Characteristics of nitrogen fixation and nitrogen release from diazotrophic endophytes isolated from sugarcane (*Saccharum officinarum* L.) stems.

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Summary

The cultures of strain JA1 and JA2 isolated from sugarcane stem (putatively identified as *Gluconacetobacter diazotrophicus*) showed $^{15}N_2$ fixation activity and acetylene reduction activity (ARA). These activities in JA1 were highest at 0.4% O_2 on solid agar culture, and at 0% O_2 in liquid culture. In both culture conditions, $^{15}N_2$ fixation activity and ARA decreased with increasing O_2 concentrations up to 20%. The growth and ARA of the strain JA1 cultivated with liquid LGIP medium were measured everyday for 10 days. Bacteria growth increased rapidly at 1st day after inoculation, and continued to increase until 6 days, then the increase was almost stopped thereafter. The strain might regulate bacterial density, possibly by quorum sensing mechanism. The ARA increased rapidly from 1st day to 5th day, but it decreased rapidly after 6th day. Very low ARA was detected after 7th day to 10th day. This suggests that nitrogen fixation is active only during early stage of proliferation of JA1, and after the bacterium growth stops nitrogen fixation activity will be 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. The nitrogen release mechanism from endophytes to sugarcane plant is very important to support N nutrition for sugarcane growth. From the experiment with JA1 strain, ^{15}N fixed during 24 hours was mainly distributed in bacteria fraction and only a little portion (about 4%) was released to the medium. However, at 10 days after one day feeding of $^{15}N_2$, a significant portion of fixed ^{15}N was distributed to the medium, especially the percentage of released ^{15}N was highest at 40% under 20% O2 conditions. This result indicates that the cultured JA1 released N after stopping growth and nitrogen fixation, possibly by their death and degradation.

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Key words : diazotrophic endophyte, $^{15}N_2$, *Gluconacetobacter diazotrophicus*, nitrogen fixation, nitrogen release, sugarcane,

INTRODUCTION

Sugarcane (*Saccharum officinarum* L.) is widely cultivated in tropical and warm-temperate regions (Reis *et al.* 2007). Recently, the use of sugarcane alcohol (ethanol) as an automotive fuel to replace gasoline has rapidly increased (Boddy *et al.* 1995, Marris 2006). Sugarcane is a C4 plant, which has an efficient photosynthetic system. 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 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). This circumstantial evidence suggests a high potential for biological nitrogen fixation (BNF) in sugarcane. Using a ^{15}N dilution technique, 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. Yoneyama *et al.* (1997) reported 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 detected. Asis *et al.* (2002) estimated the contribution of nitrogen fixation of Japanese sugarcane cultivar NiF-8 by ^{15}N dilution and natural ^{15}N abundance techniques, and total %Ndfa (percentage of N derived from atmospheric dinitrogen) were estimated 27-38%. Hiyama et al. (2013) investigated the contribution of nitrogen fixation by ^{15}N dilution in NiF-8 with different N application period. Although %Ndfa were slightly lower in N sufficient plants (15%) compared with N limited plants (20%), the absolute amount of Ndfa was higher in N sufficient plants (87 mgN plant^{-1}) than N limited plants (57 mgN plant^{-1}) at 20 weeks after transplanting. Nishiguchi *et al.* (2005) also estimated the contribution of BNF using ^{15}N dilution technique, and found that between 10% and 40% of sugarcane N was derived from biological nitrogen fixation

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(BNF) depending on the cultivars (Ni15, F172, and Nif-8) and also on the availability of mineral N. The Ni15 cultivar showed the highest BNF.

As for the presence of N_2-fixing bacteria in sugarcane, diazotrophic bacteria (nitrogen fixing 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 (Döbereiner 1961). Other diazotrophs, such as *Bacillus, Azotobacter, Derxia, Enterobacter, Erwinia, Klebsiella*, and *Azospirillum*, have also been isolated from the sugarcane rhizosphere (Reis *et al*. 2007). In 1988, a new species of *Acetobacter* was found inside the sugarcane stem and named *Acetobacter diazotrophicus* (Cavalcante and Dobereiner 1988), though this was later renamed to *Glconacetobacter diazotrophicus* (Yamada *et al*. 1997). 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 (Cavalcante and Döbereiner 1988). 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' (Reis *et al*. 1994).

