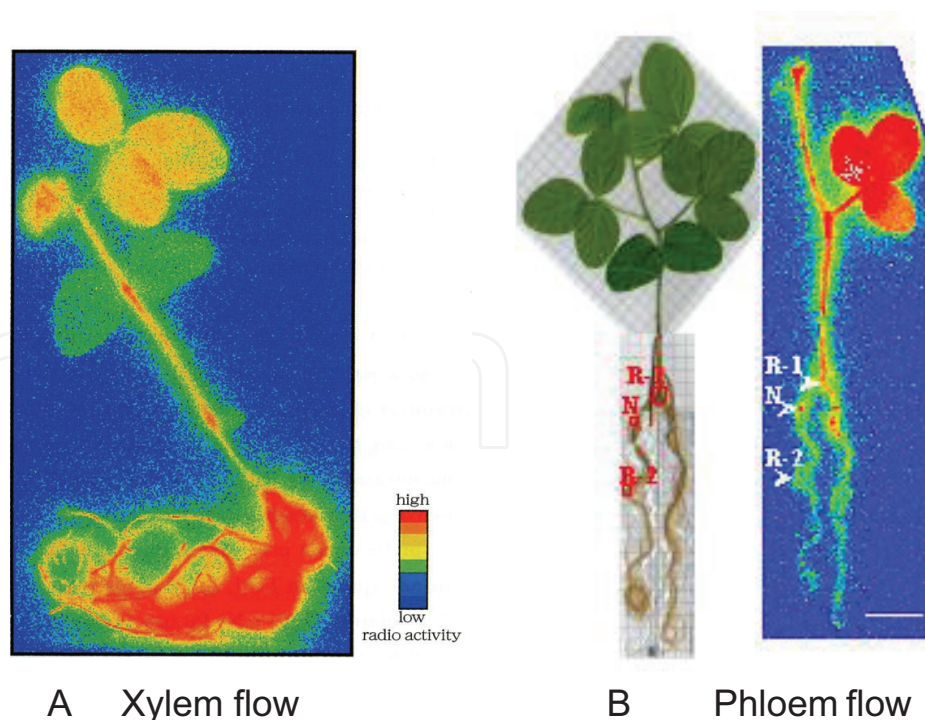


Figure 2. Positron imaging pictures of xylem flow and phloem flow in nodulated soybean plants. (A) Distribution of radioactivity after $^{13}\text{NO}_3^-$ was supplied from the roots. (B) Distribution of radioactivity after $^{11}\text{CO}_2$ was exposed to the first trifoliolate leaves of a split-rooted soybean.

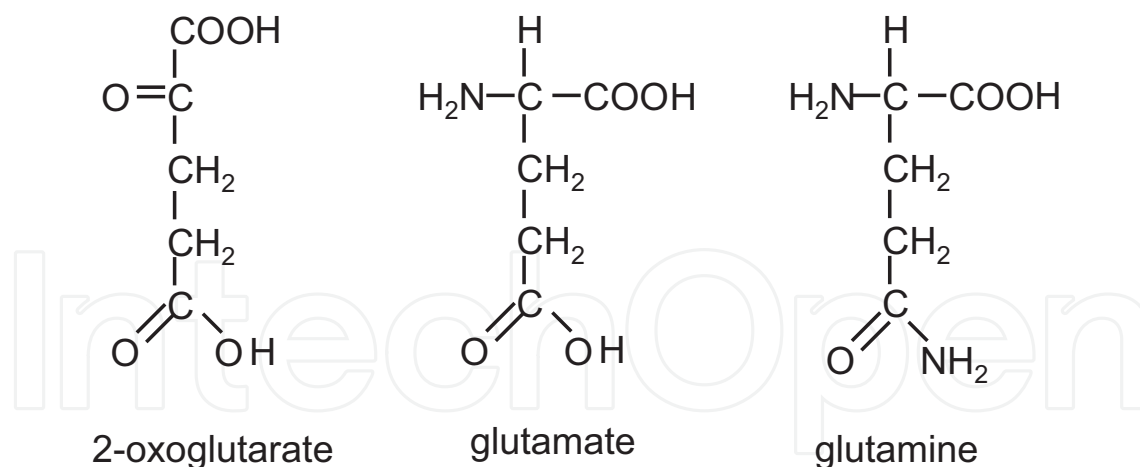


Figure 3. Chemical structures of 2-oxoglutarate, glutamate, and glutamine.

Nitrate is most abundant inorganic nitrogen in upland fields, because NH_4^+ is readily oxidized to NO_3^- by nitrifying bacteria under aerobic conditions. NO_3^- is reduced to nitrite (NO_2^-) by the enzyme nitrate reductase (NR) using one molar of NADH or NADPH as a reductant. The NO_2^- is transported to plastids and further reduced to NH_4^+ by the enzyme nitrite reductase (NiR) using 6 molar of Fd_{red} .

1.3. Role of amino acids for synthesis of proteins and nucleic acids in plants

Protein is a polymer or a complex of polymers of 20 amino acids in higher plants and plays an essential role on metabolism as enzymes, storage proteins, and structure components of the cells. The 20 amino acids consist of alanine, arginine, asparagine, aspartic acid, cysteine, glutamic acid, glutamine, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tyrosine, tryptophan, and valine. Enzyme is a kind of protein that catalyzes a specific chemical reaction in plant cells, and regulation of enzyme synthesis and the activity are essential for maintaining life and growth.

Nucleic acids, deoxyribonucleic acid (DNA), and ribonucleic acid (RNA) are a polymer of purine bases (adenine and guanine) and pyrimidine bases (thiamine, cytosine for DNA, and uracil, cytosine for RNA) with pentose (2-deoxyribose for DNA and ribose for RNA) and phosphate. DNA serves as a template of mRNA, and the mRNA is translated into amino acid sequences of protein. Purine base contains 4 N atoms in a molecule, and they are derived from two glutamines, one aspartic acid, and one glycine. Pyrimidine base contains 2 N atoms in a molecule, and they are derived from one glutamine and one aspartic acid. Amino acids are the precursors of most of N compounds in plants.

1.4. Role of amino acids for nitrogen transport and storage in plants

Amino acids and amides, especially Gln and Asn, are used for N transport through xylem and phloem in many plants. In addition, these amides are used for temporary N storage. Gln and Asn are suitable for N transport and storage, because they have two N atoms in one molecule, and the solubility is high among amino acids.

1.5. Role of amino acids for protecting the plants

In addition to the 20 protein amino acids, over 400 nonprotein amino acids are found in various natural sources, and about 240 of them have been found in plants [9]. 4-Methylene glutamine is a nonprotein amino acid found only in groundnut (*Arachis hypogaea*) [10] and tulip (*Tulipa gesneriana*) [11], and this amide is highly accumulated in the leaves and stems of the tulip plants [12], and the tentatively stored N is used for reuse of bulb storage N. Some nonprotein amino acids are toxic such as canavanine and concanavanine A in sword bean (*Canavalia gladiata*), and these toxic amino acids may contribute to protect plant from feeding damages by animals, insects, or fungi [9].

2. Concentrations of free amino acids and soluble N constituents in various parts of soybean plants

In soybean plants, ureides, allantoin, and allantoic acid are mainly used for transport of N in addition to amino acids (Figure 4). Ureides have 4 N and 4 C atoms in a molecule, and it is considered to be more efficient to transport of N than asparagine (2N and 4C) by the view of carbon economy.

Table 1 shows the total amino acid-N, ureides-N, nitrate-N, ammonium-N, and others in 80% ethanol soluble fraction of each organ. Both hydrophilic and hydrophobic low-molecular-weight compounds, such as sugars, amino acids, ureides, organic acids, and chlorophylls,

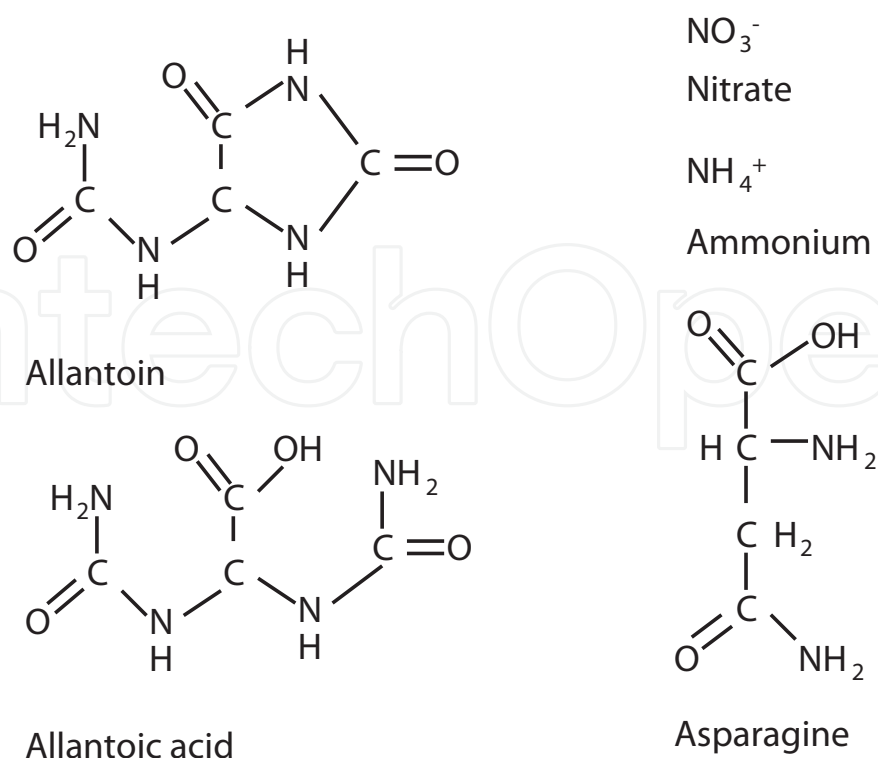


Figure 4. Chemical structures of allantoin, allantoic acid, nitrate, ammonium, and asparagine.

	Nodules	Roots	Stems (UP)	Stems (LP)	Leaves (UP)	Leaves (LP)	Pods (UP)	Pods (LP)	Seeds (UP)	Seeds (LP)
Total amino acid-N	519	147	270	134	96	96	776	415	1243	982
Ureides-N	483	19	143	62	35	36	1529	916	82	82
Nitrate-N	147	32	56	32	33	43	95	32	91	69
Ammonium-N	132	39	36	23	46	46	167	124	119	171
Others-N	4179	477	418	376	622	630	655	1281	8635	6326
Total soluble-N	5460	714	923	627	832	851	3222	2768	10170	7630

Table 1. Concentration of amino acid, ureides, nitrate, ammonium, and other 80% ethanol soluble fraction of soybean organs ($\mu\text{gN/gDW}$).

can be extracted by 80% ethanol. Macromolecules such as proteins, nucleic acids, and starch remain in the precipitate of 80% ethanol extraction. As shown in **Table 1**, the total soluble N concentration in root nodules ($5460 \mu\text{gN/gDW}$) was much higher than that in the roots ($714 \mu\text{gN/gDW}$). In the shoots, the total soluble N concentration was the highest in seeds ($10,170 \mu\text{gN/gDW}$ for upper part and $7630 \mu\text{gN/gDW}$ for lower part), followed by pods ($3222 \mu\text{gN/gDW}$ for upper part and $2768 \mu\text{gN/gDW}$ for lower part), and relatively low in stems ($923 \mu\text{gN/gDW}$ for upper part and $627 \mu\text{gN/gDW}$ for lower part) and leaves ($832 \mu\text{gN/gDW}$ for upper part and $851 \mu\text{gN/gDW}$ for lower part). The concentration of total amino acid-N was highest in seeds ($1243 \mu\text{gN/gDW}$ in upper part and $982 \mu\text{gN/gDW}$ in the lower parts), followed by pods ($776 \mu\text{gN/gDW}$ in upper part and $415 \mu\text{gN/gDW}$ in the lower parts) and nodules ($519 \mu\text{gN/gDW}$), and lowest in the leaves ($96 \mu\text{gN/gDW}$ in upper part and $96 \mu\text{gN/gDW}$ in the lower parts). Comparing nodules and roots, amino acid concentration was about 4 times higher in nodules ($519 \mu\text{gN/gDW}$) than in roots ($147 \mu\text{gN/gDW}$). The organs in the upper part were relatively high in total amino acid concentrations compared with the lower parts, and this is due to the upper part was younger than lower part. The ureide-N concentration was 25 times higher in the nodules ($483 \mu\text{gN/gDW}$) than that in the roots ($19 \mu\text{gN/gDW}$). In the shoots, ureides are highly accumulated in the pods ($1529 \mu\text{gN/gDW}$ for upper part and $916 \mu\text{gN/gDW}$ for lower part) compared with leaves and seeds. The concentrations of nitrate and ammonium were high in the nodules, but relatively low and constant among other organs.

