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Short Communication

Nitrous Oxide Emission and Microbial Community in the Rhizosphere of Nodulated Soybeans during the Late Growth Period

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We examined N_2O emissions from the rhizosphere of field-grown soybeans during the late growth stage (99–117 days after sowing). Marked emissions were detected from the nodulated root systems of field-grown soybeans, whereas a non-nodulating soybean mutant showed no emission. Degraded nodules exclusively generated the N_2O. A culture-independent analysis of microbial communities showed *Bradyrhizobium* sp., *Acidvorax facilis*, *Salmonella enterica*, *Xanthomonas* sp., *Enterobacter cloacae*, *Pseudomonas putida*, *Fusarium* sp., nematodes, and other protozoans to be more abundant in the degraded nodules, suggesting that some of these organisms participate in the N_2O emission process in the soybean rhizosphere.

Key words: nitrous oxide, microbial community, rhizosphere, soybean, rhizobia

Nitrous oxide (N_2O) is a key atmospheric greenhouse gas, which contributes to global warming and the destruction of stratospheric ozone (2, 5, 17). Agricultural land is a major source of N_2O through the microbial transformation of nitrogen in soil, namely nitrification and denitrification (7, 14, 19, 34), and contributes significantly to the net increase in atmospheric N_2O (2, 20, 21, 29). In particular, more N_2O is emitted from agricultural fields with leguminous crops than that with non-legume crops (5, 8, 18, 27). A major source of N_2O emissions from soil has been regarded as N-fertilizer so far (3, 6). Recently, however, Yang and Cai (33) reported that the amount of N_2O emitted from a soybean-cropped field was drastically increased in the late growth period, suggesting that senescence and the decomposition of roots and nodules contribute to emissions. Moreover, Kim *et al.* (16) measured N_2O flux from fields cropped with three soybean genotypes that differed in nodulating phenotypes. They found that the emission from the field with nodulating soybeans was several times higher than that from the field with non-nodulating soybean, suggesting that nodules participate in the emission process. However, the source of the N_2O from soybean-cropped fields has yet to be elucidated. In contrast, soybean nodules are known to be able to take up N_2O (26). Thus, the metabolism of N_2O in nodulated soybeans is so complex that the mechanism underlying N_2O emissions from soybean-cropped fields remains elusive. In the present study, we examined 1) identification of the source of N_2O from soybean-cropped fields, and 2) the microbial community in the soybean rhizosphere to identify organisms potentially involved in the emission of N_2O.

Soybean (*Glycine max* (L.) Merril) seeds of the nodulating cultivar Enrei and a non-nodulating mutant (En1282) derived from Enrei (13), were sown on 2 June 2005 and on 31 May 2006 in an experimental field of Tohoku University (Kashimadai, Miyagi Prefecture, Japan). The soil was classified as gray lowland soil (13).

To compare N_2O emissions from nodulating and non-nodulating soybean roots, plants were harvested 99 and 117 days after sowing (DAS) in 2005. After soil was removed from the roots by hand shaking, the root systems were cut and enclosed in a 300-mL airtight vial. Zero and 10 minutes after the vial was sealed, 40 mL of gas in the vial was sampled and transferred to a 19-mL vacuum vial. The concentration of N_2O in the gas sample was determined by a gas chromatograph (GC-14BpsE, Shimadzu, Kyoto, Japan) equipped with an electron capture detector. Six replicates were supplied for each measurement. Root samples were dried at 80°C for 48 h and weighed to calculate the rate of N_2O emission based on biomass.

To compare N_2O emissions from degraded and fresh soybean nodules, the cultivar Enrei was harvested 99 DAS in 2006. The root systems were washed in tap water and cut into roots and nodules. As some nodules were degraded, the collected nodules were further separated into 'fresh' nodules and 'degraded' nodules (Fig. S1). In the present study, fragile nodules with an obviously dark surface were defined as 'degraded' nodules. The sample aliquot (0.5 g) of each was enclosed in a 19-mL vial with a rubber stopper. Gas in the vial was sampled 0, 60, 120 and 180 minutes after sealing. The emission of N_2O was evaluated as described above.