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*. (2007) 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 predominant N_2-fixing symbiont of sugarcane or that it even expresses active nitrogenase in plants (James *et al*. 2001). In addition, the sites of colonization and the movement through xylem vessels of *G. diazotrophicus* within sugarcane plants are controversial (Dong *et al*. 1994, 1997, James *et al*. 2001).

Recently, Saito *et al*. (2008) 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*) (Mano and Morisaki, 2008, Zakria *et al*. 2007) and sweet potatoes (*Ipomoea batatas* L.) (Terakado-Tonooka *et al*. 2008).

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

To promote sugarcane growth, the transport of N from diazotrophic endophytes to plant is important in addition to the occurrence of nitrogen fixation activity. The mechanism by which N is transferred to the host sugarcane plant from N_2-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 appoplast of the host tissue and the plant cells then absorb the released N compounds. This is an 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 (Ohyama *et al*. 2009). 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.

The tracer $^{15}N_2$ was used to investigate the sites of N_2 fixation and the possible translocation of the fixed N using young sugarcane plants at 2 weeks after transplanting (Momose *et al*. 2009). Young sugarcane plants (*Saccharum officinarum* L.) from a stem cutting were exposed to $^{15}N_2$-labeled air in a 500mL plastic cylinder. Plants fed $^{15}N_2$ for 7 days were grown in normal air for 21 days of chase period. After 3 days of $^{15}N_2$ 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 (Ohyama *et al*. 2009). After 21 days of chase period, about a 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.

MATERIALS AND METHODS

Strains of diazotrophic endophytes isolated from sugarcane juice

Three srains of diazotrophic endophytes, JA1, JA2, and JA15, which have been isolated from juice of 6 month-old sugarcane (*Saccharum officinarum* L. cv. NiF-8) were kindly provided from the late Dr. Shoichiro Akao. Asis *et al*. (2000) reported that the strains JA1 and JA2 were putatively identified as *Acetobacter diazotrophicus* (renamed *Gluconacetobacter diazotrophicus*), and JA15 was putatively identified as *Herbaspirillum rubribalbicans* based on the characteristics of acetic acid production from ethanol, growth on meso-erythritol, and API 20NE diagnostic test kit (Asis *et al*. 2000).

Culture conditions of strains

Strains were maintained on a solid agar culture with 1/5 strength of PDA medium. This medium contained extract of

40g fresh potato tuber and 4 g of glucose and 15 g of agar in 1 L of distilled water. 40 g of thin slices of fresh potato tuber were boiled in 500 mL of distilled water for 20 minutes. After cooling, the soup was squeezed by two layers of cheese cloth. Then the solution of 4 g of glucose in 500 mL of distilled water was mixed with potato extract and filled up to 1 L. For long-term storage, cultured strain was suspended in 1 mL of 0.85% sodium chloride solution and mixed with 1 mL of glycerol, then stored in a freezer under -30 ℃ .

Nitrogen-free LGIP medium was employed for measurement of $^{15}N_2$ fixation and acetylene reduction activity (ARA). The LGIP medium contained the ingredients per liter as follows; K_2HPO_4 (0.2 g), KH_2PO_4 (0.6 g), $MgSO_4 \cdot 7H_2O$ (0.2 g), $CaCl_2 \cdot 2H_2O$ (0.02 g), $NaMoO_4 \cdot 2H_2O$ (0.002 g), $FeSO_4 \cdot 7H_2O$ (0.01 g), yeast extract (0.02 g), 5 mL of 0.5% bromothymol blue in 0.2 M KOH, sugarcane sugar (100 g), 5 mL of sugarcane stem juice. The pH of the solution was adjusted at 6.8. Fresh sugarcane stem surface was sterilized by ethanol and the hard epidermis was peeled, then the tissue inside was cut and crushed with a juicer. The squeezed juice with cheese cloth was separately stored in plastic tubes in freezer until used.

