Review Article

Applications of Stable Isotopes

The 1981–2000 Studies of $^{13}\text{C}/^{12}\text{C}$ and $^{15}\text{N}/^{14}\text{N}$ Discrimination in the Metabolism of Higher Plants, and the Progress Since Then

Tadakatsu YONEYAMA ^{*}, ^{**}, [†]

^{*}National Agriculture and Food Research Organization Fellow

Kannondai 3–1–1, Tsukuba, Ibaraki 305–8666 Japan

^{**}Professor Emeritus, University of Tokyo

Yayoi 1–1–1, Bunkyo-ku, Tokyo 113–8657 Japan

[†]tadakatsu_yoneyama@opal.ocn.ne.jp

During 1981–2000, my research focused on the precise determination of the natural abundances of the $^{13}\text{C}/^{12}\text{C}$ and $^{15}\text{N}/^{14}\text{N}$ ratios ($\delta^{13}\text{C}$, $\delta^{15}\text{N}$) in many samples of plant tissues and metabolites. That research benefited from the previous work performed by pioneers in this field. My studies during that period provided the followings; (1) the identification of significant differences in the $\delta^{13}\text{C}$ values in plant tissues and phloem sap affected by biotic (N acquisition, plant species and cultivars) and abiotic (drought) factors, (2) the discrimination of $^{15}\text{N}/^{14}\text{N}$ during the uptake, metabolism, and translocation of nitrate, ammonium and biologically-fixed N, and (3) the application of $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ tracing techniques for the quantification of plant residue dynamics (i.e., accumulation and degradation) in soils, plant N uptake from different sources (soils, chemical fertilizers, organic manures) and field biological N_2 fixation by legumes including trees and non-legumes such as sugarcane and sweet potato, respectively. Here I review my publications and findings which gave insight for a precise understanding of whole plant C/N metabolisms.

Key Words: ecological C and N dynamics, isotope discrimination, natural $^{13}\text{C}/^{12}\text{C}$ abundance ($\delta^{13}\text{C}$), natural $^{15}\text{N}/^{14}\text{N}$ abundance ($\delta^{15}\text{N}$), plant metabolism

1. Introduction

In 1980, soon after Professor Eitaro Wada created Biogeochemistry Laboratory in the Mitsubishi-Kasei Life Science Institute at Machida, I joined the National Institute of Agricultural Sciences (NIAS) at Tsukuba and began to study the natural abundances of the $^{13}\text{C}/^{12}\text{C}$ and $^{15}\text{N}/^{14}\text{N}$ ratios ($\delta^{13}\text{C}$, $\delta^{15}\text{N}$) in higher plants, with the goal of devising new approaches in the research field of crop nutrition and production. Before my stint at the NIAS, I had been conducting studies of nitrogen and carbon metabolisms in plants and soils using ^{13}C and ^{15}N -enriched

substrates as the tracer at the University of Tokyo Department of Agricultural Chemistry at Tokyo as a graduate student and then at the National Institute for Environmental Studies (NIES, Tsukuba) as a research fellow. At the NIAS and then at the National Institute of Agro-Biological Resources (NIAR, Tsukuba), I was able to extend my studies of nitrogen and carbon assimilation and transport in crops—in particular, in N_2 -fixing leguminous plants at the levels of $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$.

In 1981, a high-precision mass spectrometer (Finnigan Mat 250, Thermo Fisher Scientific) with dual inlets of sample and standard gases was pur-

chased for the NIAR, and I had the opportunity to use it. Due to the courtesy of Professor Wada, I learned the concept of possible global variations in the natural abundance of isotopes involved in life-related elements (C, N, O, S) at the levels from molecules to organisms and ecosystems and at temporal levels of minutes to a thousand years. I felt that a new paradigm for plant physiology and ecology would then be developed. For the first 10 years of my research at NIAS, NIAR, and National Agriculture Research Center (NARC), I had a plan to determine the isotopic fractionation during carbon fixation and nitrogen uptake and assimilation. I worked alone as a labor-intensive technician/scientist analyzing the $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values in plant tissues and metabolites using the off-line system of sample-gas (CO_2 , N_2) preparations and high-precision mass spectrometry analyses. To obtain one-sample $\delta^{13}\text{C}$ or $\delta^{15}\text{N}$ value from plant tissues (e.g., leaf, seeds, stem, roots, nodules) containing milligrams of C or N, more than one hour was required for the conversion of dry materials to gasses for a mass analysis; only six analyses could be conducted per day.¹⁾

Before and during those days, researchers had to pay attention to the $^{13}\text{C}/^{12}\text{C}$ discrimination during whole photosynthesis and its each step of the processes (stomatal CO_2 diffusion and CO_2 -fixing enzymatic reactions), the $^{15}\text{N}/^{14}\text{N}$ discrimination during plant nitrogen acquisition (nitrate uptake and N_2 fixation) and during soil nitrogen processes such as nitrogen transformation (nitrification and denitrification). I also contributed to the determination of the variations of $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values among plant tissues and the magnitudes of $^{15}\text{N}/^{14}\text{N}$ discrimination during ammonia and nitrate uptake and their assimilation and during N_2 -fixing processes.

