Estimation of contribution of nitrogen fixation in sugarcane (*Saccharum officinarum* L.) plants with ^{15}N dilution method in relation to nitrogen supply period.

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Summary

Pot experiment was conducted in warm greenhouse in Niigata University to estimate the contribution of nitrogen fixation in sugarcane (*Saccharum officinarum* L. var. NiF8) plants using ^{15}N dilution method in relation to N supply period. Sugarcane plants were grown from a cut stalk in water for 20 days and the young shoots were 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}N labeled ammonium sulfate was supplied at the rate of 100 mgN per pot a week, 3) N100N0: ^{15}N labeled ammonium sulfate was supplied at the rate of 100 mgN per por a week until 6 weeks after transplanting (WAT), then the plants were cultivated without N supply. The growth of the plants was measured every week, and plants were harvested at 12 WAT and 20 WAT. Total nitrogen content and ^{15}N abundance in each part were determined. Acetylene reduction activity was measured for each part of harvested plants. N0 plants grew very poor, and sole nitrogen fixation is not enough to support vigorous growth of sugarcane plants. N100 plants and N100N0 plants showed relatively similar shoot length and leaf number, but the N100N0 plants leaves were pale and the N concentration was almost a half of N100 plants. At 12 WAT, total N content was 408 mgN and 286 mgN per plant in N100 treatment and N100N0 treatment, respectively. The amount of nitrogen derived from nitrogen fixation (Ndfa) in N100 and N100N0 was 87 mgN (21%Ndfa) and 48 mgN (17%Ndfa) per plant respectively. At 20 WAT, total N content was 569 mgN (100N) and 292mgN (100N0N), and the amount of Ndfa was 87 mg (15%Ndfa) in 100N and 57 mgN (20%Ndfa) in 100N0N treatment. Among organs, the estimated %Ndfa tended to be higher in old leaves and stalk, and lower in green leaves and stems. From this experiment, the continuous supply of N fertilizer did not inhibit nitrogen fixation in sugarcane compared with N deficient plants.

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Key words : endophyte, ^{15}N dilution method, nitrogen fertilizer, nitrogen fixation, sugarcane,

INTRODUCTION

Sugarcane (*Saccharum officinarum* L.) is a tall, perennial grass (family Poaceae, subfamily Panicoide), and is widely 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 (Reis *et al.* 2007). Sugarcane has been used mainly for sugar and for an alcoholic drink production. Recently, the use of sugarcane alcohol (ethanol) as an automotive fuel to replace gasoline has rapidly increased (Boddy *et al.* 1995, Marris 2006).

In 2011, world production of sugarcane was 1,794 million tons. 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 the USA (267 million tons). In 2011, sugarcane was cropped over an area of 25 million hectares, and the average yield was 70.5 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).

Recently, the nitrate pollution in underground water originating from chemical nitrogen fertilizers from sugarcane fields has been a problem in Okinawa, where a major site of sugarcane production in Japan (Nakanishi 2001, Nishiguchi *et al.* 2005). In Brazil, sugarcane crops accumulate between 100 and 200 kgN per hectare per year, while N fertilization rates are relatively low, usually less than 60 kgN per hectare

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(Boddey 1995, Reis *et al.* 2007). Also, the response of sugarcane crops to N fertilizers is usually very weak (Boddey 1995, Reis *et al.* 2007). In some areas of Brazil, sugarcane has been grown continuously for more than 100 years without any N fertilizer being applied at all (Dong *et al.* 1994). These circumstantial evidences suggest a high potential for biological nitrogen fixation (BNF) in sugarcane. Using a ^{15}N dilution technique in which ^{15}N-labeled fertilizer is supplied and the amount of non-labeled N is considered to be derived from nitrogen fixation, Urquiaga *et al.* (1992) calculated the contribution of BNF in several cultivars of sugarcane, and found it to be about 70% for the most promising genotypes. Asis *et al.* (2002) estimated the contribution of nitrogen fixation of sugarcane cultivar NiF8 by ^{15}N dilution and natural ^{15}N abundance techniques, and total %Ndfa were estimated 27-38%. They also reported that the estimated percentage of nitrogen derived from atmospheric nitrogen (%Ndfa) was 26% for the roots, 14.1% for the stem and 20.5% for the leaves. Nishiguchi *et al.* (2005) also estimated the contribution of BNF using the ^{15}N dilution technique, and found that between 10% and 40% of sugarcane N was derived from biological nitrogen fixation (BNF) depending on the cultivars (Ni15, F172, and NiF8) and also on the availability of mineral N. The Ni15 cultivar showed the highest BNF. Yoneyama *et al.* (1997) examined the contribution of BNF using a ^{15}N natural-abundance method in Brazil, the Philippines and Japan, comparing the abundance of δ ^{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.* (1991) showed that 25-60% of the N assimilated in sugarcane at various sites in Brazil was derived from BNF.

