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Nitrogen Fixation and Translocation in Young Sugarcane (*Saccharum officinarum* L.) Plants Associated with Endophytic Nitrogen-Fixing Bacteria

ATSUSHI MOMOSE[1], NORIKUNI OHTAKE[1,2], KUNI SUEYOSHI[1,2], TAKASHI SATO[3], YASUHIRO NAKANISHI[4], SHOICHIRO AKAO[5], and TAKUJI OHYAMA[1,2]*

[1]*Graduate School of Science and Technology, Niigata University, 2–8050 Ikarashi, Nishiku, Niigata 950–2181 Japan;* [2]*Faculty of Agriculture, Niigata University, Niigata, 2–8050 Ikarashi, Nishiku, Niigata 950–2181, Japan;* [3]*Faculty of Bioresource Sciences, Akita Prefectural University, 241–438 Kaidobata-Nishi Shimoshinjyo-Nakano, Akita 010–0195 Japan;* [4]*Miyako Subtropical Experimental Farm, Tokyo University of Agriculture, 72–2 Aza Fukuzato, Gusukube, Okinawa 906–0103, Japan; and* [5]*Faculty of Agriculture, Miyazaki University, 1–1 Gakuen Kibanadai Nishi, Miyazaki 889–2192, Japan*

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The tracer $^{15}N_2$ was used to investigate sites of N_2 fixation and the possible translocation of the fixed N. Young sugarcane plants (*Saccharum officinarum* L.) from a stem cutting were exposed to $^{15}N_2$-labeled air in a 500 mL plastic cylinder. Plants fed $^{15}N_2$ 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 growth. After 3 days of feeding, the percentage of N derived from $^{15}N_2$ was higher in the roots (2.22%) and stem cutting (0.271%) than the shoot (0.027%). 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. The results were quite different from the fate of fixed N in soybean nodules, which is rapidly transported from nodules to roots and shoots.

Key words: $^{15}N_2$, nitrogen fixation, *Saccharum officinarum* L., sugarcane roots, translocation

Sugarcane (*Saccharum officinarum* L.) 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 (27). For many years, sugarcane has been used mainly for sugar and for alcohol production. Recently, the use of sugarcane alcohol (ethanol) as an automotive fuel to replace gasoline has rapidly increased (5, 17). In 2006, world production of sugarcane was 1,392 million tons. This is much greater than production levels for the other major crops such as maize (695 million tons), paddy rice (635 million tons), wheat (606 million tons) and potatoes (315 million tons) (http://faostat.fao.org/site/567/default.aspx). Sugarcane production is highest in Brazil (455 million tons), followed by India (281 million tons). In 2006, sugarcane was cropped over an area of 20 million hectares, and the average yield was 68 tons per hectare. Sugarcane is a C4 plant, which has an efficient photosynthetic system, and it can convert up to 2% of incident solar energy into biomass. It grows up to 4 m in height and the thick stem stores a high concentration of sucrose which is present in the expressed juice at between 12 and 20% (w/v).

In Brazil, sugarcane crops accumulate 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 (27). Also, the response of sugarcane crops to N fertilizers is usually very weak or non-existant (5, 27). In some areas of Brazil, sugarcane has been grown continuously for more than 100 years without any N fertilizer being applied at all (10). This circumstantial evidence suggests a high potential for biological nitrogen fixation (BNF) in sugarcane. Using a ^{15}N dilution technique involving the supply of a ^{15}N-labeled fertilizer, Urquiaga *et al.* (32) calculated the contribution of BNF in several cultivars of sugarcane, and found it to be about 70% for the most promising genotypes. Nishiguchi *et al.* (18) also estimated the contribution of BNF using the ^{15}N dilution, finding that between 10% and 40% of sugarcane N was derived from BNF depending on the cultivar and also on the availability of mineral N. Yoneyama *et al.* (34) examined the contribution of BNF using a ^{15}N natural-abundance method in Brazil, the Philippines and Japan, comparing the abundance of ^{15}N in sugarcane with that in neighboring weeds as control plants. At many but not all of the sites in Brazil, a contribution from BNF was indicated. Again, using the ^{15}N natural-abundance method, Boddey *et al.* (4) showed that 25–60% of the N assimilated in sugarcane at various sites in Brazil was derived from BNF.