In 1992, an international symposium entitled "Stable Isotopes in the Biosphere" was held at the Mitsubishi-Kasei Life Science Institute under the leadership of Professor Wada, and the contents of the

selected presentations were published by the Kyoto University Press (1995). Prominent scientists working in plant sciences including Drs. M. H. O'Leary, D. H. Kohl and L. L. Handley participated in the symposium, and my attendance there allowed me to both communicate with scientists from around the world and 'step up' the analytical technologies to online analyses of $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values. I learned from Dr. Handley about the direct analysis of $\delta^{13}\text{C}/\delta^{15}\text{N}$ values in dry power samples using an ANCA continuous-flow isotope ratio mass spectrometer (European Scientific); this technology enabled the analysis of the $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values of plant and environment material samples within 5 minutes if the samples contained 200–1,000 μg of C or 25–300 μg of N.²⁾ I also started to analyze $\delta^{15}\text{N}$ values in individual amino acids and polyamines extracted from plant samples³⁾ using GC/C/IRMS (gas chromatography/combustion/isotope ratio mass spectrometry).

In August 2000, I moved from Tsukuba to the University of Tokyo's Department of Applied Biological Chemistry. Although I did not conduct analyses of $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values, at that time I wrote several papers regarding the vegetation shifts based on changes in soil $\delta^{13}\text{C}$ values following the vegetation developments after several volcano eruptions in Japan's Kyushu Island and regarding the shift of vegetation from forests to sugarcane fields in the Philippines and Thailand.

From these studies, I have published 12 papers about $\delta^{13}\text{C}$ values, 26 papers about $\delta^{15}\text{N}$ values, three reviews in English^{4–6)} and six reviews in Japanese. Happily, I have found that these published data have been used in discussions in papers and reviews by scientists world-wide. Here, I have been given the pleasant opportunity to review and summarize my major research contributions to the progress and perspectives in the field of the natural abundance of $^{13}\text{C}/^{12}\text{C}$ and $^{15}\text{N}/^{14}\text{N}$ ratios in higher plants.

2. Carbon fixation and metabolism and underlying natural $^{13}\text{C}/^{12}\text{C}$ fractionations in plants

Important classic reports of the $\delta^{13}\text{C}$ values of plants are as follows: (1) Using a Nier-type mass spectrometer (analytical error, $\pm 0.1\%$), Nier and Gulbransen (1939)⁷⁾ indicated that the $^{12}\text{C}/^{13}\text{C}$ ratio of a plant (pine wood) was 91.5, which was significantly higher than that of lime stone (88.3). (2) Wickman (1952)⁸⁾ observed small but significant variations in the $^{12}\text{C}/^{13}\text{C}$ ratio among 105 plant specimens, possibly caused by ecological factors. (3) Using a high-precision double-inlet mass spectrometer (analytical error, $\pm 0.1\%$) which was developed by Urey and his co-workers,⁹⁾ Craig (1953)¹⁰⁾ analyzed the $\delta^{13}\text{C}$ values ($= [^{13}\text{C}/^{12}\text{C}_{\text{sample}} - ^{13}\text{C}/^{12}\text{C}_{\text{PDB}}] / [^{13}\text{C}/^{12}\text{C}_{\text{PDB}}] \times 1000$, ‰) of several hundred materials, ranging from -22.5% to -27.9% for timbers and from -23.6% to -29.1% for plant leaves except for one grass from S.W. Kansas (-12.2%). Pioneering work by Bender (1968)¹¹⁾ demonstrated that plant $\delta^{13}\text{C}$ relates to different photosynthetic pathways. (4) Keeling (1958)¹²⁾ measured diurnal changes in atmospheric CO_2 concentrations and their $\delta^{13}\text{C}$ values in a sea-side forest area; in the daytime, $0.32 \text{ vol}\%$ CO_2 and a -7% $\delta^{13}\text{C}$ value were obtained, and at night, $0.38 \text{ vol}\%$ and -10% were recorded. These diurnal variations might be caused by photosynthetic CO_2 fixation in the daytime (decrease of the CO_2 concentration) and by a respiratory release of ^{13}C -depleted CO_2 in the night. (5) Regarding the relative $\delta^{13}\text{C}$ values among plant chemical fractions, Park and Epstein (1961)¹³⁾ reported more negative $\delta^{13}\text{C}$ values in lipids (by $3\text{--}8\%$) compared to those in whole plant organic matter. Abelson and Hoering (1961)¹⁴⁾ observed that among the proteinogenic amino acids from photosynthetic organisms, leucine had more negative $\delta^{13}\text{C}$ values than other amino acids and that among the carbon atoms of those amino acids, carboxyl C consistently contained

less negative $\delta^{13}\text{C}$ values than the other C atoms.

Through the subsequent analyses of $\delta^{13}\text{C}$ values in leaves from many species, it has become clear that among C4-type plants, which first fix HCO_3^- by phosphoenolpyruvate carboxylase, the leaf $\delta^{13}\text{C}$ values range from -6% to -19% with a mean of -13.5% , and that among C3-type plants, which fix CO_2 by ribulose-1,5-bisphosphate carboxylase, the leaf $\delta^{13}\text{C}$ values range from -24% to -34% with a mean of -28.1% .¹⁵⁾ Farquhar et al. (1982)¹⁶⁾ proposed a theory that the variation of leaf $\delta^{13}\text{C}$ values in C3-type plants are closely related to the stomatal CO_2 concentrations; i.e., smaller stomatal apertures during leaf CO_2 fixation resulting in low internal CO_2 concentrations and also smaller $^{13}\text{C}/^{12}\text{C}$ fractionation or less negative leaf $\delta^{13}\text{C}$ values.