For the presence of endophytic diazotrophs in sugarcane juice, Bellone and Bellone (2006) concluded that in mature reasions of the sugarcane stem *Gluconacetobacter diazotrophicus* grows more abundantly than *Herbaspirillum seropedicae* or *Azospirillum brasilense*. Recently, complete genome sequence of the sugarcane nitrogen-fixing endophyte *Gluconacetobacter diazotrophicus* Pal5 was reported (Bertalan et al. 2009)

In this study, a ^{15}N dilution method was employed to investigate the effect of nitrogen fertilization period on N_2 fixation in sugarcane plants. Three fertilizer treatments were applied, 1) N0: Nitrogen free culture solution was supplied, 2) N100: ^{15}N labeled ammonium sulfate was supplied at the rate of 100 mgN per pot a week, 3) N100N0: ^{15}N labeled ammonium sulfate was supplied at the rate of 100 mgN per por a week until 6 weeks after transplanting (WAT), then plants were cultivated without N supply thereafter. The growth of the plant was measured and three plants were harvested for each treatment at 12 WAT and 20 WAT, and the total nitrogen content and ^{15}N abundance in each part were determined. Acetylene reduction activity was measured for each part of harvested plants.

MATERIALS AND METHODS

Plant cultivation

Sugarcane cut stalks were initially cultivated in water from 28th July, 2000. Stalks of sugarcane (cv. NiF8) were kindly provided from Dr. Yasuhiro Nakanishi in Miyako Subtropical Experimental Farm, Tokyo University of Agriculture, Okinawa, Japan. The stalks were washed with tap water and then with de-ionized water. Both end of the stalk were trimmed to about 12-15 cm long with one node having a shoot bud. About 2-3 cm of trimmed stalks were used for the measurement of N concentration of each stalk. Sugarcane stalks were put in a container with de-ionized water at the level of 2 cm depth, in which the upper half of stalk was exposed to the air (Figure 1A). De-ionized water was changed every day. At 16th August the shoot grew to about 30 cm high, then each plant was transplanted to 1/5000 a Wagner pot filled with vermiculite. Each stalk was buried about 3 cm below the soil surface. Nitrogen free culture solution (Table 1) was supplied every day.

The Sugaecane plants were grown in greenhouse in Niigata University, where tempreatare was kept at 20 ℃ during winter.

Fertilizer treatment

Three fertilizer treatments were conducted. The 0N treatment indicates that the plants were cultivated

Table 1 Composition of nitrogen free culture solution

	Compound Chemical formula	Concentration (mg/L)	Compound Chemical formula	Concentration (mg/L)	Compound Chemical formula	Concentration (mg/L)
Solution Ⅰ	K_2SO_4	109	KCl	0.935	K_2HPO_4	8.5
Solution Ⅱ	$CaCl_2 \cdot 2H_2O$	184				
Solution Ⅲ	$MgSO_4 \cdot 7H_2O$	123				
Solution Ⅳ	H_3BO_4	0.367	$CuSO_4 \cdot 5H_2O$	0.0315	$MnSO_4$	0.189
	$ZnSO_4 \cdot 7H_2O$	0.144	$(NH_4)_6Mo_7O_{24}$	0.004	$CoSO_4$	0.028
	$NiSO_4 \cdot 6H_2O$	0.0035				
Solution Ⅴ	EDTA・2Na	18.6	$FeSO_4 \cdot 7H_2O$	13.9		

continuously with nitrogen free culture solution (Table 1). The 100N treatment indicates that plants were supplied with 100 mgN once every week. The ^{15}N labeled ammonium sulfate (5.08 atom%^{15}N) was used to estimate the amount of N derived from fertilizer by ^{15}N dilution method. The 100N0N treatment indicates that plants were cultivated with solution containing N until 6 WAT, then they were cultivated with N free solution thereafter.