Table 2 shows the composition of free amino acids in nodules, roots, stems, leaves, pods, and seeds at pod-filling stage [13]. Soybean (cultivar Norin No. 2) seeds were inoculated with *Bradyrhizobium japonicum* (NIAS J-501) and cultivated with hydroponic solution containing nitrate (10 mgN/L). **Table 2** exhibits the amino acids composition at 67 days after planting, and the shoots were separated the upper parts and lower parts. Asparagine was abundant in every part, especially in pods and stems. Asparagine is known to play a major role in transport of nitrogen in legumes [14]. The contents of glutamate were higher in nodules, roots, and leaves, which are the primary nitrogen assimilatory organs, but relatively low in stems, pods, and seeds. Alanine was relatively high in the roots and nodules. The content of γ -amino butyric acid (GABA) was detected at a high level in most organs.

Amino acids	Nodules	Roots	Stems (UP)	Stems (LP)	Leaves (UP)	Leaves (LP)	Pods (UP)	Pods (LP)	Seeds (UP)	Seeds (LP)
Aspartate	22.317	1.764	4.59	3.618	2.688	2.112	10.864	3.735	55.935	34.37
Threonine	8.304	2.205	5.94	3.484	2.016	1.728	14.744	8.3	34.804	35.352
Serine	33.216	8.379	14.04	6.968	10.56	7.392	22.504	14.11	38.533	38.298
Asparagine	120.408	14.7	105.57	56.012	15.456	9.888	401.192	148.57	343.068	169.886
Glutamate	106.395	25.284	3.78	3.886	16.512	10.176	4.656	3.32	17.402	15.712
Glutamine	6.228	2.205	3.78	2.814	0	0	42.68	17.845	4.972	4.91
Proline	2.076	0	4.59	0	0	0	12.416	14.525	32.318	47.136
Glycine	15.57	0	0	0	0	0	2.328	0	27.346	30.442
Alanine	78.369	43.218	9.18	3.082	5.952	7.392	17.848	9.96	52.206	41.244
Citruline	7.785	0	0	0	0	0	25.608	0	31.075	25.532
Valine	8.823	3.234	6.75	3.752	3.648	3.456	20.952	16.185	48.477	47.136
Cysteine	5.19	2.058	2.97	2.948	2.4	2.784	1.552	1.66	2.486	1.964
Isoleucine	7.785	5.145	7.29	6.03	2.496	3.648	17.072	16.185	36.047	36.334
Leucine	6.747	4.263	7.02	5.628	2.4	3.84	11.64	12.45	47.234	52.046
Tyrosine	3.114	0	1.89	1.34	1.44	0	3.88	3.735	13.673	16.694
Phenylalanine	3.114	1.176	3.78	3.082	2.016	2.208	5.432	8.715	19.888	22.586
GABA	46.71	22.785	43.47	25.862	9.888	33.6	126.488	112.05	335.61	233.716
Arginine	11.937	4.557	22.95	5.628	11.904	7.872	22.504	11.62	65.879	43.208

Data from SSPN [13].

Table 2. Concnetation of free amino acids in various parts of soybean plants ($\mu\text{gN/gDW}$).

3. Amino acid metabolism in soybean nodules

Nitrogen is abundant (about 78% in volume) in the atmosphere, but plant itself cannot use the N_2 except for symbiotic association with nitrogen-fixing microorganisms. Symbiotic association by soybean and rhizobia is one of the most efficient nitrogen-fixing system, and it contributes to soybean seed yield [15]. **Figure 5** shows a photograph of nodulated soybean roots cultivated with hydroponics (A) and a model of structure of mature soybean nodule attached to the root (B). Soybean nodules have a spherical form, and they grow up to about 8 mm in diameter. Soybean nodule is classified as a determinate-type nodule, and the cell division and nodule development are completed in early stage of nodule growth. The nodule growth thereafter is mainly due to cell expansion.

Figure 6 shows the scheme of N assimilation in soybean nodules. N_2 gas is diffused into central symbiotic region of the nodule and reduced into ammonia by the enzyme “nitrogenase” in the bacteroid, a symbiotic form of rhizobia. Most of ammonia produced by N_2 fixation

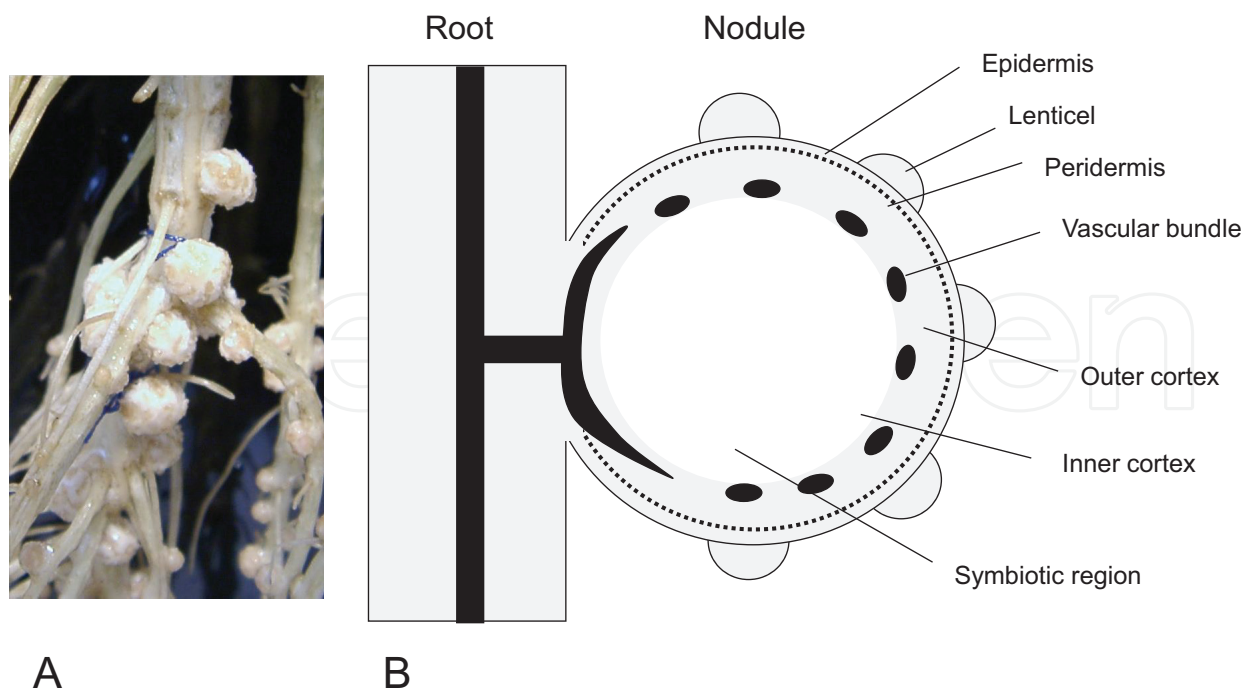


Figure 5. Photograph of nodulated soybean roots and a model of the structure of soybean nodule. (A) Photograph of nodulated soybean roots cultivated in culture solution. (B) A model of the structure of soybean nodule.

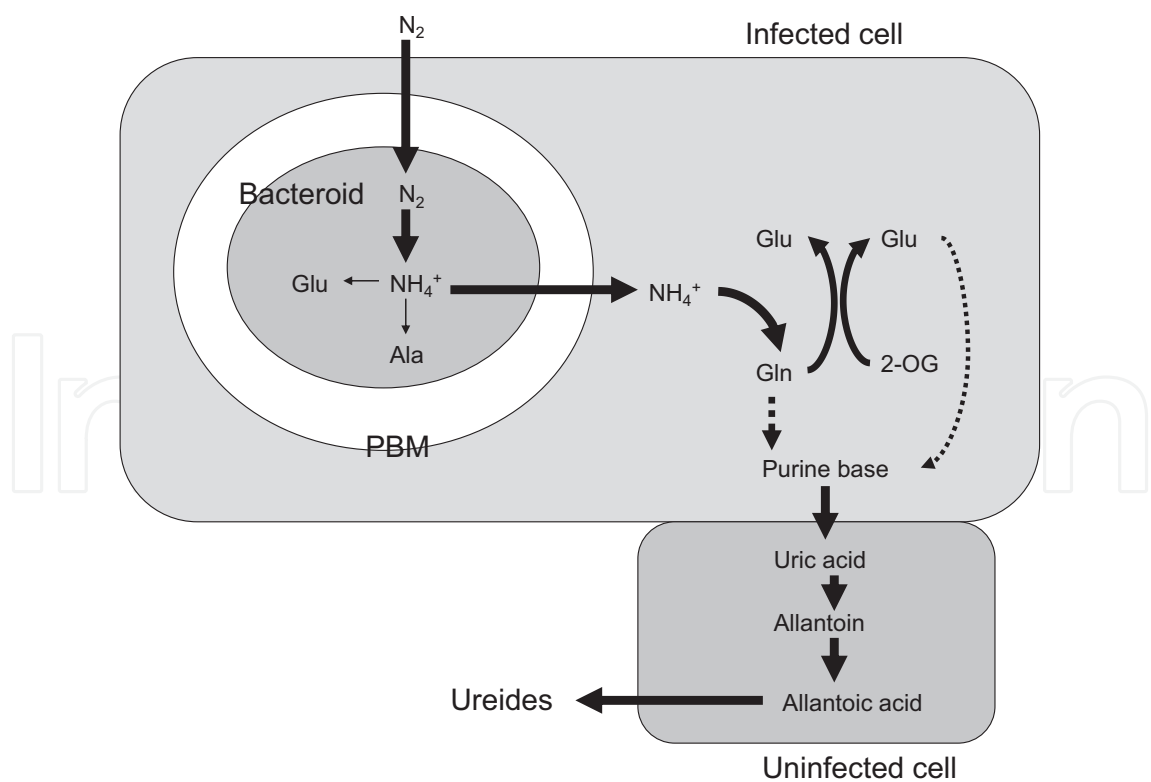


Figure 6. Model of nitrogen flow and metabolism in a soybean nodule.

is rapidly excreted into the cytosol through peribacteroid membrane (PBM) of infected cell. Based on the time course experiment with $^{15}\text{N}_2$ feedings in the nodulated intact soybean plants, the ammonia produced by nitrogen fixation is initially assimilated into amide group of Gln with Glu by the enzyme glutamine synthetase (GS) [16, 17]. Then, Gln and 2-OG produce two moles of Glu by the enzyme glutamate synthase (GOGAT). Some part of Gln is used for purine base synthesis, and uric acid is transported from the infected cells to the adjacent uninfected cells in the central symbiotic region of nodule. Uric acid is catabolized into allantoin and allantoic acid in the uninfected cells and then transported to the shoot through xylem vessels in the roots and stems. A small portion of fixed N was assimilated into alanine and Glu in the bacteroids, but it was not by GS/GOGAT pathway [18].

A small portion of fixed N is transported as amino acid-like asparagine (Asn) in addition to ureides, but the percentage is about 10–20% of total fixed N. Small amount of ureides is synthesized from NO_3^- in soybean nodules [19]. Nitrate in culture solution can be absorbed from nodule surface [20] and assimilated in the cortex of the nodules. Nitrate absorbed from lower part of roots has not readily transported to the nodules attached to the upper part of the root system [1].

4. Amino acid metabolism in soybean roots

Higher plants absorb soil inorganic nitrogen such as ammonium or nitrate from roots. **Figure 7** shows the photograph of a nodulated soybean root system (A) and a model of structure of soybean root (B). In a longitudinal direction, there are three regions in root (B): Root cell division occurs in the “apical meristem,” and the cells are differentiated and elongated in the upper “region of elongation.” Then, the root cells mature in the “region of maturation.” There are three parts, epidermis, cortex, and stele, in a cross-section of mature roots. There are two transport pathways, xylem and phloem, in the stele.

