

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

4,800

Open access books available

122,000

International authors and editors

135M

Downloads

Our authors are among the

154

Countries delivered to

TOP 1%

most cited scientists

12.2%

Contributors from top 500 universities



WEB OF SCIENCE™

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

Interested in publishing with us?  
Contact [book.department@intechopen.com](mailto:book.department@intechopen.com)

Numbers displayed above are based on latest data collected.  
For more information visit [www.intechopen.com](http://www.intechopen.com)



---

# Nitrogen Fixation in Sugarcane

---

Takuji Ohyama, Atsushi Momose, Norikuni Ohtake,  
Kuni Sueyoshi, Takashi Sato, Yasuhiro Nakanishi,  
Constancio A. Asis Jr., Soraya Ruamsungsri and  
Shotaro Ando

Additional information is available at the end of the chapter

<http://dx.doi.org/10.5772/56993>

---

## 1. Introduction

### 1.1. Biological nitrogen fixation by endophytic diazotrophic bacteria

Nitrogen (N) is a major essential element for all organisms, and generally the amount of available N (mainly inorganic nitrogen such as nitrate or ammonia) in soil is limiting factor for natural and agricultural plant production [40]. Biological nitrogen fixation (BNF) is a process by which atmospheric dinitrogen ( $N_2$ ) is reduced into 2 molecules of ammonia ( $NH_3$ ) by the enzyme nitrogenase with  $8H^+$ ,  $8e^-$  and 16 Mg ATP. BNF have important role in N cycle in both global ecosystem and agro-ecosystem. Based on the data compiled by Bezdicsek and Kennedy in 1988 [11], about 175 million metric tons of nitrogen per year is estimated to be fixed in global ecosystems, in which 90 million metric tones in agricultural land, 50 million metric tones in forest and non-agricultural land, and 35 million metric tones in sea. At that time, non-biological nitrogen fixation was estimated about 50 million metric tones per year by industrial nitrogen fixation mainly for the synthesis of ammonia fertilizer, and about 20 million metric tones by combustion, and about 10 million metric tones by lightening. In 2009, the production of N fertilizers increased to 106 million metric tones (FAOSTAT), but the amount of BNF still exceeds over non-biological nitrogen fixation.

Historically, the first experimental data to show the BNF by legumes was obtained by Boussingault (from [24]). He began crop rotation experiment including clover (legumes) in 1837, and the result clearly indicated the increase in nitrogen content was associated with cultivation of clover. Hellriegel and Wilfarth demonstrated the bacterial nitrogen fixation in

association with legumes in 1888 (from [24]). Beijerinck isolated and cultivated the bacteria from legume nodules, and then he succeeded to inoculate the isolated bacteria to the uninfected host legume plant. In 1893, Winogradsky found that the free-living strictly anaerobic bacterium, *Clostridium pasteurianum*, could fix  $N_2$  (from [24]). Beijerinck showed that the aerobic bacteria, *Azotobacter agilis* and *A. chroococcum*, could also fix  $N_2$ . Now a vast genus of bacteria (Green sulfur bacteria, Thallobacteria, Heliobacteria, Cyanobacteria, Campylobacter, Proteobacteria), and Archaea can fix  $N_2$  [56].

Several types of nitrogen fixing bacteria are recognized. First is “symbiotic bacteria”, such as *Rhizobium*, associated with leguminous plants, and frankia or cyanobacteria with non-legume plants. The second type is “free-living” (non-symbiotic) bacteria, including the cyanobacteria (or blue-green algae) *Anabaena* and *Nostoc* and such genera as *Azotobacter*, *Beijerinckia*, and *Clostridium* living in soil or water. The third type resides around the plant roots (rhizosphere) and provides fixed nitrogen to the plant. This is called “associative nitrogen fixation”. Fourth is “endophytic nitrogen fixation” associated with cereal grasses such as sugarcane.

## 2. Characteristics of sugarcane

Sugarcane (*Saccharum* spp. hybrid) is a tall, perennial grass (family Poaceae, subfamily Panicoide), and is cultivated in tropical and warm-temperate regions between 35°N and 35°S and from sea level to altitudes of 1,000 m in a wide variety of soil types [47]. Most of commercial sugarcane varieties are hybrids with *Saccharum officinarum* [1]. The optimal temperature for sugarcane cultivation is between 20 and 35 °C and the minimum rainfall requirement is 1,200mm per year [1]. The stalks (stems) of sugarcane are harvested at 9 to 18 months after planting the mother stem cutting (setts). Once planted, sugarcane can be harvested several times, because new stalks, called ratoons, repeatedly grow from the stubble. For many years, sugarcane has been used for sugar and an alcoholic drink production. Recently, the use of sugarcane alcohol (ethanol) as an automotive fuel to replace gasoline has rapidly increased [16, 35]. At the moment, sugarcane is the most economically and environmentally advantageous crop for bio-ethanol production.

In 2011, world production of sugarcane was 1,794 million tons (FAOSTAT). This is much greater than for the other major crops such as maize (883 million tons), paddy rice (723 million tons), wheat (704 million tons) and potatoes (374 million tons). Sugarcane production is highest in Brazil (734 million tons), followed by India (342 million tons), and China (115 million tons). In 2011, sugarcane was cropped over an area of 25 million hectares. Sugarcane is a C4 plant, which has an efficient photosynthetic system, and it can convert up to 2% of incident solar energy into biomass. In 2011, the average yield was 70.5 tons per hectare. It grows up to 2-6 m in height (Figure 1) and the thick stalks (stems) store a high concentration of sucrose accumulated in stalk internodes, and the expressed stalk juice contains sucrose concentration at between 12 and 20% (W/V).



**Figure 1.** Growth of sugarcane (left), and sugarcane mother stalk for planting (right).

### 3. Contribution of biological nitrogen fixation in sugarcane cultivation

In Brazil, sugarcane crops accumulate N between 100 and 200 kg N per hectare per year, while N fertilization rates are relatively low, usually less than 60 kg N per hectare [47]. Also, the response of sugarcane crops to N fertilizers is usually very weak [12,47]. The use of low N fertilizer input was justified by the results of 135 field experiments in all of main cane-growing areas of Brazil, with only 19% of the studies showing a significant increase in yield owing to the application of N fertilizer [6]. In some sites of Brazil, sugarcane has been grown continuously for more than 100 years without any N fertilizer being applied at all [20]. This circumstantial evidence suggests a high potential for BNF in sugarcane.

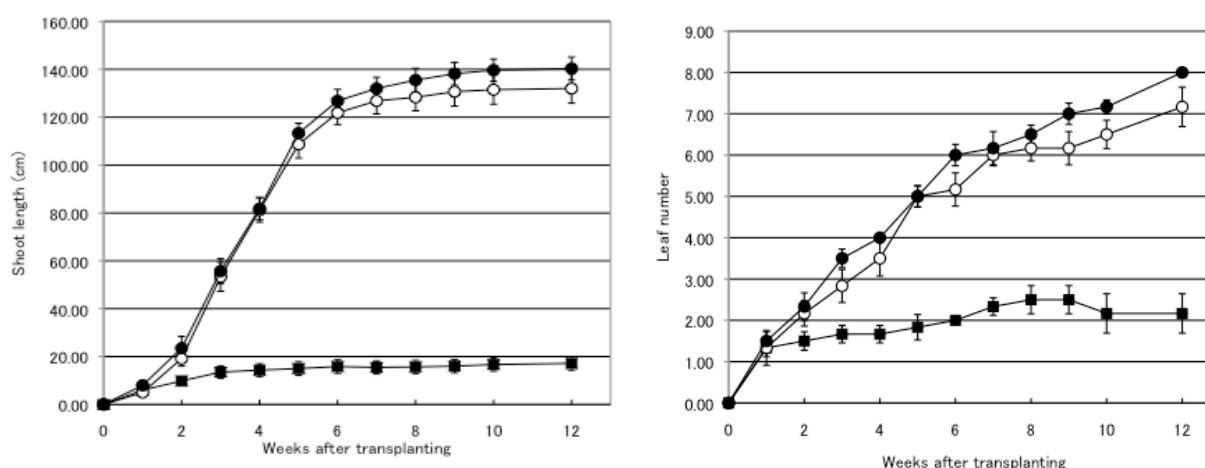
### 4. Estimation of contribution of biological nitrogen fixation by $^{15}\text{N}$ dilution technique and $^{15}\text{N}$ natural abundance method

Using a  $^{15}\text{N}$  dilution technique involving the supply of a  $^{15}\text{N}$ -labeled fertilizer, Lima *et al.* [33] evaluated the contribution of BNF in four sugarcane varieties planted in large-size pots and concrete tanks. The result showed that 40-60% of plant N was derived from BNF in the variety CB 47-89. Urquiaga *et al.* [54] also calculated the contribution of BNF in several cultivars of sugarcane, and found it to be about 70% for the most promising genotypes. Again, using the  $^{15}\text{N}$  natural-abundance method, Boddey *et al.* [13] showed that 25-60% of the N assimilated in sugarcane at various sites in Brazil was derived from BNF. The analysis showed very large BNF-inputs to several sugarcane varieties, especially the wild non-commercial species Krakatau (*S. spontaneum*) used in plant breeding in Brazil, as well as the commercial varieties SP 70-1143 and CB 45-3 in low-fertility soils. Yoneyama *et al.* [57] examined the contribution of BNF using a  $^{15}\text{N}$  natural-abundance method in Brazil, the Philippines and Japan, comparing the abundance of  $^{15}\text{N}$  in sugarcane with that in neighboring weeds as control plants. At many but not in all of the sites in Brazil, a contribution from BNF was indicated.

Asis *et al.* [5] estimated the contribution of nitrogen fixation of Japanese sugarcane cultivar NiF8 by  $^{15}\text{N}$  dilution and natural  $^{15}\text{N}$  abundance techniques, and total %Ndfa (percentage of N derived from atmospheric dinitrogen) was estimated to be 27-38%. They also reported that the estimated the %Ndfa was 26% for the roots, 14.1% for the stem and 20.5% for the leaves. Nishiguchi *et al.* [38] estimated the contribution of BNF using the  $^{15}\text{N}$  dilution in Japanese varieties of sugarcane, and found that the percentage between 10% and 40% of sugarcane N was derived from BNF depending on the cultivar and also on the availability of mineral N.

In Niigata, a pot experiment was conducted to estimate the contribution of nitrogen fixation in sugarcane (*Saccharum* spp. var. NiF8) plants using  $^{15}\text{N}$  dilution method in relation to N supply period [26]. Sugarcane plants were grown from a cut stalk in water for 20 days (Figure 1) and a young shoot was transplanted to the 1/5000 a Wagner pot filled with vermiculite. Three fertilizer treatments were applied; 1) N0: Nitrogen free culture solution was supplied, 2) N100:  $^{15}\text{N}$  labeled ammonium sulfate was continuously supplied at the rate of 100 mgN per pot a week, 3) N100N0:  $^{15}\text{N}$  labeled ammonium sulfate was supplied at the rate of 100 mgN per pot a week until 6 weeks after transplanting (WAT), then the plants were cultivated with N free solution. The growth of the plants was measured every week, and plants were harvested at 12 WAT and 20 WAT. Total N content and  $^{15}\text{N}$  abundance in each part were determined.