However, both the ^{15}N dilution method and the ^{15}N natural-abundance method are indirect and, it is possible that some of the extra N obtained is from a growth-promoting effect of endophytes rather than BNF (14). As yet little direct evidence has been presented that links diazotrophic endophytes with BNF in any grasses, including sugarcane (12). In earlier studies, Ruschel *et al.* (28) exposed soil-grown 60 or 90-day-old sugarcane plants to $^{15}N_2$ under a low oxygen concentration (pO_2=0.02) and near normal oxygen concentration (pO_2=0.16). Significant incorporation of ^{15}N was detected in roots and shoots of intact plants only under the lower of the two oxygen concentrations. In addition, the detached roots

* Corresponding author. E-mail: ohyama@agr.niigata-u.ac.jp;
Tel: +25–262–6643; Fax: +25–262–6643.

fixed $^{15}N_2$ under pO_2=0.02, but ^{15}N was not detected in the detached shoots. Based on these results, they suggested that the site of fixation was within the roots and not in the shoots but that the fixed N was rapidly translocated to the shoot within 40 hours. Again working with sugarcane, Sevilla *et al.* (30) compared plants inoculated with wild-type *Acetobacter diazotrophicus* strain PAl5, with plants inoculated with a Nif^- mutant of PAl5, and with un-inoculated (sterile) plants. Fixation of $^{15}N_2$ was observed only in the shoots and roots of the plants inoculated with the wild-type Nif^+ PAl5.

As for the presence of 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, and within a few millimeters of the roots) and in the rhizoplane (the soil:root interface) of sugarcane (9). Other diazotrophs, such as *Bacillus, Azotobacter*, *Derxia*, *Enterobacter*, *Erwinia*, *Klebsiella*, and *Azospirillum*, have also been isolated from the sugarcane rhizosphere (27). In 1988, a new species of *Acetobacter* was found inside the sugarcane stem and named *Acetobacter diazotrophicus* (7), though this was later changed to *Glconacetobacter diazotrophicus* (33). These organisms are called "endophytes" as they live inside host plant tissues without eliciting any symptoms of disease. *G. diazotrophicus* is considered a major diazotrophic bacterium 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 (7). This particular bacterium 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' (26). It is known that endophytic diazotrophic bacteria colonize the intercellular spaces and vascular tissues of sugarcane organs, without triggering any symptoms in the plant. The signaling mechanisms are as yet poorly understood but Cavalcante *et al.* (6) suggested that the ethylene signaling pathway may play a role in the establishment of the association between sugarcane and endendophytic diazotrophic bacteria. However, there is little evidence so far that *G. diazotrophicus* is actually a N_2-fixing symbiont of sugarcane or that it even expresses active nitrogenase in plants (13). In addition, the sites of colonization and the movement through xylem vessels of *G. diazotrophicus* within sugarcane plants are controversial (10, 11, 13). Recently, Saito *et al.* (29) 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_2 by endophytic bacteria has also been suggested in other crops, eg. rice (*Ozyza sativa*) (16, 35) and sweet potatoes (*Ipomoea batatas* L.) (31).

The mechanism by which N is transfered to the host sugarcane plant from N_2-fixing endophytes has not yet been elucidated. There are two possible ways for this transfer to occur. The first is that living bacteria active excrete fixed N into the appoplast of host tissue and the plant cells then absorb the released N compounds. The second is that bacteria proliferate and colonize 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.

In soybean nodules, N_2 is fixed by bacteroids (N_2-fixing rhizobia) that infect nodule cells and ammonia or ammonium is readily excreted to the cytosol of the plant cells. The ammonium is then assimilated via the glutamine synthetase/glutamate synthase (GS/GOGAT) system (20, 23), and used mainly to produce ureides, allantoin and allantoic acids, and the ureides are transported to the various plant organs via the xylem vessels (21, 22). On short-term (5 min) exposure to $^{15}N_2$, 97% of the fixed ^{15}N in the 80% ethanol-soluble fraction in the nodules was distributed in the cytosol of the nodule plant cells, while only 3% remained within the bacteroids (24).

In this study, a $^{15}N_2$ tracer experiment was conducted to investigate the sites of N_2 fixation and the possible translocation of the fixed N in young sugarcane plants. In addition, each plant part was separated into 80% ethanol-soluble and insoluble fractions, N concentrations and ^{15}N abundances were determined, and the results compared with those obtained for soybean nodule symbiosis.