For the culture-independent community analysis, the root systems of nodulating cultivar Enrei and non-nodulating mutant En1282 were carefully harvested at 99 DAS in 2005, and soil loosely attached was removed by hand shaking. The roots were transported to the laboratory on ice. After being washed in sterilized water, nodules (fresh and degraded) and root segments were separated using sterilized forceps or a cutter knife. The samples were stored at −80°C prior to the extraction of DNA. Microbial DNA was extracted from the surface of nodules or roots using a FastDNA SPIN Kit for soil (Qbiogene, Irvine, CA, USA). Briefly, the sample (0.5 g)

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Table 1. N_2O emissions from root systems of nodulating (Enrei) and non-nodulating (En1282) soybeans in the late growth period

Soybean line	Nodulation	N_2O emission (nmol hr^{-1} g fresh $weight^{-1}$)			
		99 DAS		117 DAS	
Enrei	+	2.15±0.98	**	1.85±1.05	**
En1282	–	0.00±0.02		0.01±0.03	

The values represent the means±SD for six replicates. Significant differences by t-test between Enrei and En1282 are indicated by two asterisks (**) for $p<0.01$.

was suspended in DNA extraction solution in a 2-mL screw-capped tube. After the addition of glass beads, the tube was processed in a bead beater (Micro-Dismembrator S, B. Braun Biotech International, Melsungen, Germany) for 1 min at 2,600 rpm. A ribosomal intergenic spacer analysis (RISA) was carried out to evaluate the microbial DNA as described by Ikeda *et al.* (12). As the primer sets, ITSF/ITSReub (4) was used for bacteria, while 1406f/3126T (9) and 2234C/3126T (23) were used for fungi and other eukaryotes.

The degraded nodules were further subjected to microscopic observation to check the organisms inside them. Nodules were crushed with a glass rod in a Petri dish, and then suspended in a 10 mM NaN_3 solution. Organisms in the solution were observed under Nomarski optics (BX51 and DP70 CCD camera, Olympus, Tokyo, Japan).

To examine the contribution of soybean nodules to the emission process, we compared N_2O emissions from the root systems of nodulating and non-nodulating soybean. From the roots of the normal nodulating cv. Enrei, marked N_2O emission was observed, while little N_2O was generated from the roots of the non-nodulating line En1282 (Table 1). In the late growth period, namely 99 and 117 DAS here, a significant difference between N_2O emissions from Enrei and En1282 was found ($p<0.01$). These results provided direct evidence that N_2O was generated from the soybean rhizosphere in the late growth period and that N_2O was derived from root systems with nodules.

To determine the source of N_2O in more detail, the root system of Enrei was fractionated into degraded nodules, fresh nodules and roots. Values of N_2O flux are shown in Fig. 1. The concentration of N_2O in the vials containing root samples without nodules was the same as that in the atmosphere, indicating no emission of N_2O from the root itself. In the vials with fresh nodules, very little N_2O was emitted or taken up. In contrast, degraded nodules clearly released N_2O, the amount increasing with time. The rate of emission from degraded nodules was 2.48 nmol N_2O g^{-1} hr^{-1} based on fresh weight. Thus, the emission of N_2O from the soybean root system is nodule-dependent, and the main source of N_2O is degraded nodules.

Kim *et al.* (16) reported that nodulation was related to N_2O flux from the soil surface. Moreover, Yang and Cai (33) suggested that the senescence and decomposition of soybean roots and nodules resulted in the emission of N_2O. Here, we have demonstrated for the first time that degraded nodules are the source of N_2O in the soybean rhizosphere.

The emission of N_2O from degraded nodules is probably mediated by microbial activity in the rhizosphere such as nitrification and denitrification (7, 19). Hence, we attempted to identify soil organisms that increased in abundance in degraded nodules rather than fresh nodules and roots by conducting a culture-independent community analysis. Such organisms might be involved in the emission of N_2O. Culture-independent methodologies have provided vital clues regarding the abundance and spatial distribution of microbial groups in the rhizosphere (24, 25). In particular, RISA provides highly reproducible profiles of bacterial communities (11). RISA profiles (Fig. 2) showed more bands in the profile of roots of nodulating soybean (Enrei) than non-nodulating soybean (En1282). Furthermore, several bands were enhanced in the profile of degraded nodules compared to that of fresh nodules (bands a-k with arrows in Fig. 2).