Figure 2 shows the changes in the shoot length and leaf number of each treatment for 12 weeks after transplanting (WAT). The N0 plants grew very poor, and sole nitrogen fixation is not enough to support vigorous growth of sugarcane NiF8 cultivated in Niigata. The N100 plants and N100N0 plants showed relatively similar shoot length and leaf number, but the N100N0 plants leaves were pale compared with the N100 plants. At 12 WAT, total N content was 408 mgN and 286 mgN per plant in N100 treatment and N100N0 treatment, respectively. At 20 WAT, total N content was 569 mgN (N100) and 292mgN (N100N0).



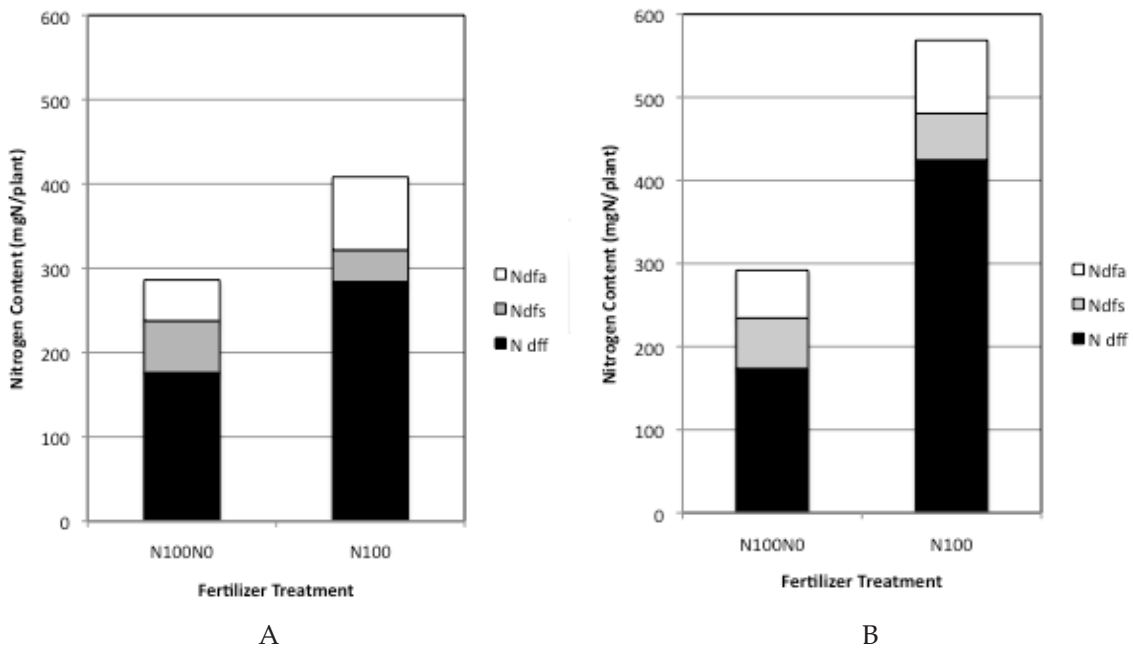
**Figure 2.** Shoot length and leaf number of sugarcane with N0 (■), N100 (●), and N100N0 (○) fertilizer treatments.

Figure 3 shows the amount of N derived from three sources of N; Ndfa (N derived from nitrogen fixation), Ndfs (N derived from a mother stalk), and NDff (N derived from fertilizer). The amount of Ndfa in N100 and N100N0 was 87 mgN (21%Ndfa) and 48 mgN (17%Ndfa) per plant respectively. At 20 WAT, the amount of Ndfa was 87 mg (15%Ndfa) in N100 and 57

mgN (20%Ndfa) in N100N0 treatment. Among organs, the estimated %Ndfa tended to be higher in old leaves and stalk, and lower in green leaves and stems (Table 1). From this experiment, the continuous supply of N fertilizer did not inhibit nitrogen fixation in sugarcane compared with N deficient plants.

12WAT		Percentage of Nitrogen (%)			20WAT		Percentage of Nitrogen (%)		
		N dff	Ndfs	Ndfa			N dff	Ndfs	Ndfa
Green Leaves	N100N0	66	21	13	Green Leaves	N100N0	62	20	18
	N100	73	10	17		N100	74	10	16
Old Leaves	N100N0	43	22	35	Old Leaves	N100N0	52	21	28
	N100	39	10	51		N100	65	10	25
Stem	N100N0	68	22	10	Stem	N100N0	66	21	13
	N100	80	8	12		N100	81	10	10
Stalk	N100N0	39	22	39	Stalk	N100N0	34	30	35
	N100	43	9	48		N100	66	9	24
Root	N100N0	57	21	21	Root	N100N0	55	19	26
	N100	60	9	31		N100	74	10	15
Total	N100N0	62	21	17	Total	N100N0	59	21	20
	N100	70	9	21		N100	75	10	15

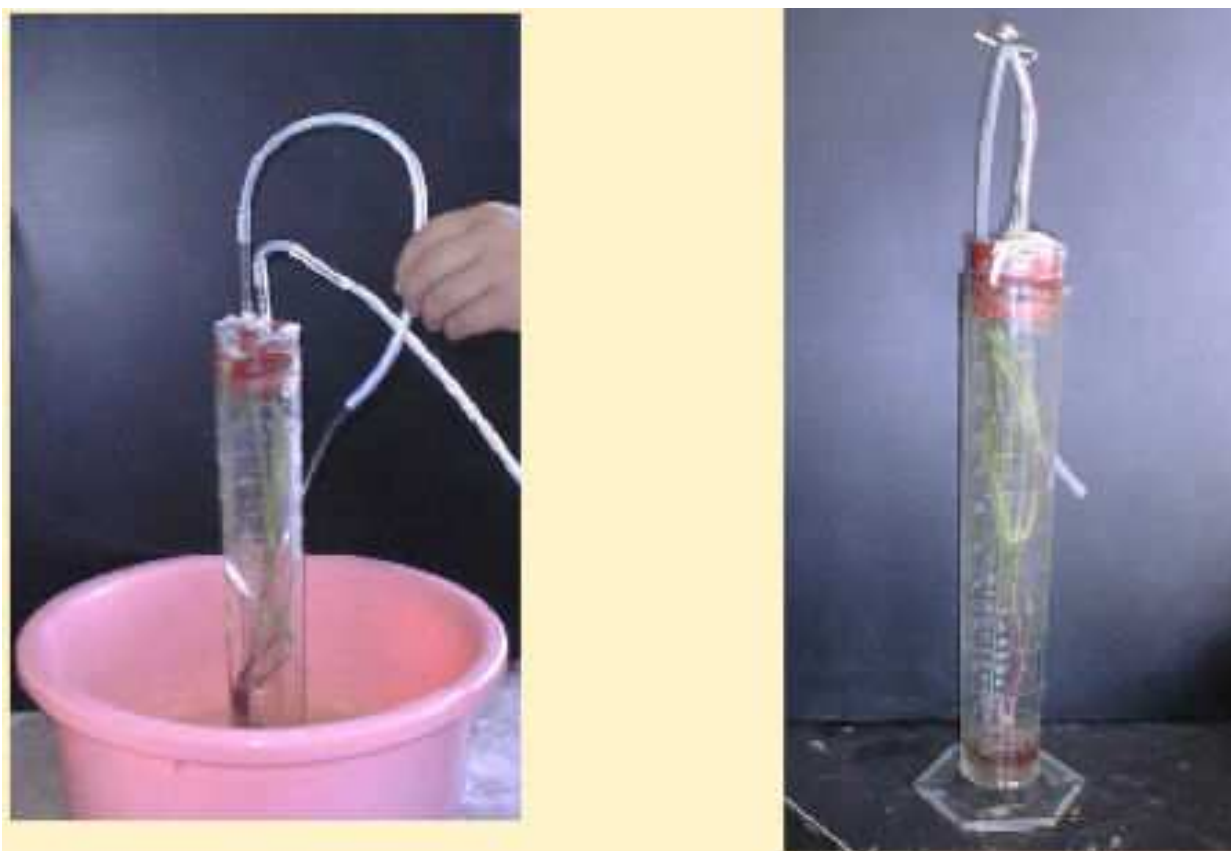
**Table 1.** Percentage of nitrogen derived from fertilizer (%Ndff), from stalk (%Ndfs), and atmospheric nitrogen (%Ndfa) at 12 WAT and 20WAT.



**Figure 3.** Nitrogen content in sugarcane plants at 12 WAP (A) and 20WAP (B).

## 5. $^{15}\text{N}_2$ fixation studies in sugarcane

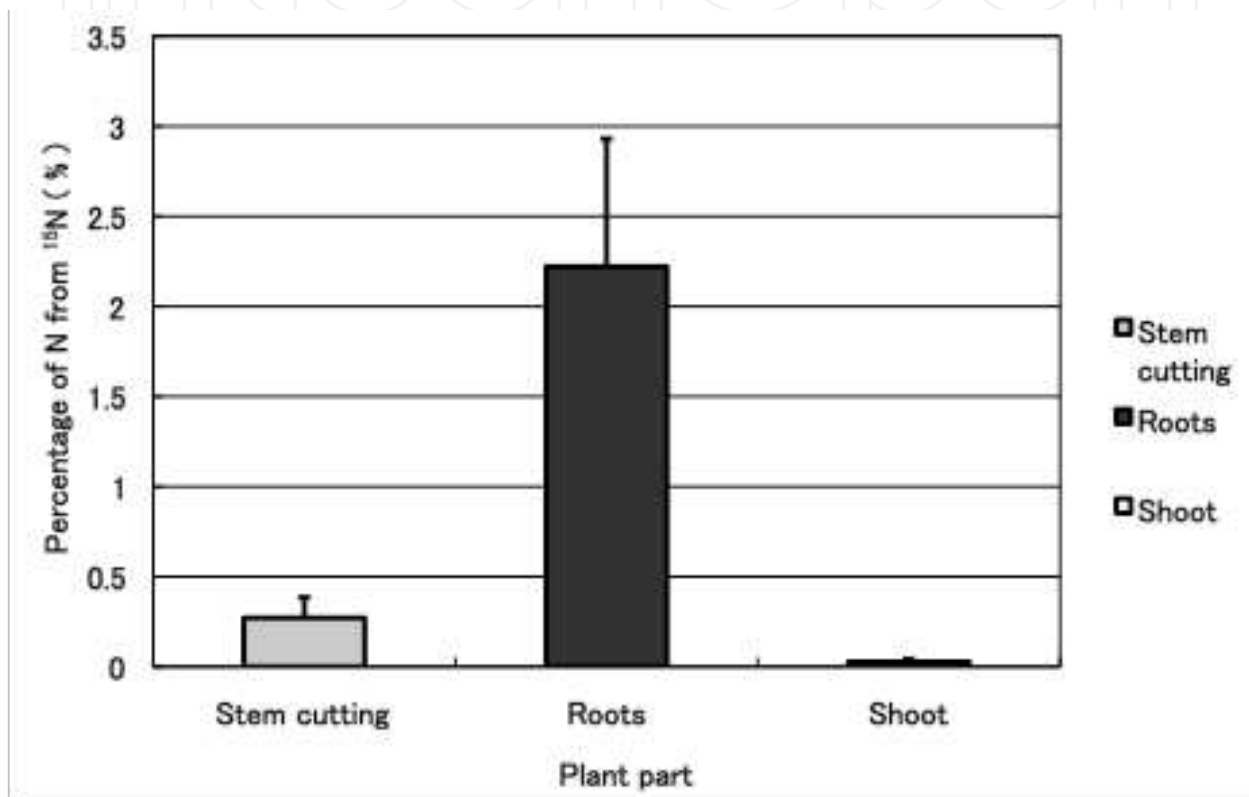
Both  $^{15}\text{N}$  dilution method and  $^{15}\text{N}$  natural-abundance method are indirect methods for estimating nitrogen fixation. Direct evidence can be obtained by  $^{15}\text{N}_2$  tracer experiment. In earlier studies, Ruschel *et al.* [48] exposed soil-grown 60 or 90-day-old sugarcane plants to  $^{15}\text{N}_2$  under a low oxygen ( $\text{O}_2$ ) concentration (2%), and near normal  $\text{O}_2$  concentration (16%). Significant incorporation of  $^{15}\text{N}$  was detected in roots and shoots of intact plants only under the lower  $\text{O}_2$  concentration (2%). In addition, the detached roots fixed  $^{15}\text{N}_2$  under 2%  $\text{O}_2$ , but  $^{15}\text{N}_2$  fixation was not detected in the detached shoots. Based on these results, they suggested that the site of fixation is within the roots and not in the shoots but that the fixed N is rapidly translocated to the shoot within 40 hours. Again working with sugarcane, Sevilla *et al.* [50] compared plants inoculated with wild-type *Acetobacter diazotrophicus* strain PA15, with plants inoculated with a  $\text{Nif}^-$  mutant of PA15, and with un-inoculated (sterile) plants. Fixation of  $^{15}\text{N}_2$  was observed only in the shoots and roots of the plants inoculated with the wild-type  $\text{Nif}^+$  PA15.