Growth measurement and sampling

The increase in the plant shoot length and new leaf number were measured every week. Plants were harvested at 12 WAT (10th November, 2000) and 20 WAT (6th January, 2001). Sugarcane plant was separated into green leaves, old senescent leaves, stems, a stalk, and roots. The stalk means the original stalk (cut stem) for planting. Plant parts were dried, ground into a fine powder, and the nitrogen concentration was determined by Kjeldahl digestion method (Ohyama *et al.* 2004). The ^{15}N abundance was determined by an emission spectrometry using Kjeldahl digested solution (Ohyama *et al.* 2004). Nitrogen derived from fertilizer (Ndff) was calculated from total N content and ^{15}N abundance in each part. Nitrogen derived from original stalk (Ndfs) was estimated by the assumption that the stalk N was distributed in proportion to total N among organs. The amount of N derived from nitrogen fixation was calculated; total N − Ndff − Ndfs.

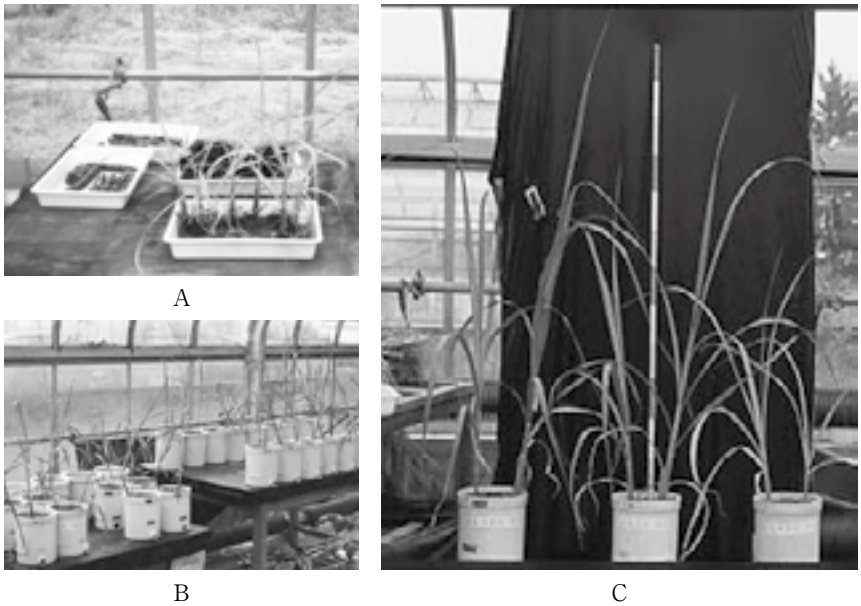
Acetylene reduction activity (ARA) was measured for each part at 12 WAT and 20 WAT. Small pieces of each part were put into a 15 mL test tube with butyl-rubber stopper. About 10% volume (1.5 mL) of air in the test tube was replaced by acetylene gas (C_2H_2). Another tube was used to measure ethylene (C_2H_4) evolution without acetylene replacement. Test tubes were incubated at 30 ℃ for 24 hours. Then 0.5 mL of gas inside were analyzed for ethylene concentration using gas chromatography.

RESULTS AND DISCUSSION

Growth of sugarcane

After stalks were cultivated in de-ionized water (Figure 1), shoots rapidly grew to about 30 cm long for 20 days

Figure 1 Cultivation of sugarcane stalks in deionized water.



A B C

Figure 2 Photographs just before transplanting (A), after transplanted to a pot (B), and measurement of shoot length (C)

(Figure 2A). Then each plantlet was transplanted to a pot filled with vermiculite (Figure 2B).

