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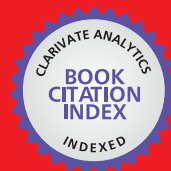
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Role of Nitrogen on Growth and Seed Yield of Soybean and a New Fertilization Technique to Promote Nitrogen Fixation and Seed Yield

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Additional information is available at the end of the chapter

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Abstract

Soybean is an important crop for human food and feed for livestock. World soybean production is increasing especially in North and South America. Soybean seeds contain a high percentage of protein about 35–40%, and they require a large amount of nitrogen compared with other crops. Soybean plants make root nodules with rhizobia, and rhizobia can fix atmospheric N₂ and give the fixed N to the host soybean plants. Also, soybean can absorb nitrogen usually nitrate from soil or fertilizers. The amount of total assimilated nitrogen in shoot is proportional to the soybean seed yield either from nitrogen fixation or from nitrogen absorption, and the nitrogen availability is very important for soybean cultivation. Maintenance of a high and long-term nitrogen fixation activity is very important for a high production of soybean. However, application of chemical nitrogen fertilizers usually depresses nodule formation and nitrogen fixation. Nitrate in direct contact with a nodule part of roots causes severe inhibition of nodule growth and nitrogen fixation, although a distant part of nodules from nitrate application gives no or little effect. Deep placement of slow-release nitrogen fertilizers, coated urea, or lime nitrogen promoted the growth and seed yield and quality of soybean without depressing nitrogen fixation.

Keywords: nitrogen fixation, soybean, deep placement, coated urea, lime nitrogen

1. Introduction

1.1. World soybean production and seed yield

Soybean (*Glycine max* (L.) Merr.) is originated in East Asia, but now it is widely cultivated in tropical, subtropical, and temperate climatic regions with an optimum mean temperature of 20–30°C. Soybean seed is one of the most important protein sources for human and livestock all over the world. In addition, soybean is a major oilseed crop in the world providing 58% of world oil seed production [1]. Soybean is cultivated over a wide range of latitudes, from the equator to high latitudes of at least 50° N [2], although each cultivar adapts to the narrow range of latitudes. Soybean is a short-day plant, and flowering is induced when the day length is shorter than a critical length. This sensitivity to photoperiod is weak or absent in soybean cultivars adapted to high latitudes, which should initiate flowering in early summer to mature in frost-free seasons [2].

Annual production of soybean is 276 M t (million tons) in 2013, and be the fourth of the major grain crops, after maize (1017 M t), paddy rice (746 M t), and wheat (713 M t) [3]. The soybean cultivation area is about 111 M ha in 2013. Recently, world soybean production has been increasing (79 M t in 1983, 115 M t in 1993, 191 M t in 2003, and 276 M t in 2013) [3]. The main soybean production countries (annual production in 2013) are the USA (89.5 M t), Brazil (81.7 M t), Argentina (49.3 M t), China (12.5 M t), and India (12.0 M t). Soybean production in Japan was only 200,000 t and it accounted for 8% of the total consumption in Japan. The annual production of soybean in the USA increased about twice during recent 30 years, and it was 5.6 times and 12.3 times in Brazil and Argentina (**Figures 1 and 2**). The annual production

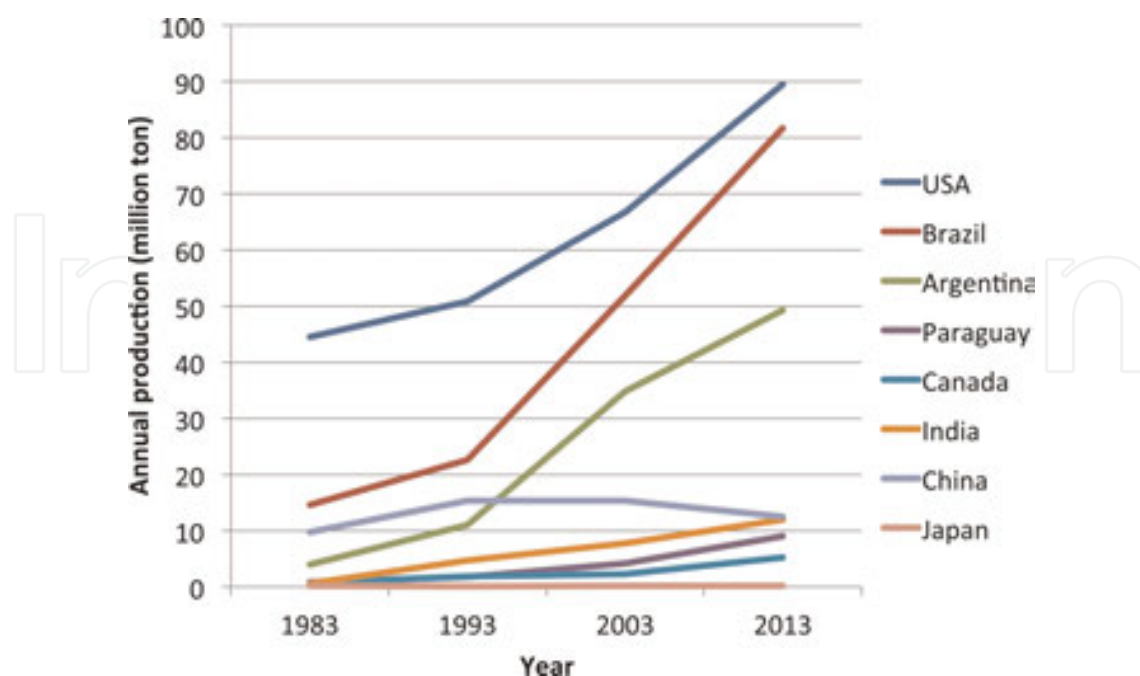


Figure 1. Changes in annual production of soybean in each country from 1983 to 2013. (Data from FAOSTAT (FAO Statistical Database (United Nations))).

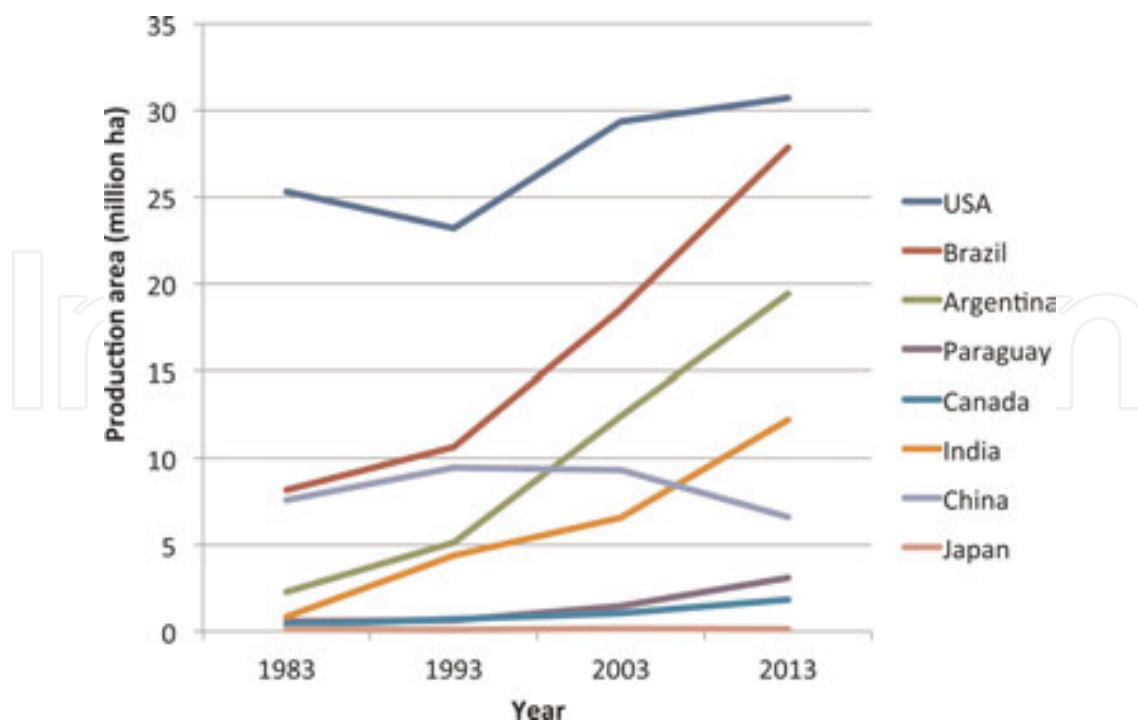


Figure 2. Changes in the production area of soybean in each country from 1983 to 2013. (Data from FAOSTAT).

| | Yield in 2013 (ton ha ⁻¹) | Yield in 1983 (ton ha ⁻¹) | Ratio (2013/1983) | Yield increase (kg ha ⁻¹ year ⁻¹) |
|-------------|---------------------------------------|---------------------------------------|-------------------|--|
| USA | 2.91 | 1.76 | 1.65 | 38 |
| Brazil | 2.93 | 1.79 | 1.64 | 38 |
| Argentina | 2.54 | 1.75 | 1.45 | 26 |
| Paraguay | 2.95 | 1.47 | 2.01 | 49 |
| Canada | 2.86 | 2.02 | 1.42 | 28 |
| India | 0.98 | 0.73 | 1.34 | 8 |
| China | 1.89 | 1.29 | 1.47 | 20 |
| Japan | 1.55 | 1.51 | 1.03 | 1 |
| World total | 2.48 | 1.62 | 1.53 | 29 |

Data from FAOSTAT.

Table 1. Changes in soybean seed yield from 1983 to 2013.

and production area in India increased during the recent 30 years, but these decreased from 2003 to 2013 in China. The world average seed yield is 2.48 t ha⁻¹ in 2013, and high in the USA (2.91 t ha⁻¹), Brazil (2.93 t ha⁻¹), Argentina (2.54 t ha⁻¹), Paraguay (2.95 t ha⁻¹), and Canada (2.86 t ha⁻¹) compared with China (1.89 t ha⁻¹), Japan (1.55 t ha⁻¹), and India (0.98 t ha⁻¹) (Table 1). The average annual increase of seed yield from 1983 to 2013 was high in the USA

(38 kg ha⁻¹ year⁻¹), Brazil (38 kg ha⁻¹ year⁻¹), Argentina (26 kg ha⁻¹ year⁻¹), Paraguay (49 kg ha⁻¹ year⁻¹), and Canada (28 kg ha⁻¹ year⁻¹) compared with China (20 kg ha⁻¹ year⁻¹), India (8 kg ha⁻¹ year⁻¹), and Japan (1 kg ha⁻¹ year⁻¹) (**Table 1**). Board and Kahlon (2011) [4] suggested that recent yield gains in the USA are 50% due to cultivar genetic improvement and 50% to improve cultural practices. Potential gains from improved cultural practices for any given locate are usually determined by comparing farmer yields with those done using recommended practice [4]. In the USA, the improvement of cultural practices can be expected to increase yield anywhere from 25 to 66% [4]. They suggested that the yield increase for Asian countries would be even greater, since their yield levels are substantially low due to many biotic and abiotic stresses [4].

1.2. Soybean seed yield potential

Soybean seed yield can be obtained at 4–6 t ha⁻¹ with well-managed fields under good climate and soil conditions [5]. Recently, a high yield over 10 t ha⁻¹ was recorded in 2008 and 2010 by an innovative farmer Mr. Kip Cullers in Missouri, the USA [6]. This fact indicates that the potential soybean productivity is much higher than we thought. Soybean yield potential has been defined as the maximum yield of a crop cultivar grown in an environment to which it is adapted, with nutrients and water non-limiting, and pests and diseases effectively controlled [7, 8]. In the USA corn-belt region, soybean yield potential has been estimated to be in the range of 6–8 t ha⁻¹ [7, 9, 10].

Table 2 shows the composition of water, macronutrients, and mineral elements in seeds of soybeans, beans, rice, wheat, and maize [11]. The nutrient composition of soybean seeds (per 100 g) produced in Japan is as follows [11]: water 12.5 g, protein 35.3 g,

| | Soybean | Bean | Rice | Wheat | Maize |
|--------------------------|---------|------|------|-------|-------|
| Macronutrients (g/100 g) | | | | | |
| Water | 12.5 | 16.5 | 15.5 | 12.5 | 14.5 |
| Protein | 35.3 | 19.9 | 6.8 | 10.6 | 8.6 |
| Lipid | 19 | 2.2 | 2.7 | 3.1 | 5 |
| Carbohydrate | 28.2 | 57.8 | 73.8 | 72.2 | 70.6 |
| Ash | 5 | 3.6 | 1.2 | 1.6 | 1.3 |
| Minerals (mg/100 g) | | | | | |
| Potassium | 1900 | 1500 | 230 | 470 | 290 |
| Phosphorous | 580 | 400 | 290 | 350 | 270 |
| Calcium | 240 | 130 | 9 | 26 | 5 |
| Magnesium | 220 | 150 | 110 | 80 | 75 |
| Iron | 9.4 | 6 | 21 | 3.2 | 1.9 |

Data from 2009 All Guide Standard Tables of Food Composition in Japan [11].

Table 2. Comparison of concentration of nutrients in various crop seeds.

lipids 19.0 g, carbohydrates 28.2 g, and minerals 5 g. The composition is quite different from the other grain crop seeds such as rice, wheat, and maize. The protein concentration in soybean seeds is about four to five times higher than that of rice, wheat, and maize, but carbohydrate concentration is lower. Bean seeds contain less protein (19.9%) and much less lipid (2.2%) than those in soybean seeds. Mineral contents in soybean seeds are also much higher than those in rice, wheat, and maize, especially potassium and calcium.

Sinclair and De Wit reported that different compositions of macronutrients in crop seeds affect biomass productivity of seeds (g of seed per g of photosynthate) [12], and the bioproductivity of soybean seed is 0.5 and rice grain is 0.75. This means 4 t of soybean seed yield is energetically equivalent to 6 t of rice grain production, which is almost the average of Japanese rice production (6.73 t ha⁻¹ in 2013). It was also reported that 1 g of carbohydrate, protein, and lipid requires 1.21, 2.48, and 3.03 g of glucose, respectively [13]. When these values are applied to the compositions of various seeds in **Table 2**, 1.79, 1.26, 1.14, 1.23, and 1.22 g of glucose are theoretically required for the production of 1 g of soybean, bean, rice, wheat, and maize seeds, respectively. Based on this calculation, 1 g of soybean seeds needs 57% more glucose, as compared with 1 g of rice grain. Therefore, over 4 t ha⁻¹ of soybean yield can be expected by the good agricultural practices under good soil and climatic conditions, although the average soybean yields remain at 1.5 t ha⁻¹ in Japan.