**Figure 4.** Exposure of a sugarcane plant to  $^{15}\text{N}_2$ . (Left)  $^{15}\text{N}_2$  gas is introduced to the cylinder displacing the nutrient solution. (Right) The plant is exposed to  $^{15}\text{N}_2$  under controlled light and temperature conditions.

Momose *et al.* reported on the nitrogen fixation and translocation in young sugarcane plants associated with endophytic nitrogen-fixing bacteria [36]. The tracer  $^{15}\text{N}_2$  was used to investi-

gate the sites of  $N_2$  fixation and the possible translocation of the fixed N. Young sugarcane plants from a stem cutting were exposed to  $^{15}N_2$ -labeled air in a 500 mL plastic cylinder (Figure 4). After 3 days of  $^{15}N_2$  feeding, the percentage of N derived from  $^{15}N_2$  was higher in the roots (2.22 %Ndfa) and stem cutting (0.271 % Ndfa) than the shoot (0.027% Ndfa) (Figure 5). At day-3, the stem cutting showed the highest quantity of fixed nitrogen per plant (about  $38 \mu g^{15}N$ ), followed by the roots ( $13 \mu g^{15}N$ ) and shoots ( $2 \mu g^{15}N$ ). The large amount of  $^{15}N$  in the stem cutting is due to the much greater N content of the cutting than young shoot and roots in the young plants at this stage.

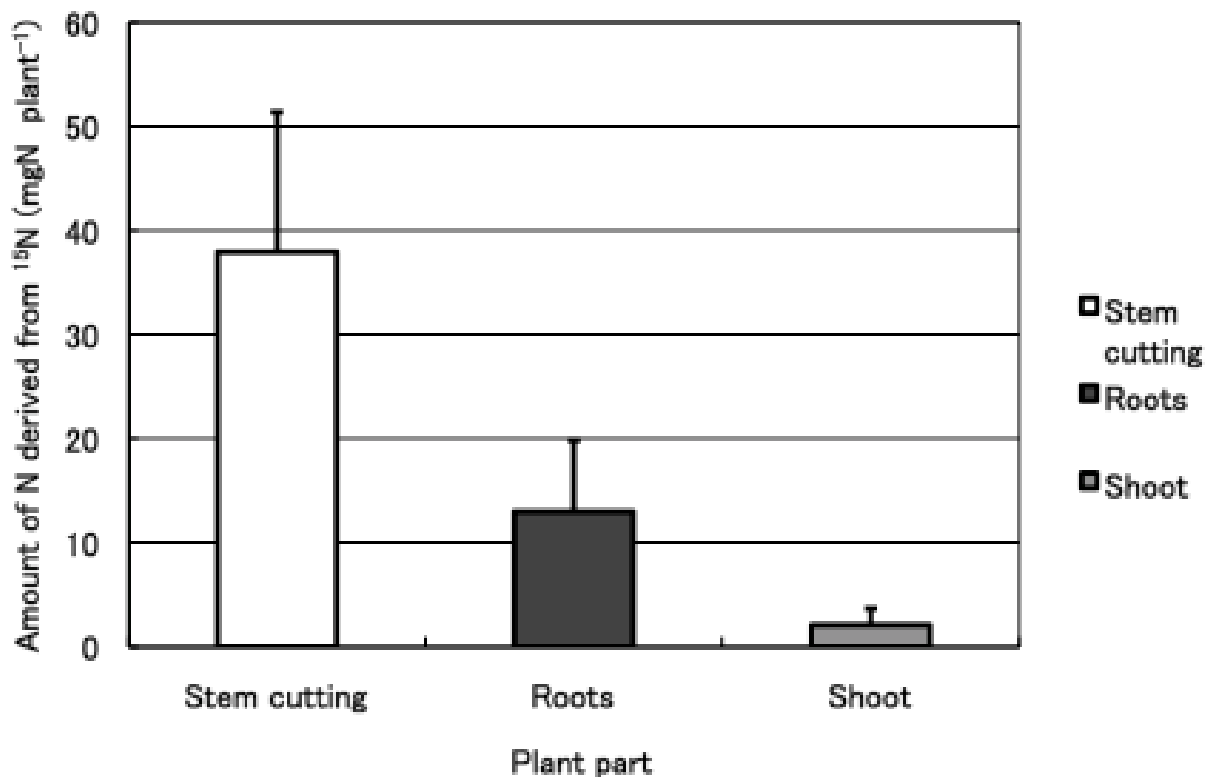


**Figure 5.** The fraction of  $^{15}N$  (expressed as a % of total N) fixed from  $^{15}N$ -labeled gas in the stem cutting, roots and shoot of sugarcane plants after three days of exposure (average with standard deviation).

The plants exposed to  $^{15}N_2$  for 7 days were grown in normal air for a further chase period. After 21 days, about half of the N originating in the stem cutting had been transported to the shoot and roots, suggesting that the cutting played a role in supplying N for the growth of shoot and roots (Figure 7). Most of the fixed N was distributed in the 80 % ethanol-insoluble fractions in each plant part, and the  $^{15}N$  fixed either in the roots or in the stem cutting remained there and was not appreciably transported to the shoot (Figure 8). The results were quite different from the fate of fixed N in soybean nodules, which is rapidly transported from nodules to roots and shoots.

From the results obtained in this experiment with young sugarcane plants, it is confirmed that the roots are the most active site of  $N_2$  fixation followed by the stem cutting. The sugarcane



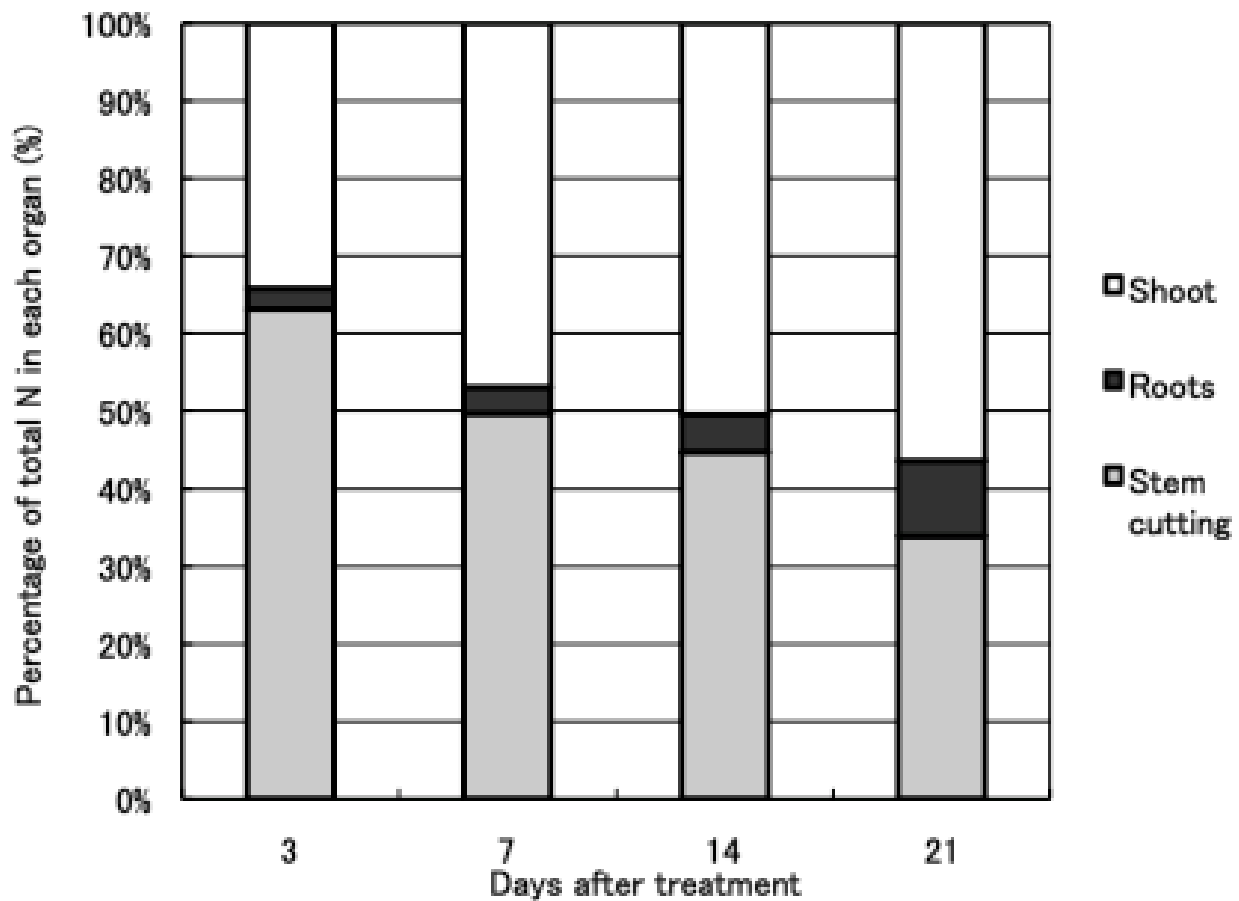


**Figure 6.** The mass of  $^{15}\text{N}$  ( $\mu\text{g}$ ) in the stem cutting, roots and shoot of sugarcane plants exposed to  $^{15}\text{N}_2$  for three days (average with standard deviation).

cuttings were initially cultured in water not in soil, so the  $\text{N}_2$ -fixing endophytes in the roots might originate from the stem cutting or root primordia. If this is the case, to support active  $\text{N}_2$  fixation, nitrogen-fixing bacteria may move into the developing roots, and colonize the intercellular space in the roots.