Figure 3 shows the increase in shoot length after transplanting. The growth was different among fertilizer treatments. The shoot length increased rapidly from 2 WAT to 6 WAT in 100N and 100N0N treatments, but it was very slow in 0N treatment. After 6 WAT, the shoot length increased only a little until 12 WAT. The shoot length at 12WAT was 140cm in 100N fertilizer treatment, 130cm in 100N0N treatment, and 18 cm in 0N fertilizer treatment. The plant growth became very slow after 6 WAT may be due to lower temperature, short daytime or the restriction of soil volume about 3L in a pot.

Figure 4 shows the increase in new leaves after transplanting. The leaf number increased until 12 WAT in both 100N and 100N0N fertilizer treatment. However, the increase almost stopped in 0N treatment. The leaf number at 12 WAT were 8 in 100N fertilizer treatment, 7 in 100N0N treatment, and 2 in 0N treatment.

As shown in Figure 3 and Figure 4, the increase in shoot length and new leaf number of sugarcane were slightly lower in 100N0N treated plants than those in 100N plants. However, the leaf color was pale in 100N0N treated plants compared with 100N plants (Figure 5).

Nitrogen content and the origins of nitrogen

Figure 6 shows the nitrogen content separated by three origins of nitrogen; N derived from fertilizer, N derived from planted stalk, and N derived from atmospheric nitrogen. At 12 WAT, total N content was 408 mgN and 286 mgN per plant in N100 treatment and N100N0 treatment, respectively. N derived from fertilizer in N100 treatment was 284 mgN and much higher than that in N100N0 treatment (176 mgN). The amount and percentage of nitrogen fixation in N100N0 and N100 was 48 mgN (17%Ndfa) and 87 (21%Ndfa) per plant, respectively. At 20 WAT, total N in N100 plants increased to 569mgN, but not in N100N0 at 292 mgN. The amount and

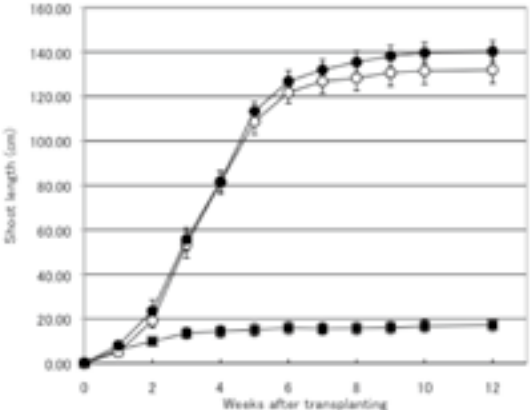


Figure 3 Comparison of sugarcane shoot length among N100, N100N0, and N0 fertilizer treatments.
● N100, ○ N100N0, ■ N0

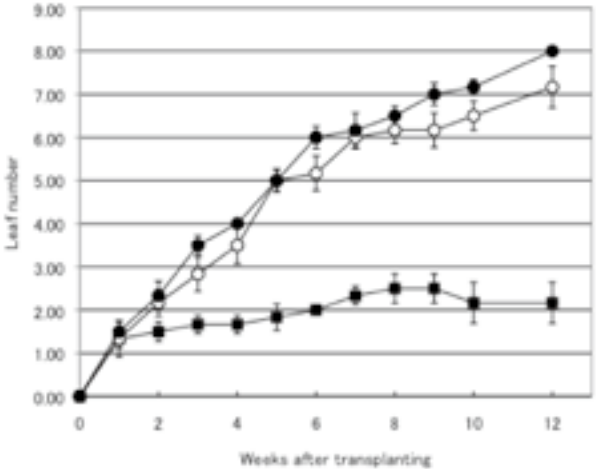


Figure 4 Comparison of sugarcane leaf number among N100, N100N0, and N0 fertilizer treatment.
● N100, ○ N100N0, ■ N0

A　　B

Figure 5 Sugarcane plants with N100 (A) and N100N0 plants (B) at 12 WAT

percentage contribution of Ndfa were almost the same from 12WAT to 20 WAT in both N100N0; 57 mgN (20%Ndfa) and N100; 88 mgN (15%Ndfa) fertilizer treatments. The low nitrogen fixation activity from 12 to 20 WAT may be due to lower temperature, short day-length or limited soil volume in soil.