The impact of weather and N_2 -fixation on the leaf ^{13}C values were examined by growing nodulating and non-nodulating isogenic lines of soybean in a Tsukuba field in 1994 (drought) and 1995 (normal weather).¹⁷⁾ An interesting correlation between leaf N concentrations and $\delta^{13}\text{C}$ values was found (Fig. 1). N_2 -fixing soybean plants subjected to drought (1994) had high leaf N concentrations and less negative

Fig. 1 The relationship between N concentrations and $\delta^{13}\text{C}$ values of individual leaf samples from nodulated (○, △) and non-nodulated (●, ▲) soybean plants harvested on days 52 and 58 in drought 1994 (○, ●) and on days 44, 48 and 63 in normal weather 1995. Adapted from Yoneyama et al. (1998).¹⁷⁾

$\delta^{13}\text{C}$ values, whereas non-nodulating soybean plants under normal weather (1995) had low leaf N concentrations and more negative $\delta^{13}\text{C}$ values. High leaf N concentrations in N_2 -fixing soybean plants may lead to high CO_2 fixing activity and low internal CO_2 concentrations¹⁸⁾ and to less negative $\delta^{13}\text{C}$ values than in non-nodulating soybean plants. The drought may have caused a reduction of stomatal aperture and

a low internal CO_2 concentration, resulting in less negative $\delta^{13}\text{C}$ values compared to the results under normal weather.

My research group compared the leaf $\delta^{13}\text{C}$ values of C3- and C4-type plants collected in Thailand crop fields during the wet season (May to October) and the dry season (November to April) as shown in Fig. 2.¹⁹⁾ The $\delta^{13}\text{C}$ values of the C3 plants under the dry season did not any increase in contrast to our expectation from the theory proposed by Farquhar et al. (1982),¹⁶⁾ whereas, in contrast, the leaf $\delta^{13}\text{C}$ values of the C4 plants were more negative under the dry season. The mechanism causing a decrease in $\delta^{13}\text{C}$ values might be the larger rate of CO_2 leakage from the bundle sheath cells to the mesophyll under drought conditions as proposed by Buchman et al. (1996).²⁰⁾

My first report about soybean $\delta^{13}\text{C}$ values (1983)²¹⁾ became the first report of the organ-specific variations in nodulating and non-nodulating legumes; a set of typical data are shown in Table 1. The phototrophic organ, i.e., the leaves, had the most negative $\delta^{13}\text{C}$ values among the organs, whereas

Fig. 2 Seasonal variation in the $\delta^{13}\text{C}$ values of C3 (○) and C4 (●) plants collected in Thailand. Adapted from Yoneyama et al. (2010).¹⁹⁾

Table 1 Natural carbon isotope abundance ($\delta^{13}\text{C}$, ‰) of nodulating (Kitamusume, Itsutsuba, A62-1) and non-nodulating (A62-2) soybean plants grown in Tsukuba

Plant part	Kitamusume	Itsutsuba	A62-1	A62-2
Premature plants (harvested on August 8, 1982)				
Grain	-27.3	-27.2	-28.6	-28.5
Pod shell	-27.1		-27.8	-28.2
Leaf	-28.7	-28.9	-29.4	-29.1
Stem	-27.4	-27.9	-27.9	-27.9
Root	-27.2	-27.9	-27.4	-27.5
Nodule	-26.3	-26.5	-26.4	
Mature plants harvested in 1981				
Grain	-26.7		-27.1	-27.5

Adapted from Yoneyama and Ohtani (1983).²¹⁾

the heterotrophic organs such as the roots, stem, and mature grains had less negative $\delta^{13}\text{C}$ values. It is noteworthy that the $\delta^{13}\text{C}$ values of the N_2 -fixing nodules were the least negative $\delta^{13}\text{C}$; 2–3‰ higher than those of the leaves. One possible reason why the nodules had less negative $\delta^{13}\text{C}$ values compared to those of the host-plant organs might be the significant contribution of active dark CO_2 fixation at nodules through phosphoenolpyruvate carboxylase (PEPC) activity, producing organic acids that supply carbon skeletons for fixed nitrogen.²²⁾ Evidence of the probable involvement of PEPC in tobacco leaf CO_2 fixation was demonstrated by the finding that Rubisco protein aspartate (carbon-4) has a much higher $\delta^{13}\text{C}$ value than other amino acids.²³⁾

To examine whether the exported carbon compounds *via* the phloem have different $\delta^{13}\text{C}$ values from those of the source organ, leaves, we analyzed the $\delta^{13}\text{C}$ values in phloem saps collected from severed aphid-stylets in wheat²⁴⁾ and from stem incisions of castor bean plants.²⁵⁾ In Table 2, part of the data for the castor bean plants is shown. The $\delta^{13}\text{C}$ value of the phloem sap was less negative than those of the leaves, and at the mature stage, the $\delta^{13}\text{C}$ values of the seeds were close to those of the phloem sap.

Therefore, the less negative $\delta^{13}\text{C}$ values observed in heterotrophic organs are determined mainly by post-photosynthetic metabolisms in green organs. A study using *Eucalyptus delegatensis* indicated that the $\delta^{13}\text{C}$ values of the phloem sap corresponded to those of carbon from starch.²⁶⁾

Over the last 20 years, $^{13}\text{C}/^{12}\text{C}$ discrimination during post-photosynthetic metabolism, particularly a large loss of CO_2 by photo- and dark respiration, has been intensively examined by many scientists to explore the reason why the $\delta^{13}\text{C}$ values of C3 plant leaves are generally most negative compared with all other organs²⁷⁾ and also why $\delta^{13}\text{C}$ values of phloem sap are less negative than those of leaves in C3 plants, as shown in Table 2.²⁵⁾ Photorespiration at the mitochondria releases CO_2 by glycine decarboxylase and a theoretical study of $^{13}\text{C}/^{12}\text{C}$ discrimination by this enzyme suggested the favor of ^{12}C in CO_2 by approx. 20%.²⁸⁾ The photorespiratory-evolved CO_2 from leaves was lower in $\delta^{13}\text{C}$ than the leaf organic matters by approx. 12%,²⁹⁾ suggesting that the remaining leaf carbon compounds could be increased in $\delta^{13}\text{C}$ values.