1.3. Nutrient acquisition by soybean plants

Figure 3 shows the fundamental physiological processes of nutrient acquisition by soybean plants related to the growth and seed yield. In order to achieve high-yield potential, soybean must sustain high photosynthesis rates and accumulate large amounts of N.

Soybean seeds are large and store nutrients in the cotyledons to support the initial growth of about 7–10 days after planting. Soybean development is separated into the vegetative development period and reproductive development period at the initial flowering stage, and the developmental stages of soybean are expressed by the descriptions by Fehr and Caviness [14]. In the case of soybean, the vegetative growth of leaves, stems, and nodes overlaps with the reproductive growth until seed initiation (R5) stage. The period of vegetative growth and reproductive growth varies by cultivars and cultivated sites, but the vegetative growth period is about 2 months and reproductive development period is about 3 months in typical Japanese soybean cultivation.

For high soybean seed yield, both the optimum vegetative growth and reproductive growth are necessary. Photosynthesis by leaves and sufficient but not excess water and nutrients absorption from roots are very important to support vigorous plant growth (**Figure 3**). In addition, soybean can fix atmospheric dinitrogen (N₂) by root nodules, which are symbiotic organ with soil bacteria called rhizobia.

Ohlorogge and Kamprath [15] calculated nutrient requirement of high-yielding soybeans, which have 8.96 t ha⁻¹ total dry matter including 3.36 t ha⁻¹ of grain and 5.60 t ha⁻¹ of vegetative parts (**Table 3**). For 1 kg of soybean seed production, about 1024 g of C, 963 g of O, and 131 g of H are required through photosynthesis from CO₂ in the air and H₂O from soil. Of the soil-derived nutrients, N, K, Ca, Mg, P, and S are required about 93, 32, 23, 10, 9, and 7 g,

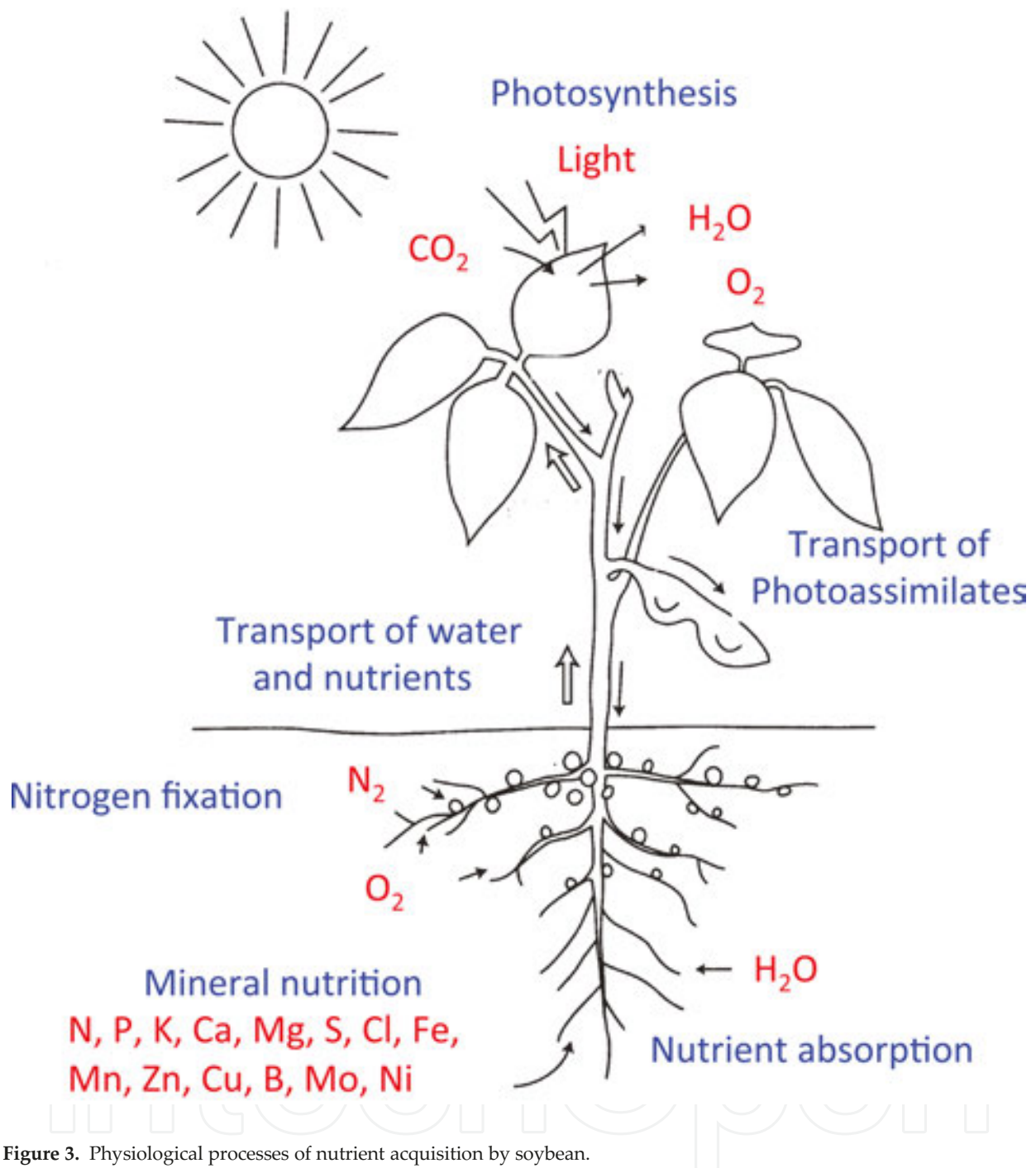


Figure 3. Physiological processes of nutrient acquisition by soybean.

respectively. Although the amounts of requirement for micronutrients, Cl, Fe, Mn, Zn, Cu, B, and Mo, are very low, these are essential for soybean growth.

1.4. Biotic and abiotic constraints to reduce soybean growth and seed yield

Among the factors inherent in agricultural production, the climatic conditions are the most difficult to control and they are of greater limiting factors in the maximum yield [16]. Abiotic stresses such as drought, excessive rain, extreme temperature, and low light can significantly

| Element | Symbol | g kg ⁻¹ of seed DW | g kg ⁻¹ of seed* |
|-------------|--------|-------------------------------|-----------------------------|
| Carbon | C | 1170 | 1024 |
| Oxygen | O | 1100 | 963 |
| Hydrogen | H | 150 | 131 |
| Nitrogen | N | 107 | 93 |
| Potassium | K | 37 | 32 |
| Calcium | Ca | 27 | 23 |
| Magnesium | Mg | 12 | 10 |
| Phosphorous | P | 10 | 9 |
| Sulfur | S | 8.3 | 7.3 |
| Chlorine | Cl | 3.3 | 2.9 |
| Iron | Fe | 0.57 | 0.496 |
| Manganese | Mn | 0.20 | 0.175 |
| Zinc | Zn | 0.07 | 0.058 |
| Copper | Cu | 0.03 | 0.029 |
| Boron | B | 0.03 | 0.029 |
| Molybdenum | Mo | 0.00 | 0.003 |
| Cobalt | Co | 0.00 | 0.001 |

Data are calculated from Ohlrogge and Kamprath [15].

The amount of nutrient requirement is in total plant dry matter including grains, stems, leaves, and roots.

*Values are calculated using seed water content of 12.5%.

Table 3. Requirement of nutrients for soybean seed production.

reduce yields of crops [16]. Next to the climatic factors, adverse soil conditions are major constraints for soybean production. In Japan, soybeans are cultivated mainly in rotated paddy rice fields and the bad drainage of water restricts nitrogen fixation and root growth and results in low productivity of soybean. In these fields, it is recommended to make open-channel drainage and under-drainage to accelerate the water drainage after a heavy rain. In addition to such physical conditions of soil, chemical conditions such as pH, contents of nutrients, and the availabilities limit soybean yield. Soil fertility is also important to support soybean growth. Among biotic constraints, weeds, insects, and diseases are serious problems to reduce plant growth and seed productivity.

2. Role of nitrogen on growth and seed yield and quality of soybean

2.1. Nitrogen requirement for seed production

A high yield of soybean requires a large amount of N, and soybean plants should continue to assimilate nitrogen during both vegetative and reproductive stages. Many field data showed that the total amount of N assimilated in a plant shoot is highly correlated with

the soybean seed yield. In **Figure 4**, the relationship between total amount of N in soybean shoot at R7 stage and seed yield is shown in a rotated paddy field in Niigata [17–21]. A linear correlation ($r = 0.855$) between seed yield and the amount of nitrogen accumulation in the shoot was observed. Salvagiotti et al. [7] reviewed the relationships among seed yield, nitrogen uptake, nitrogen fixation, and nitrogen fertilizer based on 637 published data sets [7]. A mean increase of 13-kg soybean seed yield per kg N increase in above-ground part was obtained from these data, which is equivalent to the 77 kg N required for 1 ton of seed production.

Rice crops assimilate about 80% of N until flowering, while soybean plants assimilate only about 20% of total N until initial flowering stage (R1 stage) (**Figure 5**). Therefore, continuous assimilation of nitrogen after initial flowering stage is essential for good growth and high seed yield of soybean plants.

To obtain high seed yield of soybean, good nodulation and high and long-lasting nitrogen fixation activity are very important, because the availability of soil N is generally insufficient to support soybean growth and seed N and chemical starter fertilizer N is lost in a few weeks after planting (**Figure 6**). Soybean nodule formation and nodule growth are influenced by various soil conditions such as water content, pH, nutrition, and climatic conditions such as solar radiation, temperature, rainfall, and so on. Soybean forms root nodules associated with soil bacteria, bradyrhizobia, and can fix atmospheric N_2 . Soybean can absorb and utilize inorganic nitrogen such as nitrate and ammonia from soil or fertilizer. Usually, a high yield of soybean has been obtained in a field with high soil fertility or with the application of organic manure. Supply of low and constant concentration of nitrogen from soil or organic manure

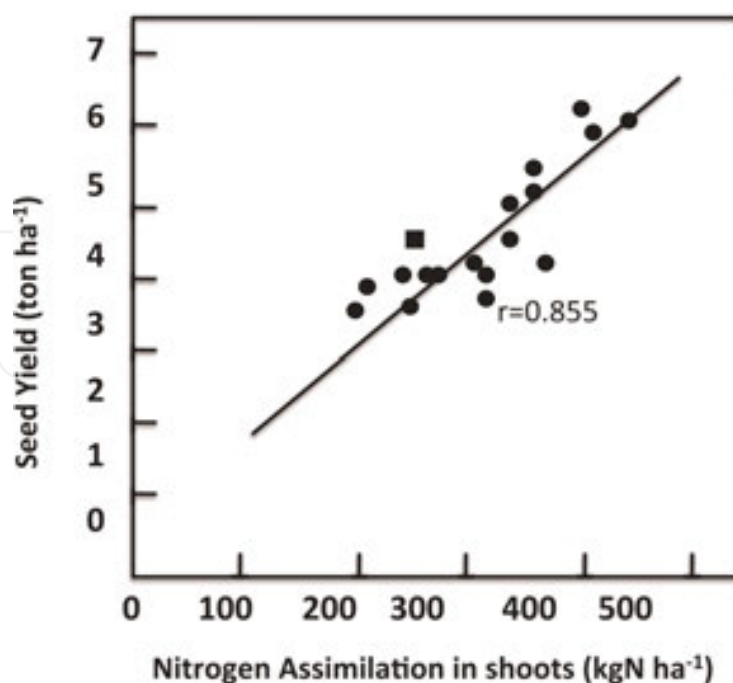


Figure 4. Relationship between the total amount of nitrogen assimilation and seed yield in soybean shoot at R7 stage (Takahashi et al. [5]).

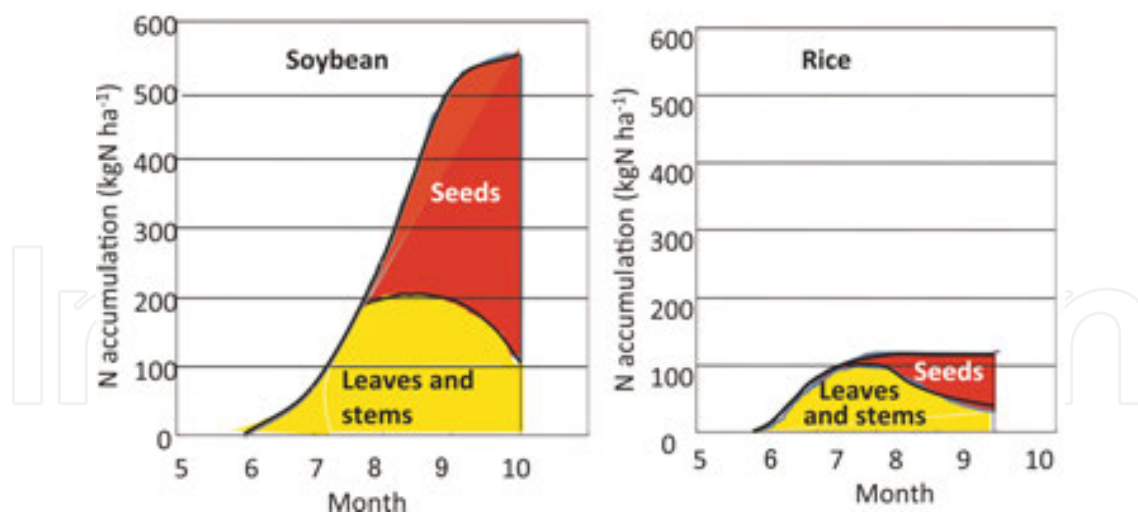


Figure 5. Comparison of N accumulation in soybean and rice plant during vegetative and reproductive stages (Ohyama et al. [38]).