Materials and Methods

Plant materials and growth of stem cuttings

Sugarcane (*Saccharum officinarum* L., cultivar NiF-8) stem cuttings about 15 cm long and 3 cm in diameter with one bud were soaked in a tray filled with tap water (Fig. 1A) and grown under a 14-h photo period at 28°C (light intensity at 228 μmol m^{-2} s^{-1}) and with a 10-h dark period at 18°C in a Biophotochamber (LX-3000, Taitec, Tokyo, Japan) for 2 weeks. The single shoot grew rapidly reaching about 40 cm (Fig. 1B).

Exposure of young sugarcane plants to ^{15}N-labeled air

Both ends of the stem cutting were trimmed and the resulting small plant, with about 4 cm of stem remaining, a good number of new roots and about 40 cm of fast-growing shoot, was placed in a 500 mL plastic measuring cylinder with an inner diameter about 5.5 cm. The cylinder was sealed at the top with a rubber stopper having both a gas-inlet tube and a water-outlet tube which reached the bottom of the cylinder. The cylinder was filled with a mineral-nitrogen-free nutrient solution. The composition of this solution was (mg L^{-1}); $CaCl_2 \cdot H_2O$ (183), $MgSO_4 \cdot 7H_2O$ (123), K_2SO_4 (109), KH_2PO_4 (8.5), KCl (0.935), $FeSO_4$ (13.9)+$EDTANa_2$ (18.6), H_3BO_3 (0.367), $MnSO_4$ (0.189), $ZnSO_4 \cdot 7H_2O$ (0.144), $CuSO_4 \cdot 5H_2O$ (0.032), $CoSO_4$ (0.028), $(NH_4)_2Mo_7O_{24}$ (0.004), and $NiSO_4 \cdot 6H_2O$ (0.0035). The pH was adjusted to 6.0.

Air labeled with $^{15}N_2$ ($^{15}N_2$:Ar:O_2=24:56:20, ^{15}N abundance=99 atom % excess, purchased from Shokotusho, Japan, gaseous impurities not detectable by gas chromatography) was introduced through the inlet tube and the displaced nutrient solution was drained from the outlet tube, until only about 50 mL of solution remained. The inlet and outlet tubes were immediately sealed with pinch cocks applied to short lengths of silicon tubing (Fig. 2). To maintain photosynthesis of the plants, 2.5 mL of CO_2 gas was injected each day. The solution and gas inside were otherwise not changed. Just after this injection, the initial concentration of CO_2 was about 0.5% (v/v), however, the CO_2 was depleted in one day. In a preliminary experiment without supplementing CO_2, plants exhibited chlorosis. After 7 days of exposure to $^{15}N_2$, some plants were transplanted to 0.02 m^2 pots filled with vermiculite (3.5 L), and grown in a greenhouse for an additional two-week chase period with daily irrigation with a nitrogen-free nutrient solution of the same composition as above. Replication throughout the experiment was in triplicate.

Sampling and analysis

Sampling was done four times. The first and second samples were taken at day-3 and day-7 from the start of the $^{15}N_2$ exposure period. The third and fourth samples were obtained after the trans-

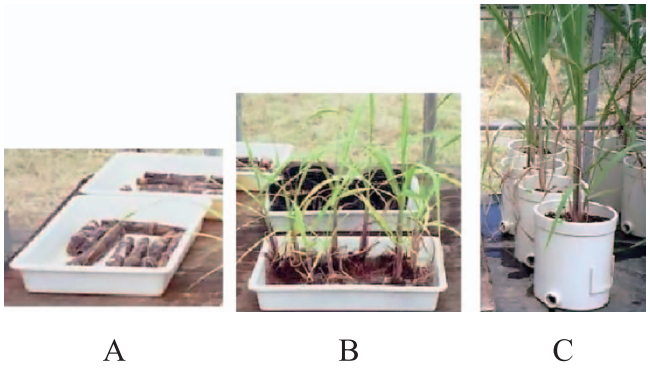

Fig. 1. A photograph of sugarcane plants. A: on the planting of stem cuttings in a tray. B: at 2 weeks after planting. C: after 5 months of cultivation with a vermiculite medium irrigated daily with a nutrient solution.