$^{13}\text{C}/^{12}\text{C}$ discrimination during the active dark respiratory release of CO_2 from sucrose-derived TCA

Table 2 Natural carbon isotope abundance ($\delta^{13}\text{C}$, ‰) in plant organs (leaves, stem+petioles, seeds), xylem and phloem saps from castor bean plants grown in a Tsukuba field in 1994 (drought and high-temperature) and in 1995 (normal weather)

Year	Days after seeding	Leaves	Stem + Petioles	Seeds	Xylem sap	Phloem sap
1994	52	–28.9	–28.3		–27.4	–27.1
	62	–29.0	–28.3		–26.7	–27.0
	77	–27.7	–27.7	–24.5	–26.1	–24.2
1995	51	–29.9	–28.6		–28.3	–28.8
	66	–29.1	–28.0		–28.0	–29.0
	80	–29.1	–28.2	–27.3	–26.4	–28.6

Adapted from Yoneyama et al. (1998).²⁵⁾

cycle organic acids is favor of ^{12}C . Respiration by pyruvate decarboxylase using pyruvate (which is at the metabolic branching point) as the substrate releases CO_2 containing less negative $\delta^{13}\text{C}$ values than the leaf $\delta^{13}\text{C}$ value, because part of the pyruvate is preferentially used to synthesize acetyl CoA and lipids.³⁰⁾ The latter product lipids contain more negative $\delta^{13}\text{C}$ values, as reported previously.¹³⁾ Thus, the $^{13}\text{C}/^{12}\text{C}$ discrimination processed in overall leaf carbon metabolisms results in a slight decrease in the leaf $\delta^{13}\text{C}$ value but an increase in the $\delta^{13}\text{C}$ values of carbon sources (starch/sucrose) for the export or phloem-sap carbon compounds.^{24, 25)}

The individual organic compounds in plants contain intramolecular poly carbon atoms, and individual carbon $\delta^{13}\text{C}$ values can be determined through the enzymatic metabolism of their synthesis and degradation. To understand the $^{13}\text{C}/^{12}\text{C}$ discrimination happening in individual carbons, measurements of their specific carbon-atom $\delta^{13}\text{C}$ values in specific organs, tissues, and pools are required.³¹⁾

3. Ecosystem vegetation studies using $\delta^{13}\text{C}$ values as an indicator

An exogenous supply (deposition and incorporation) of new dry and wet plant-residue organic matter into the soil and the decomposition of the endogenous organic matter cause the turnover (synthesis and degradation), or exchange of soil organic carbon and subsequent nutrient release; the released nutrients are efficient for plant growth but also prone to loss. If the exogenously-supplied organic matter is from a C4 plant and the endogenous soil organic matter is composed of C3 plant species, the magnitude of the input of the C4-plant organic matter can be detected from the rate of the observed increase in the $\delta^{13}\text{C}$ values in the soil organic matter,^{32, 33)} because the $\delta^{13}\text{C}$ values of the C4 plant species are definitely higher than those of the C3 plants.

During the 20-year period from 1981 to 2000,

my research group collected samples of wild plants, crops, and soil from various sites across Japan. Analyses of the soil $\delta^{13}\text{C}$ values, which ranged from -17‰ to -27‰ , indicated that Andisols (volcanic ash soils) accumulated considerable organic matter that originated from the C4-type Japanese Pampas grass (i.e., *Miscanthus sinensis*, -10‰).³⁴⁾ The highest contributions from C4 plants were observed on Kyushu island at the southwest end of Japan and the least but still significant contribution was found in the southeast part of the northern island (Hokkaido). The involvement of C4 plants was not observed in the northern part of Hokkaido. Such a distribution pattern of soil $\delta^{13}\text{C}$ values is in accord with the distribution of C3 and C4 plant species.³⁵⁾ The long term retention as much as hundred years of *Miscanthus sinensis* residue as humic substances in the Andisols is explained as resulting from a conjugate/complex formation with active heavy metals such as aluminum and iron in humid acidic soils³⁶⁾ and by the transformation of grasses like C4 *Miscanthus sinensis* and C3 herbaceous plants to charred fragments by fires in dry soils.³⁷⁾

The analyses of $\delta^{13}\text{C}$ values of the vertical soil layers (69 samples in the 4.0-m depth) from Miyakonojo Basin in Kyushu island clearly indicated that after every corruption of volcanos and subsequent ash-falls, the wild plants, which were firstly C4 plants having deep-root development and then intensively C3 plants having shallow root mats, invaded the tephra, forming soil humas accumulation (called Kuroniga soil); in particular, the high-dense upper tephra layers.³⁸⁾ The possible plant species causing humas accumulation were identified by a phytolith analysis.