A sequence analysis of the RISA amplicons revealed that *Bradyrhizobium* sp., *Acidvorax facilis*, *Salmonella enterica*, *Xanthomonas* sp., *Enterobacter cloacae* and *Pseudomonas putida* as bacteria, and *Fusarium* sp. as a fungus were enhanced in degraded nodules (Table 2). Nematodes (*Zeldia* sp.) and other protozoans (*Tetrahymena thermophila*) were characteristically detected in degraded nodules. These organisms in the degraded nodule are the potential players which are related to the emission of N_2O. In fact, among the bacterial species described above, *Acidvorax* sp. (10), *Enterobacter* sp. (1) and *Bradyrhizobium* sp. (15) are known to be

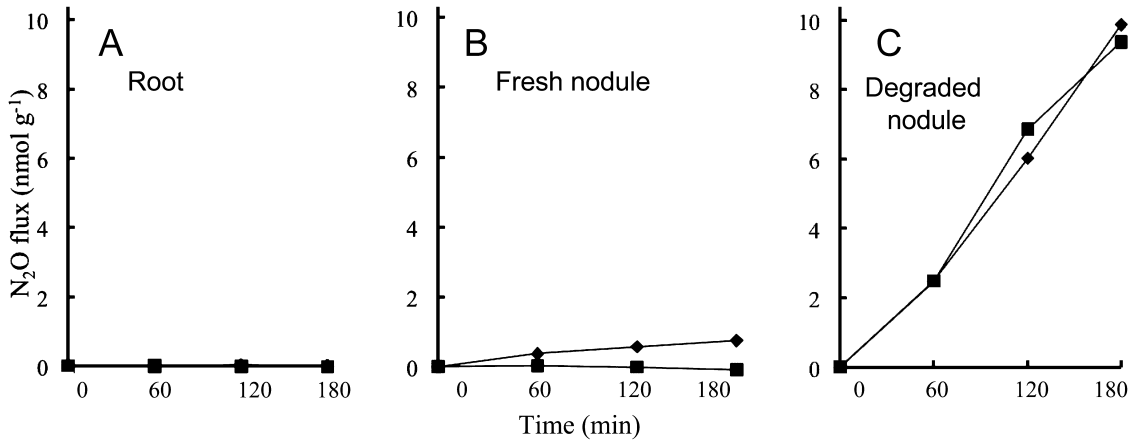

Fig. 1. N_2O flux from the roots (A), fresh nodules (B) and degraded nodules (C) of a field-grown soybean (Enrei) root system at 99 days after sowing (DAS). N_2O flux was expressed as nmol N_2O based on tissue gram fresh weight. Duplicated results were plotted.

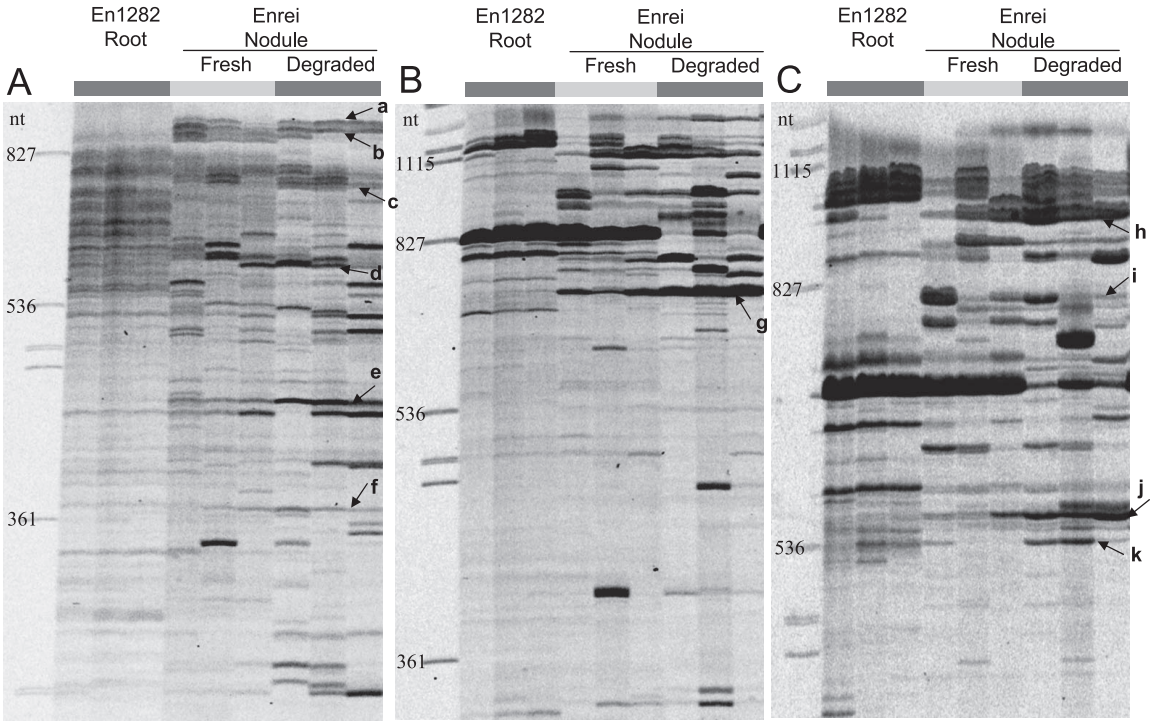

Fig. 2. RISA fingerprinting profiles of DNA extracted from roots, fresh nodules, and degraded nodules of field-grown soybeans. Bacterial RISA with primers ITSF/ITSReub (A) and fungal RISA with primers 1406f/3126T (B) and 2234C/3126T (C). Letters were assigned to characteric bands in the profile of degraded nodules. Triplicate results for each sample are shown.