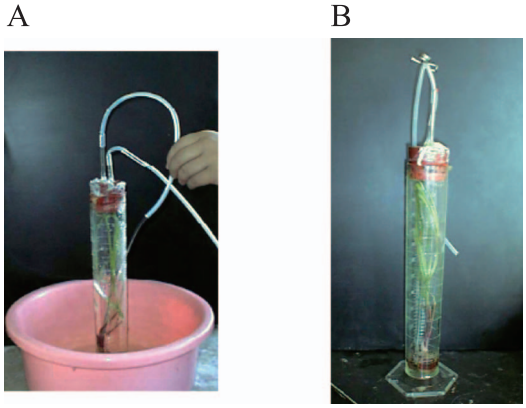

Fig. 2. Exposure of a sugarcane plant to $^{15}N_2$. A: $^{15}N_2$ gas is introduced to the cylinder by displacing the nutrient solution. B: The plant is exposed to $^{15}N_2$ ($^{15}N_2$:Ar:O_2=24:56:20) under controlled light and temperature conditions.

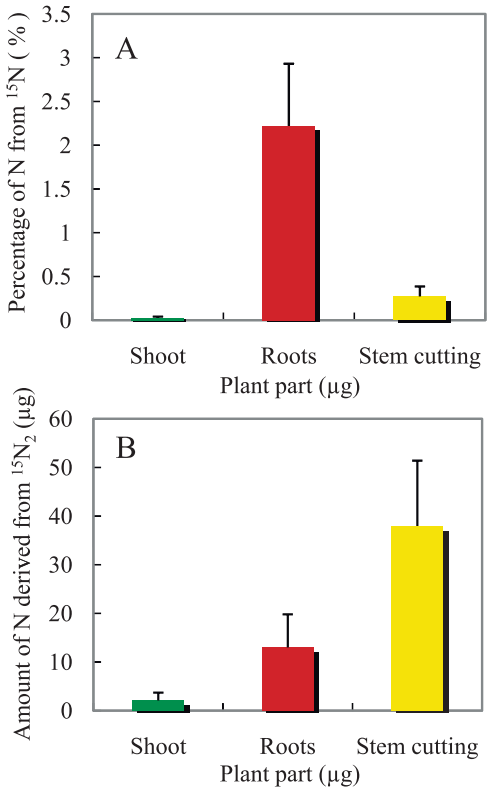

Fig. 3. Percentage and amount of N derived from $^{15}N_2$ in a shoot, roots and a stem cutting of sugarcane at day-3 after $^{15}N_2$ exposure. A: The percentage of N derived from $^{15}N_2$ (expressed as ^{15}N%) fixed from ^{15}N-labeled gas in the shoot, roots and stem cutting of sugarcane plants after three days of exposure (average with standard error). B: The amount of N derived from $^{15}N_2$ in the shoot, roots and stem cutting of sugarcane plants exposed to $^{15}N_2$ for three days (average with standard error).

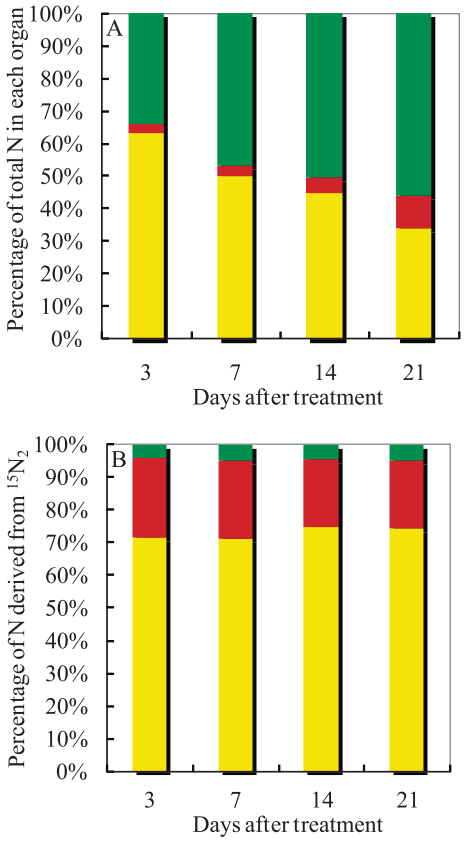

Fig. 4. Changes in the percentage distribution of N and ^{15}N in the shoot, roots and stem cutting of sugarcane plants at 3, 7, 14, and 21 days. $^{15}N_2$ was supplied during the initial 7 days. A: Changes in the percentage distribution of N in the shoot (green), roots (red) and stem cutting (yellow) of sugarcane plants. B: Changes in the percentage distribution of ^{15}N in the shoot, roots and stem cutting of sugarcane plants.

plantation to pots at day-14 (7 days post-transplant) and at day-21 days (14 days post-transplant). Plants were divided into three parts, shoot, roots and stem cutting, dried in a ventilated oven at 80°C and ground into a fine powder with a vibrating mill (CMT, Tokyo, Japan).