Figure 7 and Table 2 show the distribution of %Ndff, %Ndfs, and %Ndfa in N100N0 and N100 fertilizer treatments at 12 WAT and 20 WAT. The old leaves (35% in N100N0 and 51% in N100 treatment) and stalks (39% in N100N0 and 48% in N100 treatment) showed the highest %Ndfa at 12WAT followed by roots (21% in N100N0 and 31% in 100N). The %Ndfa in green leaves (17% in N100, and 13% in N100N0) and stem (10% in N100N0 and 12% in N100) were relatively lower. The similar trends were observed at 20 WAT. The old leaves (28% in N100N0 and 25% in N100 treatment) and stalks (35% in N100N0 and 24% in N100 treatment) showed the highest %Ndfa at 12 WAT followed by roots (26% in N100N0 and 15% in N100 treatment). The %Ndfa in green leaves (18% in N100N0 and 16% in N100 treatment) and stem (13% in N100N0, and 10% in N100 treatment) were relatively lower.

Table 3 shows acetylene reduction activity of each part of sugarcane in N100N0 and N100 fertilizer treatments at 12 WAT and 20 WAT. ARA was detected in all part of sugarcane. In N100N0 treatment at 12 WAT, stem showed the highest activity followed by the roots and stalk. In N100 treatment at 12 WAT, root showed the highest activity among organs. At 20 WAT the ARA increased from 12 WAT, and old leaves and stalk showed higher ARA in N100 fertilizer treatment. On the other hand, green leaves and roots showed highest ARA in N100N0 fertilizer treatment.

Effect of nitrogen supply on nitrogen fixation

From the present study nitrogen (ammonium sulfate) supply to soil did not depress nitrogen fixation at 12 WAT and 20 WAT. The amount of N derived from nitrogen fixation was higher in N100 treatment than in N100N0 treatment, indicating that the supply of nitrogen fertilizer might promote plant growth and gave positive effect on nitrogen fixation activity consequently. The depression of nodule growth and nitrogen fixation activity in soybean by combined nitrogen, especially nitrate, is well known (Ohyama *et al.* 2011). Compared with legume-rhizobium symbiosis, sugarcane-endphyte symbiosis seems to be not strictly regulated by nitrogen supply.

Nishiguchi et al. (2005) reported that the effect of 45 days interruption of nitrogen supply after 0.5mN nitrate application for 45 days was different among sugarcane cultivars. Continuous supply of nitrate for 90 days decreased the amount of nitrogen derived from BNF in sugarcane variety, Ni15, but it increased in variety NiF8. Response of NiF8 variety to nitrate was similar to our results in which ammonium sulfate was supplied.

Muthukumarasamy *et al.* (1999) investigated the effect of N fertilizer application on the number of sugarcane endophytes, *Acetobacter diazotrophicus* (renamed *Gluconacetobacter diazotrophicus*) and *Herbaspirillum* spp. isolated from sugarcane plants. The application of N fertilizer deceased the number of *A. diazotrophicus*, but not the number of *Herbaspirillum* spp. The nitrogen effect may be different among endophyte species.

Nitrogen transport from various N sources

In this study Ndfa was distributed in all part of sugarcane plants, although %Ndfa was higher in old leaves