Figure 8 shows a model of nutrient flow from soil to xylem vessel in the roots. Nutrients, such as NH_4^+ and NO_3^- in soil solution, are absorbed by epidermal cells or root hairs and are transported cell to cell by the symplastic pathway. Nutrients also enter into the free space of the root cortex by the apoplastic pathway and are absorbed by cortical cells. The apoplastic pathway is blocked by a water proof Casparian strip, so the nutrients should be passed through endodermis by a symplastic pathway into stele. Then, the nutrients are released from parenchima cells of stele to the free space in the stele and loaded into the xylem vessel, which is the vertically connected dead cell walls. The nutrients and water are transported from roots to the shoots by transpiration in leaves and root pressure.

Figure 9 shows a model of ammonium and nitrate assimilation in a plant cell. NH_4^+ is transported into cell cytoplasm by the ammonium transporter, which is located in a plasma membrane. NH_4^+ is first assimilated into glutamine (Gln) combined with glutamic acid (Glu) by the enzyme glutamine synthetase (GS) consuming one molar of ATP. Then, the amide group of Gln is transferred to an organic acid, 2-oxoglutarate (2-OG), by glutamate synthase (GOGAT)

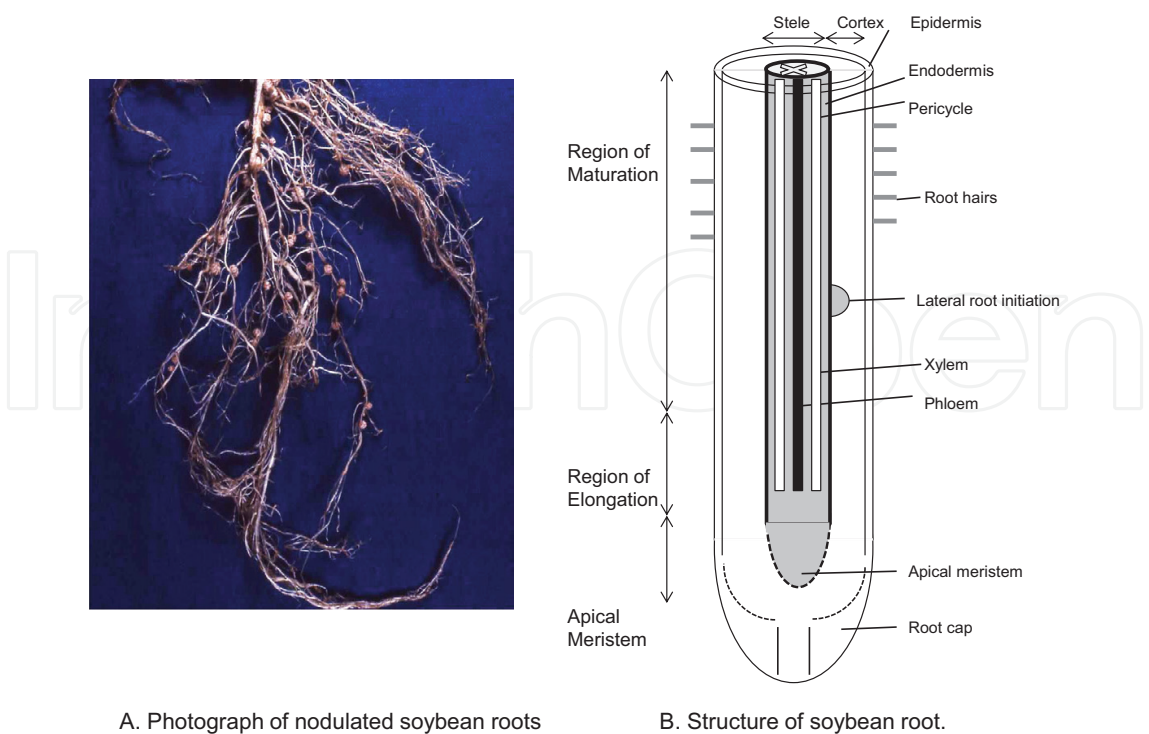


Figure 7. Photograph of nodulated soybean roots and a model of the structure of soybean root. (A) Photograph of nodulated soybean roots cultivated in soil. (B) A model of the structure of soybean root.

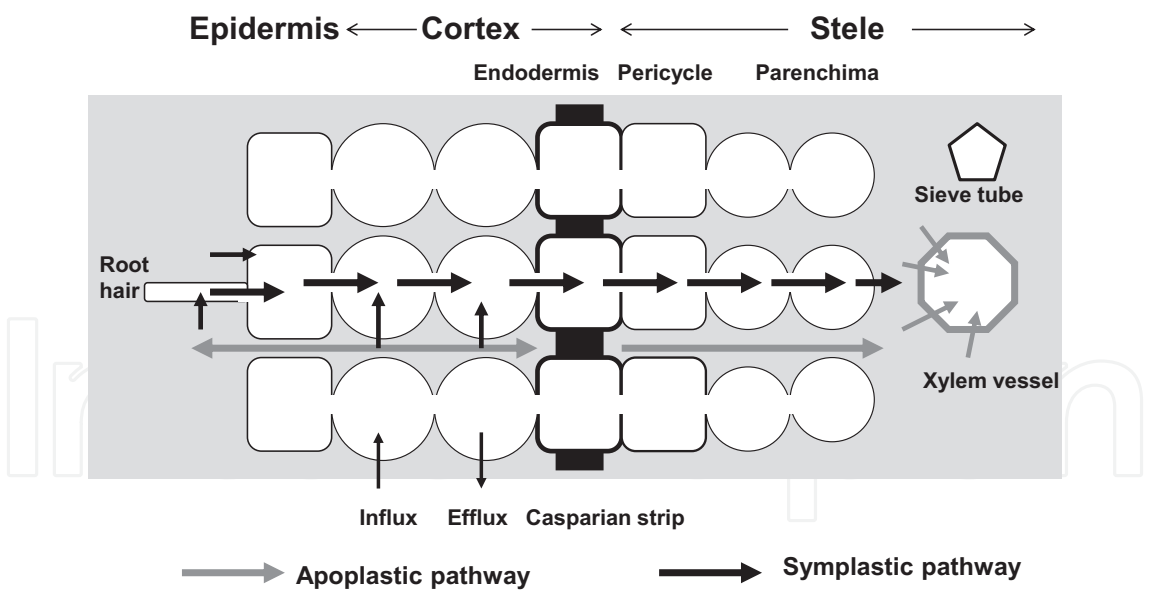


Figure 8. A model of nutrients and water flow in the root.

in plastids using two molar of reduced Ferredoxin (Fd_{red}). Various amino acids are synthesized by amino transferases or amino acid metabolism. Amino acids are used for the synthesis of proteins, nucleic acids, etc. Some parts of amino acids are exported outside the cell to transport to the other organs.

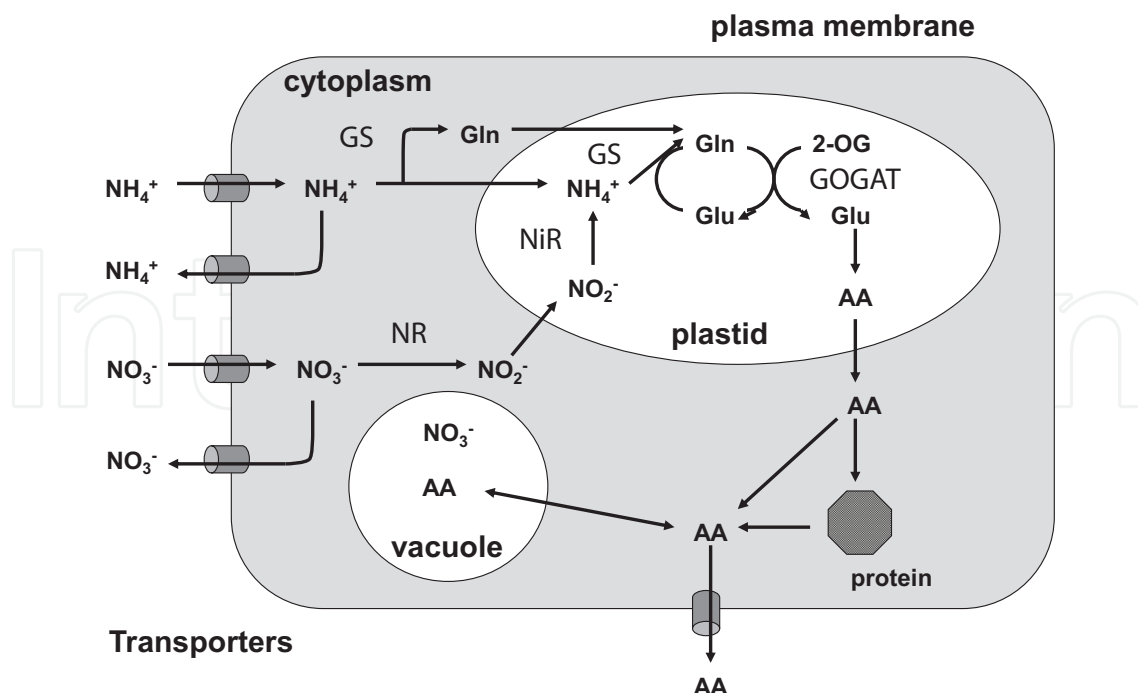


Figure 9. A model of ammonium and nitrate transport and metabolism in root cells. Gln: glutamine, Glu: glutamate, 2-OG: 2-oxo-glutarate, AA: amino acids, GS: glutamine synthetase, GOGAT: glutamate synthase.

Nitrate is most abundant inorganic nitrogen in upland fields under aerobic conditions. NO_3^- is transported into cell cytoplasm by nitrate transporters on plasmamembrane. Two types of nitrate transporters are present in plants, a high-affinity transport system (HATS) and a low-affinity transport system (LATS). Affinity to nitrate is high in HATS (K_m is about 10–100 μM) but low in LATS (K_m is over 0.5 mM) [21]. The nitrate uptake is driven by a proton motive force and mediated by $2\text{H}^+/\text{NO}_3^-$ -symport. By analyzing circulation system of culture solution, the nitrate absorption rate of soybean roots was measured. The kinetics between nitrate concentration versus nitrate absorption rate indicated that soybean root has only one high-affinity nitrate transporter in the roots, which K_m was 19 μM . NO_3^- is reduced to nitrite (NO_2^-) by the enzyme nitrate reductase (NR) using one molar of NADH or NADPH as a reductant. The NO_2^- is transported to plastids and further reduced to NH_4^+ by the enzyme nitrite reductase (NiR) using six molar of Fd_{red} . Then, NH_4^+ is assimilated into Gln by GS located in plastids. Finally, Gln and 2-OG are converted to 2 Glu by the enzyme GOGAT. Soybean roots absorb NO_3^- not only during day but also during night. The absorption rate in night was about 2/3 compared with that in day time [22].

In order to compare the assimilation and transport of nitrate, nitrite, and ammonium, $^{15}\text{NO}_3^-$, $^{15}\text{NO}_2^-$, or $^{15}\text{NH}_4^+$ was supplied to the nodulated soybean plants [23]. The N from nitrate and ammonium was rapidly absorbed, and 70% of ^{15}N absorbed was distributed in the shoots at 24 hours after ^{15}N treatments. The partitioning of ^{15}N among the organs was similar between $^{15}\text{NO}_3^-$ and $^{15}\text{NH}_4^+$ treatments. However, ^{15}N absorption was low, and most of ^{15}N remained in the roots after $^{15}\text{NO}_3^-$ treatment.

Figure 10 shows the scheme of N flow from roots and nodules.