Powder samples (50 mg) were extracted in 80% ethanol and divided into residue and extract by centrifugation. The total N-content of powder samples and the 80% ethanol extract and residue was determined by Kjeldahl digestion. The abundance of ^{15}N in a sample was determined by gas-chromatography mass-spectrometry (GC-MS) with a DELTA plus XP (Thermo Fisher Scientific, Bremen, Germany). The percentage of N derived from $^{15}N_2$ (^{15}N%) in total N was calculated as follows. The ^{15}N atom % excess of $^{15}N_2$ gas was 99. Each part of control sugarcane plants not subjected to $^{15}N_2$ fixation was analyzed and the data were used for natural abundance of ^{15}N in the control plants by calculating the percentage

Table 1. Changes in percentage of N derived from $^{15}N_2$ in total N and the amount of labeled N in each part of sugarcane plants supplied with $^{15}N_2$

Part	Average (standard error)			
	Day-3	Day-7	Day-14	Day-21
Percentage of N derived from $^{15}N_2$ (^{15}N%)				
Shoot	0.027 (0.014)	0.034 (0.022)	0.014 (0.011)	0.014 (0.009)
Roots	2.221 (0.709)	2.286 (0.373)	0.699 (0.278)	0.369 (0.106)
Stem cutting	0.271 (0.114)	0.478 (0.135)	0.273 (0.032)	0.381 (0.104)
Amount of labeled N (μg N/plant)				
Shoot	2.1 (1.6)	2.4 (1.3)	1.5 (1.2)	1.9 (1.3)
Roots	13.0 (6.8)	12.1 (2.3)	6.8 (4.0)	8.6 (2.8)
Stem cutting	37.9 (13.5)	35.9 (11.7)	24.8 (4.0)	30.8 (6.7)

of N derived from $^{15}N_2$ (^{15}N%).

$^{15}N\%=(^{15}N \text{ atom \% of sample}-{}^{15}N \text{ atom\% of control plant})/0.99$

Results and Discussion

Direct evidence of the fixation of N_2 and subsequent translocation of the fixed N within the plant requires the use of $^{15}N_2$ gas as a tracer. We have used $^{15}N_2$ to study the assimilation and translocation of fixed N in soybean plants (19, 25). In these experiments, a water displacement method was used to introduce the $^{15}N_2$ gas into a cylinder in which the nodulated roots of an intact plant are enclosed. Water displacement is considered to be less harmful to plants than vacuum displacement. We used a similar water displacement method here to expose sugarcane plants grown from stem cuttings to ^{15}N-labeled air. Because N_2 fixation has been suggested to occur in the shoot as well as in the roots, a whole sugarcane plant including the roots, a small stem cutting and a single shoot was enclosed in a 500 mL plastic cylinder for exposure to $^{15}N_2$ gas.

The site of nitrogen fixation in young sugarcane plants associated with endophytic nitrogen-fixing bacteria

The shoot dry weight increased about 2 fold from day-3 (750 mg) to day-21 (1350 mg) (Table S1). The root dry weight increased about 6 fold from day-3 (59 mg) to day-21 (360 mg). The dry weight of stem cuttings did not changed markedly during the 21 days of the experiment.

The N content of the shoot at day-21 was approximately 13.5 mg, about twice that at day-7 just after the exposure to $^{15}N_2$. The N content of the roots at day-21 was about 2.3 mg, nearly 4-times that at day-7. On the other hand, the total N content of the stem cutting decreased from 14 mg at day-3 to 8 mg at day-21 (Table S1).

Fig. 3A shows the percentage of N derived from $^{15}N_2$ (^{15}N%) in total N of the shoot, roots and stem cutting after 3 days of exposure. The ^{15}N% in the roots was highest at 2.22%, suggesting that the roots are the most active site for N_2 fixation. The stem cutting also exhibited ^{15}N incorporation but only at about one tenth of this level (0.271%), while the shoot contained only about one hundredth as much (0.027%). Table 1 shows the changes in ^{15}N% in each part of the plant at 3, 7, 14 and 21 days. The ^{15}N% in the roots at day-7 (2.29%) was almost the same as at day-3 (2.22%), suggesting ^{15}N fixation in the roots to be inhibited after 3 days possibly due to deleterious conditions in the closed environment of the cylinder without a change of air and culture solution for 7 days. This indicates that actual N_2-fixing activity in sugarcane under natural conditions might be higher than the results obtained in this experiment.