In an earlier study with NiF8 sugarcane using a  $^{15}\text{N}$  dilution technique [5], after 5 months of cultivation in a pot supplied with  $^{15}\text{N}$ -labeled mineral fertilizer, it was estimated that the roots contributed greater proportions of BNF (26%) than the stem (14%) and leaves (21%). Compared with the stem and shoot, the roots offer certain advantages as sites of  $\text{N}_2$  fixation. These are first that the host plant provides carbohydrates to the root endophytes, and second that oxygen concentrations are usually lower in the roots and soil than in the atmosphere ( $p\text{O}_2$  0.21). Low  $p\text{O}_2$  is beneficial to protect nitrogenase from oxygen damage, and for optimum nitrogen fixation activity.

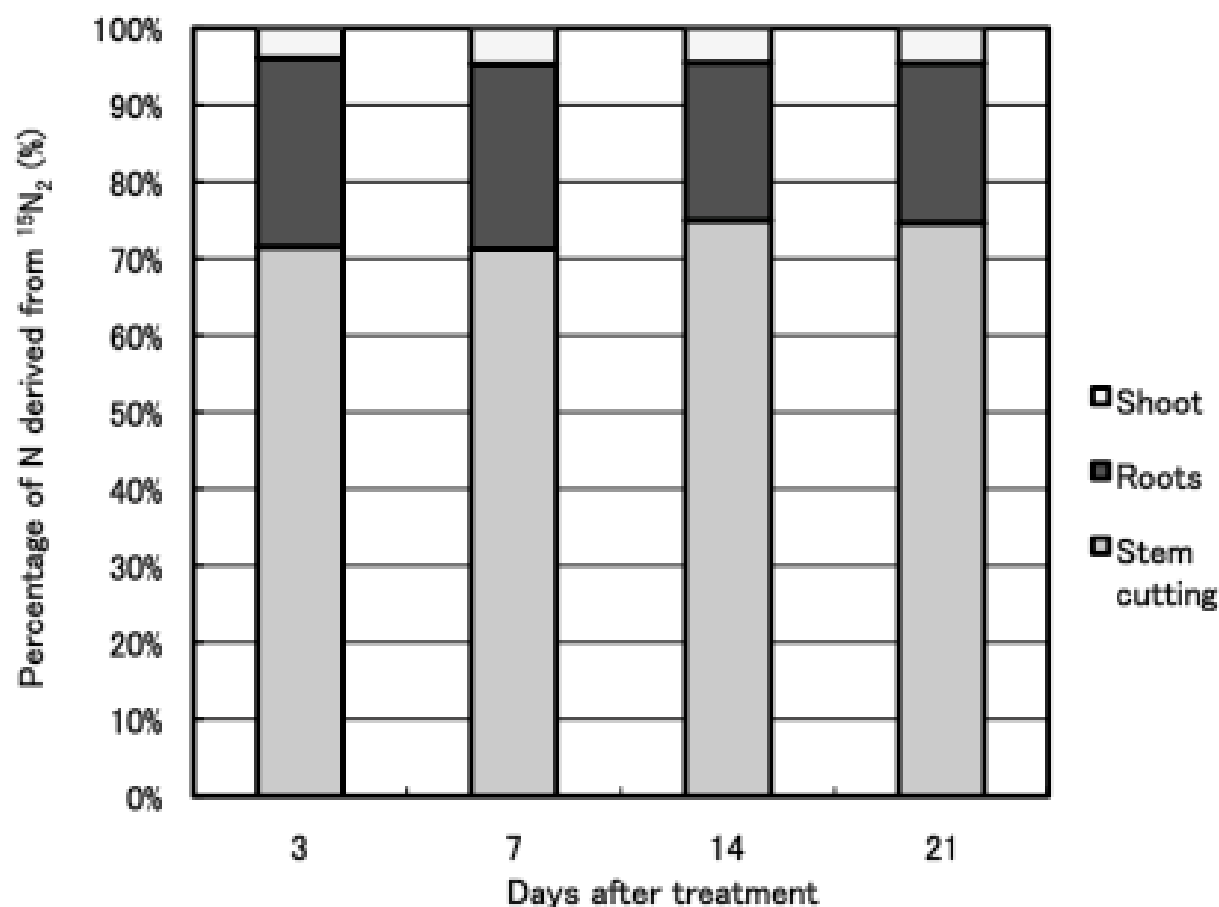
The fixation of nitrogen in stem cuttings of sugarcane is probably due to the activity of endophytes. Because very young sugarcane plants were used in our experiments, the finding of extremely limited  $\text{N}_2$  fixing activity in the shoots should not necessarily be taken to imply an insignificant stem endophytic contribution to the N economies of mature field-grown sugarcane plants. Many sugarcane endophytes (e.g. *Gluconacetobacter diazotrophicus*) are adapted to high sucrose concentrations while our very young shoots did not have mature stems that offered these conditions. However, there are some evidences that BNF in sugarcane stems



**Figure 7.** Changes with time in the fractional (%) distribution of total N ( $^{14}\text{N}+^{15}\text{N}$ ) in the stem, roots and shoot of sugarcane plants exposed to  $^{15}\text{N}_2$  for 7 days.

are very low. Zakria *et al.* reported that  $^{15}\text{N}_2$  was exposed to matured sugarcane stem with vacuum infiltration, the  $^{15}\text{N}$  incorporation was not observed in non-inoculated stems [59]. Ando *et al.* [2] analyzed *nifH* genes, and they could not detect the *nifH* gene with *G. diazotrophicus*.

The absence of any significant translocation of fixed  $^{15}\text{N}$ , most of it remaining in the 80% ethanol-insoluble fraction, in young sugarcane plants supports the possibility that fixed N may be used after the disintegration of dead endophytes, rather than being rapidly transported to the host plant as in the case of soybean nodules [44, 45]. However, it does not mean that there is no contribution of  $\text{N}_2$  fixation in the roots to shoot growth. When the  $\text{N}_2$ -fixing bacteria in the roots eventually die, the products of their decomposition may well contribute to the growth of the roots and shoots of the plant. Even if this does not occur, then the N fixed in the roots will at least contribute to soil fertility in the field after the natural processes of root turnover and decomposition. Ando *et al.* [3] calculated  $\text{N}_2$  fixation rates, fertilizer efficiency and the turnover of organic matter in sugarcane production in Thailand. Using the  $^{15}\text{N}$  natural-abundance method, they estimated that the contribution of crop  $\text{N}_2$  fixation to the overall N economy of many of the plantations was about 0-30%. Meanwhile, the contributions of N from



**Figure 8.** Changes with time in the fractional distribution (%) of  $^{15}\text{N}$  in the stem, roots and shoot of sugarcane plants exposed to  $^{15}\text{N}_2$  for 7 days.

applied mineral fertilizers were about 18-31%. This implies that N supplied from other soil-N sources such as from decomposing crop organic matter is important for supporting the fertility of the soil under sugarcane production.

## 6. Diazotrophic endophytes in sugarcane

As for the presence of  $\text{N}_2$ -fixing bacteria in sugarcane, diazotrophic bacteria belonging to the *Beijerinckia* genera have been found in large numbers in the rhizosphere (the soil volume adjacent to, or within a few millimeters from the root surface) and in the rhizoplane (the soil:root interface) of sugarcane [19]. Other diazotrophs, such as *Bacillus*, *Azotobacter*, *Derxia*, *Enterobacter*, *Erwinia*, *Klebsiella*, and *Azospirillum*, have also been isolated from the sugarcane rhizosphere [47]. In 1988, a new species of *Acetobacter* was found inside the sugarcane stem and named *Acetobacter diazotrophicus* [15], though this was later renamed to *Gluconacetobacter diazotrophicus* [55]. These microorganisms are called “endophytes” as they live inside host plant tissues without eliciting any symptoms of disease [8]. Recently, Saito *et al.* [49] reported a broad

distribution and phylogeny of anaerobic endophytes of cluster XIVa clostridia in various plant species including the leaves, stems, stem cuttings and roots of sugarcane. The fixation of N<sub>2</sub> by endophytic bacteria has also been suggested in other crops, eg. rice (*Oryza sativa*) [34,58], sweet potatoes (*Ipomoea batatas* L.) [51,1]. Recently, endophytic bacteria associated with nitrogen fixation and indole acetic acid synthesis were identified in ornamental bulb flower, curcuma (*Curcuma alismatifolia* Gagnep.) to promote plant growth, and the endophytes were corresponded to *Bacillus drentensis*, *Sphingomonas pseudosanguinis*, and *Bacillus methylotrophicus* using 16S rDNA sequence analysis [53].

*G. diazotrophicus* is considered to be a major diazotrophic endophyte in sugarcane and has been isolated from leaves, stems and roots of sugarcane plants collected from a number of sites in Brazil and also in other countries [15]. *G. diazotrophicus* is clustered in the alpha subclass of Proteobacteria, and *Herbaspirillum seropedicae*, *Herbaspirillum rubrisubalbicans* and *Burkholderia* sp, clustered in the beta subclass of the Proteobacteria [14,8]. Rhizobia, which are symbiotic nitrogen fixing bacteria with legumes, survive as a free living state in soil after symbiotic state in root nodules. Different from rhizobia, *G. diazotrophicus* does not survive free in the soil, and it is thought that it is mainly transmitted in the course of vegetative propagation, which is usually done from stem cuttings or 'setts' [46].

Recently, Ando *et al.* [2] detected the *nifH* gene sequences, encoding the homodimer Fe protein of nitrogenase, in sugarcane stem and pineapple leaves to detect unculturable strains of bacteria as well as culturable strains. Their result showed that the sequences of the *nifH* clones were homologous to those of bacteria in the genera *Bradyrhizobium*, *Seratia*, and *Kebsiella*. On the other hand, no *nifH* sequence related to *G. diazotrophicus* was detected in sugarcane. This result indicates the absence or the presence of few *G. diazotrophicus* in the stems of the sugarcane plants used in their study. The expression of *nifH* gene in the stems and roots of sugarcane plants was investigated and the *nifH* RNA sequences similar to those of *Bradyrhizobium* sp. and *Azorhizobium caulinodans* were detected [52]. The *nifH* expression of *Bradyrhizobium* sp. and *Rhizobium* sp. in roots of field-grown Brazilian sugarcane was also found [23].

It is known that endophytic diazotrophic bacteria colonize in the vascular tissues or intercellular spaces and of sugarcane organs. For the presence of endophytic diazotrophs in sugarcane juice, and Bellone and Bellone [9] concluded that in the mature region of the sugarcane stem *G. diazotrophicus* grows more abundantly than *H. seropedicae* or *Azospirillum brasilense*. However, the sites of colonization and the movement through xylem vessels of *G. diazotrophicus* within sugarcane plants are controversial [20,21,28]. James *et al.* [28] reported that *G. diazotrophicus* may reside in xylem of sugarcane stem, however, Dong *et al.* [21] pointed out that xylem is not suitable habitat and the intercellular space apoplast is the probable location of the bacteria.