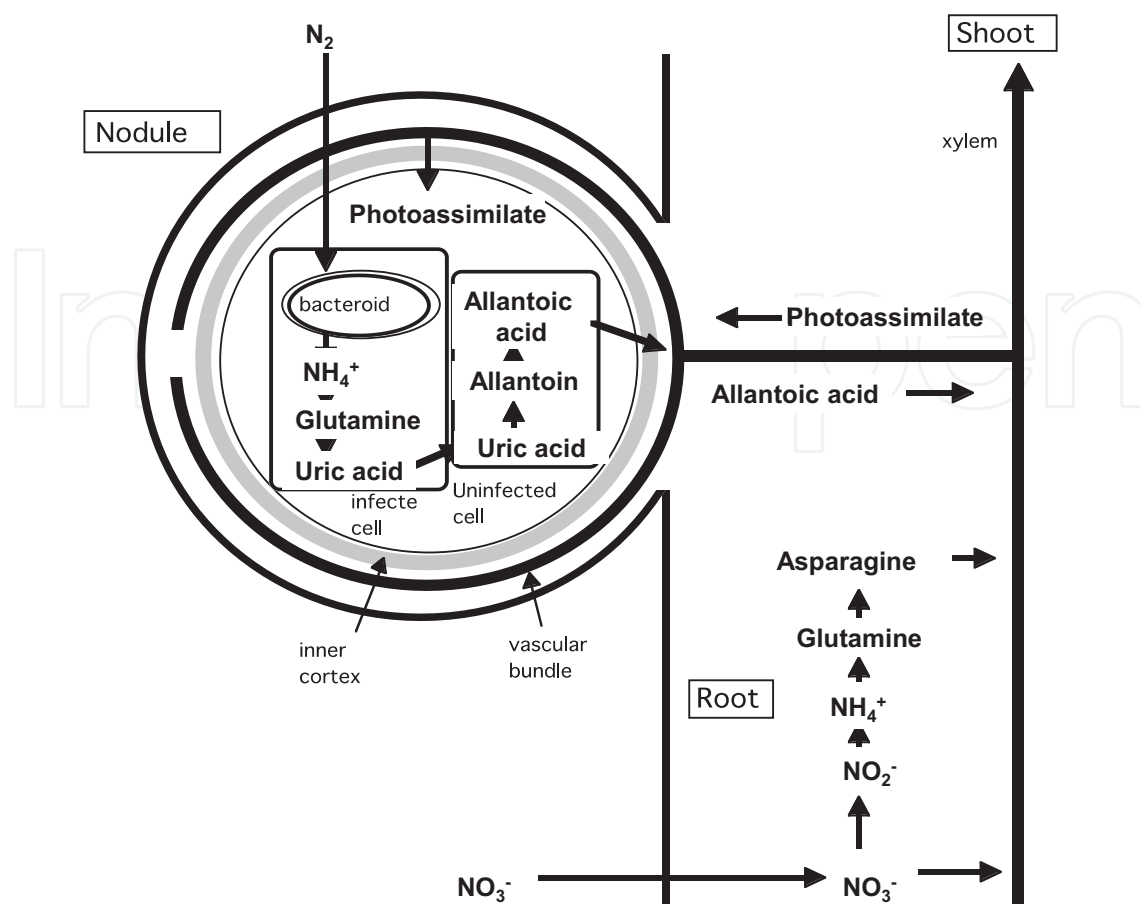


Figure 10. Nitrogen metabolism and transport in nodules and roots of soybean plants.

5. Amino acid metabolism in soybean stems

Stems support the upright structure of shoots, and they connect among roots, leaves, flowers, and fruits in higher plants. Soybean has a main stem and several lateral stems. The structure of the soybean stem was shown in **Figure 11**. In the bark of the stem, there are epidermis and cortex including vascular bundles with phloem. In the central woody part of the stem, there are xylem vessels and pith. Xylem sap comes up through xylem vessels in the stem from root xylem vessels via transpiration stream and root pressure. As shown in **Figure 12**, xylem vessel is not closed pipe, but they have pits on the wall and water and solutes move to the apoplast from xylem vessels and also liquid in apoplast come back to xylem vessels [24].

Stems play a role in storing nutrients temporary or long term in perennial plants. In soybean, ureides and amino acids can be temporarily stored in the stems, and these compounds are eventually transported to the leaves, pods, and seeds. For analyzing concentrations of nitrogen constituents, such as amino acids, ureides, and nitrate, stem extract and stem fluid collected by centrifugation or xylem bleeding sap were used. Stem extract contains all the extractable compounds in the cells including cytoplasm and vacuoles, apoplast fluid outside of the cells, and xylem sap in xylem vessels. Stem extract contains a large amount of cellular

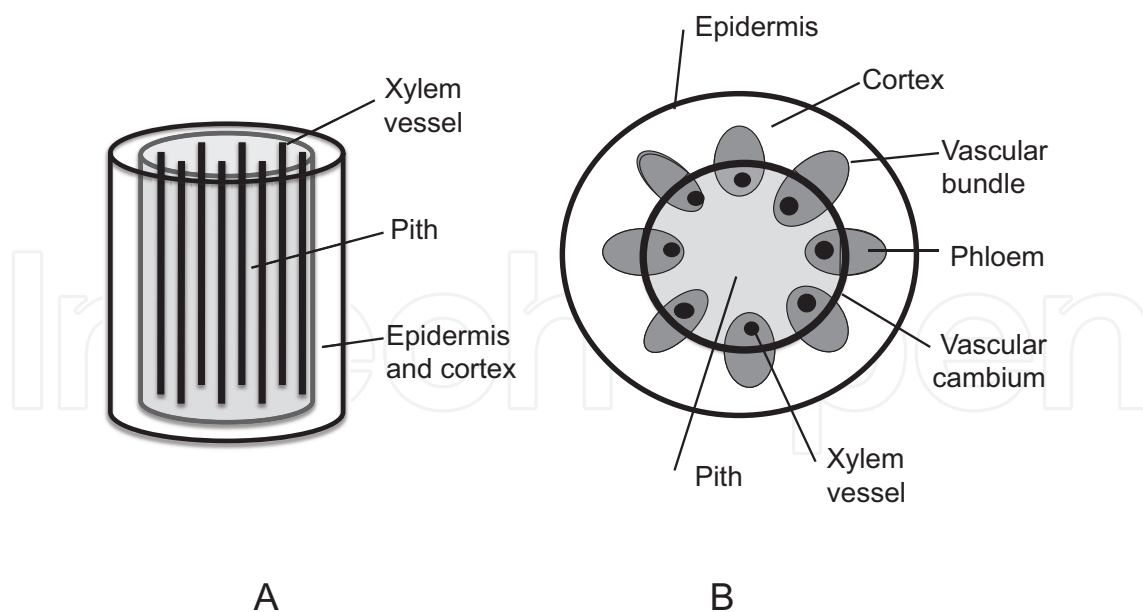


Figure 11. Model of the structure of soybean stem. (A) 3-D image of stem. (B) Cross-section of soybean stem.

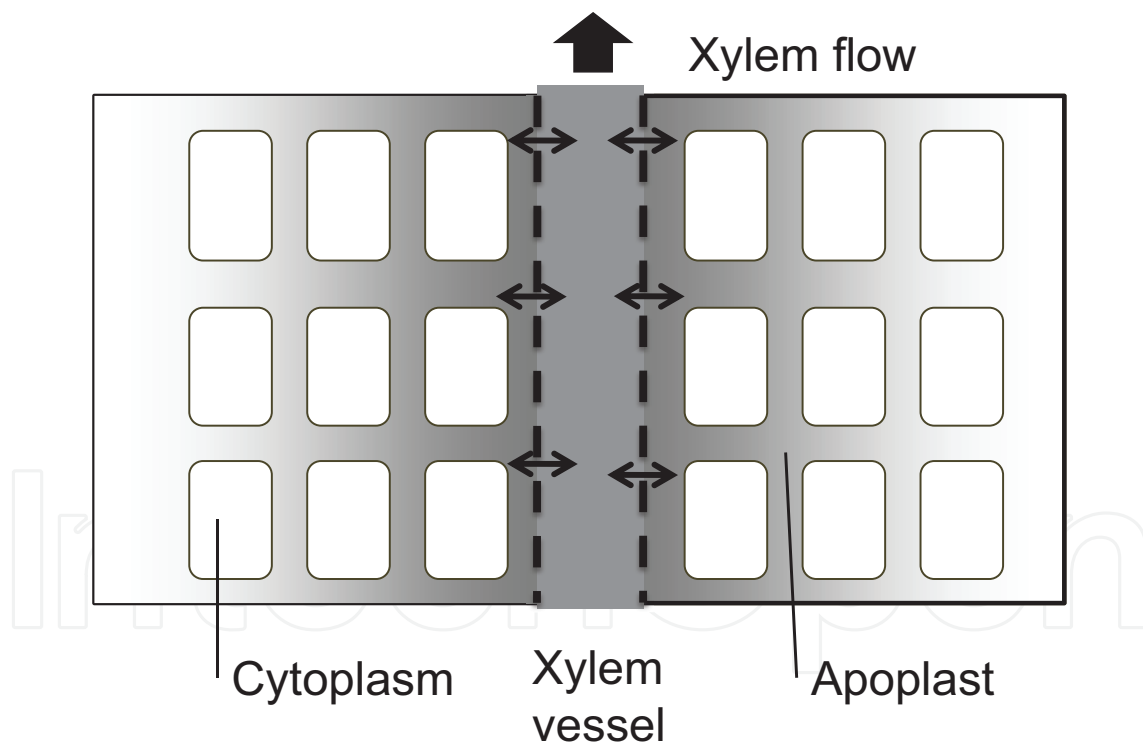


Figure 12. A model of xylem flow in soybean stem.

components such as temporary storage N compounds. When the apoplast fluid collected by centrifugation and xylem bleeding sap were compared, the concentrations of nitrate and ureides are relatively same between two fluids, but the concentrations of amino acids are several times higher in apoplast fluid compared with xylem bleeding sap [25]. Therefore, for the

estimation of the percentage dependence of nitrogen fixation in total nitrogen assimilation by relative ureide method, we use the xylem bleeding sap from cut basal stump. Ohtake et al. [26] reported that Asn is the major amino acid in soybean xylem sap, and the average N atoms per an amino acid in xylem sap was about 2.0, irrespective of growth stages.

Sometimes, it is not successful for collecting xylem sap, especially when soil is dry or during late growth stage. Herridge and Peoples [27] used a vacuum extracted stem exudate from lower part of the main stem of the cut shoot or hot-water extraction of the stems.

A relative ureide method is a simple and reliable method for estimating the percentage of nitrogen depending on nitrogen fixation (%Nd_{fa}) in a field-grown soybean [27–29]. **Figure 13** shows the concept of the simple relative ureide method estimating ratio of N₂ fixation and N absorption in the fields. Although a small portion of ureides are transported from non-nodulated soybean grown with nitrate and small portion of amino acids are transported from nodulated soybean without nitrate, we simply estimate the percentage of ureide-N in total N of ureide-N, amide-N plus nitrate-N in root bleeding sap. Takahashi et al. [29] reported the comparison of ureide-N concentration in xylem sap between nodulating and nonnodulating isoline (**Figure 14**), indicating that ureides concentration is much higher in nodulated soybean compared with nonnodulated soybean at any stage [29]. To confirm the origin of ureides in the stems, two treatments have been done for 8 hours. ¹⁵N₂ gas was exposed to a group of soybean plants, and ¹⁵NO₃⁻ was added to the culture solution to another group, and then the ratios of ¹⁵N abundance from ¹⁵N₂/¹⁵NO₃⁻ in each compounds in stems and nodules were calculated (**Figure 15**). The ratios in most amino acids in stems were between 0.1 and 0.5, and