Fig. 3B shows the amount of N derived from $^{15}N_2$ in each organ at day-3. All the data for 3, 7, 14 and 21 days are listed in Table 1. At day-3, the stem cutting showed the highest value (about 38 μg^{15}N), followed by the roots (13 μg^{15}N) and shoots (2 μg^{15}N). The large amount of ^{15}N in the stem cutting is due to the much greater N content of the cutting in these young plants compared to that of the shoot and roots. The stem cutting, roots and shoot contained total N amounts of 14 mgN, 0.59 mgN and 7.6 mgN, respectively (Table S1).

These results offer direct evidence of the sites of active biological nitrogen fixation in young sugarcane plants. At this early stage of development, the root system was very small and so its contribution to the fixation of N_2 was lower than that of the much more substantial stem cutting (Fig. 3B). However, in later growth stages, and especially in the field, the root system becomes much more extensive in comparison with the stem cutting. Its relatively greater biomass will likely enable it to contribute more to the plant's total N_2 fixation than the stem cutting. This is indicated in these experiments where, five months after the rooted cuttings were transplanted to pots (Fig. 1C), the roots contained 85 mgN, which accounted for about 15% of the total N in the whole plant (570 mgN).

The stem cutting showed N_2 fixation, possibly due to endophytic diazotrophs such as *Glconacetobacter diazotrophicus*. The sugarcane (*Saccharum officinarum* L.) cultivar NiF-8 used in our experiments is grown commercially on Miyako-jima Island, Japan. Analyzing the stem juice of this cultivar, Asis *et al.* (2) found 21 bacterial isolates with acetylene-reducing activity (indicative of N_2 fixation), of which 13 were putatively characterized as *Acetobacter diazotrophicus* and 4 as *Herbaspirillum seropedicae*. The N_2-fixing microorganisms in the roots may have originated from the stem cutting because the cutting initially did not have roots, although it is possibile that some N_2-fixing microorganisms infected the plant during the cultivation. In contrast to the roots and stem cutting, the young shoot contained very little ^{15}N. In the early growth stages from 2 to 3 weeks after bud break, the number of N_2-fixing microorganisms in the shoots might be too low to detect appreciable nitrogen fixation.

Translocation of N and fixed N in young sugarcane plants during $^{15}N_2$ feeding and the chase period

Fig. 4A shows the changes in the distribution of total N in the young sugarcane plants. At three days after the start of $^{15}N_2$ treatment, the stem cutting contains 63% of total N and the roots and shoots each contain 2.7% and 34%, respectively. The percentage of N in the stem cutting decreased gradually to 34% at day-21, while that in the roots and the shoot increased to 9.8% and 56%, respectively at day-21. Fig. 4A clearly shows that the storage of N in the stem cutting contributes to the growth of the shoot and roots from planting to 21 days after ^{15}N treatment (35 days after bud break). This suggests that the stem cutting serves as a storage organ supporting the initial growth of the roots and shoot and supplementing supplies of carbohydrate, N and other nutrients.

Fig. 4B shows the changes in the distribution of the fixed ^{15}N among the shoot, roots and stem cutting. The distribution of ^{15}N among these organs was relatively constant and did not change significantly from day-3 to day-21 after exposure of $^{15}N_2$. On average, the stem cutting, roots and shoot account for 73%, 22.5% and 4.5% of fixed N respectively. This result is quite different from the results of experiments with soybeans exposed to $^{15}N_2$. After 10 h of exposure, only 36% of the fixed ^{15}N remained in the nodules, while the balance was located as follows; roots 9%, stems 17%, leaves 18%, pods 10% and seeds 10% (19).