The change between C4 and C3 vegetation and their record in soil organic matter along with altitude from 1,630 m to 1,920 m in the Kirigamine grassland in Japan's central island (Honshu) were investigated for their biomass of present vegetation and

the $\delta^{13}\text{C}$ values of plants and soils (a record of old vegetation).³⁹⁾ At the low altitudes, the current and old vegetation was dominated by C4 plants, whereas at altitudes over 1800 m, C3 plants prevailed as present vegetation but the old vegetation recorded in the soil showed a considerable biomass production of C4 plants (*Miscanthus sinensis*), which was used to keep horses.

The turnover of soil organic matter during the shift of vegetation from forests (C3 plants) to sugarcane (C4 plant) cultivation was investigated in the Philippines⁴⁰⁾ and in Thailand.⁴¹⁾ The half-lives of the degradation of forest residue in the Philippines and Thailand were 1.2–1.8 years and 0.7 years, respectively. These short half-lives in humid tropics are in contrast to the longer half-lives (15–50 years) in temperate areas.⁴²⁾ Another important observation is that the plant residue component (as hardly-decomposable humus) which remained after rapid degradation is small in humid tropics, but much larger in temperate areas. With such different degradation and retention under tropical and temperate climates, diverse organisms and mechanisms may be involved. Their compositions and functions should be examined in detail using molecular and isotope-tracing technologies.⁴³⁾

4. Nitrogen acquisition and metabolism and the underlying $^{15}\text{N}/^{14}\text{N}$ discrimination in plants

Important classic works for $\delta^{15}\text{N}$ values ($= [^{15}\text{N}/^{14}\text{N}_{\text{sample}} - ^{15}\text{N}/^{14}\text{N}_{\text{air}}] / ^{15}\text{N}/^{14}\text{N}_{\text{air}} \times 1,000, \text{‰}$) of plants are as follows: (1) Hoering (1955)⁴⁴⁾ analyzed the $\delta^{15}\text{N}$ values of many natural samples including the first analyses of plant samples such as white clover leaves ($\delta^{15}\text{N} = -6.5\text{‰}$), dandelion leaves (-2.8‰), red oak leaves (-0.9‰), cedar leaves ($+1.3\text{‰}$), American elm leaves ($+1.9\text{‰}$), and seaweed from Tokyo Bay ($+8.1\text{‰}$). (2) Hoering and Ford (1960)⁴⁵⁾ examined the $^{15}\text{N}/^{14}\text{N}$ discrimination during N_2 fixation by cultured *Azotobacter* species in nitrogen-free media: Ratio of the rates of $^{14}\text{N}^{14}\text{N}$ and $^{15}\text{N}^{14}\text{N}$ fixa-

tion by several *Azotobacter* species was 1.000, which implicated that no $^{15}\text{N}/^{14}\text{N}$ discrimination occurred during the biological N_2 fixation. They discussed possible biochemical reasons of the un-expectedly insignificant $^{15}\text{N}/^{14}\text{N}$ discrimination. (3) Delwiche and Steyn (1970)⁴⁶⁾ reported that nodulating clover plants had a lower ^{15}N abundance than grass, and that the ^{15}N abundance of the grass was close to the soil ^{15}N abundance. Rennie et al. (1976)⁴⁷⁾ observed that barley plants had a higher ^{15}N abundance than fava bean plants, and they suggested that the fractional contribution of N_2 fixation in legumes may be estimated from the difference in the ^{15}N abundance between N_2 -fixing and non-fixing plants.

During the latter half of the 1960s and all of the 1970s, intensive measurement of the $\delta^{15}\text{N}$ values of various environment materials such as soils, soil nitrate/ammonium, atmospheric N compounds (N_2O , NH_3 , NO_3^-) and inorganic (ammonium, nitrate) and organic (urea, manure, composts) fertilizers were taken. It was found that the environment's nitrogen molecules contained mostly positive $\delta^{15}\text{N}$ values, indicating that a bacterial formation (by enzyme reactions) of nitrate from ammonium (nitrification) and N_2 and N_2O from nitrate (denitrification) induced a great $^{15}\text{N}/^{14}\text{N}$ discrimination; $\delta^{15}\text{N}$ values of the product became negative and the remaining substrate $\delta^{15}\text{N}$ values became positive compared to the initial $\delta^{15}\text{N}$ values of the source substrate, whereas there was insignificant $^{15}\text{N}/^{14}\text{N}$ discrimination during the microbial or industrial N_2 -fixation (reductive conversion of N_2 to NH_3). Original works by Miyake and Wada^{48, 49)} regarding the $\delta^{15}\text{N}$ values of the marine ecosystem gave important hints: (1) The $\delta^{15}\text{N}$ values of marine nitrate (approx. 6‰) were more enriched than those of terrestrial organisms, whose N is first available from rain or nitrogen fixation and low in $\delta^{15}\text{N}$ values and thereafter becomes enriched with ^{15}N due to nitrification-denitrification processes. (2) Trophic levels of the diet's chain accompany the

elevation of $\delta^{15}\text{N}$ values.

The $\delta^{15}\text{N}$ values in whole plants are determined depending on the two factors, (i) the $\delta^{15}\text{N}$ values of the source materials and (ii) the $^{15}\text{N}/^{14}\text{N}$ discrimination during nitrogen acquisition. The N sources include amino acids ($\delta^{15}\text{N}$ value = +2‰ to +8‰) released from plant, animal and microorganism residues, ammonia (+2‰ to +12‰) produced by mineralization from the amino acids, nitrate (+1‰ to +6‰) nitrified from ammonia, fixed nitrogen (−2‰ to −0.2‰) and for crops, inorganic chemical fertilizers (−1‰ to +2‰) and organic composts (+8‰ to +15‰). The nitrate in rain, ammonia from cattle barns, and nitrogen oxides from fuel combustion are partly absorbed through leaves and/or indirectly taken up after they are captured by the soil solution/particles.