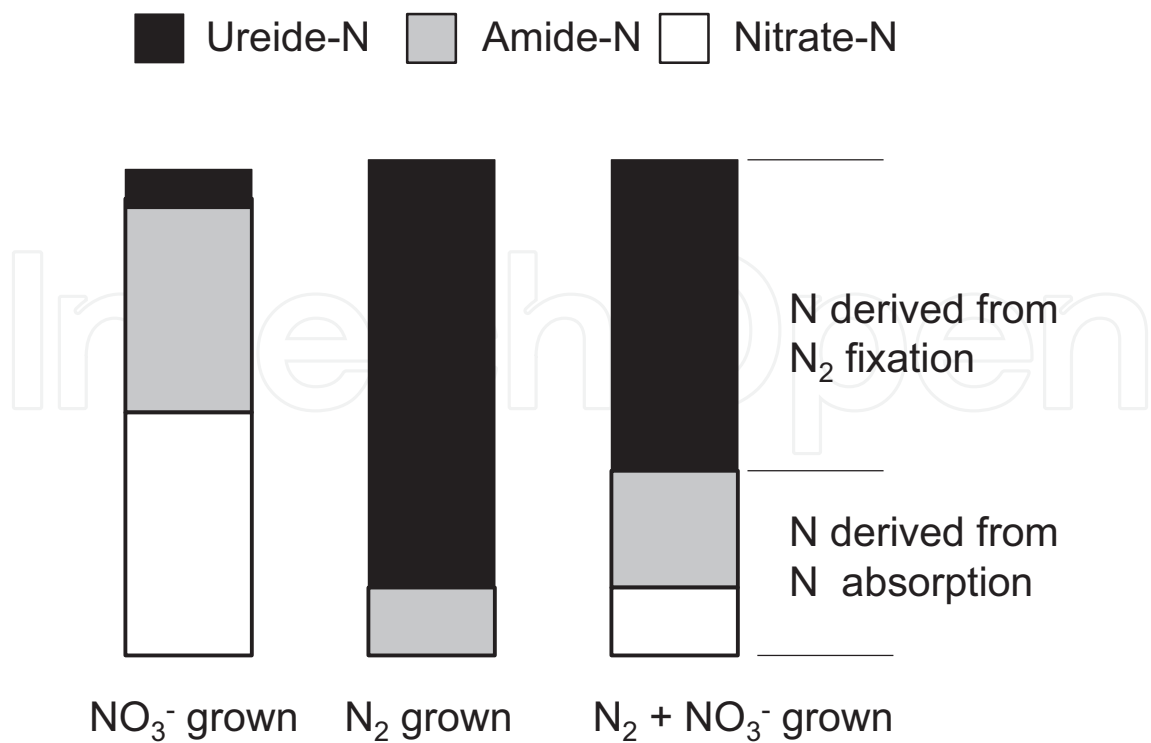


Figure 13. Concept of the simple ureide method for estimating N derived from nitrogen fixation and soil + fertilizer N.

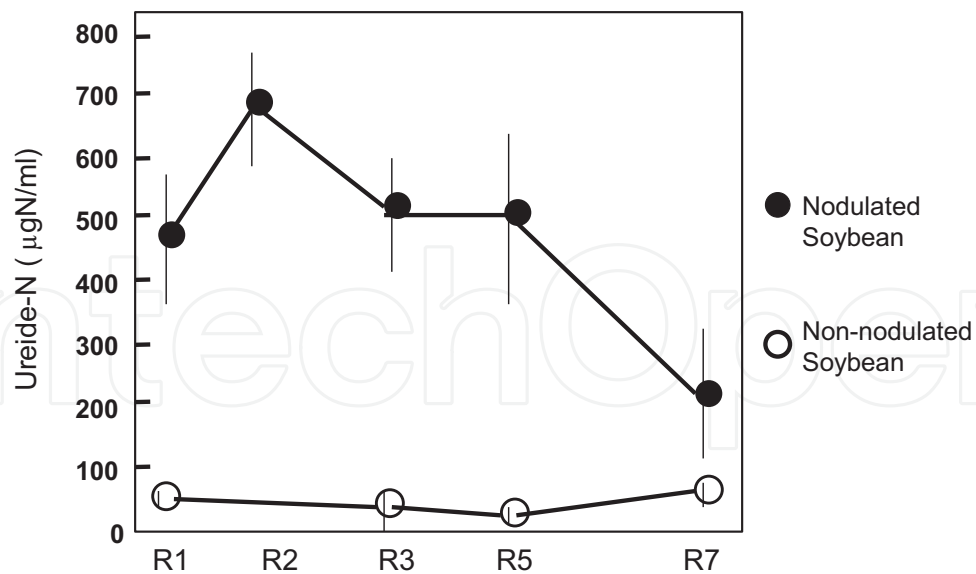


Figure 14. Changes in ureide-N concentration in xylem sap collected from nodulated and nonnodulated soybean.

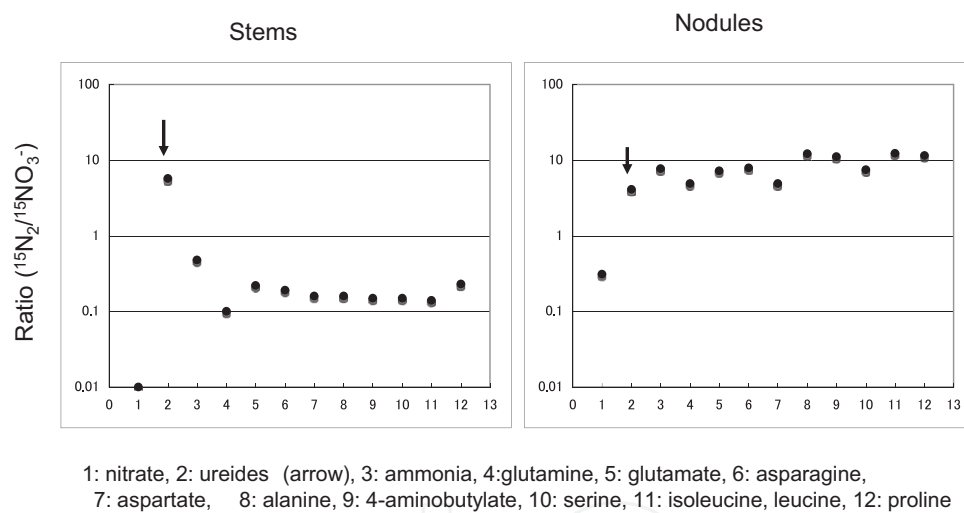


Figure 15. Comparison of the ratios of ^{15}N abundance from $^{15}\text{N}_2$ and $^{15}\text{NO}_3^-$ in stems and nodules. (A) Ratios in stems. (B) Ratios in nodules.

only ureides showed a high value nearly 10. This indicated that most of ureides in stems are derived from nitrogen fixation and amino acids in stem derived from nitrate absorption [19]. The ratios of ureides and amino acids in the nodules showed about 10.

6. Amino acid metabolism in soybean leaves

Soybean has trifoliolate leaves, in addition to a first pair of green cotyledons, the second pair of primary leaves, and the prophylls [30]. **Figure 16** shows the model of soybean leaflet of trifoliolate leaves (A) and the internal structure of soybean leaf tissue (B) [31]. Each leaflet is

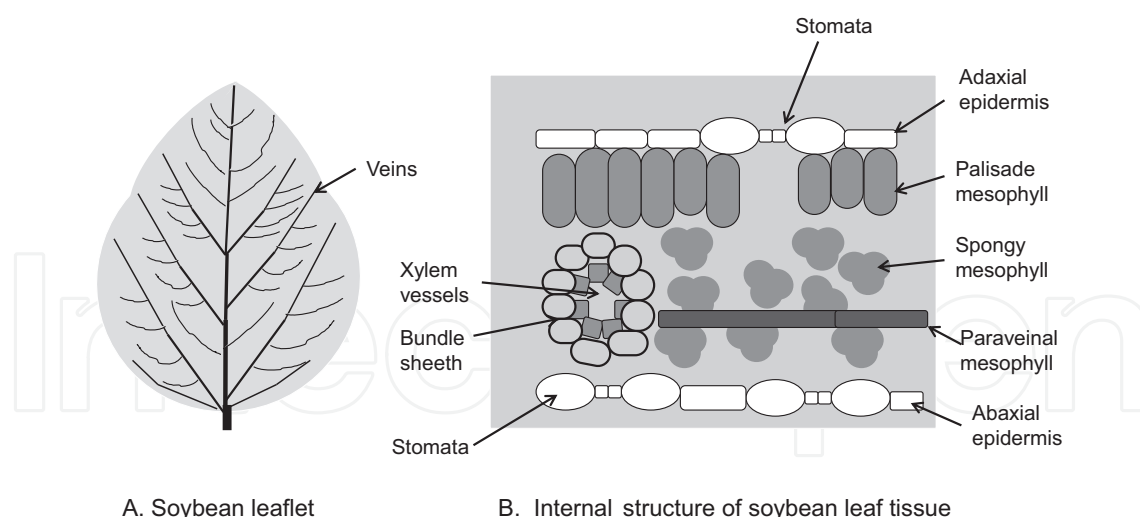


Figure 16. A model of soybean leaflet of trifoliolate leaves and the internal structure of soybean leaf. (A) Mature soybean leaflet of trifoliolate leaves with network of veins. (B) Model of the internal structure of soybean leaf tissue.

highly vasculated and as many as six orders of veins have been observed. All vascular bundles in a leaf are collateral, with adaxial xylem and abaxial phloem. The adaxial layer of mature leaflet is the upper epidermis, and the second and third layers are palisade tissue containing chloroplasts. A portion of fourth layer differentiates into veins and the paraveinal mesophyll which is flanked by minor vein. The fifth and sixth layers become spongy mesophyll, and the abaxial layer becomes the lower epidermis [31].

Leaves are the organ of photosynthesis-producing sugar from carbon dioxide (CO_2) in the atmosphere and H_2O absorbed from roots. Also, leaves play an important role in N metabolism such as nitrate reduction and amino acid metabolism. The metabolic products in leaves are transported to the roots and apical buds to support their nutrition through phloem vessels. Evapotranspiration of water through stomata or leaf surface helps upward water flow and nutrient transport from root to shoots via xylem vessels. Xylem vessels are dead cell wall, but phloem vessels are living cells. Therefore, when petiole was treated by heat, phloem transport can be blocked (petiole-girdling treatment).

Petioles of upper or lower soybean leaves cultivated with solution culture with 10 mgN-NO_3^- at 69 days after planting were girdling treatment by hot steam. Then, after 10 hours of $^{15}\text{N}_2$ or $^{15}\text{NO}_3^-$ treatment, leaf blades are harvested for analysis [32].

Table 3 shows the concentration of total amino acid, ureides, nitrate, ammonium, and total soluble N in intact and girdled leaves. The ratios of ureides and nitrate were almost 1.0, indicating that the concentrations of these compounds did not change by the petiole girdling. This may be due that all the ureides and nitrate are metabolized in the leaf blades and not retransported via phloem. **Table 4** shows the sugar concentration in petiole girdled and intact leaves. The concentrations of fructose, glucose, and sucrose in the petiole-girdled leaves are 1.8–3.8 times higher than those in the intact leaves, irrespective of upper or lower leaves. These accumulations of sugars were due to a blockage of phloem transport from leaves to stems. On the other hand, the concentration of amino acids and ammonia (**Table 5**) increased about 1.5–2.6 times by petiole-girdling treatment, which is similar to the sugar concentration.

	Upper leaves			Lower leaves		
	Intact leaves	Stem girdling	Ratio	Intact leaves	Stem girdling	Ratio
Total amino acid-N	16	41	2.6	15	34	2.3
Ureides-N	36	36	1	45	50	1.1
Nitrate-N	36	44	1.2	37	32	0.9
Ammonium-N	60	88	1.5	56	92	1.6
Total soluble-N	769	1024	1.3	981	1065	1.1

Table 3. Concentration of amino acid, ureides, nitrate, ammonium, and 80% ethanol soluble fraction of intact and girdled leaves ($\mu\text{gN/gDW}$).

	Upper leaves			Lower leaves		
	Intact leaves	Stem girdling	Ratio	Intact leaves	Stem girdling	Ratio
Fructose	2.78	9.35	3.4	2.35	8.43	3.6
Glucose	2.84	7.31	2.6	1.86	7.1	3.8
Sucrose	4.74	8.35	1.8	6.03	11	1.8

Table 4. Concentration of 80% ethanol soluble sugar of soybean leaves (mgN/gDW).