Recently, complete genome sequence of the sugarcane nitrogen-fixing endophyte *G. diazotrophicus* Pal5 was reported [10]. *G. diazotrophicus* Pal5 was the third diazotrophic endophytic bacterium to be completely sequenced, followed by *Azoarcus* sp. strain BH72 [30] and *Klebsiella pneumoniae* strain 342 [25]. Its genome is composed of a 3.9 Mb chromosome and 2 plasmids of 16.6 and 38.8 Kb, respectively. 3,938 coding sequences are annotated and those are related to the endophytic lifestyle such as nitrogen fixation, plant growth promotion, sugar metabo-

lism, transport systems, synthesis of auxin and the occurrence of bacteriocins [10]. Gene clusters for gum-like polysaccharide biosynthesis, tad pilus, quorum sensing, for modulation of plant growth by indole acetic acid and mechanisms involved in tolerance to acidic conditions were identified and may be related to the sugarcane endophytic and plant-growth promoting traits of *G. diazotrophicus*.

A broad proteomic description of *G. diazotrophicus* identified 583 proteins, and potential metabolic pathways for nucleotides, amino acids, carbohydrates, lipids, cofactors and energy production were described [31]. A differential protein expression analysis was carried out to study *G. diazotrophicus* interaction with sugarcane [22].

The mechanisms for the association between sugarcane plants and diazotrophic endophytes are as yet poorly understood.

De Carvalho *et al.* [18] reviewed sugarcane-endophytes association. Besides biological nitrogen fixation, these endophytes exhibit growth promoting traits by mechanisms involving nutrient solubilization, plant hormone production and pathogens antagonistic activity. Cavalcante *et al.* [16] suggested that the ethylene signaling pathway may play a role in the establishment of the association between sugarcane and endophytic diazotrophic bacteria.

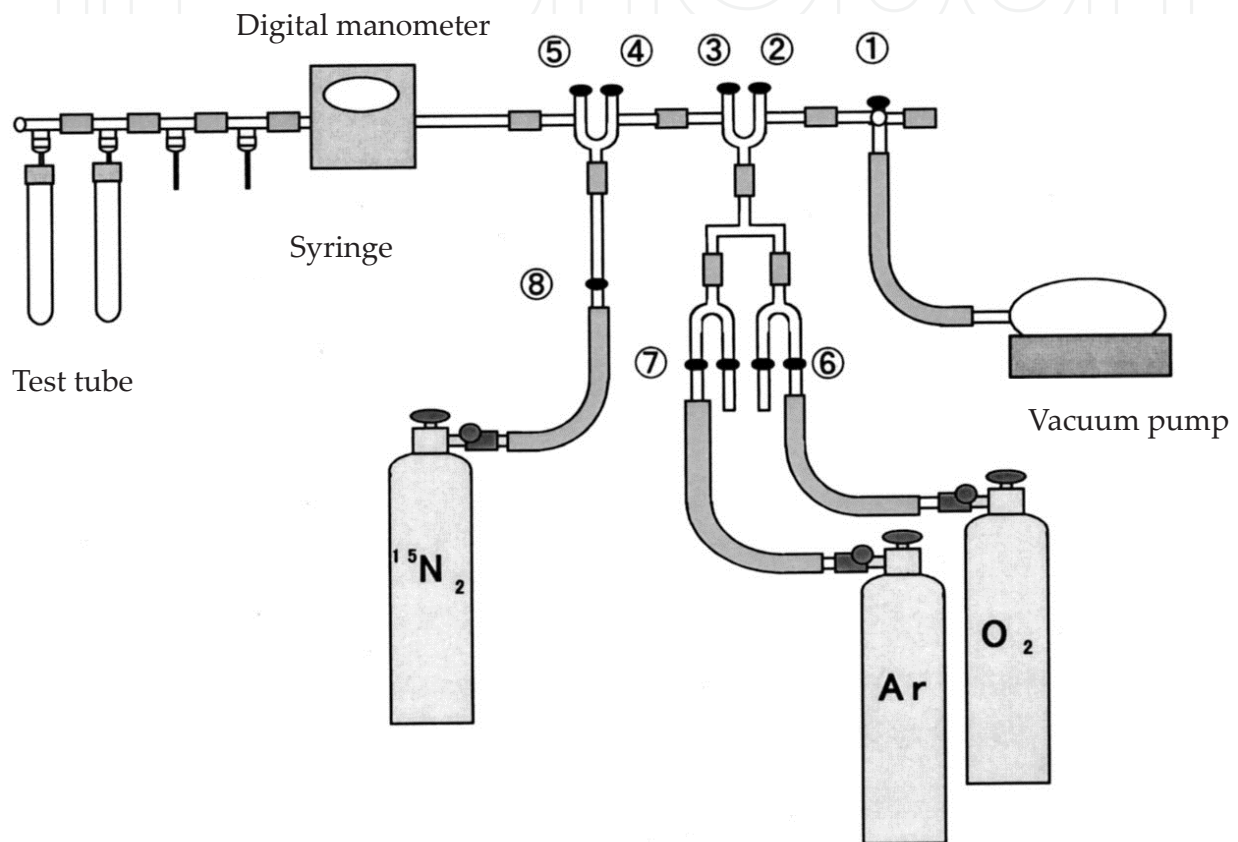
## **7. Mechanism by which N is transferred to the host plant from endophytic nitrogen-fixing bacteria**

To promote sugarcane growth and high yield of sugar, the transport of N from diazotrophic endophytes to the host plant is important in addition to the occurrence of high nitrogen fixation activity. The mechanism by which N is transferred to the host sugarcane plant from N<sub>2</sub>-fixing endophytes has not yet been fully elucidated. There are two possible ways for this transfer to occur. The first is that living bacteria actively excrete fixed N into the apoplast of the host tissue and the plant cells then absorb the released N compounds. This is analogous to legume-rhizobia symbiosis, in which fixed ammonia is rapidly excreted from bacteroid (a symbiotic state of rhizobia) to cytosol of infected cells in soybean root nodules [45]. The second is that bacteria proliferate and colonize in the host tissue and the fixed N is released to the host cells only after their death and disintegration. No direct evidence has yet been obtained.

There is little direct evidence on how N<sub>2</sub> fixed is supplied from endophyte to sugarcane plants. When an amylolytic yeast was used to mimic the plant, *G. diazotrophicus* was capable to excrete part of the fixed N into the medium [17]. There are also evidences that ammonium is the product excreted by this bacterium under N<sub>2</sub> fixation conditions [7]. Data obtained on sugarcane EST expression analysis indicated that nitrogen metabolism was active in plants colonized by diazotrophic endophytes [39]. It suggests that the sugarcane N assimilation apparatus can play a role on the incorporation of the N compound released by the diazotrophic bacteria during association [39].

The characteristics of nitrogen fixation and transport in endophytic bacteria isolated from sugarcane stem were investigated [37]. The strains JA1 and JA2 were putatively identified as

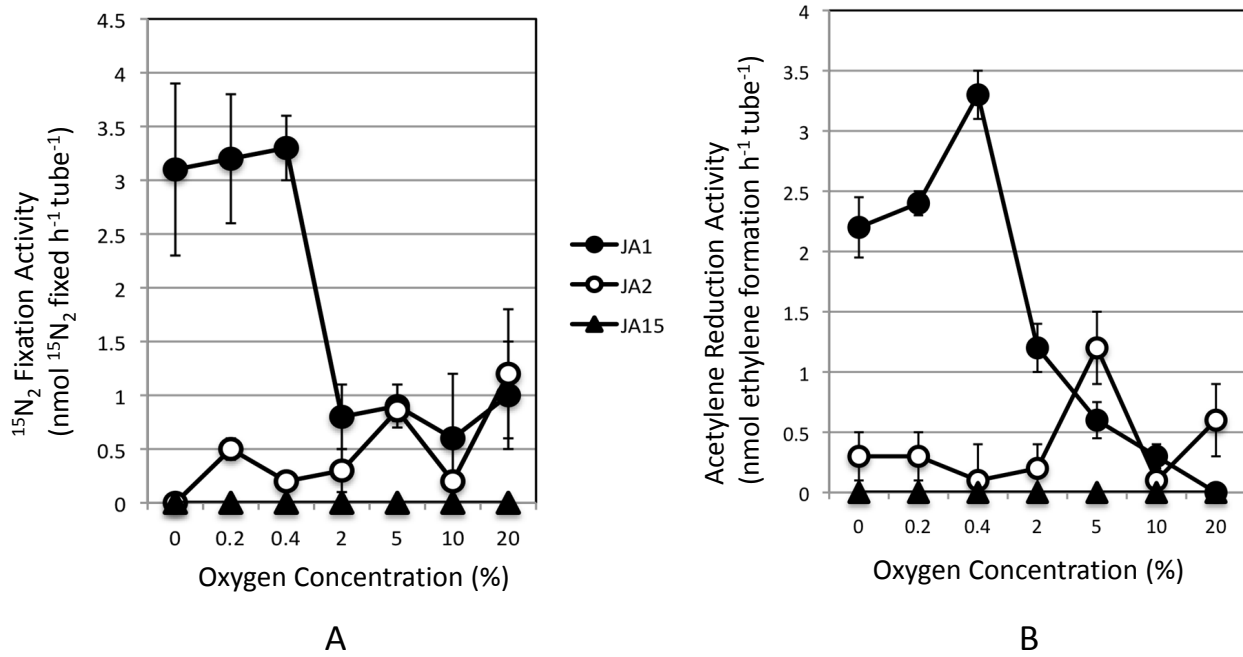
*G. diazotrophicus* [4]. The cultures of strain JA1 and JA2 in N free LGIP medium were exposed to the  $^{15}\text{N}$  labeled air with different  $\text{O}_2$  concentrations using vacuum system (Figure 9). The result showed  $^{15}\text{N}_2$  fixation activity and acetylene reduction activity (ARA) in both culture, although optimum  $\text{O}_2$  concentration were different (Figure 10, 11). These activities in JA1 were highest at 0.4%  $\text{O}_2$  on solid agar culture (Figure 10), and at 0%  $\text{O}_2$  in liquid culture (Figure 11). In both culture conditions,  $^{15}\text{N}_2$  fixation activity and ARA decreased with increasing  $\text{O}_2$  concentrations up to 20%.