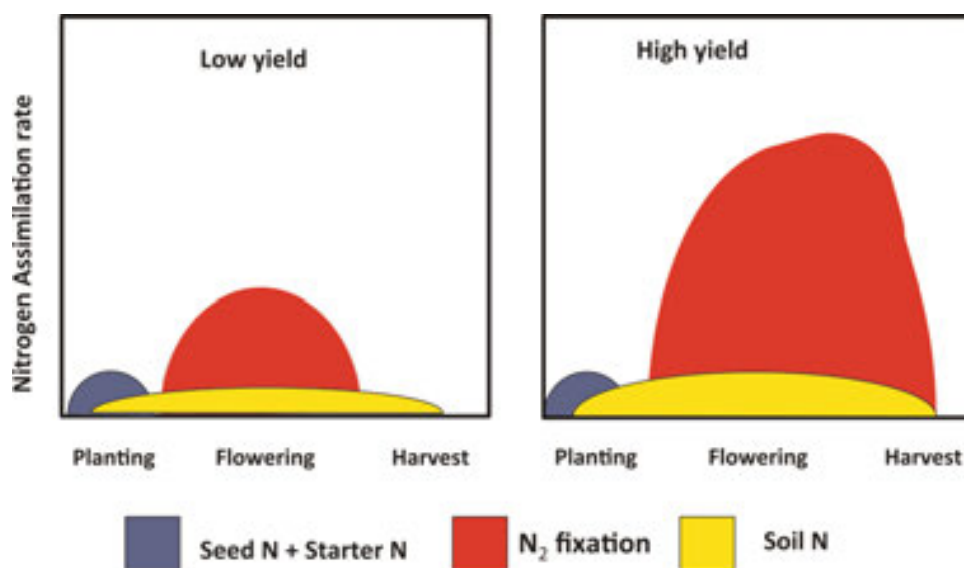


Figure 6. Comparison of the nitrogen assimilation patterns coming from seed N + starter N, soil N, and N₂ fixation between low-yield and high-yield model [38].

may support the soybean growth without depressing nodulation and nitrogen fixation activity. Nevertheless, a high concentration of mineral N depresses nodule formation and nitrogen fixation activity, especially nitrate, the most abundant inorganic nitrogen in upland fields, severely inhibits nodulation and nitrogen fixation [22–24].

Soybean plants assimilate the N from three sources, N derived from symbiotic N₂ fixation by root nodules (Ndfa), N absorbed from soil mineralized N (Ndfs), and N derived from fertilizer when applied (Ndff) (**Figure 7**). One ton of soybean seed requires about 70–90-kg N assimilation, which is about four times more than in the case of rice [25]. It is necessary to use both N₂ fixation by root nodules and absorbed N from roots for the maximum seed yield of soybean [26, 27]. Sole

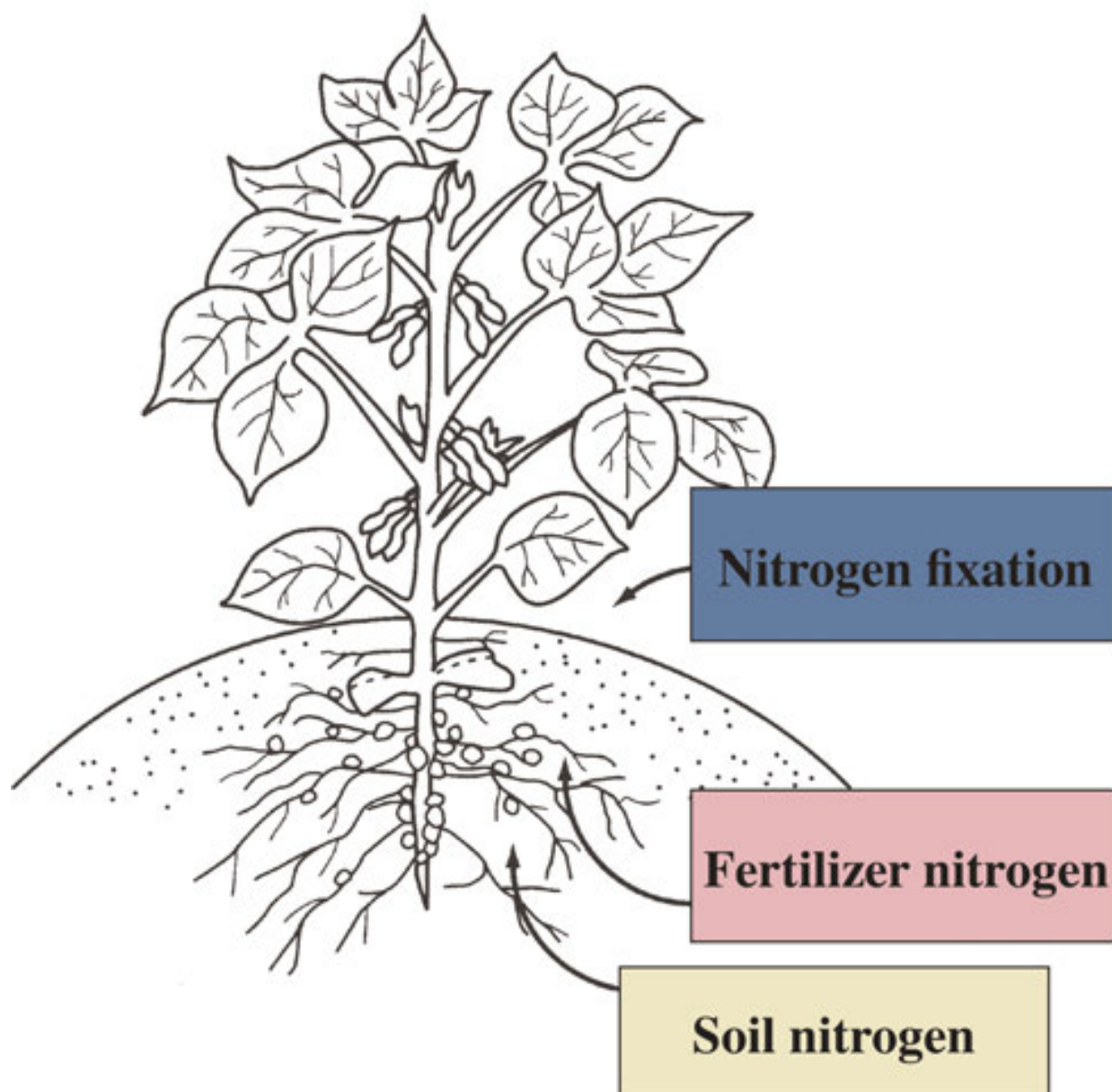


Figure 7. Three sources of N assimilated in soybean plants.

N_2 fixation is often insufficient to support vigorous vegetative growth, which results in the reduction of seed yield. On the other hand, a heavy supply of N often depresses nodule development and N_2 fixation activity, and accelerates nodule senescence, which also results in the reduction of seed yield. Moreover, a heavy supply of N from fertilizer or from soil causes the over-luxuriant growth of shoot, resulting in lodging and poor pod formation. Therefore, no nitrogen fertilizer is applied or only a small amount of N fertilizer is applied as a “starter N.”

Initial nodulation mainly occurs at the basal part of the main roots, but these nodules are broken down earlier, and many nodules are formed at the lateral roots during reproductive stage (**Figure 8**) [28].

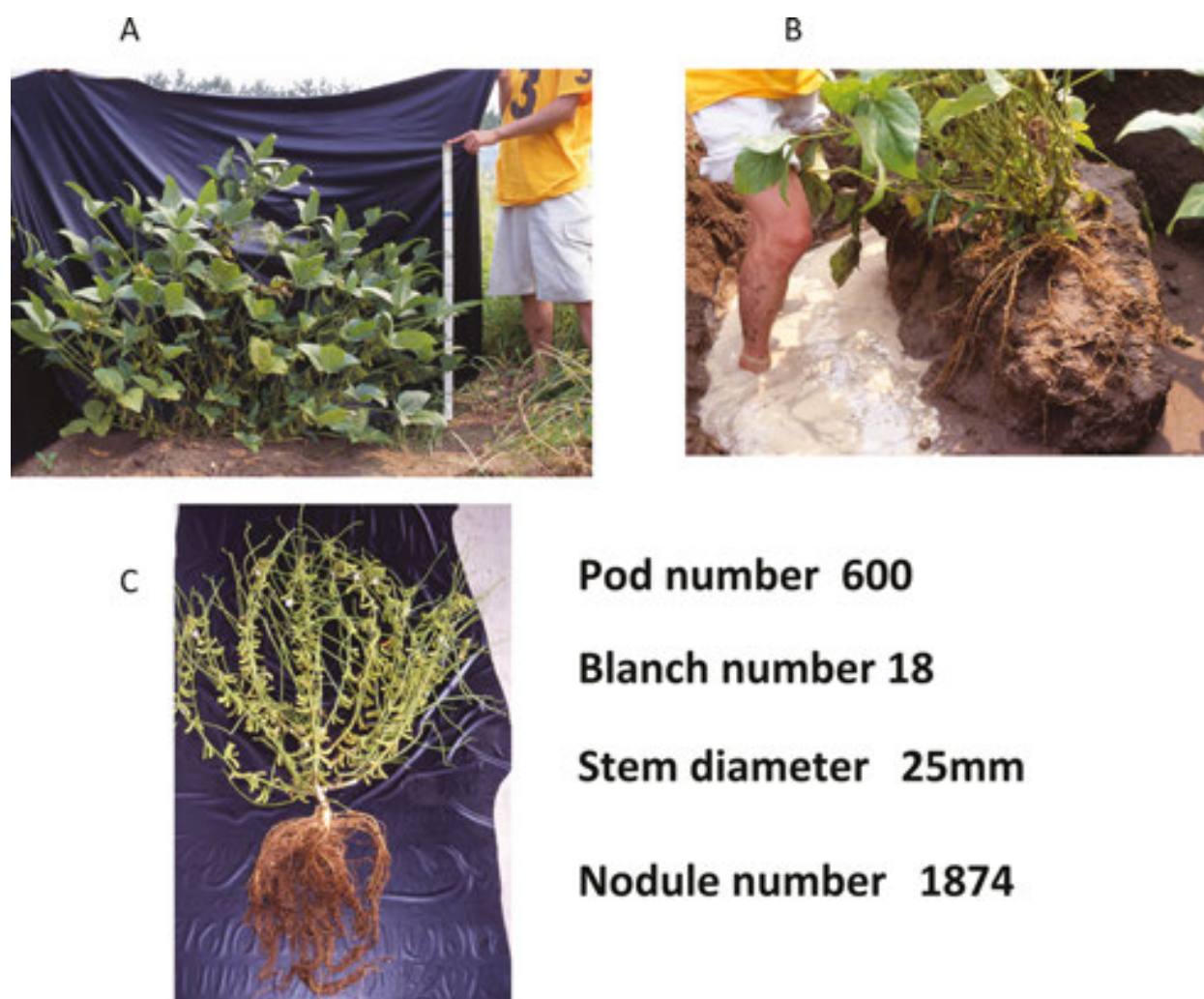


Figure 8. A giant soybean (cultivar Williams) plant cultivated at planting density of 2 plants m^{-2} (from Sukanuma et al. [28]). A: One big plant. B: Roots are recovered from soil. C: Whole body of giant soybean.

2.2. Field assessment of nitrogen fixation and nitrogen absorption by simple relative ureide method

There are several methods for field assessment of nitrogen fixation by nodules and nitrogen absorption by soybean roots, such as N balance method comparing nodulated and non-nodulated isolines, ^{15}N dilution method, ^{15}N natural abundance method, and relative ureide method [29]. Simple relative ureide method is the most convenient and reliable with a low-cost and inexpensive apparatus.

Kushizaki et al. discovered that nodulated soybean plants contain a large amount of ureides (allantoin and allantoic acid) in stems, while non-nodulating isolate contains much less amount of ureides [30]. The $^{15}N_2$ was exposed to the nodulated roots of soybean plants, and the initial assimilation of ^{15}N was investigated in cytosol (plant cytoplasm) and bacteroid (a symbiotic state of rhizobia in nodules) fractions [31–33]. The result suggested that most of the fixed ^{15}N is immediately exported from bacteroid to plant cytosol and initially assimilated into glutamine and glutamate via GS/GOGAT pathway in cytosol, then metabolized into

| Fertilizer application | Year | Cultivar | Stage | Method | %Ndfa | Reference |
|------------------------|------|-----------|-------|-----------------------|-------|-----------------------|
| Control | 1984 | T202 | Seed | $\delta^{15}\text{N}$ | 75 | Yoneyama et al. [40] |
| Control | 1990 | Enrei | R7 | RU | 74 | Takahashi et al. [19] |
| Control | 1990 | T202 | R7 | RU | 75 | Takahashi et al. [20] |
| Control | 1990 | T201/T202 | R7 | N-balance | 75 | Takahashi et al. [20] |
| Deep placement | 1990 | Enrei | R7 | RU | 64 | Takahashi et al. [19] |
| Deep placement | 1990 | T202 | R7 | RU | 59 | Takahashi et al. [20] |
| Deep placement | 1990 | T201/T202 | R7 | N-balance | 65 | Takahashi et al. [20] |
| Top dressing | 1990 | Enrei | R7 | RU | 65 | Takahashi et al. [19] |
| Top dressing | 1990 | T202 | R7 | RU | 60 | Takahashi et al. [20] |
| Top dressing | 1990 | T201/T202 | R7 | N-balance | 62 | Takahashi et al. [20] |

Data from Takahashi et al. [39].

$\delta^{15}\text{N}$: ^{15}N natural abundance method, RU: relative ureide method, N-balance: N-balance method using nodulating and non-nodulating soybean isolines.

Table 4. Estimated percentage of Ndfa in soybean cultivation in the rotated paddy field in Niigata Agricultural Experiment Station.

various amino acids via transamination from glutamate. Then, ureides, allantoin and allantoic acid, are synthesized from amino acids and amides in cytosol.

On the other hand, after adding $^{15}\text{NO}_3^-$ in the culture solution of hydroponic soybean, the ^{15}N concentration of asparagine increased markedly, indicating that asparagine is a major assimilatory compound of NO_3^- in soybean roots [34].