Measurement of $^{15}N_2$ fixation and acetylene reduction activity of JA1, JA2 and JA15 strains under various oxygen concentrations

Each strain was cultured in a 9 mL test tube with a butyl-rubber stopper. 2 mL of LGIP medium with 1.5% agar were put into the tube and strain was culture on the slant at 30 ℃ for 6 days.

$^{15}N_2$ gas (30% N_2, 99.4 atom%^{15}N), Ar and O_2 were mixed in tubes using a vacuum system (Figure1, 2, 3). The cultured test tube was connected with vacuum system with a hypodermic needle, and gas inside was evacuated by a vacuum pump. Then 0%, 0.2%, 0.4%, 2%, 5.0%, 10%, or 20% of O_2 was introduced in the tube, and $^{15}N_2$ gas and Ar was introduced until the gas pressure inside becomes 100 kPa (1 atmosphere). The tubes were separated from vacuum system and incubated at 30 ℃ for 1 day (24 hours).

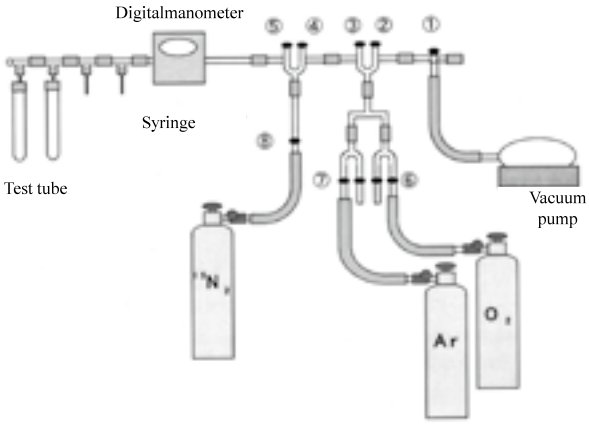
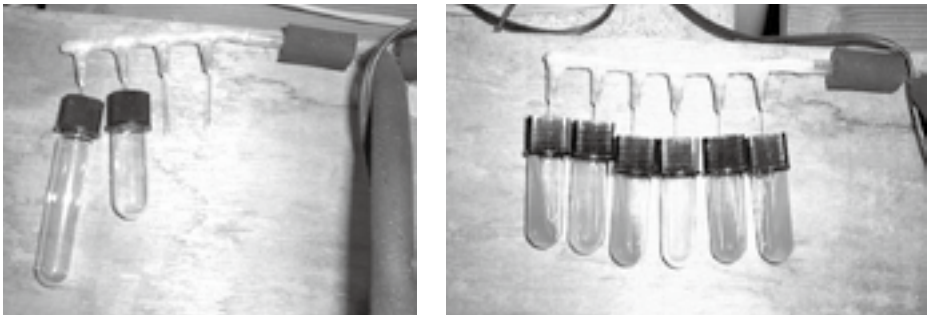


Figure 1 Outline drawing of vacuum system for exchanging the gas in test tubes.
Test tubes are connected with hypodermic needles on the left side. The air in test tubes are evacuated by vacuum pump. $^{15}N_2$ gas, Ar, O_2 was introduced from cylinders by monitering the pressure inside with digital manometer. Numbers show cocks.

Figure 2 Photograph of vacuum system for exchanging the gas in test tubes.



A **B**

Figure 3 Photographs of test tubes connected to the hypodermic needles.
A: 15 mL and 10 mL test tubes with butyl rubber stopper were connected
B: 10 mL test tubes with cultured bacteria with LGIP solid medium were connected to hypodermic needles.

After $^{15}N_2$ gas exposure, the test tubes were evacuated again with vacuum system, and 10% acetylene was introduced into the tubes at the same concentration of O_2 as those for $^{15}N_2$ exposure. The tubes were incubated at 30 ℃ for 1 hour, and the ethylene concentration in 0.5 mL of the gas inside was analyzed by Gas chromatography.