	Upper leaves			Lower leaves		
	Intact leaves	Stem girdling	Ratio	Intact leaves	Stem girdling	Ratio
Asparagine	347	781	2.3	390	669	1.7
Threonine	287	772	2.7	222	677	3
Serine	782	1440	1.8	649	1283	2
Asparagine	928	8089	8.7	875	7760	8.9
Glutamate	1729	3290	1.9	1888	2597	1.4
Glutamine	0	914		0	774	
Proline	653	4.837	7.4	1008	2597	1.4
Alanine	492	1587	3.2	579	1106	1.9
Valine	775	1918	2.5	585	1509	2.6
Cysteine	270	294	1.1	221	282	1.3
Isoleucine	758	2674	3.5	730	2270	3.1
Leucine	733	2196	3	641	1824	2.8
Tyrosine	156	579	3.7	123	380	3.1
Phenylalanine	536	1426	2.7	404	896	2.2
GABA	3332	6267	1.9	3035	4683	1.5
Arginine	1582	1036	0.7	1043	1022	1

Data from SSPN [32].

Table 5. Composition of free amino acids in intact and girdled leaves of soybean plants.

Among amino acids, Asn was the highest ratio about 9.0 by the petiole-girdling treatment. Most of the other amino acids show the ratios 2–4, but only arginine showed the ratio 0.7 and 1.0 in upper leaves and lower leaves, respectively. Pate et al. [33] reported that asparagine and glutamine are predominant in phloem exudate obtained by phloem bleeding technique from legume fruits.

Figure 17 summarizes the flow of N in soybean leaves. Ureides, nitrate, and Asn transported to the leaf blades via xylem vessels are metabolized in leaves and assimilated into leaf protein. Then, the degradation products of leaf protein are retransported to the apical buds, roots, and pods via phloem.

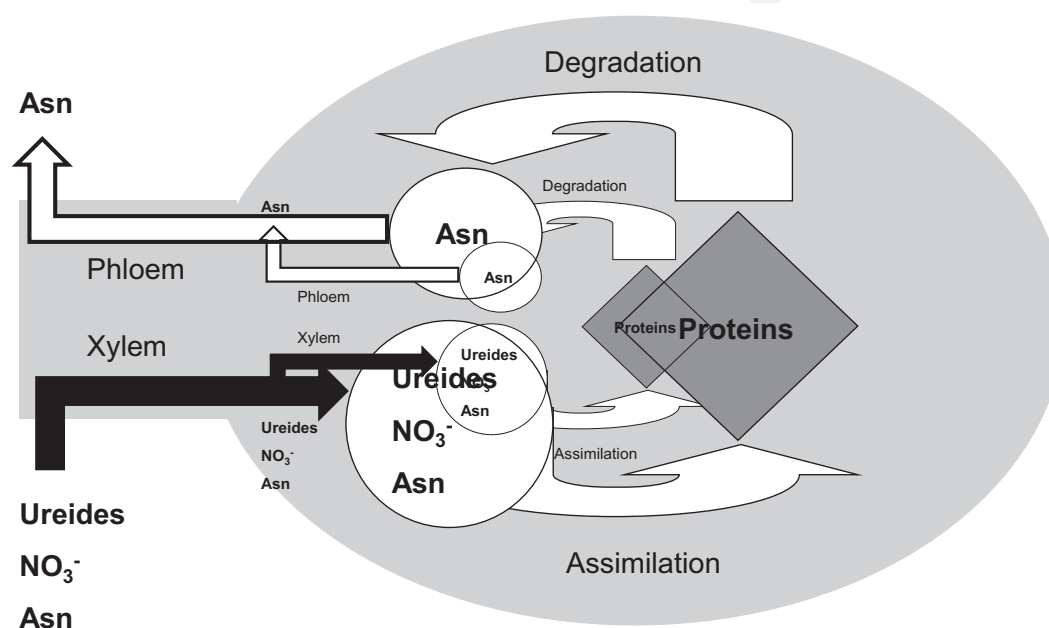


Figure 17. A model of nitrogen flow in soybean leaves.

7. Amino acid metabolism in soybean pods and seeds

Figure 18 shows the top (A) and side (B) views of a mature soybean seed and growing cotyledons in a pod (C, D) [31]. The mature soybean seed consists of a seed coat surrounding a large embryo. Seed coat has a hilum (seed scar), and there is a tiny hole (micropyle) at the end of the hilum. The tip of the hypocotyl radical axis is located just below the micropyle. There is a main vein at the dorsal part of a pod, and nutrients such as sugar and ureides and amino acids are transported through the vein. Seed coat has a funiculus connecting main vein and hilum. Nutrients are transported through vasculature bundles in seed coat; however, the vascular systems are not directly connected to the cotyledons. The cotyledons are cultured in a seed coat cavity. Therefore, nutrients are excreted to the cavity, and cotyledons absorb them by themselves.

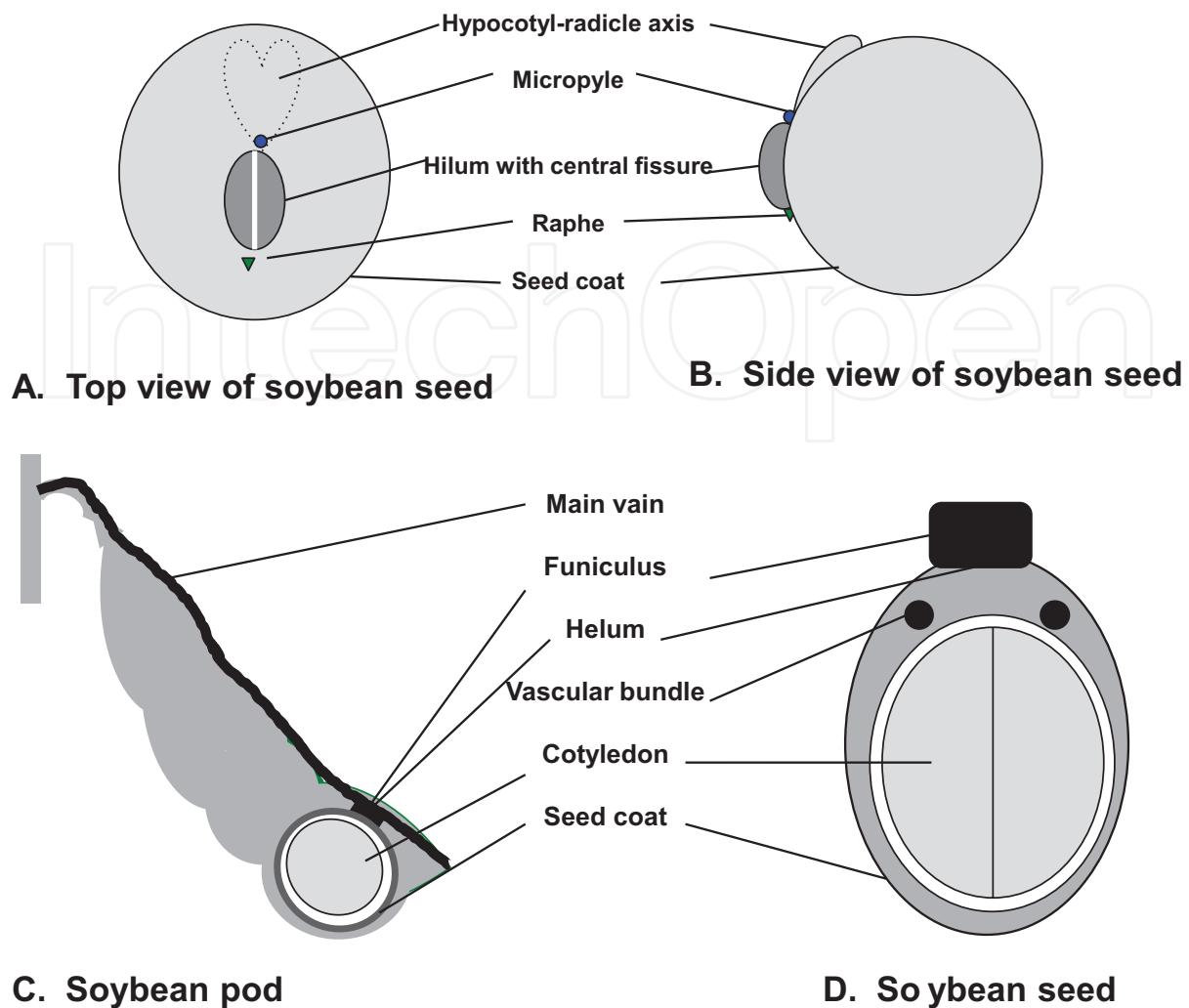


Figure 18. Structures of soybean seed and pod. (A) Top view of soybean seed. (B) Side view of soybean seed. (C) Soybean pod with seeds inside. (D) Soybean seed.

As shown in **Table 1**, young pods contained a high concentration of ureides both in the upper and the lower pods. The high accumulation of ureides in the pods may be due to that ureides are tentatively stored in the pods before transporting to the seeds. Seeds contain a high concentration of amino acids especially Asn and GABA (**Table 2**).

Figure 19 shows the changes in ureide-N and amino acid-N in seeds and pods of nodulated and nonnodulated soybean [34]. The concentrations of ureides in the pods of nodulated soybean were high at 1st September and decreased after 15th September. The ureide-N concentration kept low in the pods of nonnodulated soybean plants. Amino acid-N concentrations were similar between nodulated and nonnodulated soybeans and decreased linearly from 1st September to 10th October at maturing stage. On the other hand, the ureide-N concentrations in the seeds of nodulated and nonnodulated plants were constantly low. The amino acid-N concentrations were similar between nodulated and nonnodulated soybeans, decreased from 1st September to 22nd September, and then constant until maturity at 10th October.

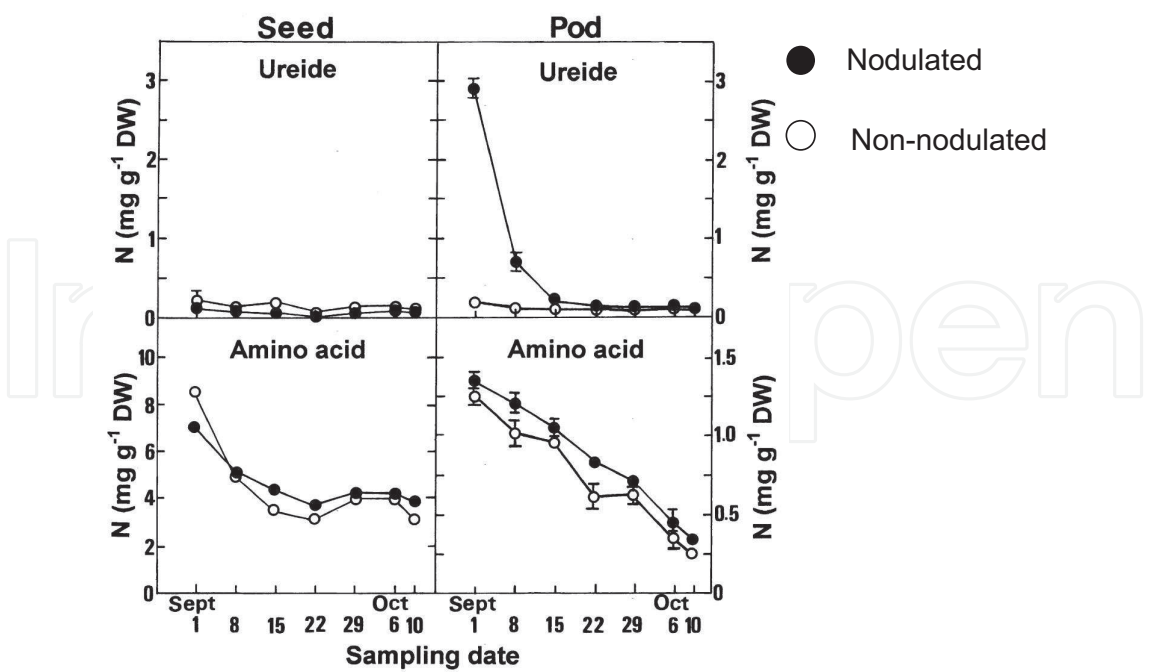


Figure 19. Changes in udeide-N and amino acid-N in pods and seeds of nodulated and nonnodulated soybean plants.

Most parts of N stored in matured seeds are storage proteins in the cotyledons (Figure 20A) [35]. Soybean seed storage protein consists of glycinin and β -conglycinin (Figure 20B). The glycinin is the hexamer, which has acidic and basic subunits. The β -conglycinin is the trimer, which has α' , α , and β subunits.