Many kinds of tropical grain legumes, such as soybean, common bean, cowpea, pigeon pea, and mung bean that have spherical determinate type of nodules, transport the bulk of fixed N as ureides (allantoin and allantoic acid). On the other hand, nitrate and amino acids (especially asparagine) are the major transport forms of N absorbed by soybean roots [34, 35]. Herridge et al. [36, 37] developed the “relative ureide method” for evaluating the % Ndfa (percentage of nitrogen derived from atmospheric dinitrogen) by analyzing the concentrations of nitrogen compounds in xylem sap obtained from bleeding sap of a cut stump or by vacuum collection from stems. The concentrations of ureide-N, nitrate-N, and α -amino-N can be determined by colorimetry [38].

This method is reliable in the field assessment of % Ndfa of soybeans, without any requirement of reference plants such as non-nodulated isolines. This method is also applicable for experiments with variable N fertilizer application (Table 4), and no preparation before sampling is necessary. The modified equation can be adopted for the estimation of % Ndfa in field experiment [38].

2.3. Quantitative estimation of daily N_2 fixation and N absorption rate by modification of xylem-sap sampling and colorimetric methods for relative ureide method

By periodical sampling of soybean shoots and xylem sap, a quantitative estimation of the seasonal changes in N_2 fixation activity and N absorption rate is possible [29, 39, 41]. Soybean

plants are harvested at R1, R3, R5, and R7 stages, or R1, R3, and R7 stages, and relative ureide-N in xylem sap and plant N is analyzed. **Figure 9** shows the example of the evaluation of Ndfa by simple relative ureide method [42].

McClure and Israel [43] analyzed the transport of nitrogen in the xylem sap of soybean plants, and proposed the percentage of ureide-N in xylem sap may be used as an indicator of the relative contribution of N₂ fixation to the total input of plant N. Herridge and Peoples [36] compared the ureide assay and ¹⁵N dilution method and proposed the calibration equations. Regarding the equations, we proposed an equation as $100 \times \text{ureide-N} / (\text{ureide-N} + \text{amide-N} + \text{nitrate-N})$, where amide-N is "2 × α-amino-N," because major form of amino acids and amides is asparagine which has two N atoms in a molecule [43–45]. When plants are periodically harvested, the nitrogen fixation activity and nitrate absorption rate can be estimated from the relative ureide percentage and total N increase in the shoots [19, 21, 29, 39, 41].

For relative ureide analysis, xylem-sap bleeding from the cut surface of the lower part of the main stem is collected in a tube (**Figure 10A**), and the concentrations of ureides, amides, and nitrates in sap are determined by an optical spectrometer. Sometimes, xylem sap cannot be collected in the tube, especially when soil is dry or during late growth stages. Herridge and Peoples [36] used a vacuum-extracted exudate from the lower part of the main stem of the cut shoot or hot-water extraction of the stems [36].

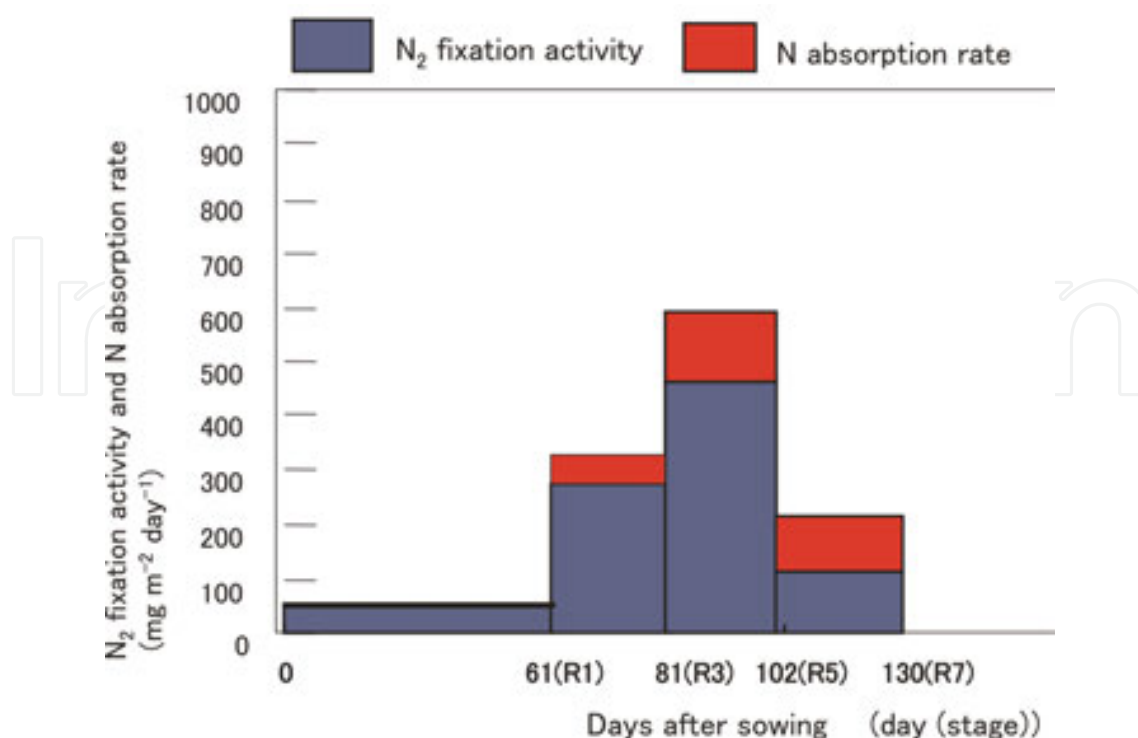


Figure 9. Changes in daily N₂ fixation activity and N absorption rate in soybean plants cultivated in Niigata with paper pot inoculation method (Tewari et al. [42]).

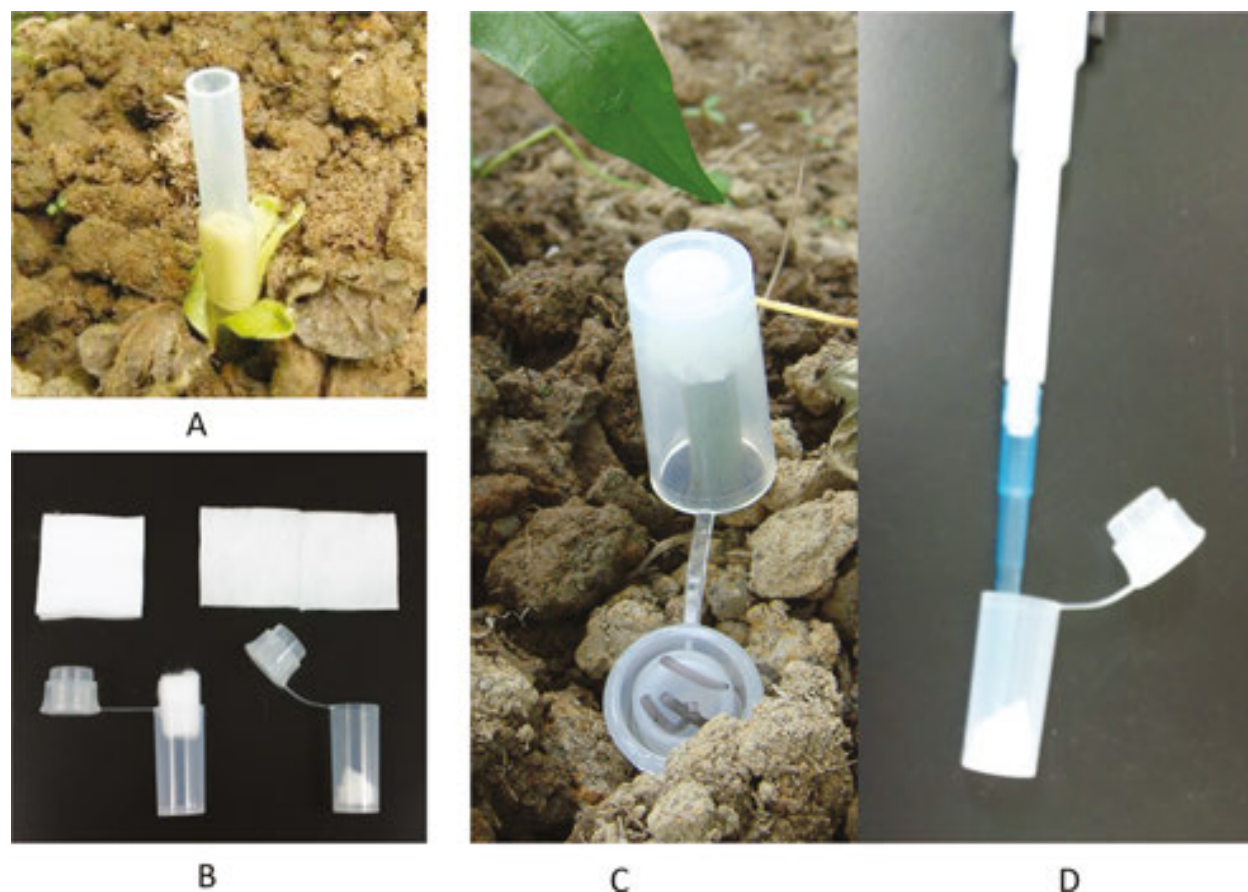


Figure 10. Collection of root-bleeding xylem sap (Sakazume et al. [46]). A: Xylem sap is collected in a tigon tube inserted to the woody part of the stem. B: Preparation of plastic cup with absorbent cotton. C: The plastic cup is put on a cut stem and xylem sap can be absorbed in cotton. D: The sap is recovered from cotton by sucking with an auto-pipette.

Recently, we collected the root-bleeding xylem sap as shown in **Figure 10C** [46]. The lower part of the main stem just below the node with cotyledons was cut by a pair of pruning shears. We used a tigon tube being inserted to the woody part of the stem (**Figure 10A**). Xylem sap started to exude at several minutes after cutting by a root pressure. However, the tube sometimes escaped from the stem or xylem sap leaked when the stem shape was irregular. Recently, we used a 6-mL plastic cup with absorbent cotton (**Figure 10B**). The plastic cup was put on the cut stem and xylem sap could be absorbed in the cotton (**Figure 10C**), and the sap was recovered by sucking with an auto-pipette (**Figure 10D**).

For each standard analysis of ureides, amides, and nitrate, 50 μL of sample solution was necessary for estimating relative ureide-N percentage. In addition, it was time-consuming to analyze each component by using glass test tubes. We developed a micro-scale analysis of these components in xylem sap using 2.5 μL of xylem sap instead of 50 μL for standard assay. The colorimetric reaction was carried out in a 1.5-mL Eppendorf centrifuge tube, and the 200 μL of reaction mixture was put into the well of a 96-well microplate, and the optical absorbance was measured by a microplate reader (**Figure 11**) [46].

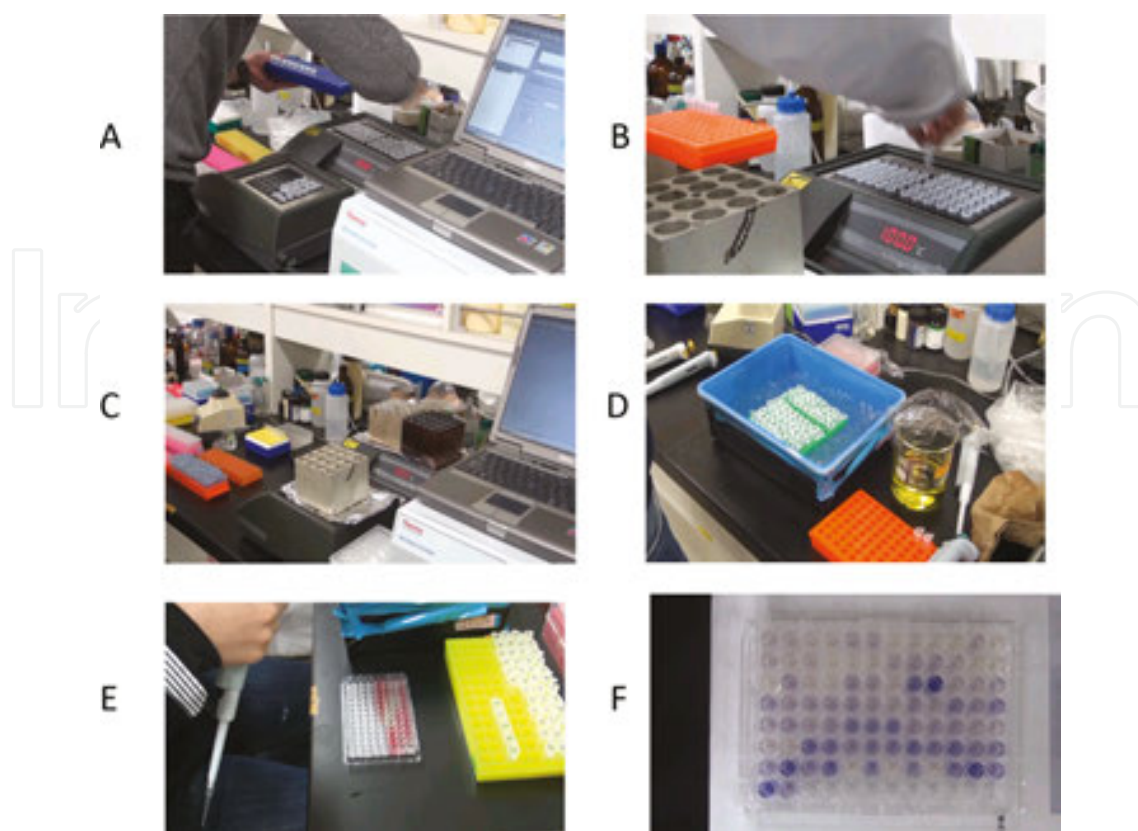


Figure 11. A micro-scale analysis of components in xylem sap of 2.5 μL (Sakazume et al. [46]). A–D: The colorimetric reaction was carried out in a 1.5-mL Eppendorf centrifuge tube. E, F: 200 μL of reaction mixture was put into the well of a 96-well microplate, and the optical absorbance was measured by a microplate reader.