We grew paddy rice under flooded conditions, and upland rice and sorghum under drained conditions with and without the application of ammonium sulfate (+0.3‰) in the three types of potted soils

(Toyosato alluvial, Yawara alluvial, and Tsukuba Andosols) with different $\delta^{15}\text{N}$ values for 75 days (Table 3). The $\delta^{15}\text{N}$ values of the nitrogen derived from different soils, which are the $\delta^{15}\text{N}$ values of the tops without fertilizer application, and the $\delta^{15}\text{N}$ values derived from chemical fertilizer in the fertilizer-applied pots were calculated from the ^{15}N mass-balance in the plants with and without chemical fertilizer application.⁵⁰⁾ The paddy rice plants took up the soil-derived ammonia (whose $\delta^{15}\text{N}$ values were varied between +4‰ and +6‰ and close to the original soil $\delta^{15}\text{N}$ values) in the pots without fertilizer application and the fertilizer-derived ammonia, whose $\delta^{15}\text{N}$ values were varied between +2‰ and +3‰ and higher than that of the original fertilizer $\delta^{15}\text{N}$ (+0.3‰).

The significant ^{15}N enrichment of the taken-up nitrogen might imply that the nitrogen may be the remained-N after the partial nitrification-denitrification of the fertilizer nitrogen. The upland rice and sorghum plants in the pots without fertilizer ap-

Table 3 Dry matter (DM, g/pot) and nitrogen accumulation (mg/pot) by paddy rice, upland rice, and sorghum plants and $\delta^{15}\text{N}$ values (‰) of soil derived N (without fertilizer) and fertilizer-derived N (with N fertilizer) using Toyosato alluvial (Ta), Yawara alluvial (Ya) and Tsukuba Andosols (TA). The $\delta^{15}\text{N}$ value of the fertilizer (ammonium sulfate) was +0.3‰

Plant	Soil	Without N fertilizer			With N fertilizer		
		DM	Nitrogen	$\delta^{15}\text{N}$ derived from soil	DM	Nitrogen	$\delta^{15}\text{N}$ derived from fertilizer
Paddy rice	Ta (+4.1)	3.7	34.1	+5.5	58.0	606.3	+1.8
	Ya (+2.8)	2.2	17.5	+3.6	49.1	614.5	+2.3
	TA (+8.0)	1.8	15.6	+5.9	30.1	566.3	+2.9
Upland rice	Ta (+4.1)	3.1	32.9	+1.4	10.3	259.1	+4.5
	Ya (+2.8)	2.1	17.9	−0.6	14.7	371.1	+0.8
	TA (+8.0)	0.8	9.0	+0.9	5.7	126.1	−0.6
Sorghum	Ta (+4.1)	6.9	33.3	+0.8	69.8	743.7	+1.8
	Ya (+2.8)	3.6	16.6	−1.0	41.5	545.6	+1.8
	TA (+8.0)	1.6	8.7	+1.9	4.6	128.3	−1.2

Adapted from Yoneyama et al. (1990).⁵⁰⁾

plication took up soil-derived ^{15}N -depleted nitrogen (whose $\delta^{15}\text{N}$ values were from -1‰ to $+2\text{‰}$) and lower than that of the $\delta^{15}\text{N}$ values of the original soils. The lower $\delta^{15}\text{N}$ values of the taken-up nitrogen might imply that the plants took up nitrate after the nitrification of the soil-derived ammonium. The upland rice and sorghum took up fertilizer-derived nitrogen, whose estimated $\delta^{15}\text{N}$ values were from -1‰ to $+5\text{‰}$. The variation of the taken-up nitrogen $\delta^{15}\text{N}$ values may indicate that the plant uptake of a mixture of ^{15}N -enriched ammonia and ^{15}N -depleted nitrate resulted from the partial nitrification rates depending on the soil types.

When tea plants,⁵¹⁾ vegetables,^{52, 53)} and paddy rice⁵⁴⁾ were grown in organic manure-amended soils, the $\delta^{15}\text{N}$ values of the shoots and rice grains were enriched in ^{15}N . This high $\delta^{15}\text{N}$ value revealed the major contribution of manure-derived nitrogen. The loss of N from soils, fertilizers, and composts to underground was also traced using the $\delta^{15}\text{N}$ tracing technique.⁵⁵⁾

When rice plants were grown in hydroponics with ammonium as a single source of nitrogen, the plant nitrogen contained ^{15}N -depleted $\delta^{15}\text{N}$ values compared to the $\delta^{15}\text{N}$ values of the initial hydroponic ammonium, although the $\delta^{15}\text{N}$ values of the shoots and roots were similar to each other, and the $\delta^{15}\text{N}$ values of the ammonium that remained in the hydroponics were enriched in ^{15}N , suggesting $^{15}\text{N}/^{14}\text{N}$ discrimination during the ammonium/ammonia uptake by the ammonia uptake transporter.^{56, 57)} The ammonia taken up into the root cells is assimilated to the amide of glutamine by glutamine synthetase. During this process, when isolated spinach leaf GS2 was employed *in vivo*, $^{15}\text{N}/^{14}\text{N}$ discrimination of approx. -16.5‰ was observed.⁵⁸⁾

Plants take up also nitrate by nitrate transporters and assimilate to ammonia by a tight series of nitrate and nitrite reductases. The uptake of nitrate by pearl millet,⁵⁹⁾ a leafy vegetable,⁶⁰⁾ and rice plants⁵⁷⁾

causes small $^{15}\text{N}/^{14}\text{N}$ discrimination. The nitrate reduction to ammonia/amino acids (the limiting step is nitrate reductase reaction) caused a large $^{15}\text{N}/^{14}\text{N}$ discrimination—as much as 15‰ in spinach leaf extract *in vitro*⁶¹⁾ and 18 to 21‰ in leaf blade and 9 to 17‰ in the petioles and midribs *in vivo* of a vegetable⁶⁰⁾ resulting in ^{15}N depletion in the products ammonia and amino acids and ^{15}N enrichment in the remaining nitrate.