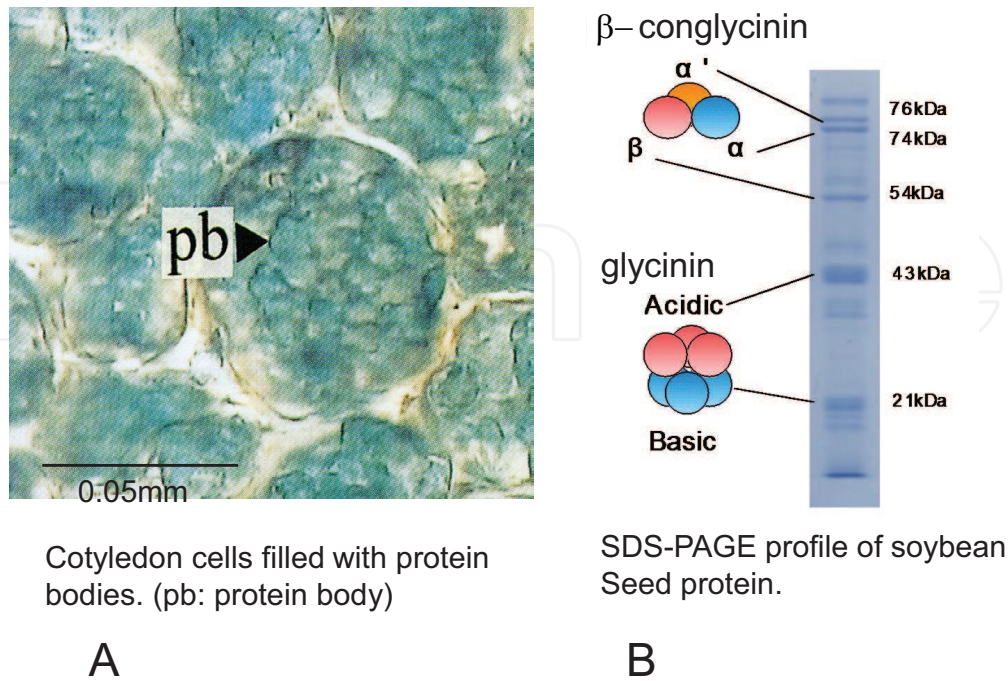


Figure 20. Microscopic picture of soybean cotyledon and soybean seed storage proteins. (A) Microscopic picture of thin slice of soybean cotyledon. Pb: protein body. (B) Soybean seed storage protein separated by SDS-PAGE and stained with CBB.

Figure 21 summarizes the metabolism and transport of N from pod into seeds. The ureides, allantoin, and allantoic acid, transported from nodules, are tentatively accumulated in the young pods, and these are metabolized into amino acids such as Gln, Asn, and then, these amino acids are secreted into the cavity of seed coat. Then, cotyledons absorb amino acids and synthesize storage protein and sort to the protein body. Asn and amino acids from roots and leaves are also transported to the pods and secreted into seed coat, and then, cotyledons absorb them for storage protein synthesis.

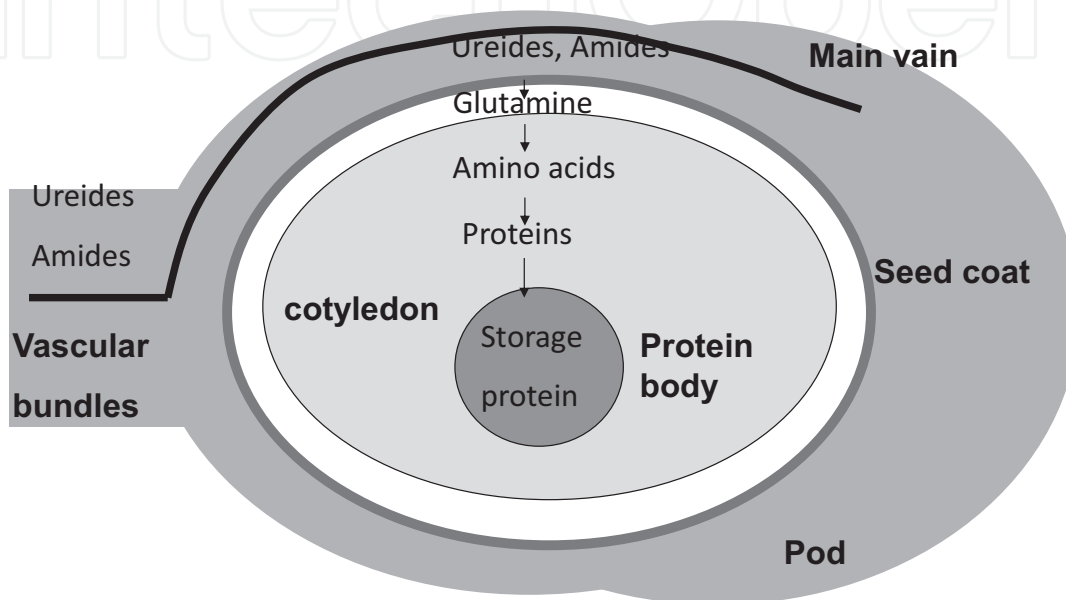


Figure 21. Model of the flow of ureides and amino acids in soybean pod to a seed.

8. Recycling of nitrogen from shoot to roots

Recycling of nitrogen from shoot to roots via phloem supports the initial growth of roots and nodules that need external nitrogen nutrients until nitrogen absorption and nitrogen fixation start to meet the N demand. The quantitative measurement of recycling of N in soybean cultivar Williams and hypernodulation mutant lines, NOD1-3, NOD2-4, and NOD3-7, was carried out by split root experiment in which a half root system was in the pot with ^{15}N -labeled solution and the other was in nonlabeled solution (**Figure 22**) [36]. The roots of soybean plants cultivated in culture solution were separated into two pots at 33 days after planting. At the next day, ^{15}N -labeled nitrate (10 mgN L^{-1}) was added in the one side of pot and non-labeled nitrate (10 mgN L^{-1}) in the other side of pot. After 2 days of treatment, plants were harvested and percentage of N from ^{15}N -labeled source ($^{15}\text{N}\%$) was determined in each part. The ^{15}N was highest in the roots in ^{15}N -labeled pot (14.0%), followed by stems (6.0%) and leaves (3.9%). A small portion of recycling ^{15}N was detected both in roots (0.7%) and nodules (0.3%) in the nonlabeled pot after 2 days of split root treatment in Williams. The distribution of ^{15}N in sum of nodules and roots was the same in NOD1-3, NOD2-4, and NOD3-7 hypernodulation lines.

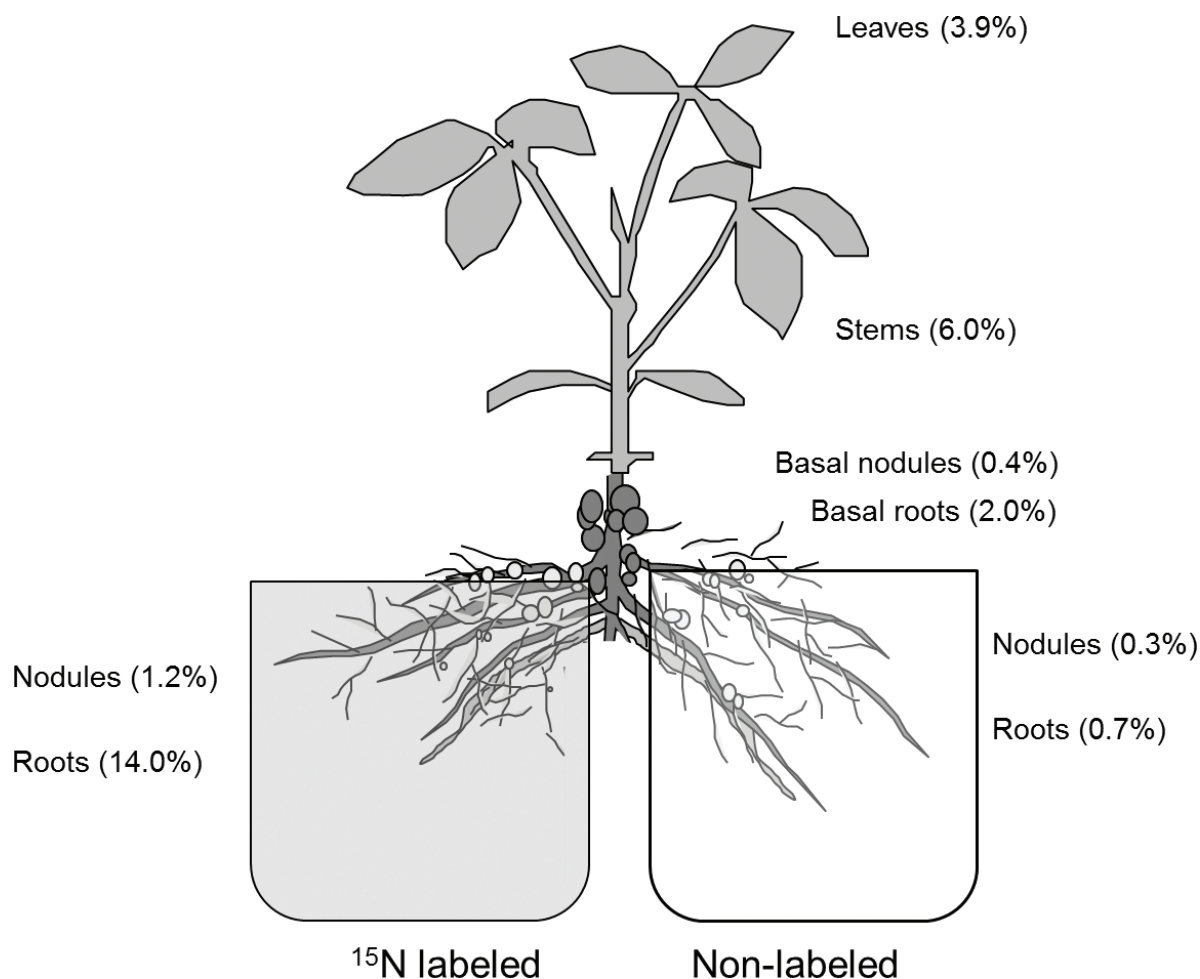


Figure 22. Recycling of N derived from a half root of soybean plants with ^{15}N -labeled nitrate. Percent in parenthesis is percentage of labeled N in total N in each part.

9. Overall nitrogen transport from fixed N and absorbed N

Figure 23 shows the model of initial flow of N in soybean plants originated from N_2 fixation (A) in nodules and NO_3^- absorption from roots (B).

The ureides produced by nitrogen fixation in nodules are transported to leaves and used for the leaf growth and metabolism. Mature leaves do not retransport ureides from phloem, but these are transported as amino acids, especially Asn. Some ureides are directly transported to the pods, and young pods accumulate a large amount of ureides, then it is used for seed growth via seed coat after decomposition into amino acids, such as Gln and Asn.

The absorbed nitrate in the roots is transported as NO_3^- or amino acids especially Asn after assimilated in the roots. The absorbed NO_3^- is transported to the leaves, then reduced by leaf nitrate reductase and nitrite reductase, then assimilated into amino acids. The amino acids are transported to the pods, and then seeds from the leaves.