3. Deep placement of slow-release nitrogen fertilizer promotes nitrogen fixation, and increases soybean seed yields

3.1. Deep placement of coated urea

Efficient fertilizer application is critical to crop production, economical benefit, and ecological advantages. The chemical formula, amount, size, as well as timing and placement of fertilizer affect the fertilizer use efficiency and consequently crop yield. For soybean cultivation in Japan, a basal dressing of compound fertilizers containing ammonium sulfate or urea at the rate of 20–40 kgN ha^{-1} is generally applied as “starter N.”

Takahashi et al. [17–21] investigated the effect of deep placement of coated urea (CU) to promote soybean seed yield in a rotated paddy field in Niigata, Japan. The soil is a fine-textured gray lowland soil: texture; CL (clay loam), $\text{pH}(\text{H}_2\text{O})$; 6.6, CEC (cation exchange capacity); 28.8 ($\text{cmol}(+) \text{kg}^{-1}$), total carbon content; 10.9 g kg^{-1} , total N content; 1.02 g kg^{-1} , amount of mineralized N determined by the incubation of air dry soil under upland conditions for 4 weeks at 30°C; 47 mg kg^{-1} . Field experiment was conducted with three fertilizer N treatments. Control was conventional basal dressing of 16 kgN ha^{-1} as ammonium sulfate in

surface layer about 0–10 cm supplemented with $60 \text{ kg P}_2\text{O}_5 \text{ ha}^{-1}$, $80 \text{ kg K}_2\text{O ha}^{-1}$, and $1000 \text{ kg Ca(OH)}_2 \text{ ha}^{-1}$. Deep placement was 100 kgN ha^{-1} using 100-day type of coated urea, and top dressing was 100 kgN ha^{-1} by using 70-day type of coated urea at initial flowering stage. The seeds of soybean were grown by single stem training ($75 \times 15 \text{ cm}$) at the planting density of $8.9 \text{ plants m}^{-2}$.

Hundred-day-type coated urea hyperbolically releases urea and 80% of which is released in 100 days in water at 25°C . The deep placement of nitrogen fertilizer was carried out using the fertilizer injector devised by Shioya (**Figures 12 and 13**). Hundred-day-type-coated urea was applied just under the seed placement lines at a depth of about 20 cm. Top dressing of 70-day

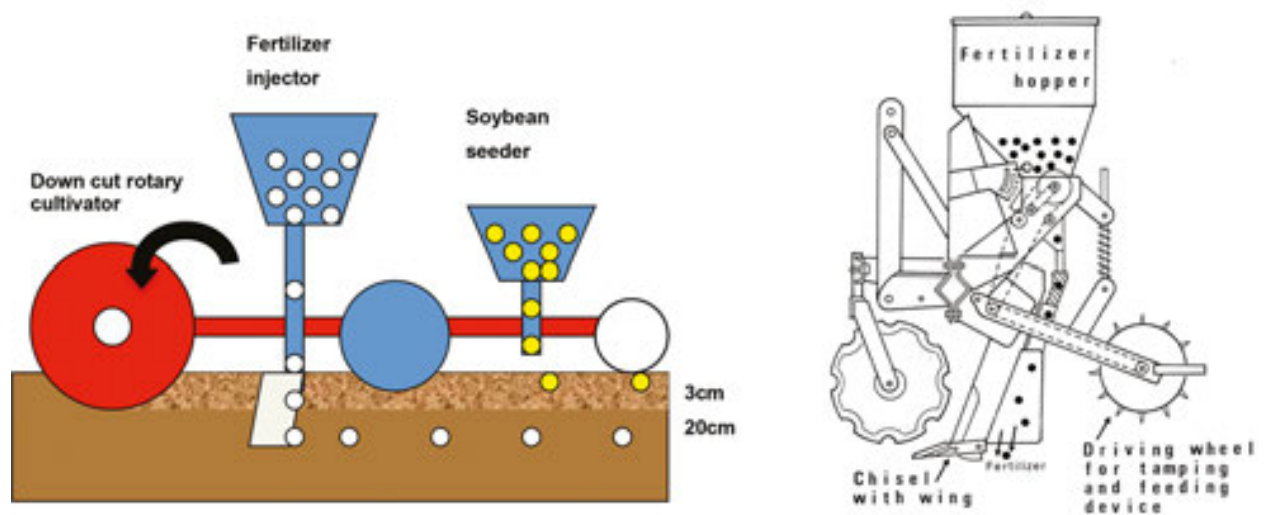


Figure 12. Fertilizer injector used for deep placement of coated urea (Takahashi et al. [39]).



Figure 13. The photograph of deep placement of coated urea taken in 1988.

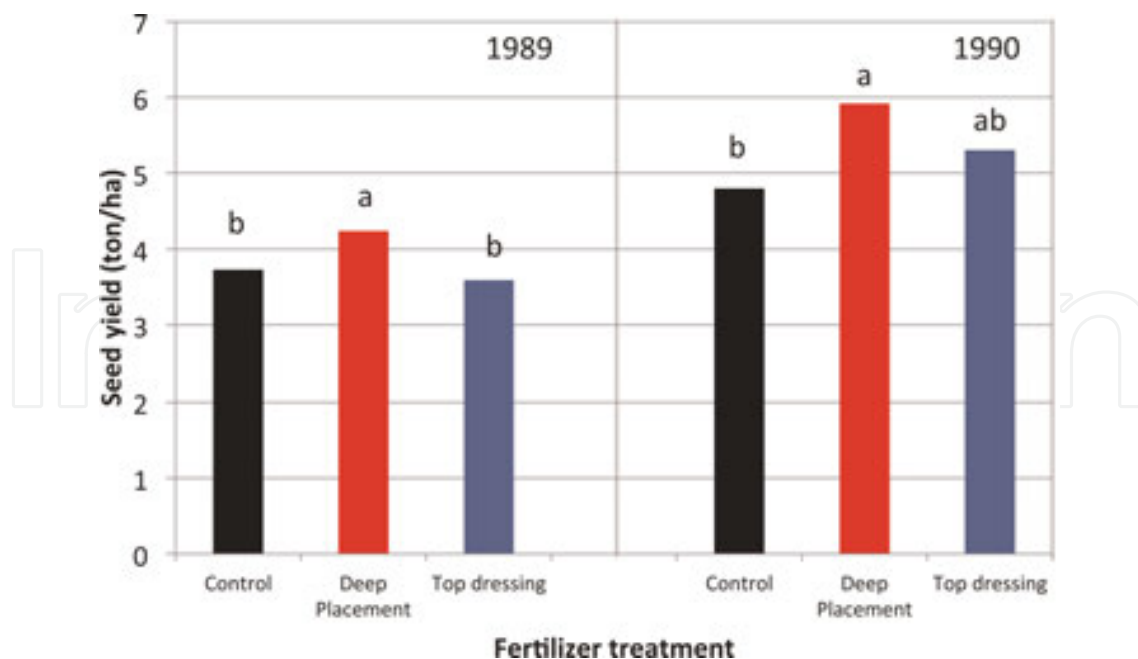


Figure 14. Seed yield of soybean in control, deep placement of 100-day-type-coated urea or top dressing of 70-day-type-coated urea (Takahashi et al. [17, 19]).

type of coated urea was carried out at R1 stage by broadcasting the fertilizer just before the earthing up intertillage.

Figure 14 shows the seed yield in 1989 and 1990. In 1989, seed yield by deep placement was 4.24 t ha^{-1} and 14% higher than the conventional control treatment (3.73 t ha^{-1}). The top-dressing treatment decreased seed yield about 4% and the yield was 3.59 t ha^{-1} . In 1990, seed yield by deep placement was 5.92 t ha^{-1} and 23% higher than the conventional treatment (4.80 t ha^{-1}). The top-dressing treatment increased seed yield about 11% and the yield was 5.31 t ha^{-1} .

Figure 15 shows the seasonal changes in daily N_2 fixation activity and N absorption rate calculated by a simple relative ureide method described before. Control plants mainly used fixed N_2 until R5 stage, and N absorption rate was higher after R5–R7 stage. In deep placement N_2 fixation activity was not depressed and N absorption rate was higher than control plants. As a result, the total amount of assimilated N in deep placement was 39.3 g m^{-2} and higher than control treatment (33.0 g m^{-2}). In the case of top-dressing treatment, N absorption rate after R3 stage became higher, but N_2 fixation activity after R5 stage became lower, and the total N was 36.3 g m^{-2} . The fertilizer use efficiency was evaluated by ^{15}N -labeled fertilizers, and the N recovery rate in the deep placement of 100-day-type-coated urea was 62% and much higher than top-dressed 70-day-type-coated urea (33%) and basal dressing of ammonium sulfate (9%) at R7 stage. The leaf area index (LAI) was higher in deep placement of 100-day-type-coated urea (2.99) than control (1.96) and top dressing of 70-day-type-coated urea (2.28) at R7 stage. The higher LAI might support the nodule activity during the pod-filling stage [19].

Takahashi et al. analyzed the accumulation of ammonium-N, nitrate-N, and urea-N in surface soil of 0–10 cm and deep soil of 15–25 cm in the field at R1, R3, R5, and R7 stages (**Figure 16**)

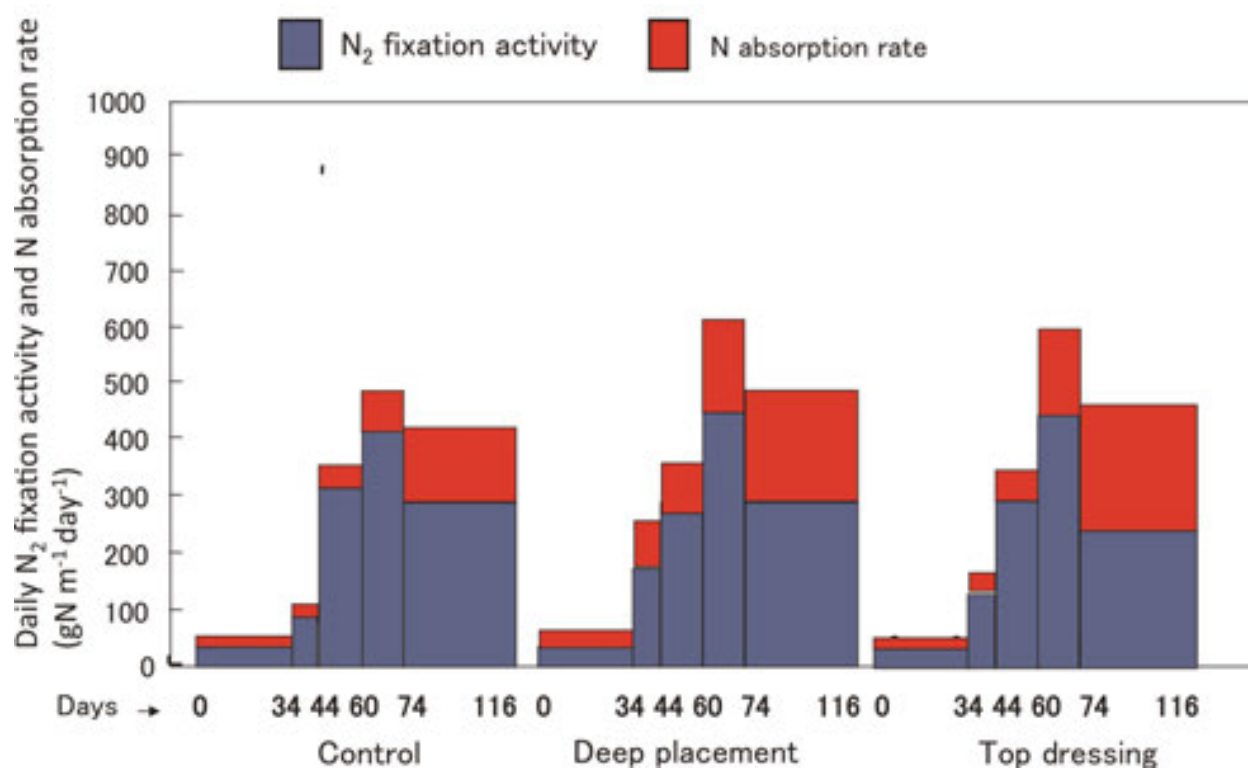


Figure 15. Changes in daily N₂ fixation activity and N absorption rate in control, deep placement of 100-day-type-coated urea or top dressing of 70-day-type-coated urea (Takahashi et al. [19]).

[20]. A high accumulation of ammonium-N was observed in the deep layer (15–25 cm) of soil at R3 and R5 stages of deep placement of 100-day-type-coated urea, although ammonium and nitrate accumulation was not observed in the surface layer at any stage of deep placement of coated urea. The result indicates that deep placement of coated urea slowly released urea and urea inside the particle was rapidly degraded to ammonium and it remained in the deep layer of soil for relatively long time. The slow nitrification rate might be due to low oxygen concentrations and low nitrification activities in deeper layers in rotated paddy fields in Niigata [20]. When 70-day type of coated urea was applied in the surface layer by top dressing at R1 stage, ammonium and nitrate concentration in the surface layer at R3 stage was significantly higher than control treatment. The accumulation of ammonium and nitrate in the surface layer where nodulation occurs might inhibit nodulation and nitrogen fixation.