Table 2. Microbial community characteristically appeared on the surface of degraded nodules

Primer set	Band[a]	Closest relative in known species[b]	Organism[c]	Score[d]
Bacteral RISA (ITSF/ITSR eub)	a	*Bradyrhizobium* sp.	B	343
	b	*Bradyrhizobium japonicum*	B	755
	c	*Acidovorax facilis*	B	523
	d	*Aeromonas hydrophila*	B	418
	d	*Salmonella enterica*	B	607
	d	*Xanthomonas* sp.	B	846
	e	*Enterobacter cloacae*	B	519
	f	*Pseudomonas putida*	B	323
Fungal RISA (1406f/3126T)	g	*Fusarium oxysporum*	F	1140
	g	*Oxytricha granulifera*	P	375
Fungal RISA (2234C/3126T)	h	*Pythium inflatum*	F	777
	i	*Zeldia* sp.	N	545
	j	*Fusarium solani*	F	1019
	j	*Uroleptus pisces*	P	648
	k	*Tetrahymena thermophila*	P	739

[a] Corresponds to the letters in Fig. 2.
[b] Sequence similarities between amplicons of RISA and known microbial species.
[c] Classified as B, bacteria; F, fungi; P, protozoa; N, nematoda.
[d] BLAST search score.

capable of denitrification. Moreover, potentially functional genes for denitrification have been reported for *Salmonella* sp. (30), *Xanthomonas* sp. (32) and *Pseudomonas* sp. (22). Among fungi, some strains of *Fusarium* sp. have a denitrifying capability (28, 31). It is likely that such microbes are involved in the emission of N_2O from degraded nodules. No nitrifying bacterium was detected in the degraded nodules in this work. Nematodes were frequently observed in the degraded

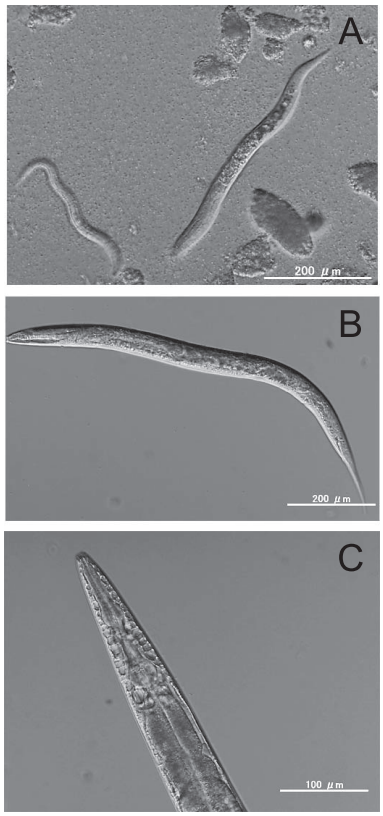

Fig. 3. Microscopic observation of nematodes in degraded soybean nodules. Nodules were sampled on 21 August, 2005 from soybeans grown at Kashimadai field. (A) An observation of crushed soybean nodules (Enrei) in a 10 mM NaN_3 solution. Bacteroid cells were observed around the nematodes. Higher magnified view of the whole body (B) and pharynx (C) of a nematode picked from a specimen of crushed soybean nodules.

nodules (10 positive fields in 10 degraded nodules) as shown in Fig. 3. RISA indicated the existence of nematodes as well (Table 2). Nematodes may be initial invaders of nodules, promoting further decomposition by physical destruction of the nodule's surface.

In conclusion, we showed that N_2O was exclusively emitted from degraded nodules of field-grown soybeans in the late growth period. In addition, we identified organisms potentially relevant to the emission of N_2O in rhizosphere soil. Because N_2O is generated by the complex processes of microbial nitrogen transformation including ammonification, nitrification and denitrification (7, 19), the mechanisms of its emission in degraded soybean nodules remain to be elucidated.

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