After acetylene reduction assay, colony of bacteria on the agar slant was washed to a pylex test tube and dried with aluminum block heater at 140℃. Dry weight was measured and it was digested by Kjeldahl digestion method (Ohyama *et al*. 2004). The digested solution was filled up to 10 mL. Nitrogen concentration in diluted solution was determined by Indophenol method (Ohyama *et al*. 2004).

The ammonia in 4 mL of diluted solution was concentrated in 0.4 mL of 0.2M HCl using Conway's micro-diffusion method. Aliquot of concentrated solution was put into a pylex-glass tube and a discharge tube was made for ^{15}N analysis by an emission spectrometry (JASCO N-150) (Ohyama *et al*. 2004).

Changes in the growth and ARA of JA1 strain during culture period

The suspension of JA1 strain was inoculated in 300 mL of LGIP liquid medium in a 500 mL flask, and incubated at 30 ℃ . For 10 days from inoculation, ARA and cell density at OD_{560} were measured everyday.

Nitrogen release from fixed nitrogen in bacteria to the medium

Nitrogen release from bacteria after nitrogen fixation was investigated using liquid culture of JA1 strain under various oxygen concentrations. The liquid culture of JA1 was preincubated for 4 days in LGIP medium, and $^{15}N_2$ gas with 0%, 0.2%, 0.4%, 2%, 5.0%, 10%, or 20% of O_2 were exposed to the liquid culture of JA1 for 1day. At 24 hours after starting $^{15}N_2$ exposure, the suspension was washed into a centrifuge tube, and separated into bacteria (precipitate) and medium (supernatant) by centrifugation with 12,000 g for 10 min. The precipitate was washed by distilled water several times and separated by centrifugation with 12,000 g for 10 min. Both precipitate and supernatant plus washings were digested by Kjeldahl digestion method, and the N concentration and ^{15}N abundance were measured (Ohyama *et al*. 2004).

The similar experiment was done under 0%, 0.4%, 5%, and 20% O_2 conditions. After feeding $^{15}N_2$ for 24 hours, bacteria and medium fractions were separated by centrifugation. Also bacteria culture continued for 3 days and 10 days after exposed to $^{15}N_2$ for 24 hours. Then N content and ^{15}N abundance of bacteria and medium fraction were determined.

RESULTS AND DISCUSSION

Nitrogen fixation activity of JA1, JA2 and JA15 strains under different oxygen concentration.

Figure 4A and Figure 4B show the nitrogen fixation activity (nmole fixed $^{15}N_2$ h^{-1} $tube^{-1}$) and acetylene reduction activity (ARA) (nmole ethylene formed h^{-1} $tube^{-1}$), respectively. The JA1 strain showed the highest $^{15}N_2$ fixation activity (3.5 nmole fixed $^{15}N_2$ h^{-1} $tube^{-1}$) and ARA (3.3 nmole ethylene