During N_2 fixation by the symbiotic system of legumes and nodule-resided rhizobia, the $^{15}\text{N}/^{14}\text{N}$ discrimination were small as much as -2‰ to -0.2‰ .⁶²⁾ Interestingly, the $\delta^{15}\text{N}$ values of soybean, kidney bean, winged bean, pea and fava bean nodules were enriched by as much as $+3\text{‰}$ to $+9\text{‰}$, whereas the leaf values were depleted by as much as -2‰ .^{62, 63)} In contrast, the $\delta^{15}\text{N}$ values of white clover, common vetch, Chinese milk vetch, and alfalfa nodules were not enriched at all and their leaf values were -1‰ to -0.4‰ .^{1, 62)} The enrichment in the nodules of soybean, cowpea, mungbean and swordbean occurred at the bacteroids, the N_2 -fixing site,^{64, 65)} and the ^{15}N enrichment in the nodules of pea and fava bean was observed at the nodule cytosol, the site of fixed-N assimilation.^{66, 67)} The most ^{15}N -enriched molecule ($\delta^{15}\text{N}$ value = $+40\text{‰}$) in the nodules, determined by GC/C/MS, was identified as homospermidine, a nodule-specific polyamine.⁶⁸⁾ Such ^{15}N -enrichment in the symbiotic organ and ^{15}N -depletion in the plant-side foliar parts were also found in the mycorrhiza-plant system.^{69, 70)}

The $\delta^{15}\text{N}$ values of individual amino acids, polyamines, and other N-containing molecules showed varied signatures.^{68, 71)} Determining the origin and product relations and different pathways are complex but probably highly regulated, like a puzzle. Between source and sink organs, N-containing compounds are transported *via* the phloem and xylem, targeting the growing organs and storages like seeds and tubers. The $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ values of phloem sap

were close to those of seeds in wheat²⁴⁾ and castor bean plants.²⁵⁾

After these studies by our group, increased attention was paid to $^{15}\text{N}/^{14}\text{N}$ discrimination during plant nitrogen metabolism by focusing on individual molecules/metabolites^{72, 73)} and enzyme reactions,^{72, 74)} and the following developments occurred: (1) The determination of $\delta^{15}\text{N}$ values of individual amino acids has been extended with investigations into the relationship of amino acid $\delta^{15}\text{N}$ values between the environment (soils) and several species of plants.⁷⁵⁾

(2) The $^{15}\text{N}/^{14}\text{N}$ discrimination (approx. 15‰) during uptake and assimilation of nitrate by plants (e.g., Yoneyama and Kaneko, 1989⁶⁰⁾) were theoretically examined by Robinson et al. (1998)⁷⁶⁾; the site of their $^{15}\text{N}/^{14}\text{N}$ discrimination was at the branched pathway as the nitrate in the roots is partially reduced and incorporated into root protein amino acids whose $\delta^{15}\text{N}$ values are ^{15}N -depleted compared to that of the original nitrate, and the unreduced nitrate, whose $\delta^{15}\text{N}$ value is enriched in ^{15}N , is transported to the shoot. Thus, the reduction and assimilation of nitrate occur in the leaves and the ^{15}N -depleted amino acids are transported to growing organs and roots *via* the phloem.⁷⁷⁾ In a report by Wilson et al. (2011)⁷⁸⁾ on the low $\delta^{15}\text{N}$ values of aphids, those authors hypothesized that in aphid-colonized plants, the plant nitrate reductase activity might be enhanced, resulting in an increase in the plant $\delta^{15}\text{N}$ values and a decrease in the aphid-diet amino acids' $\delta^{15}\text{N}$ values.

(3) In soybean and cowpea plants, the ^{15}N -enrichment in the nodulated roots is closely related to the ^{15}N -depletion in the shoots, and as such the extent of the $\delta^{15}\text{N}$ difference between the nodulated roots and shoots can be an index of the N use efficiency of fixed N (equivalent to the contribution to the plant N accumulation, %Ndfa, percent nitrogen derived from the atmosphere).^{79, 80)} In this method, the parallel cultivation of non-nodulating reference plants is not required.

(4) In the leaf cell mitochondria, the glycine decarboxylase reaction ($2 \text{ glycine} \rightarrow \text{serine} + \text{NH}_3 + \text{CO}_2$) involved in photorespiratory metabolism produces NH_3 at the rate of 10 times the rate of nitrate reduction.⁸¹⁾ The $^{15}\text{N}/^{14}\text{N}$ discrimination of the ammonia released from the stomata was predicted to be as much as -18% .⁸²⁾ The NH_3 is largely re-assimilated to glutamine (amido) by chloroplast glutamine synthetase, which use ATP as the driving force and causes -16.5% discrimination.⁵⁸⁾ More detailed searches are required for the isotopic discrimination of $^{15}\text{N}/^{14}\text{N}$ and $^{13}\text{C}/^{12}\text{C}$ during photorespiratory metabolism.