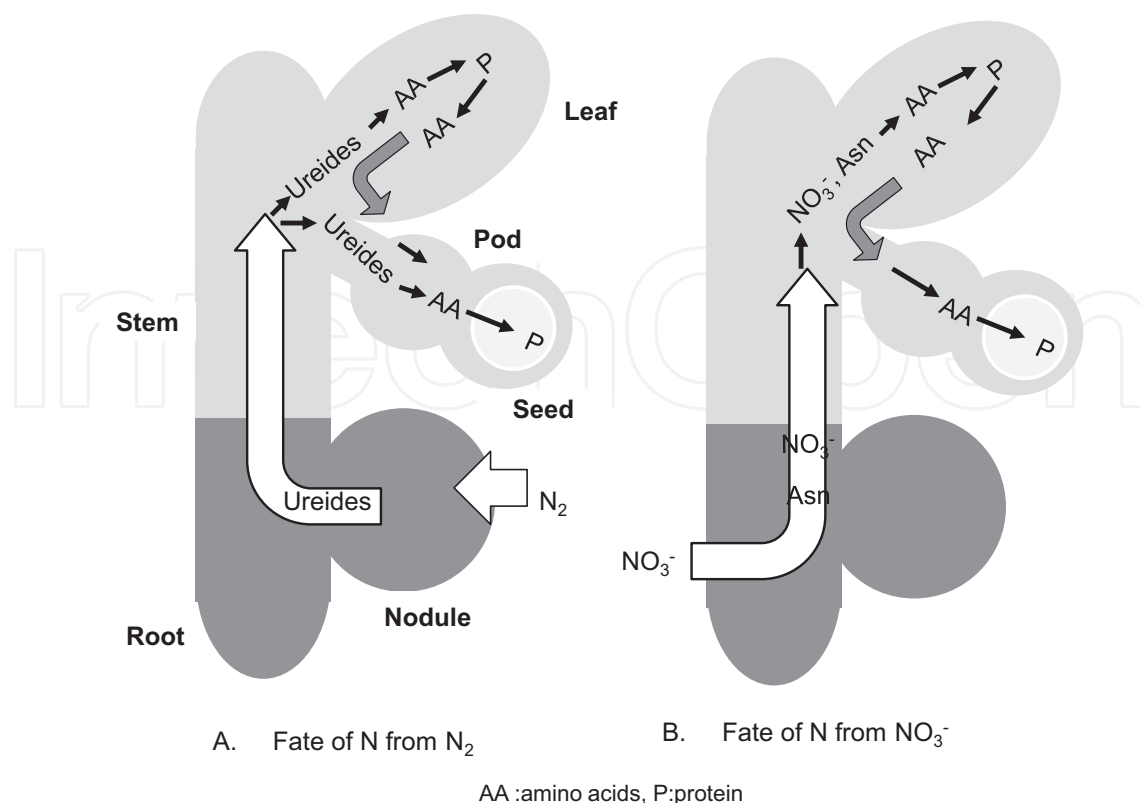


Figure 23. Model of the fate of N from N_2 fixation and NO_3^- absorption in soybean plants. (A) Fate of N from N_2 . (B) Fate of N from NO_3^- .

Author details

Takuji Ohyama^{1*}, Norikuni Ohtake¹, Kuni Sueyoshi¹, Yuki Ono¹, Kotaro Tsutsumi¹, Manabu Ueno¹, Sayuri Tanabata², Takashi Sato³ and Yoshihiko Takahashi¹

*Address all correspondence to: ohyama@agr.niigata-u.ac.jp

1 Faculty of Agriculture, Niigata University, Niigata, Japan

2 Agricultural Research Institute, Ibaraki University, Ibaraki, Japan

3 Faculty of Bioresource Sciences, Akita Prefectural University, Akita, Japan

References

- [1] Sato T, Ohtake N, Ohyama T, Ishioka NS, Watanabe S, Osa A, Sekine T, Uchida H, Tsuji A, Matsubashi S, Ito T, Kume T. Analysis of nitrate absorption and transport in non-nodulated and nodulated soybean plants with $^{13}NO_3^-$ and $^{15}NO_3^-$. *Radioisotopes*. 1999;**48**:450–458

- [2] Fujikake H, Yamazaki A, Ohtake N, Sueyoshi K, Matsushashi S, Ito T, Mizuniwa C, Kume T, Hashimoto S, Ishioka NS, Watanabe S, Osa A, Sekine T, Uchida H, Tsuji A, Ohyama T. Quick and reversible inhibition of soybean root nodule growth by nitrate involves a decrease in sucrose supply to nodules. *Journal of Experimental Botany*. 2003;**54**:1379–1388
- [3] Tempest DW, Meers JL, Brown CM. Synthesis of glutamate in *Aerobacter aerogenes* by hitherto unknown route. *Biochemical Journal*. 1970;**117**:405–407
- [4] Kumazawa K, Yoneyama T, Arima Y, Muhammad SS. Assimilation of nitrogen by rice plant as revealed with ^{15}N . *Proceedings of the Japan Academy Series B*. 1987;**63**:219–222
- [5] Yoneyama T, Kumazawa K. A kinetic study of the assimilation of ^{15}N -labelled ammonium in rice seedling roots. *Plant & Cell Physiology*. 1974;**15**:655–661
- [6] Yoneyama T, Kumazawa K. A kinetic study of the assimilation of ^{15}N -labelled nitrate in rice seedling roots. *Plant & Cell Physiology*. 1975;**16**:21–26
- [7] Arima Y, Kumazawa K. Evidence of ammonium assimilation via glutamine synthetase-glutamate synthase system in rice seedling roots. *Plant & Cell Physiology*. 1977;**18**:1121–1129
- [8] Muhammad S, Kumazawa K. Assimilation and transport of nitrogen in rice. I. ^{15}N -labelled ammonium nitrogen. *Plant & Cell Physiology*. 1974;**15**:747–758
- [9] Rosenthal GA. *Plant Nonprotein Amino and Imino Acids: Biological, Biochemical, and Toxicological Properties*. New York: Academic Press; 1982. pp. 1–273
- [10] Done J, Fowden L. A new amino-acid amide in the groundnut plant (*Arachis hypogea*): Evidence of the occurrence of γ -methylene glutamine and γ -methylene glutamic acid. *Biochemical Journal*. 1952;**51**:451–458
- [11] Zacharius RM, Polland JK, Steward FC. γ -methylene glutamine and γ -methylene glutamic acid in the tulip (*Tulipa gesneriana*). *Journal of the American Chemical Society*. 1954;**76**:1961–1962
- [12] Ohyama T, Kera T, Ikarashi T. Occurrence of 4-methyleneglutamine and 2-oxo-4-methyl-3-pentene-1,5-dioic acid in leaves and stem of tulip plants. *Soil Science and Plant Nutrition*. 1988;**34**:613–620
- [13] Ohyama T. Comparative studies on the distribution of nitrogen in soybean plants supplied with N_2 and NO_3^- at the pod filling stage. II. Assimilation and transport of nitrogenous constituents. *Soil Science and Plant Nutrition*. 1984;**30**:219–229
- [14] Lea PJ, Mifflin BJ. Transport and metabolism of asparagine and other nitrogen compounds within the plants. In: *The Biochemistry of Plants*. Vol. 5. Academic Press; San Diego, California, USA. 1980. pp. 569–607
- [15] Ohyama T, Minagawa R, Ishikawa S, Yamamoto M, Hung NVP, Ohtake N, Sueyoshi K, Sato T, Nagumo Y, Takahashi Y. Soybean seed production and nitrogen nutrition. In: El-Shemy HA, editor. *Soybean Physiology and Biochemistry*. Rieka, Croatia: InTech; 2011

- [16] Ohyama T, Kumazawa K. Incorporation of ^{15}N into various nitrogenous compounds in intact soybean nodules after exposure to $^{15}\text{N}_2$ gas. *Soil Science and Plant Nutrition*. 1978;**24**:525–533
- [17] Ohyama T, Kumazawa K. Nitrogen assimilation in soybean nodules I. The role of GS/GOGAT system in the assimilation of ammonia produced by N_2 fixation. *Soil Science and Plant Nutrition*. 1980;**26**:109–115
- [18] Ohyama T, Kumazawa K. Nitrogen assimilation in soybean nodules II. $^{15}\text{N}_2$ assimilation in bacteroid and cytosol fractions of soybean nodules. *Soil Science and Plant Nutrition*. 1980;**26**:205–213
- [19] Ohyama T, Kumazawa K. Assimilation and transport of nitrogenous compounds originated from $^{15}\text{N}_2$ fixation and $^{15}\text{NO}_3^-$ absorption. *Soil Science and Plant Nutrition*. 1979;**25**:9–19
- [20] Mizukoshi K, Nishiwaki T, Ohtake N, Minagawa R, Ikarashi T, Ohyama T. Nitrate transport pathway into soybean nodules traced by tungstate and $^{15}\text{NO}_3^-$. *Soil Science and Plant Nutrition*. 1995;**41**:75–88
- [21] Sueyoshi K, Ishikawa S, Ishibashi H, Abdel-Latif S. Nitrate transport in barley. In *Nitrogen Assimilation in Plants*. Research Signpost; Kerala, India. 2010. pp. 67–81
- [22] Ohyama T, Kato N, Saito K. Nitrogen transport in xylem of soybean plant supplied with $^{15}\text{NO}_3^-$. *Soil Science and Plant Nutrition*. 1989;**35**:131–137
- [23] Ohyama T, Saito K, Kato N. Assimilation and transport of nitrate, nitrite, and ammonia absorbed by nodulated soybean plants. *Soil Science and Plant Nutrition*. 1989;**35**:9–20
- [24] Ohya T, Tanoi K, Hamada Y, Okabe H, Rai H, Hojo J, Suzuki K, Nakanishi TM. An analysis of long-distance water transport in the soybean stem using H_2^{15}O . *Plant & Cell Physiology*. 2008;**49**(5):718–729
- [25] Sakazume T, Tanaka K, Aida H, Ishikawa S, Nagumo Y, Takahashi Y, Ohtake N, Sueyoshi K, Ohyama T. Estimation of nitrogen fixation rate of soybean (*Glycine max* (L.) Merr.) by micro-scale relative ureide analysis using root bleeding xylem sap and apoplast fluid in stem. *Bulletin of the Faculty of Agriculture, Niigata University*. 2014;**67**:27–41
- [26] Ohtake N, Nishiwaki T, Mizukoshi K, Minagawa R, Takahashi Y, Chinushi T, Ohyama T. Amino acid composition in xylem sap of soybean related to the evaluation of N_2 fixation by the relative ureide method. *Soil Science and Plant Nutrition*. 1995;**41**:95–102
- [27] Herridge DF, Peoples MB. Ureide assay for measuring nitrogen fixation by nodulated soybean calibrated by ^{15}N methods. *Plant Physiology*. 1990;**93**:495–503
- [28] Takahashi Y, Chinushi T, Nakano T, Ohyama T. Evaluation of N_2 fixation activity and N absorption activity by relative ureide method in field-grown soybean plants with deep placement of coated urea. *Soil Science and Plant Nutrition*. 1992;**38**:699–708
- [29] Takahashi Y, Chinushi T, Ohyama T. Quantitative estimation of N_2 fixation activity and N absorption rate in field grown soybean plants by relative ureide method. *Bulletin of the Faculty of Agriculture, Niigata University*. 1993;**45**:91–105

- [30] Lersten NR, Carlson JB. Vegetative morphology. In: Wilcox JR, editor. Soybeans: Improvement, Production, and Uses. 2nd ed. Madison, Wisconsin, USA: ASA, CSSA, SSSA publishers; 1987
- [31] Carlson JB, Lersten NR. Reproductive morphology. In: Wilcox JR, editor. Soybeans: Improvement, Production, and Uses. 2nd ed. Madison, Wisconsin, USA: ASA, CSSA, SSSA publishers; 1987
- [32] Ohyama T, Kawai S. Nitrogen assimilation and transport in soybean leaves: Investigation by petiole girdling treatment. Soil Science and Plant Nutrition. 1983;**29**:227–231
- [33] Pate JS, Sharkey PJ, Lewis OAM. Phloem bleeding from legume fruits-A technique for study of fruit nutrition. Planta. 1974;**120**:229–243
- [34] Ohtake N, Suzuki M, Takahashi Y, Fujiwara T, Chino M, Ikarashi T, Ohyama T. Differential expression of β -conglycinin genes in nodulated and non-nodulated isolines of soybean. Physiology Plant. 1996;**96**:101–110
- [35] Ohtake N, Ikarashi Y, Ikarashi T, Ohyama T. Distribution of mineral elements and cell morphology in nodulated and non-nodulated soybean seeds. Bulletin of the Faculty of Agriculture, Niigata University. 1997;**49**:93–101
- [36] Ohyama T, Ueno M, Ono Y, Ohtake N, Sueyoshi K, Sato T, Tanabata S. Recycling of nitrogen from shoots to underground parts in hypernodulation mutant lines of soybean by split-root experiment. Bulletin of the Faculty of Agriculture, Niigata University. (in press) 2017.