3.2. Deep placement of lime nitrogen

Coated urea is suitable for deep placement, because it gradually releases urea until reproductive growth stage in accordance with soybean N requirement. However, as the price of coated urea is relatively expensive about five times higher than urea, the cost of it may be a burden for farmers. Tewari et al. compared the effect of deep placement of lime nitrogen (LN) with coated urea [41, 42, 47–51]. Lime nitrogen has been produced by artificial nitrogen fixation, which was done by Frank and Caro in 1901 prior to the establishment of Harber and Bosch process to convert atmospheric N₂ to ammonia in 1906. Lime nitrogen contains 60% of calcium cyanamide (CaCN₂) with calcium oxide (CaO) and free carbon (C). The fertilizer-grade lime nitrogen

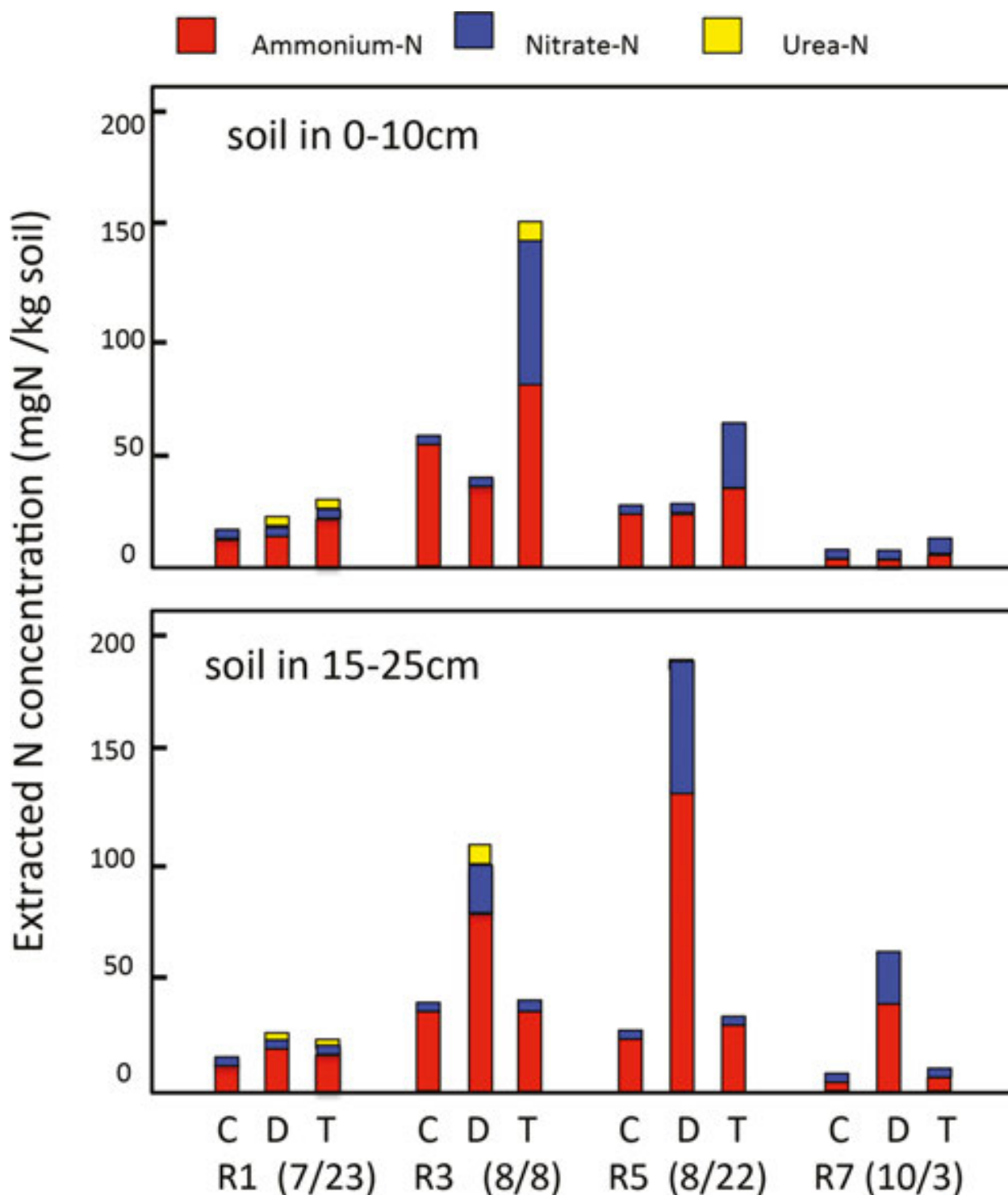


Figure 16. Changes in ammonium, nitrate, and urea concentration in the upper layer (0–10 cm) and deep layer (15–25 cm) of soybean field (Takahashi et al. [20]). C: control. D: Deep placement of 100-day-type-coated urea. T: Top dressing of 70-day-type-coated urea.

contains 21% N, 11% Ca, 11% C, 5% oil, 2–4% water, and oxides of aluminum, iron, and silicon [52]. CaCN_2 is converted to urea in soil, then the urea is hydrolyzed to ammonium and carbon dioxide. In the presence of moisture and air, dicyandiamide is formed from cyanamide of

CaCN₂, and this is a potent nitrification inhibitor, which inhibits the oxidation of ammonium to nitrate. Therefore, the ammonium produced by CaCN₂ decomposition persists for a long period of time and the nitrate concentration remains low in soil.

In 2001, fertilizer experiments were conducted in three sites in Niigata, Japan, of a rotated paddy field [46], a newly reclaimed wet land piled up with about 40-cm depth of surplus soil [48], and a sandy dune field [48]. Four fertilizer treatments of control without deep placement and deep placements of urea, 100-day-type-coated urea, and lime nitrogen were conducted. In addition, three different inoculation methods of *Bradyrhizobia* were carried out. They were non-inoculated paper pot (NIPP), direct inoculation transplanting (DT), and inoculated paper pot (IPP). Paper pot was made of biodegradable paper in soil. The pot was opened at the bottom to allow root expansion below the pot. The paper pot used was filled with vermiculite and inoculated with *B. japonicum* USDA110. Plants were grown in a paper pot for 10 days after planting, and transplanted to the field. Direct inoculation was seed inoculation by suspension of *B. japonicum* without using paper pot. Other plants were germinated in non-inoculated paper pot, and transplanted at 10 days after planting.

Table 5 shows the seed yields of three sites in which fertilizers and inoculation treatments were carried out. In the rotated paddy field, significant higher yield was observed by deep placement of coated urea and lime nitrogen compared with control without deep placement of urea. Among the same fertilizer treatments, the seed yields with IPP and DT inoculation methods tended to exceed that with NIPP.

| Inoculation method | Fertilizer treatment | Rotated paddy field | Reclaimed field | Sandy dune field |
|--------------------|----------------------|---------------------|-----------------|------------------|
| NIPP | Control | 288b | 78b | 172b |
| | Urea | 453a | 286a | 246a |
| | Coated urea | 429a | 358a | 249a |
| | Lime nitrogen | 460a | 340a | 250a |
| DT | Control | 314b | 194b | 191b |
| | Urea | 422ab | 336a | 262a |
| | Coated urea | 535a | 397a | 271a |
| | Lime nitrogen | 541a | 356a | 267a |
| IPP | Control | 331b | 201c | 183b |
| | Urea | 467b | 290b | 273a |
| | Coated urea | 604a | 400a | 305a |
| | Lime nitrogen | 612a | 419a | 332a |

NIPP: Non-inoculated paper pot. DT: Direct transplanting of inoculated seedlings. IPP: Inoculated paper pot. Means followed by the same letter are not significantly different at 5% level in the same inoculation methods in the same field.

Table 5. Seed yield of soybean cultivated with deep placement of N fertilizers and different inoculation methods in three fields of Niigata in 2001. Data from Tewari et al. [47–49].

| Fertilizer treatment | Total N (g m ⁻²) | Ndfa (g m ⁻²) | Ndfs + Ndff (g m ⁻²) | %Ndfa |
|----------------------|------------------------------|---------------------------|----------------------------------|-------|
| Control | 22.4b | 13.6b | 8.8b | 61 |
| Urea | 28.3b | 13.2b | 15.1a | 47 |
| Coated urea | 31.0a | 21.3a | 9.7b | 69 |
| Lime nitrogen | 33.4a | 21.4a | 12.0ab | 64 |

Data from Tewari et al. [47].

Table 6. Nitrogen origin of soybean cultivated with various N fertilizers and inoculated paper pot in rotated paddy field of Niigata (2001).

Similar results were observed in the newly reclaimed field and the sandy dune field, although seed yield levels were lower than those in rotated paddy field, possibly due to lower soil fertility in these fields. Irrespective of field types and inoculation methods, deep placement of lime nitrogen and coated urea gave the promotive effect on seed yield of soybean. The application of lime nitrogen tended to give higher seed yield than that of coated urea, although data were statistically not significant. The results of three field experiments confirmed that deep placement of coated urea can be replaced by lime nitrogen.

Table 6 shows the nitrogen origin of soybean shoots at R7 stage. Total N assimilation was higher in deep placement of coated urea (31.0 g m⁻²) and lime nitrogen (33.4 g m⁻²) than in urea (28.3 g m⁻²) and control (22.4 g m⁻²) treatment. Based on the analyses of Ndfa and Ndfs + Ndff, it was confirmed that Ndfa from deep placement of coated urea (21.3 g m⁻²) and lime nitrogen (21.4 g m⁻²) was higher than that in urea (13.2 g m⁻²) and control (13.6 g m⁻²) treatment. This indicates that deep placement of lime nitrogen and coated urea promoted nitrogen fixation without inhibiting it.

3.3. Effect of the depth of placement of lime nitrogen on seed yield and nitrogen assimilation

The effects of different depths of placement of lime nitrogen at 10, 15, and 20 cm were compared in a rotated paddy field at the Niigata Agricultural Research Institute in 2003 [49]. In addition to conventional basal application of mixed chemical fertilizer (ammonium sulfate 16 k kgN ha⁻¹, P₂O₅ 60 kg ha⁻¹, K₂O 80 kg ha⁻¹, and Ca(OH)₂ 1000 kg ha⁻¹ in the plow layer at a depth of 0–10 cm), lime nitrogen (100 kgN ha⁻¹) was placed in 10, 15, or 20 cm depth just under sowing line.

Figure 17 shows the seed yield classified with seed quality. The total seed yield was highest in the deep placement of lime nitrogen at 20 cm (617 g m⁻²) compared with 15-cm depth (526 g m⁻²), 10-cm depth (515 g m⁻²), and control without deep placement (428 g m⁻²). The yield of good seeds was significantly higher in deep placement of lime nitrogen at 20-cm depth.

The daily N₂ fixation activity and N absorption rate were calculated using the simple relative ureide method (**Figure 18**). Average daily N₂ fixation activity and N absorption rate were relatively low until 71 days after sowing (R2 stage). From 71 days (R1) to 102 days (R5) after sowing, both the N₂ fixation activity and N absorption rate were high in lime nitrogen

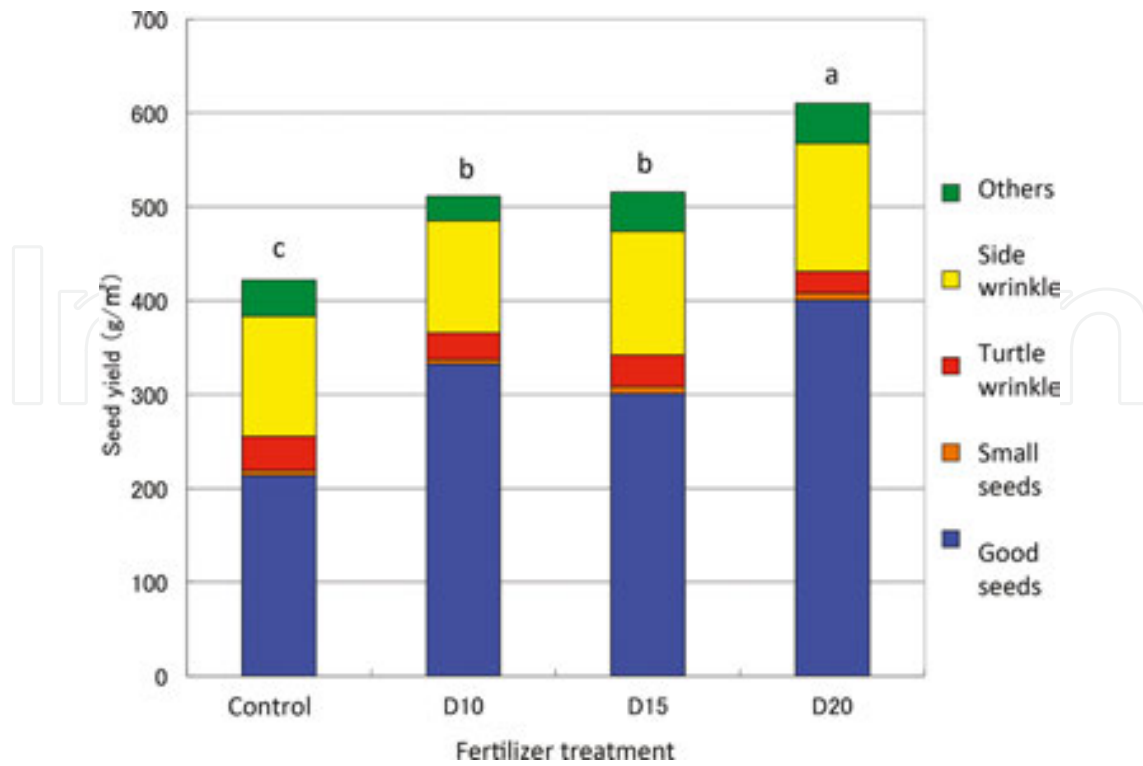


Figure 17. Seed yield classified with seed quality of soybeans cultivated with different depths of lime nitrogen placement (Tewari et al. [50]). Control: lime nitrogen was not applied. D10: lime nitrogen was applied at 10-cm depth. D15: lime nitrogen was applied at 15-cm depth. D20: lime nitrogen was applied at 20-cm depth.