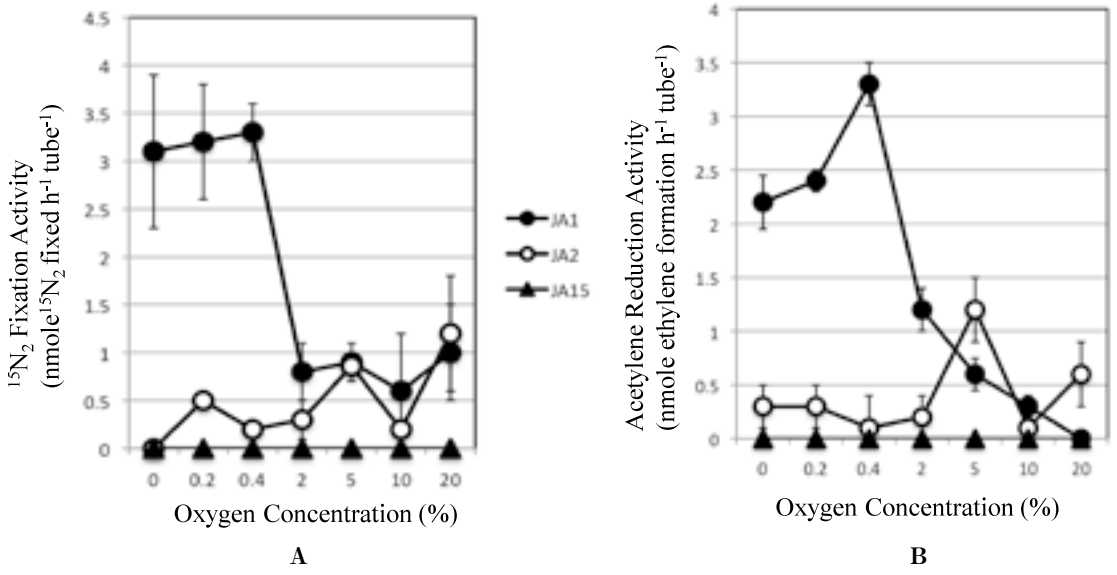


Figure 4 $^{15}N_2$ fixation activity and acetylene reduction activity of strains JA1, JA2 and JA15 under 0, 0.2, 0.4, 2, 5, 10, 20% O_2 concentrations.
$^{15}N_2$ fixation activity was measured at first for 24 h, and then acetylene reduction activity was measured for 1 h.
A: $^{15}N_2$ fixation activity
B: Acetylene reduction activity.

formed h^{-1} $tube^{-1}$) under 0.4% O_2. Both activities decreased under higher O_2 concentration above 2%. On the other hand, JA2 strain showed the high ARA under 5% O_2 and under 20% O_2. Neither ^{15}N fixation activity nor ARA was detected in JA15 under any O_2 conditions.

Changes in the growth and ARA of JA1 strain during culture period

Figure 5 shows the changes in bacteria density (OD_{560}) and ARA during incubation at 30 ℃ after inoculation to the LGIP liquid medium. Optical density increase rapidly at 1st day, and increased until 6 days after inoculation, then the increase was very slow thereafter. The ARA increased rapidly from 1st day to 5th day (1.53 nmole ethylene formed h^{-1} $tube^{-1}$), but it decreased rapidly after 6th day. Very low ARA was measured after 7th day.

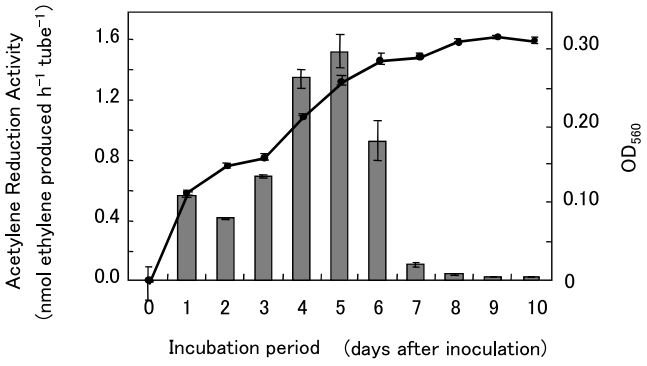


Figure 5 Changes in growth and acetylene reduction activity after inoculation to the liquid LGIP medium.
Optical density OD_{560} of the culture liquid (line graph) and the acetylene reduction activity (bar graph) were measured everyday.

Nitrogen release from fixed nitrogen in bacteria to the medium

Figure 6A and 6B shows the nitrogen fixation activity (nmole fixed $^{15}N_2$ h^{-1} $tube^{-1}$) and ARA (nmole ethylene formed h^{-1} $tube^{-1}$) of strain JA1 cultured with liquid LGIP medium, respectively. Both $^{15}N_2$ fixation (1.7 nmole fixed $^{15}N_2$ h^{-1} $tube^{-1}$) and ARA (1.9 nmole ethylene formed h^{-1} $tube^{-1}$) were highest under 0% O_2 conditions in liquid culture. This is different from solid medium shown in Figure 4A and 4B, showing the highest value at 0.4% O_2. Both $^{15}N_2$ fixation and ARA decreased with increasing O_2 concentration.

Most of the fixed ^{15}N remained in bacteria compared with medium. At 0% O_2, fixed ^{15}N in bacteria was (1.7 nmole fixed $^{15}N_2$ h^{-1} $tube^{-1}$), and fixed ^{15}N in medium was (0.065 nmole fixed $^{15}N_2$ h^{-1} $tube^{-1}$), which accounted for about 4% of total fixed $^{15}N_2$.