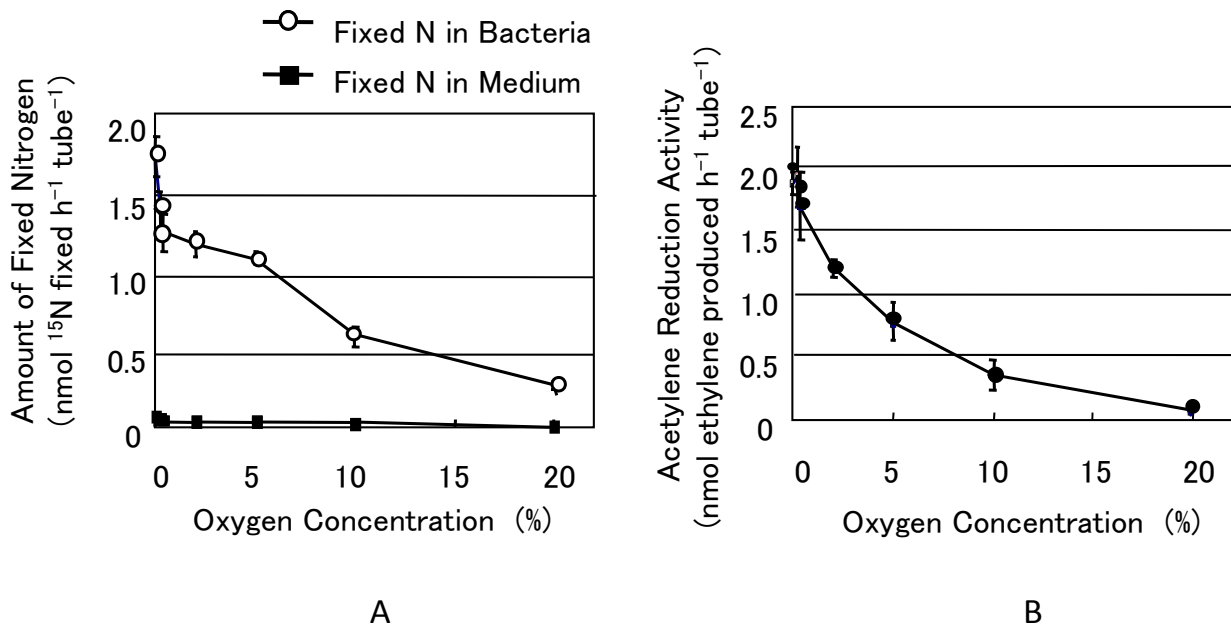
**Figure 9.** Vacuum system for exchanging the gas inside the test tubes.

The growth and ARA of the strain JA1 cultivated with liquid LGIP medium were measured every day for 10 days (Figure 12). Bacteria growth increased rapidly at day-1 after inoculation, and continued to increase until day-6, then the increase was almost stopped thereafter. The strain might regulate bacterial density, possibly by quorum sensing mechanism. The ARA increased rapidly from day-1 to day-5, but it decreased rapidly after day-6. Very low ARA was detected after day-7 to day-10.

This result suggests that nitrogen fixation is active only during early stage of proliferation of JA1. After the bacterium growth stops, nitrogen fixation activity is inactivated. If the situation is the same inside the sugarcane organs, the continuous proliferation should be essential to keep nitrogen fixation activity of the diazotrophic endophyte.

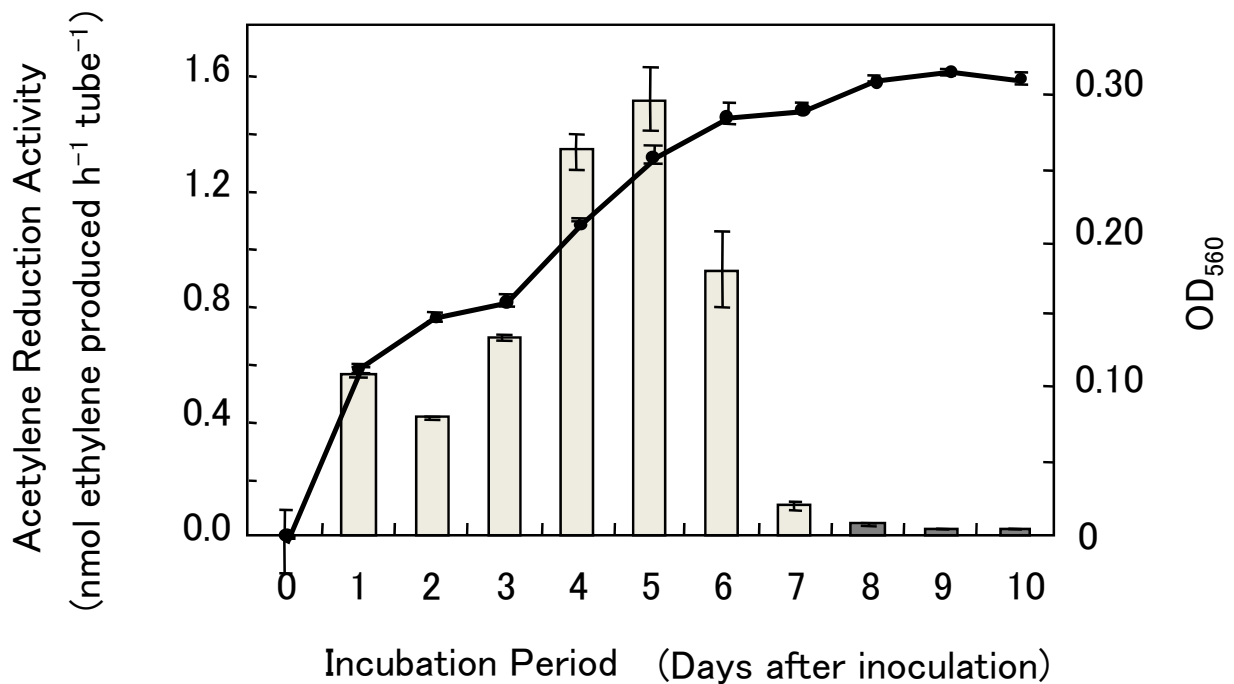


**Figure 10.**  $^{15}\text{N}_2$  fixation activity (A) and acetylene reduction activity (B) of isolated strains from sugarcane endophytes cultivated on agar medium under various concentration of  $\text{O}_2$ . (average with standard deviation)



**Figure 11.**  $^{15}\text{N}_2$  fixation activity (A) and acetylene reduction activity (B) of isolated strain JA1 cultivated in liquid medium under various concentration of  $\text{O}_2$ . (average with standard deviation)

The N release mechanism from endophytes to sugarcane plant is very important to support N nutrition for sugarcane growth. From the experiment with JA1 strain,  $^{15}\text{N}$  fixed during 24 hours was mainly distributed in bacteria fraction and only a little portion (about 4%) was released



**Figure 12.** Growth (OD<sub>560</sub>, line graph) and acetylene reduction activity (bar graph) of isolated strain JA1 cultivated in liquid medium. (average with standard deviation)

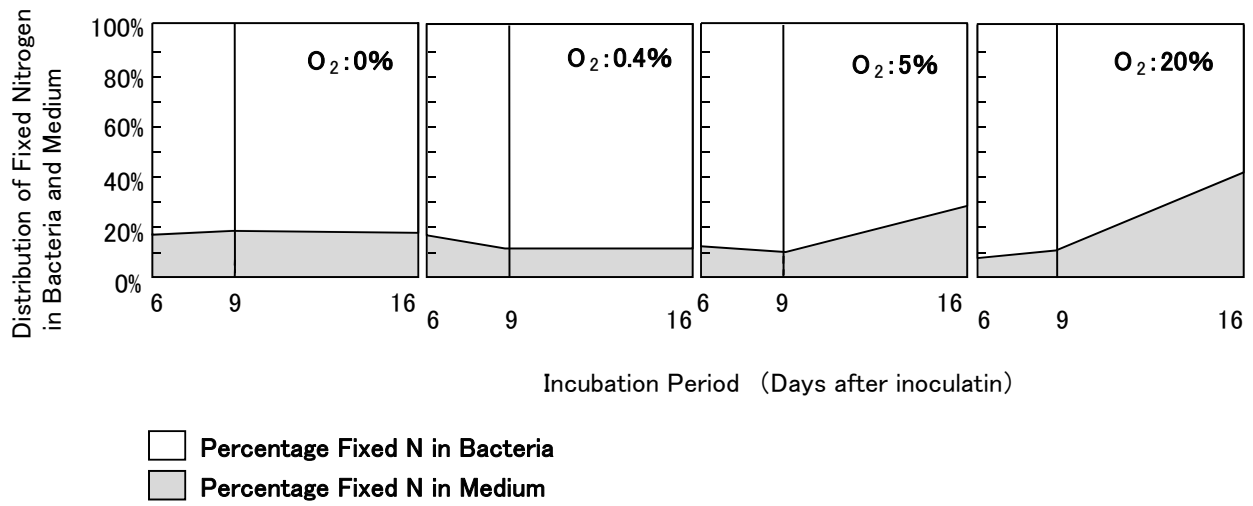
to the medium. However, at 10 days after one day feeding of <sup>15</sup>N<sub>2</sub>, a significant portion of fixed <sup>15</sup>N was distributed to the medium, especially the percentage of released <sup>15</sup>N was highest at 40% under 20% O<sub>2</sub> conditions (Figure 13). This result indicates that the cultured JA1 released N after stopping growth and nitrogen fixation, possibly by their death and degradation. Lethbridge and Davidson [32] suggested that endophytic bacteria only transferred fixed N to the plant when they died and were eventually decomposed.

## 8. Conclusion

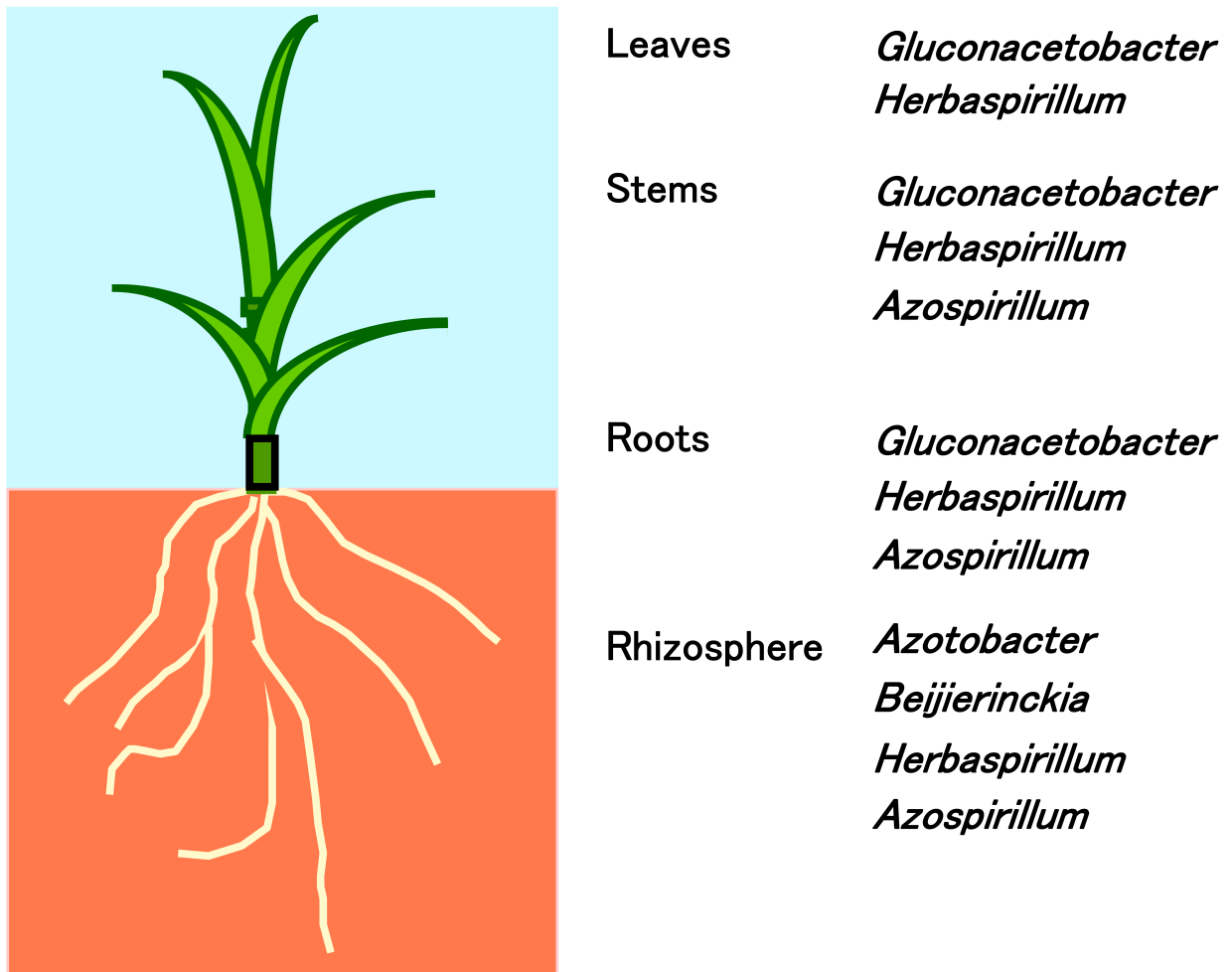
In our studies, a <sup>15</sup>N<sub>2</sub> tracer experiment was conducted to investigate the sites of N<sub>2</sub> fixation and the possible translocation of the fixed N in young sugarcane plants. Most active nitrogen fixation was observed in the roots, followed by planted stalk. The young shoot showed very little nitrogen fixation. Although many diazotrophic endophytic bacteria are reported in all parts of sugarcane plants (Figure 14), the roots may be a principal part of nitrogen fixation.