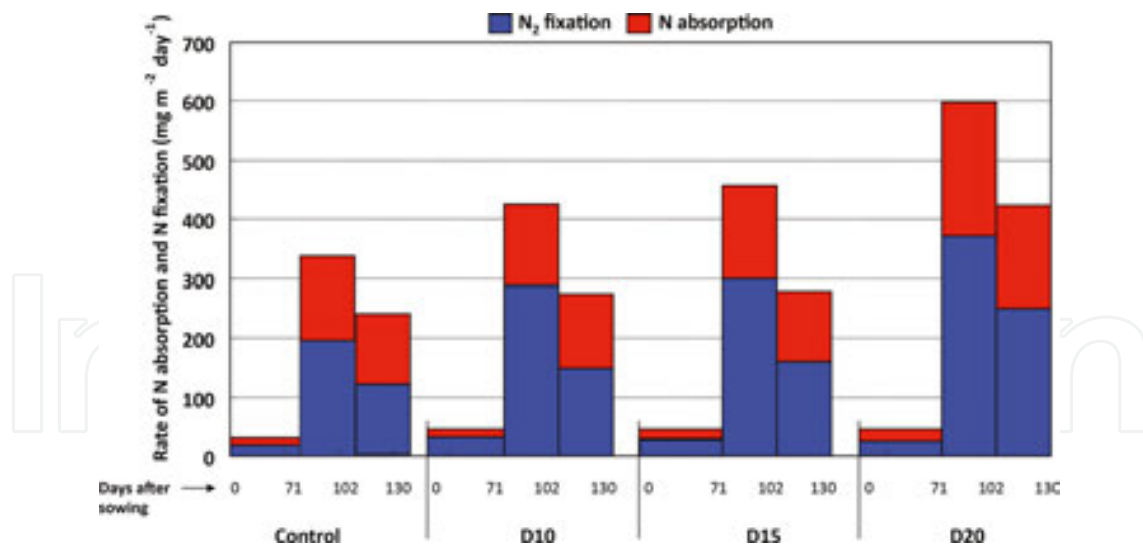


Figure 18. Changes in daily N₂ fixation activity and N absorption rate in soybean plants grown with different depths of lime nitrogen placement (Tewari et al. [50]). Control: lime nitrogen was not applied, D10: lime nitrogen was applied at 10 cm, D15: lime nitrogen was applied at 15 cm, D20: lime nitrogen was applied at 20 cm.

treatment of especially 20-cm depth compared with control plants. From 102 days (R5) to 130 days after sowing (R7), the N₂ fixation activity declined in all fertilizer treatments, but D20 plants (20-cm depth) kept the highest N₂ fixation activity and N absorption rate.

3.4. Effect of the amount of placement of lime nitrogen on seed yield and nitrogen assimilation

The effect of different amounts of placement of lime nitrogen at 50, 100, or 200 kgN ha⁻¹ was compared in a rotated paddy field at the Niigata Agricultural Research Institute in 2003 [51]. In addition to conventional basal application of mixed chemical fertilizers in the plow layer of a depth of 0–10 cm, lime nitrogen (50, 100, or 200 kgN ha⁻¹) was placed in 20-cm depth under soil surface just below sowing line.

Figure 19 shows the seed yield classified with seed quality. The total seed yield was highest in the deep placement of 100 kgN ha⁻¹ lime nitrogen (570 g/m²) compared with 50 kgN ha⁻¹ (510 g/m²), 200 kgN ha⁻¹ (500 g/m²), and control without deep placement (470 g/m²). The yield of good seeds increased in deep placement of lime nitrogen at 20-cm depth.

The daily N₂ fixation activity and N absorption rate were calculated using the simple relative ureide method (**Figure 20**). The average daily N₂ fixation activity and N absorption rate were relatively low until 71 days after sowing (R2 stage). From 71 to 102 days (R5 stage) after sowing, both the N₂ fixation activity and N absorption rate were higher in LN treatment of especially 100 kgN ha⁻¹ compared with control plants.

From 102 to 130 days after sowing (R7 stage), the N₂ fixation activity declined in all fertilizer treatments, but plants of 100 kgN ha⁻¹ kept high N₂ fixation activity and N absorption rate during this period. When 200 kgN ha⁻¹ was applied, N₂ fixation activity was lower than that at 100 kgN ha⁻¹ of lime nitrogen.

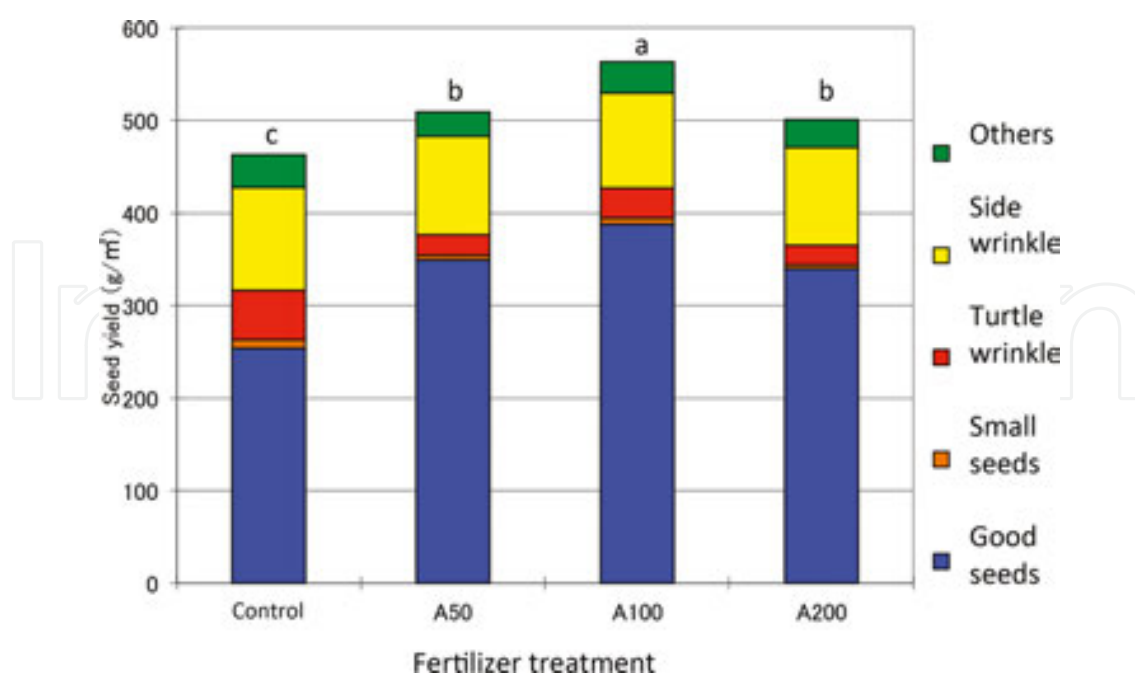


Figure 19. Seed yield classified with seed quality of soybeans cultivated with different rates of lime nitrogen at 20-cm depth (Tewari et al. [51]). Control: lime nitrogen was not applied, A50: 50 kgN ha⁻¹ lime nitrogen was applied, A100: 100 kgN ha⁻¹ lime nitrogen was applied, A200: 200 kgN ha⁻¹ lime nitrogen was applied.

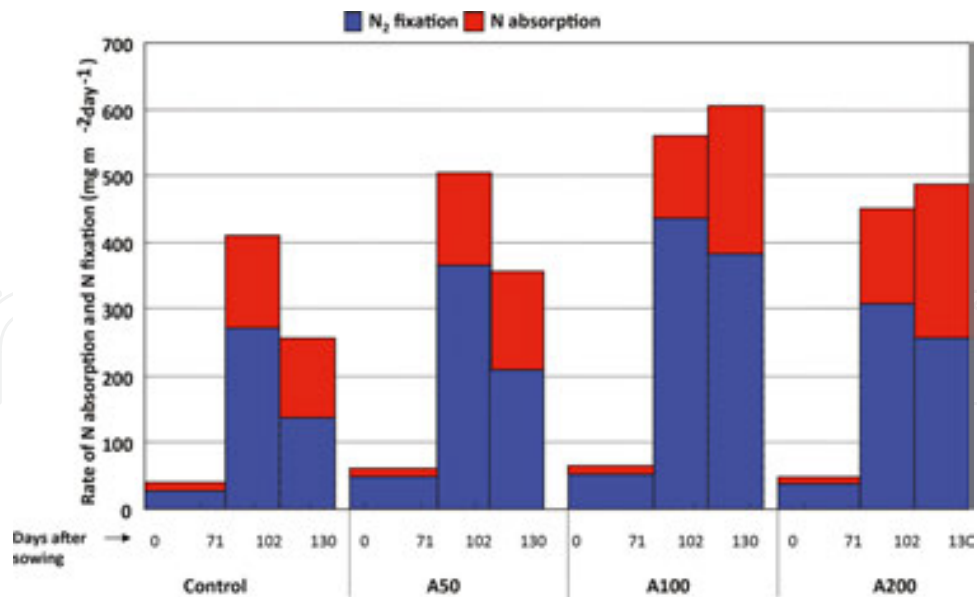


Figure 20. Changes in daily N₂ fixation activity and N absorption rate in soybean plants grown with different rates of lime nitrogen placed at 20-cm depth (Tewari et al. [51]). Control: lime nitrogen was not applied, A50: 50 kgN ha⁻¹ lime nitrogen was applied, A100: 100 kgN ha⁻¹ lime nitrogen was applied, A200: 200 kgN ha⁻¹ lime nitrogen was applied.

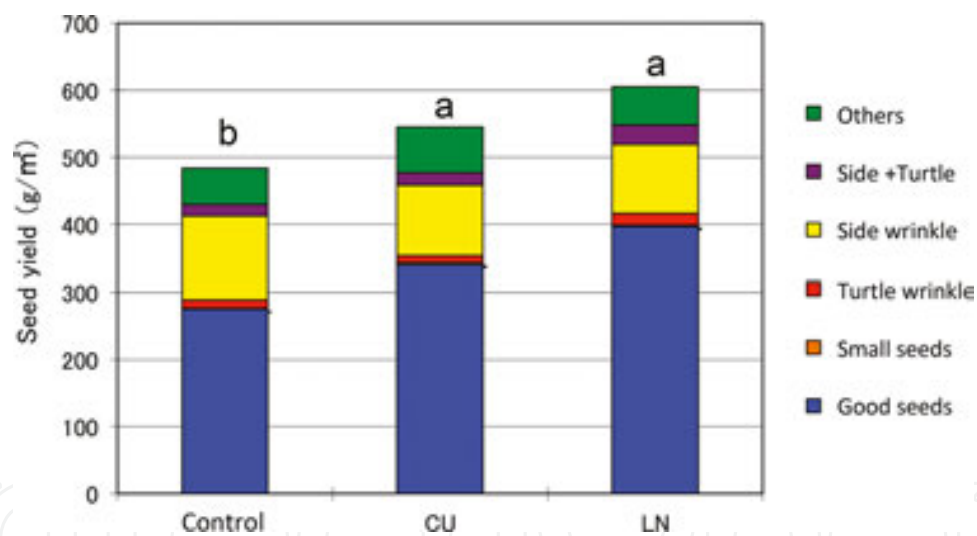


Figure 21. Seed yield classified with seed quality of soybeans grown with deep placement of coated urea and lime nitrogen (Tewari et al. [42]). Control: lime nitrogen was not applied, CU: 100-day-type-coated urea was applied at 20-cm depth, LN: lime nitrogen was applied at 20-cm depth.

3.5. Comparison of seasonal absorption patterns of coated urea and lime nitrogen

The experiment was carried out in a rotated paddy field in Niigata Agricultural Research Institute in 2004, and the utilization of deep placement of ¹⁵N-labeled coated urea (CU) and lime nitrogen (LN) was investigated at R1, R3, R5, and R7 stages [42]. In this experiment, seed yield was higher in deep placement of LN or CU than in control treatment (**Figure 21**). **Figure 22** shows changes in daily N₂ fixation activity and N absorption rate. From R1 (61 DAS (days after sowing)) to R5 (102 DAS) stage, both N₂ fixation activity and N absorption rate were higher in deep placement of CU or LN compared with control treatment. From R5 to

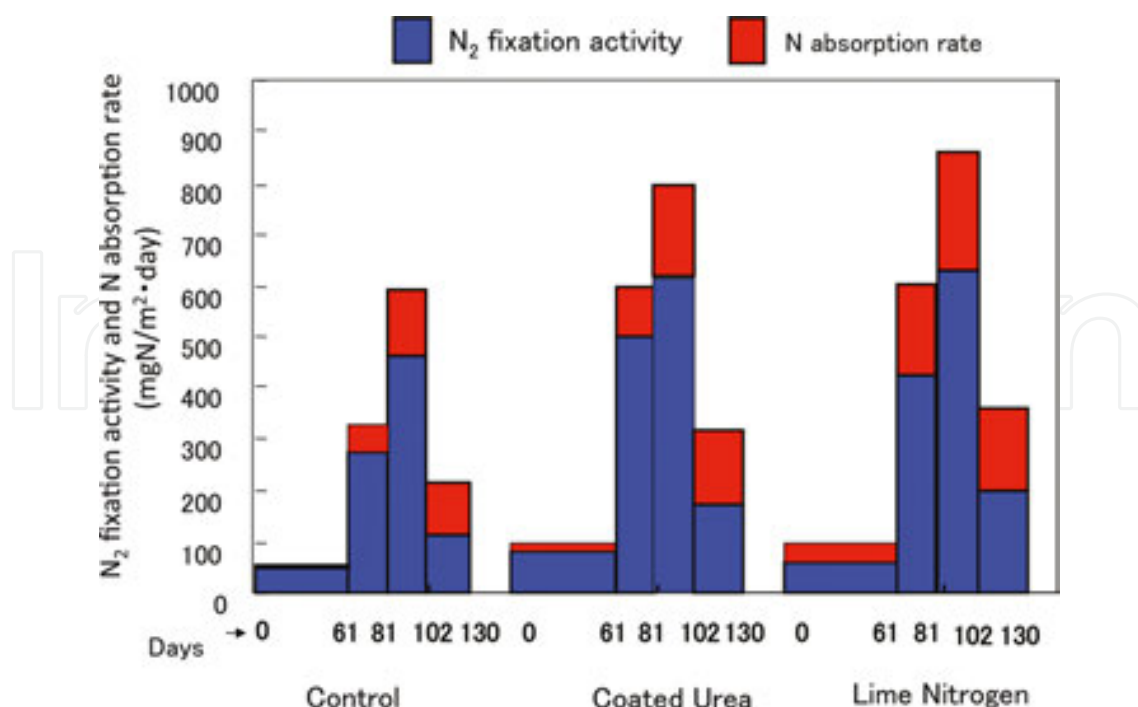


Figure 22. Changes in daily N₂ fixation activity and N absorption rate in soybean plants cultivated in Niigata (Tewari et al. [42]). Control: lime nitrogen was not applied, CU: 100-day-type-coated urea was applied at 20 cm, LN: lime nitrogen was applied at 20-cm depth.