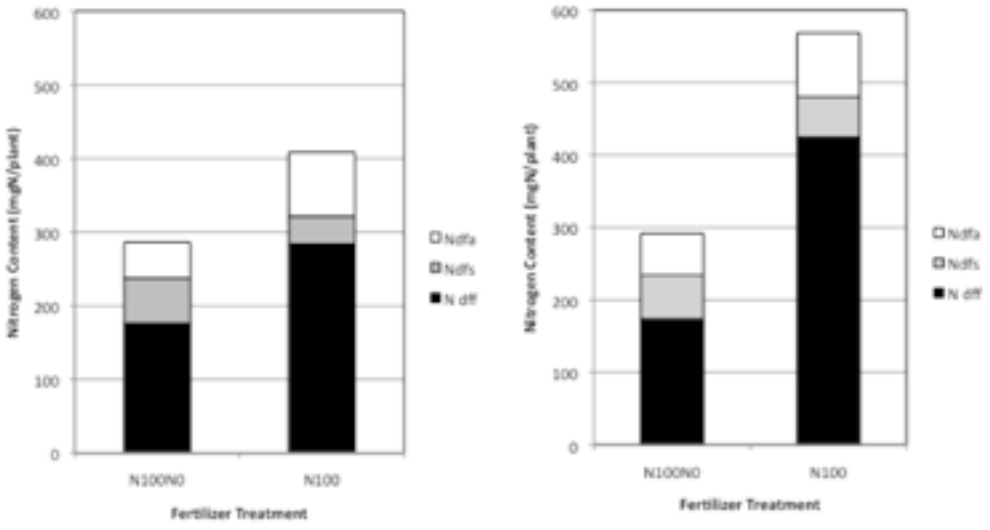


Figure 6 Nitrogen content derived from atmospheric nitrogen (Ndfa), from planted stalk (Ndfs), and from fertilizer (NDff) at 12 WAT (A) and 20 WAT (B).

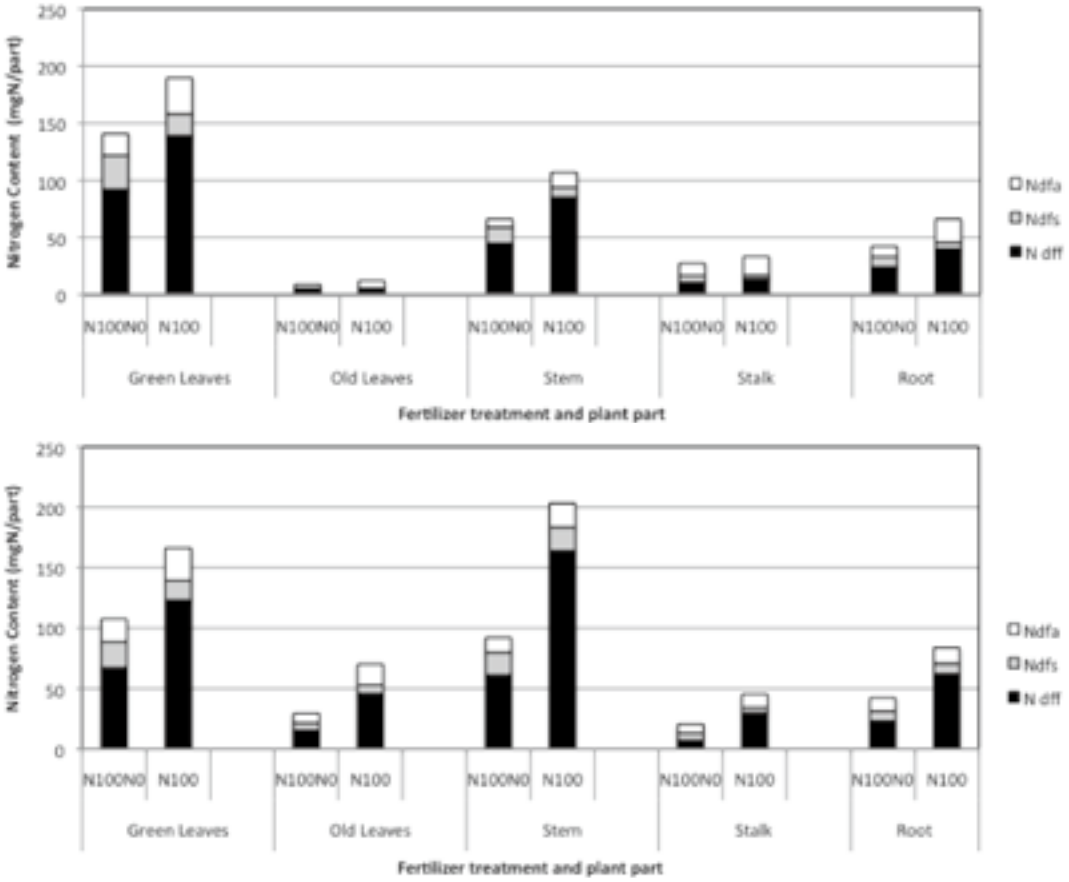


Figure 7　Nitrogen content in each part of sugarcane plant derived from atmospheric nitrogen (Ndfa), planted stalk (Ndfs), and fertilizer (Ndff) at 12 WAP (upper) and 20 WAP (lower).