Figure 7 shows the changes in the fixed ^{15}N in bacteria (strain JA1), medium, and sum of bacteria plus medium. Figure 8 shows the percentage distribution of fixed ^{15}N between bacteria and medium. The percentage of ^{15}N in the medium was high at first day at 0% O_2 (17%). The percentage distribution of ^{15}N was constant at 9th and 16th days after inoculation. On the other hand, the distribution of fixed ^{15}N at 6th day was lowest (7%) at 20% O_2, but it increased to 40% at 16th days.

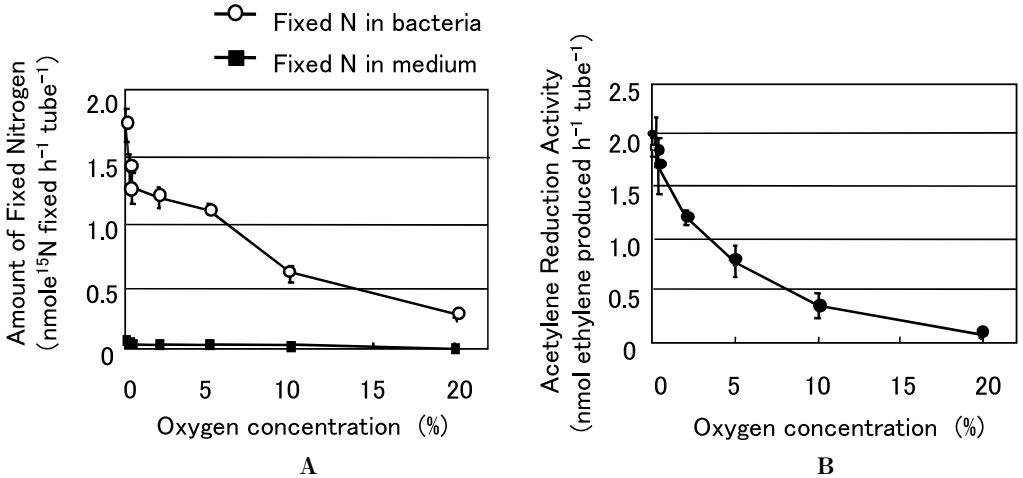


Figure 6 Amount of $^{15}N_2$ fixed and acetylene reduction activity of strains JA1, in LGIP liquid culture under 0, 0.2, 0.4, 2, 5, 10, 20% O_2 concentrations.
$^{15}N_2$ fixation activity was measured at first for 24 h, and then acetylene reduction activity was measured for 1 h.
A: Amount of $^{15}N_2$ fixed in bacteria and medium
B: Acetylene reduction activity.