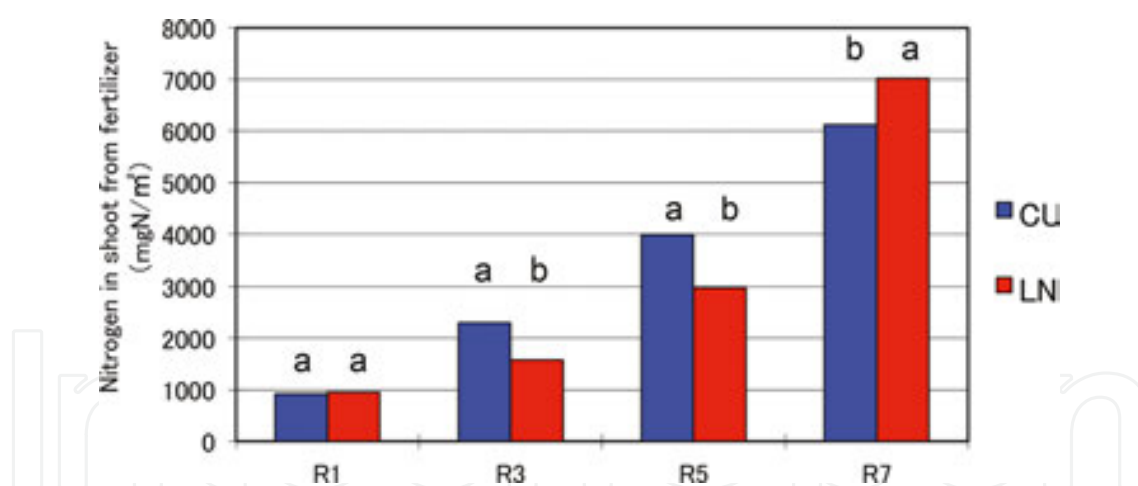


Figure 23. Nitrogen recovered in soybean shoot grown with deep placement of 100-day-type-coated urea or lime nitrogen (Tewari et al. [42]). CU: Deep placement of 100-day-type-coated urea, LN: deep placement of lime nitrogen.

R7, the daily N₂ fixation activity declined in all treatments, but plants grown with deep placement of CU and LN maintained the higher activity than control plants.

Figure 23 shows the changes in the content of labeled N in soybean plants cultivated with ¹⁵N-labeled CU or LN. At R1 stage, the absorption rates of N from CU and LN were almost the same, but those were higher with CU than with LN at R3 and R5 stages. However, at the R7 stage the labeled N content increased markedly with LN and exceeded that with CU. At R7, fertilizer use efficiencies were 70% in LN and 61% in CU.

3.6. Effect of deep placement of lime nitrogen on distribution of root nodules

The experiment was conducted in a rotated paddy field of Niigata Agricultural Research Institute, Nagaoka [53]. Conventional basal dressing of chemical fertilizer containing ammonium sulfate (16 kg N ha^{-1}), fused magnesium phosphate ($60 \text{ kg P}_2\text{O}_5 \text{ ha}^{-1}$), and potassium chloride ($80 \text{ kg K}_2\text{O ha}^{-1}$) was plowed in a soil layer at 0–10-cm depth. A soybean plant was planted in the center of the wooden box (length, 45 cm; width, 4.5 cm; depth, 30 cm) filled with the field soil (**Figure 24**). The boxes were embedded in the field. Two fertilizer treatments were conducted. Control was no

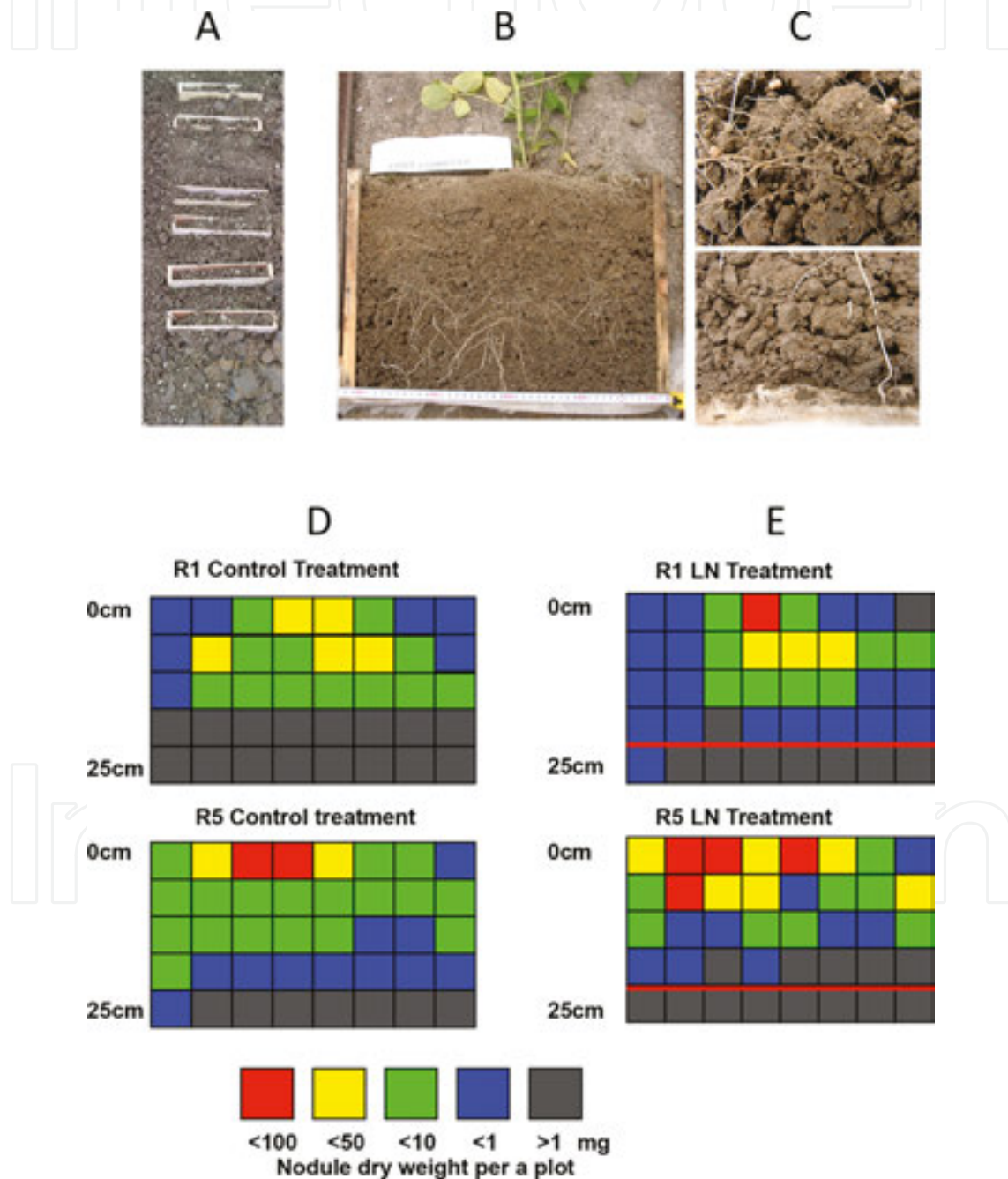


Figure 24. Distribution of nodules in the root box (rhizobox) (Ohyama et al. [53]). A: Placement of root box in soybean field; B: inside of root box; C: nodules and roots; D: control treatment without deep placement; E: deep placement of lime nitrogen at 20-cm depth.

additional fertilizer, and for deep placement of lime nitrogen, lime nitrogen ($1.12 \text{ gN plant}^{-1}$) was applied at 20-cm depth in the box. At R1 (initial flowering) stage and R5 (pod-filling) stage, the rhizoboxes were dug out. The soil in the box was separated into blocks with each profile of 5×5 cm, and the soils were washed out. The dry weights of roots and nodules were measured.

Total nodule weight of the plants treated with deep placement of lime nitrogen was $0.57 \text{ g plant}^{-1}$ and lower than the control nodule weight of $0.73 \text{ g plant}^{-1}$ at initial flowering stage (R1 stage). The nodule distribution was not different between deep placement of lime nitrogen and control treatment at R1 stage. At R5 stage, the total nodule dry weight of deep placement of lime nitrogen was $1.17 \text{ g plant}^{-1}$ and much higher than that in control plants ($0.73 \text{ g plant}^{-1}$). The nodule weight in the upper layer of soil in lime nitrogen treatment was higher than that in control treatment. This result supported the promotion of nitrogen fixation by deep placement of lime nitrogen estimated by relative ureide method. The dry weight of the shoot was also higher in deep placement of lime nitrogen (37 g plant^{-1}) compared with control plants (28 g plant^{-1}) at R5 stage. These results demonstrated that deep placement of lime nitrogen provides

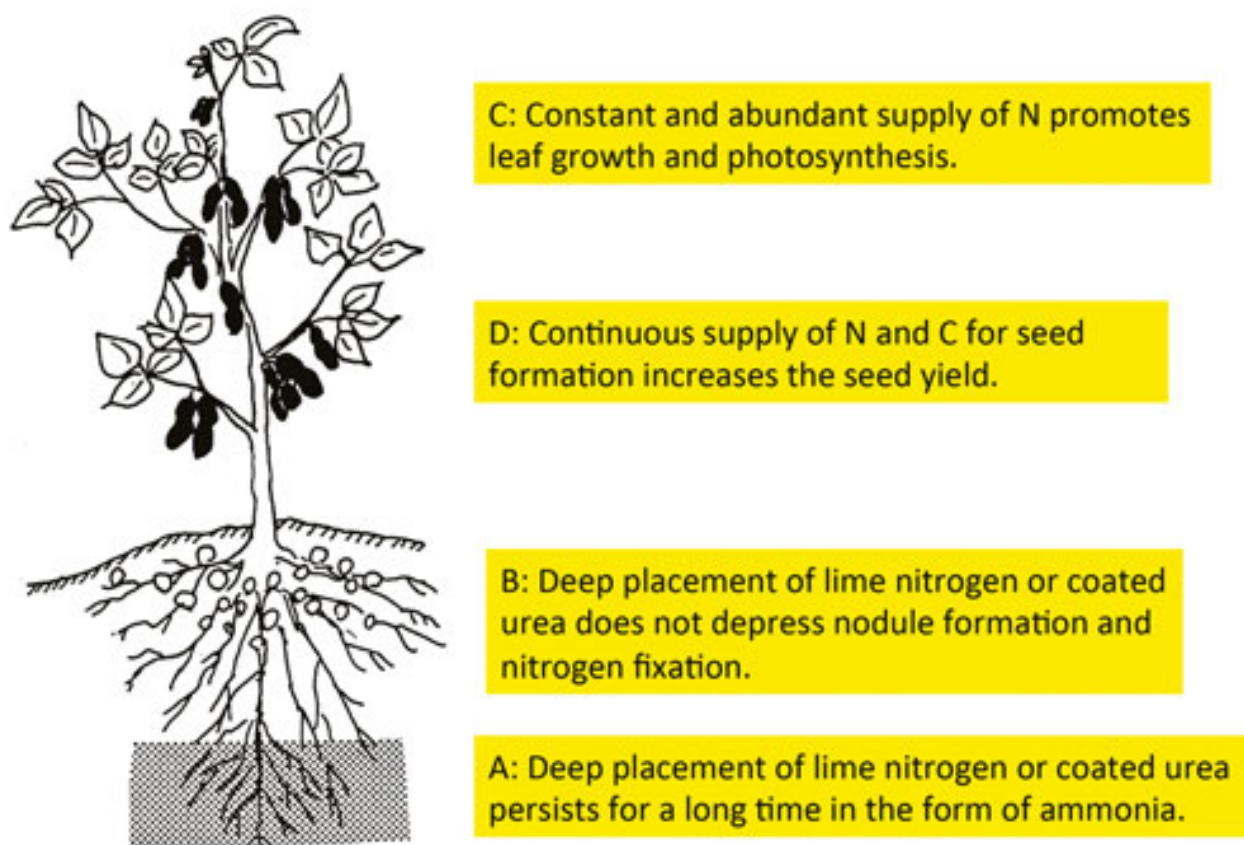


Figure 25. Promotive effects of deep placement of lime nitrogen or coated urea. (A) Basal dressing of deep placement of lime nitrogen or coated urea persists in the form of ammonia for a long time until reproductive stages. Plant roots can absorb N from lower soil layer, and the root growth and water and nutrient absorption may be increased [18]. Lime nitrogen contains Ca, and the Ca application may be beneficial for soybean growth, because soybean requires much Ca. Making a slit in soil under planting may improve water drainage. (B) Deep placement of lime nitrogen or coated urea did not increase the concentration of nitrate or ammonium in the upper layer where nodules are mainly formed. So this method does not inhibit nodulation and nitrogen fixation. (C) Continuous supply of N from lower soil promotes leaf photosynthetic activity until seed-maturing stage. Therefore, abundant photosynthate may be transported to nodules as well as seeds. (D) Continuous supply of N and C for seed growth increases the seed yield and improves the seed quality.

the nitrogen needed in the reproductive stage and vigorous photosynthetic activity of the leaves promotes nodule growth and nitrogen fixation. Takahashi et al. [5] reported that deep placement of coated urea increased the total dry weight of shoots and leaf area index in R7 compared with control treatment without deep placement. The abundant supply of C and N decreased the flower and pod shedding, and the seed number and seed weight increased.

3.7. Summary of the effect of deep placement of coated urea or lime nitrogen

Figure 25 summarizes the effect of deep placement of lime nitrogen or coated urea to promote seed yield of soybean.

4. Conclusions and perspectives

For maintaining agricultural production and protecting the environment, efficient use of N fertilizer is crucial. To optimize the chemical form, rate, timing, and placement of the fertilizer are important for the demand of various crops grown under various conditions. Deep placement of lime nitrogen or coated urea promoted the soybean growth and seed yield through promotion of nitrogen fixation after initial flowering stage. The promotive effect on nodulation by deep placement of lime nitrogen was confirmed by rhizobox experiment.

The effects of deep placement of N-, P-, and K-fertilizers on growth, nodulation, and yield of soybean have been investigated by Groneman [54]. Recently, deep placement of lime nitrogen has been tested in many agricultural fields, and the seed yield and quality are mostly improved. However, this technique has not been used in a large-scale farming. The agricultural machines, which can efficiently put fertilizers in deep place with high speed drive, should be developed. In addition, as the price of coated urea and lime nitrogen is relatively expensive, it is beneficial to use cheap nitrification inhibitors with urea or ammonia fertilizers.

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