Table 2 Percentage of Nitrogen derived from fertilizer (%Ndff), from stalk (%NDfs), and atmospheric nitrogen (%Ndfa) at 12 WAT and 20WAT

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 3 Acetylene reduction activity in each part of sugarcane plant at 12 and 20 WAT (nmole C_2H_4 production h^{-1} g^{-1}DW)

Plant part	Fertilizer treatment	12WAT	20WAT
Green Leaves	N100N0	1.77	7.05
	N100	1.3	2.1
Old Leaves	N100N0	0.97	3.89
	N100	1.67	4.21
Stem	N100N0	4.43	1
	N100	1.5	1.22
Stalk	N100N0	1.83	3.78
	N100	1.03	3.51
Root	N100N0	2.3	6.19
	N100	2.27	3.1

and stalk than green leaves and stem (Figure7, Table 2).

In our previous paper (Momose et al. 2009), the tracer $^{15}N_2$ was used to investigate sites of N_2 fixation and the possible translocation of the fixed N in young sugarcane plants at 2-5 weeks after planting. Sugarcane plants 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 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%). At 21 days after $^{15}N_2$ exposure, most of ^{15}N fixed either in the roots or in the stem cutting remained there and was not appreciably transported to the shoot. On the other hand, about a half of the N originating in the stalk had been transported to the shoot and roots, suggesting that the cutting played a role in supplying N for growth. The results were quite different from the fate of fixed N in soybean nodules, which is rapidly transported from nodules to roots and shoots.

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施肥窒素供給期間によるサトウキビ（*Saccharum officinarum* L.）の生育に対する窒素固定寄与の^{15}N 希釈法による推定

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要　約

施肥窒素供給期間の違いによるサトウキビ（*Saccharum officinarum* L., 品種農林８号）の窒素固定寄与率を^{15}N 希釈法で調べる目的で新潟大学の温室内でポット試験を実施した。サトウキビの種茎を水中で発芽させたのち、バーミキュライトを充填した1/5000 a のワグネルポットで栽培した。窒素施肥処理は、３区設けた。１) N0区 : 無窒素培養液のみ供与、２) N100: ^{15}N 標識の硫酸アンモニウム水溶液（100 mgN）を週１回ポットに添加、３) N100N0: 移植から移植後６週目まで、^{15}N 標識の硫酸アンモニウム水溶液（100 mgN）を週１回ポットに添加し、その後無窒素栽培した。移植後植物の地上部の長さと葉数を毎週測定した。また、移植12週間後と20週間後に植物を採取し、各部位の窒素含有量、^{15}N 濃度とアセチレン還元活性を測定した。N0区の植物はほぼ生長が停止し、窒素固定だけでは生育を支えるには不十分であることが確認された。N100区と N100N0 区の植物は、地上部の長さや葉の数は、大きな差はなかったが、N100N0区の葉は葉色が薄く、窒素含有量も N100区の半分程度であった。移植12週間後には , N100 区と N100N0 区の株あたり全窒素含有率は、それぞれ408 mgN と286 mgN であった。^{15}N 希釈法で推定した窒素固定量は、N100 区と N100N0 区でそれぞれ株あたり87 mgN（窒素固定由来窒素割合 %Ndfa, 21%）、48 mgN（17%Ndfa）であった。移植20週間後では、N100 区と N100N0 区の株あたり全窒素含有率は、それぞれ569 mgN と292 mgN であった。窒素固定推定量は、N100 区と N100N0 区でそれぞれ株あたり87 mgN（%Ndfa, 21%）、57 mgN（33% Ndfa）であった。器官別窒素固定割合では、枯葉、種茎が高く、緑葉、茎が低い傾向がみられた。

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