N derived from $^{15}N_2$ in 80% ethanol-soluble and insoluble fractions

The samples were extracted with 80% ethanol, and separated into ethanol-soluble and ethanol-insoluble fractions. The ethanol-soluble fraction contains low molecular weight compounds such as amino acids and ammonia, and the ethanol-insoluble fraction contains large molecules such as proteins and nucleic acids. The amount of soluble N in shoots was relatively constant during the 21 days, while the amount of insoluble N increased from 7 mg to 13 mg (Table S2). The 80% ethanol-soluble N accounted for a small fraction in roots around 5–10% of total N (Table S2). On the other hand, soluble N was relatively abundant at 3.28 mg in the stem cutting, and accounted for about 23% at day-3, after which 80% ethanol-soluble and insoluble N decreased during growth (Table S2).

In the sugarcane experiment, the abundance of ^{15}N was lower in the soluble than insoluble fractions at day-3 and at day-7 of exposure to $^{15}N_2$ (Table S2). This suggests that the fixed N is primarily incorporated into the cell components of the endophytic bacteria, rather than being transferred to the cells of the host plant. This contrasts with soybean nodules where the abundance of ^{15}N in the 80% ethanol-soluble fraction just after $^{15}N_2$ exposure was about 5-times higher than that in the insoluble fraction (20, 25). This enhancement is due to an active turnover and to the small size of the soluble fraction in the nodules.

The abundance of ^{15}N in the free amino acid pool was determined after separation by two-dimensional thin layer chromatography (22). In the stem cutting, asparagine and alanine were the major free amino acids, while 4-amino butyric acid (GABA) and valine were also detected. In the shoot, asparagine and alanine again dominated the free amino acid pool, while GABA, valine, hydroxyproline, glycine, glutamate and glutamine were also detected. After 7 days of exposure, the abundance of ^{15}N in alanine and asparagine in the stem cutting and shoot was lower than 0.04% (data not shown). These very low ^{15}N% values support the interpretation that the endophytes do not excrete low molecular weight compounds such as ammonia or amino acids. Lethbridge and Davidson (15) suggested that the endophytic bacteria only transferred fixed N to the plant when they died and were eventually decomposed. In contrast, Cohjo *et al.* (8) suggested that 50% of the N fixed by *Gluconacetobacter diazotrophicus* was transferred to the co-cultured yeast in an *in vitro* system, so a small portion of the fixed N may be used by the host plant tissue at the site of fixation.

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

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 cuttings were initially cultured in water not in soil, so the N-fixing endophytes in the roots might originate from the stem cutting or root primordia. If this is the case, to support active N_2 fixation, nitrogen-fixing bacteria may move into the developing roots, and colonize the intercellular space. In an earlier study with NiF 8 sugarcane using a ^{15}N dilution technique (3), after 5 months of cultivation in a pot supplied with ^{15}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 N fixation. These are [1] that the host plant provides carbohydrates to the root endophytes, and [2] that oxygen concentrations are usually lower in the roots and soil than in the atmosphere (pO_2 0.21). Low pO_2 is beneficial to protect nitrogenase from oxygen damage, this enzyme being very sensitive to high pO_2.

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 N-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. Glconacetobacter diazotrophicus*) are adapted to high sucrose concentrations while our very young shoots did not have mature stems that offered these conditions.

However, Zakria *et al.* (36) recently reported very low levels of $^{15}N_2$-fixing activity in pieces of mature sugarcane stem (of NiF-8 and Ni15) in which sugar concentrations were as high as 20%. These stems showed inconsistent acetylene-reducing activities. Therefore, the fixation of N_2 in mature sugarcane stems may be influenced also by the types and numbers of endopytes present, by stem age/maturity, and by other undefined physiological conditions.

The absence of any significant translocation of fixed ^{15}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. However, it does not mean that there is no contribution of N fixation in the roots to shoot growth. When the N_2-fixing bacteria in the roots eventually die, the products of their decomposition may well contribute to the growth of the roots and other parts 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.* (1) calculated N_2 fixation rates, fertilizer efficiency and the turnover of organic matter in sugarcane production in Thailand. Using the ^{15}N natural-abundance method, they estimated that the contribution of crop N_2 fixation to the overall N economy of many of the plantations was about 0–30%. Meanwhile, the contributions of N from 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.

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 (14). By promoting biological N fixation through associative diazotrophs, the cost associated with N fertilizer usage in sugarcane production can be reduced and environmental problems such as NO_3^- leaching or N_2O gas emission, consequent upon the use of excessive chemical fertilizers, can be avoided. Further research will be important for promoting more efficient N_2 fixation rates in sugarcane production. Further study of $^{15}N_2$ fixation with larger plants in pots or fields is required to clarify whether the results obtained for young plants apply at later stages.

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