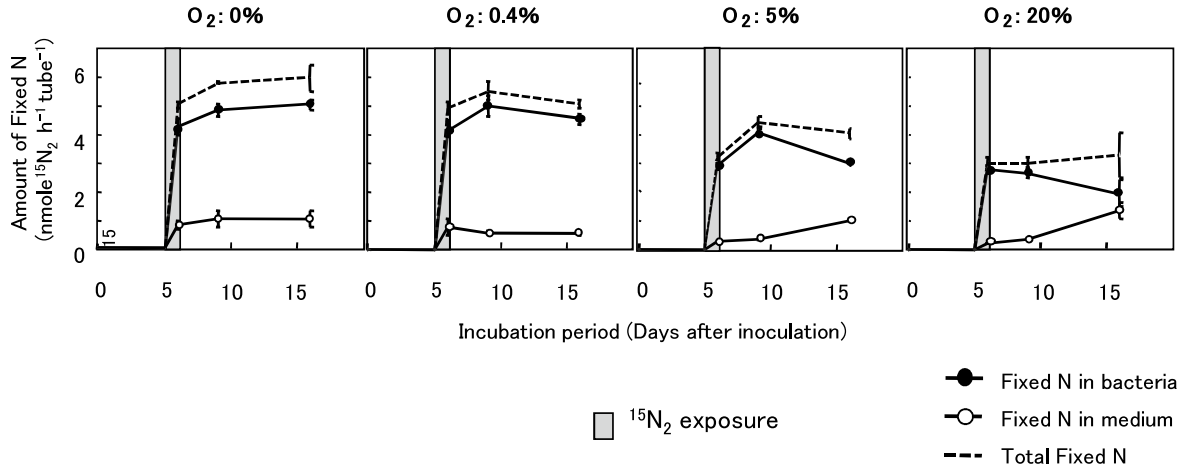


Figure 7　The amount of $^{15}N_2$ fixed in bacteria (strain JA1) and the medium under 0, 0.4, 5, 20% O_2 concentrations. $^{15}N_2$ was exposed to the test tubes for 24 h after 5th day after inoculation, and then bacteria and the medium was analyzed at 6th, 9th, and 16th days after inoculation.

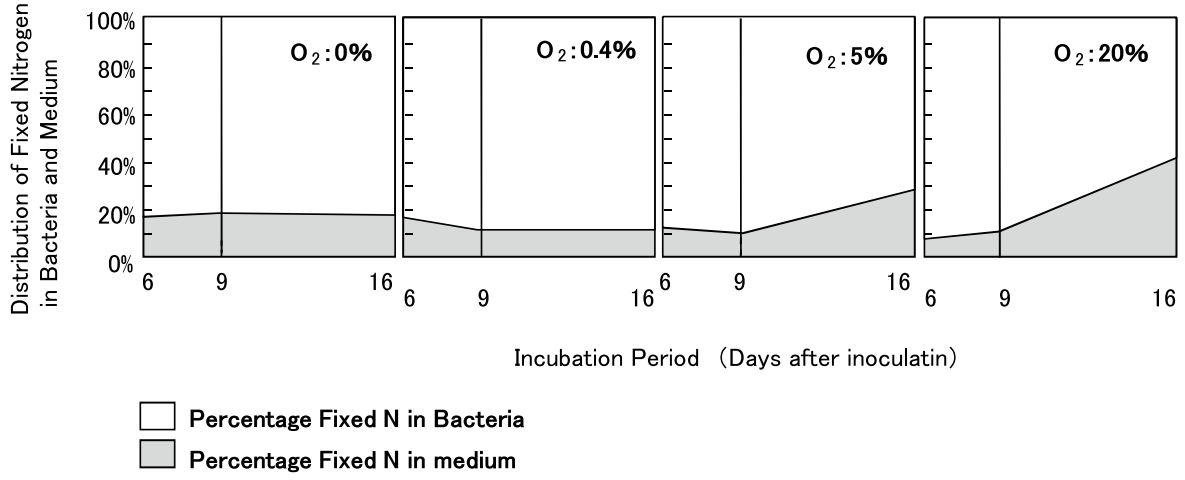


Figure 8　Percentage distribution of fixed ^{15}N in bacteria (strain JA1) and the medium under 0, 0.4, 5, 20% O_2 concentrations.

Characteristics of nitrogen fixation by isolated bacteria from sugarcane stem

The solid and liquid culture of strain JA1 and JA2 (putatively identified as *G. diazotrophicus*) with LGIP medium showed $^{15}N_2$ fixation activity and ARA. The activities of JA1 were highest at 0.4% O_2 on solid agar culture (Figure 4), and 0% O_2 in liquid culture (Figure 6). In both culture conditions, $^{15}N_2$ fixation activity and ARA in JA1 decreased with increasing O_2 concentrations up to 20%. The solid culture of JA15 (putatively identified as *H. rubribalbicus*) did not show any $^{15}N_2$ fixation activity and ARA under any O_2 concentrations (0%-20%) (Figure 4).