The fixed <sup>15</sup>N either in roots or stalk were not readily transported to the shoots. In addition, the fixed N was mainly located in the 80% ethanol insoluble fractions, which contains high molecular weight compounds such as protein. The isolated diazotrophic endophyte fixed <sup>15</sup>N<sub>2</sub> in N free liquid and solid cultures. They fixed <sup>15</sup>N<sub>2</sub> only during their active growth period, and nitrogen fixation stopped thereafter. It is possible that the endophytic bacteria can fix N during proliferation stage, and they will release N after their death and decomposition.





**Figure 13.** Distribution of fixed N in bacteria and medium fractions after <sup>15</sup>N<sub>2</sub> exposure at day-6 of incubation period under different O<sub>2</sub> concentrations.



**Figure 14.** Presence of major diazotrophic endophytes in sugarcane

The translocation of fixed N is quite different from legume-rhizobia symbiosis. In soybean nodules, N<sub>2</sub> is fixed by bacteroids (N<sub>2</sub>-fixing rhizobia) in the infected cells and ammonia or ammonium is readily excreted from bacteroid to the cytosol of the infected cells. On short-term (5 min) exposure to <sup>15</sup>N<sub>2</sub>, 97% of the fixed <sup>15</sup>N in the 80% ethanol-soluble fraction, which contain low molecular weight compounds such as amino acids, in the nodules was distributed in the cytosol of the nodule plant cells, while only 3% remained within the bacteroids [44]. The ammonium is then assimilated via the glutamine synthetase/glutamate synthase (GS/GOGAT) system [41,43], and used mainly to produce ureides, allantoin and allantoic acids, and the ureides are transported to the various plant organs via the xylem vessels [42,45].

Other than in Brazil, 150-250 kg of urea-N per hectare per year is usually applied to sugarcane, the actual amount depending on soil fertility, on genotype and on target yield [29]. By promoting BNF through endophytic or associative diazotrophs, the cost associated with N fertilizer usage in sugarcane production can be reduced and environmental problems such as NO<sub>3</sub><sup>-</sup> leaching or N<sub>2</sub>O gas emission, consequent upon the use of excessive chemical fertilizers, can be avoided. Further research will be important for promoting more efficient N<sub>2</sub> fixation rates in sugarcane production.

## Acknowledgements

We greatly appreciate the late Prof. Dr. Shoichiro Akao for giving us the chance to do the experiment on sugarcane-endophyte association. This research was supported by a grant from a Research Project of MAFF, Japan.

## Author details

Takuji Ohyama<sup>1,2\*</sup>, Atsushi Momose<sup>2</sup>, Norikuni Ohtake<sup>1,2</sup>, Kuni Sueyoshi<sup>1,2</sup>, Takashi Sato<sup>3</sup>, Yasuhiro Nakanishi<sup>4</sup>, Constancio A. Asis Jr.<sup>5</sup>, Soraya Ruamsungri<sup>6</sup> and Shotaro Ando<sup>7</sup>

\*Address all correspondence to: [ohyama@agr.niigata-u.ac.jp](mailto:ohyama@agr.niigata-u.ac.jp)

1 Faculty of Agriculture, Niigata University, Niigata, Japan

2 Graduate School of Science and Technology, Niigata University, Niigata, Japan

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

4 Faculty of International Agriculture and Food Studies, Tokyo University of Agriculture, Tokyo, Japan

5 Philippine Rice Research Institute, Science City of Muñoz, Nueva Ecija, Philippines

6 Faculty of Agriculture, Chiang Mai University, Chiang Mai, Thailand

7 Japan International Research Center for Agricultural Sciences, Okinawa, Japan

## References

- [1] Ando, S. 2010. Nitrogen fixation associated with endophytic bacteria. In *Nitrogen Assimilation in Plants*, Ed. Ohyama, T. and Sueyoshi, K. (Research Signpost, Kerala, India), pp. 215-231.
- [2] Ando, S., Goto, M., Meunchang, S., Thongra-ar, P., Fujiwara, T., Hayashi, H., and Yoneyama T., 2005. Detection of *nifH* sequences in sugarcane (*Saccharum officinarum* L.) and Pineapple (*Ananas comosus* (L.) Merr.), *Soil Sci. Plant Nutr.*, 51: 303-308.
- [3] Ando, S., Meunchang, S., Thippayarugs, S., Prasertsak, P., Matsumoto, N., and Yoneyama, T. 2002. Evaluation of sustainability of sugarcane production in Thailand based on nitrogen fixation, efficiency of nitrogen fertilizer and flow of organic matters. *JIRCAS Working Report*, 30:61-64.
- [4] Asis, C.A. Jr., Kubota, M., Ohta, H., Arima, Y., Chebotar, V.K., Tsuchiya, K., and Akao, S. 2000. Isolation and partial characterization of endophytic diazotrophs associated with Japanese sugarcane cultivar. *Soil Sci. Plant Nutr.*, 46:759-765.
- [5] Asis, C.A. Jr., Kubota, M., Ohta, H., Arima, Y., Ohwaki, Y., Yoneyama, T., Tsuchiya, K., Hayashi, N., Nakanishi, Y., and Akao, S. 2002. Estimation of the nitrogen fixation by sugarcane cultivar NiF-8 using  $^{15}\text{N}$  dilution and natural  $^{15}\text{N}$  abundance techniques. *Soil Sci. Plant Nutr.*, 48:283-285.
- [6] Azeredo, D.F, Bolsanello, J., Wever, M., and Vieira, J.R., 1986. Nitrogenio em cana-planta, doses e fracionamento. *STAB-Acucar Alcool e Subproductos*, 4: 26-32.
- [7] Baldani, J.I., Caruso, L., Baldani, V.L.D., Goi, S.R., and Döbereiner, J. 1997. Recent advances in BNF with non-legume plants. *Soil Biol. Biochem.*, 29: 911-922.
- [8] Barnett, M.J., Toman C.J., Fisher R.F., and Long S.R. (2004) A dual-genome symbiosis chip for coordinate study of signal exchange and development in a prokaryote-host interaction. *Proc. Natl. Acad. Sci. USA*, 101:16636–16641.
- [9] Bellone de, S.C., and Bellone, C.H. 2006. Presence of endophytic diazotrophs in sugarcane juice. *World J. Microbiol. Biotech.*, 22, 1065-1068.
- [10] Bertalan, M., et al., 2009, Complete genome sequence of the sugarcane nitrogen-fixing endophyte *Gluconacetobacter diazotrophicus* Pal5. *BMC Genomics*, 10, 450.
- [11] Bezdicek, D.F., and Kennedy, A.C. 1988. Symbiotic nitrogen fixation and nitrogen cycling in terrestrial environments, In *Micro-organisms in Action; Concepts and Applications in Microbiol Ecology*, Ed. Lynch, J.M. and Hobbie, J.E., Blackwell Scientific Publications, Oxford, UK. 241-260
- [12] Boddey, R.M. 1995. Biological nitrogen fixation in sugar cane: A key to energetically viable biofuel production. *CRC Crit. Rev. Plant Sci.*, 14:263-279.

- [13] Boddey, R.M., Urquiaga, S., Reis, V., and Döbereiner, J. 1991. Biological nitrogen fixation associated with sugar cane. *Plant Soil*, 137:111-117.
- [14] Boddey, R.M., Urquiaga, S., Alves, B.J.R., and Reis, V. 2003. Endophytic nitrogen fixation in sugarcane: present knowledge and future applications. *Plant Soil*, 252:139–149.
- [15] Cavalcante, V.A., and Döbereiner, J. 1988. A new acid-tolerant nitrogen-fixing bacterium associated with sugarcane. *Plant Soil*, 108:23-31.
- [16] Cavalcante, J.J.V., Vargas, C., Nogueira, E.M., Vinagre, F., Schwarcz, K., Baldani, J.I., Ferreira, P.C.G., and Hemerly, A.S. 2007. Members of the ethylene signalling pathway are regulated in sugarcane during the association with nitrogen-fixing endophytic bacteria. *J. Exp. Bot.*, 58:673-686.
- [17] Cohjo, E.H., Reis, V.M., Schenberg, A.C.G., and Döbereiner, J. 1993. Interactions of *Acetobacter diazotrophicus* with an amyolytic yeast in nitrogen-free batch culture. *FEMS Microbiol. Lett.*, 106:341-346.
- [18] De Carvalho, T.L.G., Ferreira, P.C.G, Hemerly, A.S. 2011. Sugarcane genetic controls involved in the association with beneficial endophytic nitrogen fixing bacteria, *Tropical Plant Biol.* 4:31–41.
- [19] Döbereiner, J. 1961. Nitrogen-fixing bacteria of the genus *Beijerinckia* Derk in the rhizosphere of sugar cane. *Plant Soil*, 15:211-216.
- [20] Dong, Z., Canny, M.J., McCully, M.E., Roboredo, M.R., Cabadilla, C.F., Ortega, E., and Rodes, R. 1994. A nitrogen-fixing endophyte of sugarcane stems. *Plant Physiol.*, 105:1139-1147.
- [21] Dong, Z., McCully, M.E., and Canny, M.J. 1997. Does *Acetobacter diazotrophicus* live and move in the xylem of sugarcane stems? Anatomical and physiological data. *Ann. Bot.*, 80:147-158.
- [22] Dos Santos, M.F., De Pádua, V.L.M., Nogueira, E.M., Hemerly, A.S., and Domont, G.B. 2010. Proteome of *Gluconacetobacter diazotrophicus* co-cultivated with sugarcane plantlets. *J. Proteomics*, 73:917–931.
- [23] Fischer, D., Pfitzner, B., Schmid, M., Simões-Araújo, J.L., Reis, V.M., Pereira, W., Ormeño-Orrillo, E., Hai, B., Hofmann, A., Schloter, M., Martinez-Romero, E., Baldani, J.I., and Hartmann, A., 2012. Molecular characterization of the diazotrophic bacterial community in uninoculated and inoculated field-grown sugarcane (*Saccharum* sp.), *Plant Soil*, 356, 83-99.
- [24] Fisher, K., and Newton, W.E. 2004. Nitrogen fixation: An historical perspective. In *Canalystes for nitrogen fixation: Nitrogenases, relevant chemical models, and commercial processes*. Ed Smith B.E., Richard, R.L., Newton, W.E., (Kluwer Academic Publisher, Dordrecht.) pp. 1-31.