5. Ecosystem studies of symbiosis using $\delta^{15}\text{N}$ values as an indicator: N_2 fixation and mycorrhizal association

In various ecosystems, plant physiology adapts to the availability of nitrogen in the environment. Although it consumes much energy (i.e., photosynthates), the biological N_2 fixation is an efficient source, if the plant's demand is very high (for rapid growth) but reduced nitrogen in its environment is scant. When the nitrogen of plant tops contains $\delta^{15}\text{N}$ values close to -2% to -0.2% , the nitrogen totally originates from fixed N. The nitrogen originates totally from soil nitrogen, the plant $\delta^{15}\text{N}$ values would be close to $+4\%$ to $+8\%$, which can be precisely determined using non N_2 -fixing plants as the reference. From the $\delta^{15}\text{N}$ values of the supposing N_2 -fixing plants, we can determine the possible proportion of fixed N (%Ndfa) in N_2 -fixing plants. These N_2 -fixing and non-fixing reference plants are harvested at sites remote from the δ values-measuring laboratories.^{83–85)}

Estimates of the percentages of N derived from fixed N (%Ndfa) in soybean grains at 52 sites in Japan were $52 \pm 26\%$.⁸⁶⁾ Such %Ndfa of groundnuts grown with N-fertilizer (urea) treatments at 0 to 200 kg ha^{-1} in India were 85 to 70%,⁸⁷⁾ and those of

pigeonpeas harvested in India were 44 to 63% in sole cropping and 65 to 86% in the intercropping with sorghum.⁸⁸⁾ We applied this $\delta^{15}\text{N}$ method to measure the %Ndfa in sugarcane plants harvested at many sites in Brazil, the Philippines and Miyako Island (Japan)⁸⁹⁾ and in sweet potato plants harvested at Tsukuba (Japan)⁹⁰⁾; both non-nodule-forming plants might gain fixed N by as much as 0 to 70% of the total plant N depending on sites and cultivars. After 15-years long molecular investigations, we successfully identified the possible candidates of endophytic free-living N_2 -fixing rhizobia habiting in the plant tissues such as sugarcane stems⁹¹⁾ and sweet potato petioles.⁹²⁾

Wild plants, whose roots form the symbiotic system with arbuscular mycorrhiza, show a preference for the uptake of ammonia and amino acids, which are released from decomposing plant residues and whose $\delta^{15}\text{N}$ values are higher than that of the nitrate produced from them by nitrification. Therefore, arbuscular mycorrhiza-infected plants have $\delta^{15}\text{N}$ values, higher than those of non-infected plants,⁹³⁾ but other types of fungi, i.e., ectomycorrhizal and saprotrophic fungi, do not follow this rule.^{69, 70)} If N_2 -fixing legumes form the hyphal network by arbuscular mycorrhiza with other N-deficient weeds, some ^{15}N -depleted fixed-N can be transferred from legumes to neighboring plants—as much as 30 to 80% of the weed N.⁹⁴⁾

Our investigations of the $\delta^{15}\text{N}$ values in leguminous and non-leguminous plants in Thailand and Brazil indicated that the $\delta^{15}\text{N}$ values of leguminous trees other than shrub trees were close to those of non-leguminous trees, indicating only small N_2 -fixation in humid tropics.⁹⁵⁾ A hypothesis proposed by Martinelli et al. (1999)⁹⁶⁾ stated that tree leaves' $\delta^{15}\text{N}$ values in ^{15}N -enriched tropical and ^{15}N -depleted temperate ecosystems suggest that temperate ecosystems under a closed N cycle system have depleted N availability for trees, whereas humid tropical

ecosystems, which provide open N cycle system, are saturated with nitrogen so that the mature tropical ecosystem and plants including leguminous trees do not require extra nitrogen through biological N_2 fixation. The deficient element in the humid tropical forests could be phosphorus but not nitrogen. An estimate of annual input through biological N_2 fixation to high-N and low-P available neotropical rainforests in Guyana was approx. 6% of the total forest N.⁹⁷⁾

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要 旨

安定同位体利用技術

1981年から2000年の間に植物の代謝における $^{13}\text{C}/^{12}\text{C}$, $^{15}\text{N}/^{14}\text{N}$ 同位体分別の研究をどう展開したか、その後どう展開されたか？

米山忠克^{*, **, †}: *農業・食品産業技術総合研究機構フェロー, 305-8666 茨城県つくば市観音台3-1-1, **東京大学名誉教授, 113-8657 東京都文京区弥生1-1-1, [†]tadakatsu_yoneyama@opal.ocn.ne.jp

1981年から2000年の筆者の $^{13}\text{C}/^{12}\text{C}$, $^{15}\text{N}/^{14}\text{N}$ 同位体

分別の研究では植物組織や代謝物など多数試料の精密分析を行った。成果は：(1)植物組織や篩管液の $\delta^{13}\text{C}$ 値とそれらのN獲得や乾燥などによる変動, (2)植物による窒素の獲得, 代謝, 体内移行での $^{15}\text{N}/^{14}\text{N}$ 同位体分別, (3) $\delta^{13}\text{C}$ と $\delta^{15}\text{N}$ トレーサー法の生態学的適用；日本のクロボク土壌生成やタイとフィリピン[†]の森林からサトウキビ土壌への移行における植物残渣由来Cの集積と代謝の解析ならびに植物における各種窒素源からの吸収量の推定である。これらの研究がその後どのように展開されたかをレビューした。