The bacteria growth and ARA of the strain JA1 was investigated everyday from inculation to liquid LGIP medium for 10 days (Figure 5). Optical density increase rapidly at 1st day, and increased until 6th days after inoculation, then the increase was slow thereafter. The strain JA1 regulates bacterial density, possibly by quorum sensing mechanism. Quorum sensing is a system of regulating population density by many species of bacteria. The ARA increased rapidly from 1st day to 5th day (1.53 nmole ethylene formed h^{-1} $tube^{-1}$), but it decreased rapidly at 6th day. Very low ARA was measured after 7th day. This suggests that nitrogen fixation activity is only during early stage of growth and after the growth of bacteria becomes slow, the nitrogen fixation activity is inactivated. If the situation is the same inside the sugarcane organs, the continuous proliferation is necessary to keep nitrogen fixation activity of diazotrophic endophytes.

Nitrogen release from bacteria to medium

Concerning to the nitrogen release from endophytes to sugarcane plant is very important to support N nutrition for

sugarcane growth. From this experiment with JA1 strain, ^{15}N fixed during 24 hours was mainly distributed in bacteria fraction and only a little portion was released to the medium, about 4% of the total fixed ^{15}N (Figure 6). However at 10 days after feeding $^{15}N_2$ a significant portion of fixed ^{15}N was distributed to the medium (Figure 7). Especially the percentage of released ^{15}N was highest at 40% under 20% O_2 conditions (Figure 8). This result indicates that N release from cultured JA1 release N after stopping growth and nitrogen fixation, possibly due to their death and degradation. The high concentration of O_2 may be toxic to JA1 strain by oxidation stress.

In soybean nodules, N_2 fixed by the bacteroids (N_2-fixing rhizobia) is readily excreted to the cytosol of the plant cells in the form of ammonia. The ammonia is then assimilated via the glutamine synthetase/glutamate synthase (GS/GOGAT) system (Ohyama and Kumazawa 1978), and used mainly to produce ureides, allantoin and allantoic acids, and the ureides are transported to the various plant organs via the xylem vessels (Ohyama and Kumazawa 1979 ab). 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 (Ohyama and Kumazawa 1980b).

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サトウキビ（*Saccharum officinarum* L.）から分離した窒素固定内生菌の窒素固定と窒素放出機構

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要　約

サトウキビ（*Saccharum officinarum* L.，品種農林８号）の茎から分離された内生窒素固定菌（JA1株、JA2株、菌の性質から *Gluconacetobacter diazotrophicus* と予測された）の$^{15}N_2$固定活性とアセチレン還元活性（ARA）を調べた。試験管内で固形LGIP培地または液体LGIP培地で培養した菌を、酸素濃度を０から20％まで変化させて、24時間$^{15}N_2$固定活性を測定し、その後同じ試験管で１時間ARAを測定した。固形培地では、O_2　0.4％で最大活性を示したが、液体培地では、O_2　0％で最大活性を示した。どちらも、O_2　20％まで、酸素濃度が高まるにつれて活性が低下した。JA1株の生育密度（吸光度）とARAをLGIP液体培地に植継いでから10日後まで毎日測定した。菌密度は、植継１日後に急速に高まり６日後までは増加したが、それ以降10日目までは増加が抑制された。JA1株の増殖抑制はクオラムセンシング機構によると予想される。ARAも植継５日目までは上昇したが、６日目以降急激に低下した。７日目から10日目までは、ほとんど活性を示さなかった。この結果は、本菌の窒素固定活性は、菌の増殖中にのみ発現し、増殖が止まると窒素固定活性も停止することが示唆された。このことが、サトウキビ植物体内でも同様であるとすると、内生菌が増殖を続けることが、窒素固定の維持に必要であると推定される。内生菌から、植物への窒素の放出は、サトウキビの生育促進に重要である。液体培養したJA1株は、24時間の$^{15}N_2$固定直後では、大部分の固定窒素が菌体内に残存しており、培地中には、固定窒素総量の４％程度のみ放出していた。一方、24時間$^{15}N_2$を固定させた後、酸素濃度0-20％で10日間培養を続けたところ、酸素濃度が高い程、放出^{15}N量の割合が増加し、O_2　20％では、固定窒素総量の40％が培地に放出されていた。これらの結果は、JA1株は、窒素固定停止後、増殖を停止し、菌の死に伴う分解により、窒素を放出するのではと予想された。

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