- [25] Fouts, D.E., Tyler, H.L., De Boy, R.T., Daugherty, S., Ren, Q., Badger, J.H., Durkin, A.S., Huot, H., Shrivastava, S., Kothari, S., et al. 2008. Complete genome sequence of the N<sub>2</sub>-fixing broad host range endophyte *Klebsiella pneumoniae* 342 and virulence predictions verified in mice. *PLoS Genet.*, 47:e1000141.
- [26] Hiyama, T., Momose, A., Nishimura, K., Ishizaki, N., Ishikawa, S., Yamamoto, M., Hung, N.V.P., Chamaiporn, A., Ruamrungsri, S., Ohtake, N., Sueyoshi, K., Ohyama, T. 2013. Estimation of contribution of nitrogen fixation in sugarcane (*Saccharum officinarum*) plants with <sup>15</sup>N dilution method in relation to nitrogen supply period. *Bull. Facul. Agric. Niigata Univ.*, 66:11-19
- [27] James, E.K., and Olivares, F.L. 1997. Infection and colonization of sugar cane and other graminaceous plants by endophytic diazotrophs. *CRC Crit. Rev. in Plant Sci.*, 17:77-119.
- [28] James, E.K., Olivares, F.L., de Oliveira, A.L.M., dos Reis, Jr. F.B., da Silva, L.G. and Reis, V.M. 2001. Further observations on the interaction between sugar cane and *Gluconacetobacter diazotrophicus* under laboratory and greenhouse conditions. *J. Exp. Bot.*, 52:747-760.
- [29] Kennedy, I.R., Choudhury, A.T.M.A., and Kecskes, M.L. 2004. Non-symbiotic bacterial diazotrophs in crop-farming systems: can their potential for plant growth promotion be better exploited? *Soil Biol. Biochem.*, 36:1229-1244.
- [30] Krause, A., Ramakumar, A., Bartels, D., Battistoni, F., Bekel, T., Boch, J., Boehm, M., Friedrich, F., Hurek, T., Krause, L., et al. 2008. Complete genome of the mutualistic, N<sub>2</sub>-fixing grass endophyte *Azoarcus* sp. strain BH72. *Nat Biotechnol.*, 24:1385-1391.
- [31] Lery, L.M.S., Coelho, A., von Kruger, V.M.A. et al. 2008. Protein expression profile of *Gluconacetobacter diazotrophicus* PAL5, a sugarcane endophytic plant growth-promoting bacterium. *Proteomics*, 8:1631-1644
- [32] Lethbridge, G., and M.S. Davidson. 1983. Root-associated nitrogen-fixing bacteria and their role in the nitrogen nutrition of wheat estimated by <sup>15</sup>N isotope dilution. *Soil Biol. Biochem.*, 15:365-374.
- [33] Lima, E., Boddey, R.M., and Döbereiner, J., 1987. Quantification of biological nitrogen fixation associated with sugar cane using a <sup>15</sup>N aided nitrogen balance. *Soil Biol. Biochem.*, 19:165-170.
- [34] Mano, H., and Morisaki, H. 2008. Endophytic bacteria in the rice plant. *Microbes Environ.*, 23:109-117.
- [35] Marris, E. 2006. Drink the best and drive the rest. Brazil's sugar-cane ethanol industry is the world's best and able to get better. *Nature*, 444:670-672.
- [36] Momose, A., Ohtake N., Sueyoshi, K., Sato, T., Nakanishi, Y., Akao, S., and Ohyama, T. 2009. Nitrogen fixation and translocation in young sugarcane (*Saccharum officina-*

- rum L.) plants associated with endophytic nitrogen-fixing bacteria. *Microbes Environ.*, 24, 224-230
- [37] Momose, A., Hiyama, T., Nishimura, K., Ishizaki, N., Ishikawa, S., Yamamoto, M., Hung, N.V.P., Ohtake, N., Sueyoshi, K., and Ohya, T. 2013. Characteristics of nitrogen fixation and nitrogen release from diazotrophic endophytes isolated from sugarcane (*Saccharum officinarum* L.) stem. *Bull. Facul. Agric. Niigata Univ.*, 66 (1) 66:1-9
- [38] Nishiguchi, T., Shimizu, T., Njoloma, J., Oota, M., Saeki, Y., and Akao, S. 2005. The estimation of the amount of nitrogen fixation in the sugarcane by  $^{15}\text{N}$  dilution technique. *Bull. Facul. Agric. Univ. Miyazaki*, 51:53-62.
- [39] Nogueira, E.M., Vinagre, F., Masuda, H.P., et al. 2001. Expression of sugarcane genes induced by inoculation with *Gluconacetobacter diazotrophicus* and *Herbaspirillum rubrisubalbicans*. *Gen. Mol. Biol.*, 24:199-206.
- [40] Ohya, T. 2010. Nitrogen as a major essential element of plants. In *Nitrogen Assimilation in Plants*, Ed Ohya, T., Sueyoshi, K. Research Signpost, Kerala India, pp. 1-17.
- [41] Ohya, T., and Kumazawa. K., 1978. Incorporation of  $^{15}\text{N}$  into various nitrogenous compounds in intact soybean nodules after exposure to  $^{15}\text{N}_2$  gas. *Soil Sci. Plant Nutr.*, 24:525-533.
- [42] Ohya, T., and Kumazawa. K. 1979. Assimilation and transport of nitrogenous compounds originated from  $^{15}\text{N}_2$  fixation and  $^{15}\text{NO}_3$  absorption. *Soil Sci. Plant Nutr.*, 25:9-19.
- [43] Ohya, T., and Kumazawa. K. 1980. Nitrogen assimilation in soybean nodules I. The role of GS/GOGAT system in the assimilation of ammonia produced by  $\text{N}_2$ -fixation. *Soil Sci. Plant Nutr.*, 26:109-115.
- [44] Ohya, T., and Kumazawa. K. 1980. Nitrogen assimilation in soybean nodules II.  $^{15}\text{N}_2$  assimilation in bacteroid and cytosol fractions of soybean nodules. *Soil Sci. Plant Nutr.*, 26:205-213.
- [45] Ohya, T., Ohtake, N., Sueyoshi, K., Tewari, K., Takahashi, Y., Ito, S., Nishiwaki, T., Nagumo, Y., Ishii, S., Sato, T. 2009. Nitrogen fixation and metabolism in soybean plants. Nova Science Publishers, Inc., New York, pp.1-131.
- [46] Reis, V.M., Olivares, F.L., and Döbereiner, J. 1994. Improved methodology for isolation of *Acetobacter diazotrophicus* and confirmation of its endophytic habitat. *World J. Microbiol. Biotechnol.*10:101-104.
- [47] Reis, V., Lee, S., and Kennedy, C. 2007. Biological nitrogen fixation in sugarcane. p. 213-232. In: *Associative and Endophytic Nitrogen-fixing Bacteria and Cyanobacterial Associations*, Ed. Emerich, C. and Newton W.E., Springer, Dordrecht, The Netherlands. pp. 213-232.

- [48] Ruschel, A.P., Henis, Y., and Salati, E. 1975. Nitrogen-15 tracing of N-fixation with soil-grown sugarcane seedlings. *Soil Biol. Biochem.*, 7:181-182.
- [49] Saito A., Kawahara, M., Ikeda, S., Ishimine, M., Akao, S., and Minamisawa, K. 2008. Broad distribution and phylogeny of anaerobic endophytes of cluster XIVa clostridia in plant species including crops. *Microbes Environ.*, 23:73-80.
- [50] Sevilla, M., Burris, R.H., Gunapala, N., and Kennedy, C. 2001. Comparison of benefit to sugarcane plant growth and  $^{15}\text{N}_2$  incorporation following inoculation of sterile plants with *Acetobacter diazotrophicus* wild-type and Nif mutant strains. *Mol. Plant-Microbe Int.*, 14:358-366.
- [51] Terakado-Tonooka, J., Ohwaki, Y., Yamakawa, H., Tanaka, F., Yoneyama, T., and Fujiwara, S. 2008. Expressed *nifH* genes of endophytic bacteria detected in field-grown sweet potatoes (*Ipomoea batatas* L.). *Microbes Environ.*, 23:89-93
- [52] Thaweenut, N., Hachisuka, Y., Anso, S., Yanagisawa, S., Yoneyama, T. 2011. Two seasons' study on *nifH* gene expression and nitrogen fixation by diazotrophic endophytes in sugarcane (*Saccharum* spp. hybrids): expression of *nifH* genes similar to those in rhizobia. *Plant Soil*, 338, 435-449.
- [53] Thepsukhon, A., Choonluchanon, S., Tajima, S., Nomura, M., and Ruamrungsri, S., 2013, *Journal of Plant Nutrition*, 36, 1424-1438
- [54] Urquiaga, S., Cruz, K.H.S., and Boddey, R.M. 1992. Contribution of nitrogen fixation to sugarcane: Nitrogen-15 and nitrogen-balance estimates. *Soil Sci. Soc. Am. J.*, 56:105-114.
- [55] Yamada, Y., K. Hoshino, and T. Ishikawa. 1997. The phylogeny of acetic acid bacteria based on the partial sequence of 16S ribosomal RNA: The elevation of the subgenus *Gluconoacetobacter* to the generic level. *Biosci. Biotech. Biochem.*, 61:1244-1251.
- [56] Yokoyama, T. 2010 Biological nitrogen fixation: Nitrogen cycle in agriculture. In *Nitrogen Assimilation in Plants*, Ed. Ohyama, T., Sueyoshi, K. Research Signpost. Kerala, India, pp. 169-174.
- [57] Yoneyama, T., Muraoka, T., Kim, T.H., Dacanay, E.V., and Nakanishi, Y. 1997. The natural  $^{15}\text{N}$  abundance of sugarcane and neighbouring plants in Brazil, the Philippines and Miyako (Japan). *Plant Soil*, 189:239-244.
- [58] Zakria, M., Njoloma, J., Saeki, Y., and Akao, S. 2007. Colonization and nitrogen-fixing ability of *Herbaspirillum* sp. strain B501gfp1 and assessment of its growth-promoting ability in cultivated rice. *Microbes Environ.*, 22:197-206.
- [59] Zakria, M., Udonishi, K., Saeki, Y., Yamamoto, A., and Akao, S. 2008. Infection, multiplication and evaluation of the nitrogen-fixing ability of *Herbaspirillum* sp. strain B501 gfp1 in sugarcane stems inoculated by the vacuum infiltration method. *Microbes Environ.*, 23:128-133.