Effect of some cover crops and their secondary metabolites on nitrous oxide (N_2O) emission by *Pseudomonas* denitrifiers isolated from chemically fertilized corn farm soil

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Abstract*—Using a Pseudomonas denitrifying bacterium, which had been isolated from Japanese Andisol corn farm as an active nitrous oxide (N_2O) emitter and likely to be missing nosZ gene, we investigated denitrification-regulating activity of some cover crops or green manure plants against this N_2O emitter. In the preliminary screening, root exudates from the 10 seedlings of yellow flowering leaf mustard (*Brassica juncea*) and crimson clover (*Trifolium incarnatum*) showed 50% repression of the N_2O emission by an incomplete denitrifier* Pseudomonas *sp. 05CFM15-6D. When direct extracts of the seedling roots with MeOH were assayed, however, only the seedlings of* B. juncea *showed a remarkable inhibition of bacterial cell growth and N_2O emission at concentration equivalent to 10 seedlings. The root extract from* B. juncea *equivalent to 2 to 4 seedlings maintained inhibiting activity toward N_2O emission, while it did not affect bacterial cell growth. Conversely, water-soluble fraction from aboveground of European small radish (*Raphanus sativus *var.* sativus*) sprouts showed statistically significant acceleration of N_2O emission ($P<0.01$) with slight but insignificant cell growth activation. As some secondary metabolites are uniquely contained in these cover crops, suppressing or accelerating activity of such phytochemicals in N_2O emission was also investigated. Allyl isothiocyanate at 30 μM markedly inhibited N_2O emission of the* Pseudomonas *denitrifier but not suppressed its cell growth. In contrast, methyl isothiocyanate sinigrin, cyanamide, and betanin did not affect on N_2O emission of the denitrifier at 150-300 μM.*

Keywords*—cover crops,* Brassica juncea*, N_2O emission, allyl isothiocyanate,* Raphanus sativus *var.* sativus*.

I. INTRODUCTION

Nitrous oxide (N_2O) is an active greenhouse gas contributing more than 7% of global warming [1]. N_2O, one of the most active greenhouse gases, has a strong global warming potential and contributes to depleting ozone layer in atmosphere [2]. In global scale, agricultural soils are recognized as major anthropogenic N_2O emission sources. In particular, agriculture soil plays dominant roles in N_2O emission with widespread use of nitrogenous fertilizers and manure to drive emission increment [3,4], in which N cycle, biological denitrification is the most important process [5]. Denitrification is a microbial nitrate respiration process, in which the oxidized nitrogen compounds served as electron acceptors for energy production in anaerobic conditions [6,7].

Cover crops, including some Brassicaceae and Fabaceae plants are often introduced into farmland to be subjected to crop rotation or maintain soils before leaving idle. The cover crops often play a role of green manure to reserve mineral nutrition in the soil, while they are often cultivated for the purpose of repression and regulation of weeds [8,9] or soil-borne disease causative soil microorganisms [10,11]. In particular, yellow flowering leaf mustard (*Brassica juncea*), white mustard (*Sinapis alba*) [12], and hairy vetch (*Vicia villosa*) [13] are cover crops used worldwide for biofumigation and weed control. If any annual plants can suppress N_2O emission from the farmland soil, such cover crops are useful for cleaning of denitrifiers from the cropland soils. As cover crops are generally plowed into soil for fertilization, it may affect on denitrification processes in soil if plant tissues contained secondary metabolites active against denitrification-associated enzymes. In this paper, we wish to report unique responses of methanolic extracts from seedlings of some cover crops on an active N_2O emitter isolated from Andisol of corn farmland in Shicizunai, Japan. Also, we further discuss on soil management strategy to minimize N_2O emission from fertilized soils in croplands.

II. MATERIAL AND METHOD

2.1 Plant seeds and seedlings: Plant seeds used in this study are as follows: yellow flowering leaf mustard (*Brassica juncea* cv. Kibananochikara) and crimson clover (*Trifolium incarnatum* cv. Strawberry Candle) were purchased from Takii Seed Ltd. (Kyoto, Japan), and European small radish (*Raphanus sativus* var. *sativus* cv. Sakuranbohatsukadaikon) was from Atariya Noen Ltd. (Katri, Chiba Prefecture, Japan). Hairy vetch (*Vicia villosa* cv. Mamesuke) was from Snow Seed Co (Sapporo, Japan). As phytochemicals used, allyl isothiocyanate, methylisothiocyanate, and cyanamide were purchased from

Wako (Osaka, Japan), whereas sinigrin hydrate was from TCI (Tokyo, Japan). Betanin was extracted from red beetroot cultivated by ourselves and isolated using a reverse-phase column chromatograph technique [14].

2.2 N_2O emission assay: To investigate the main causative microorganisms of N_2O production in autumn, Winogradsky's mineral solution-based, a gellan gum soft gel medium [15] was used. As mineral N for the substrate of N_2O production, KNO_3 (each 500 mg L^{-1}) was added to the Winogradsky's mineral solution [16]. The pH of the solution was adjusted to 6.0 with 1 M H_2SO_4, followed by filtration through a polytetrafluoroethylene (PTFE) membrane (pore size, 0.45 µm; Merck Millipore, Billerica, MA, USA) to remove insoluble mineral residues. Gellan gum powders was 0.3 % (w/v), and powders of the gelling matrix was heated until melted, mixed well, and then cooled to room temperature. A 10.0 mL portion of the medium was poured into a 30-mL gas chromatographic vial (Nichiden-Rika Glass Co., Kobe, Japan) sealed with a butyl rubber plug and screw cap septum, and then autoclaved at 121 °C for 15 min. The headspace volume is 22.6 mL [17].

To obtain fresh inoculates for N_2O emission assay, Pseudomonas sp. was shake-cultured in 50 mL of Winogradsky's medium supplemented with 0.5% sucrose at 110 rpm at 20 °C for 24 h in dark. Inoculates were collected from 50 mL cultured medium by centrifuging at 8000 ×*g* at 4 °C for 10 min, washed with Milli-Q water several times, and then dissolved with sterilized water. Only for the preliminary assay, bacterial cells pre-cultured on potato-dextrose agar (Nissui, Tokyo, Japan) for 2 days at 25 °C were suspended into sterilized MilliQ water. The bacterial cell suspension (approximately 10^6 cells mL^{-1}, OD_{660} of 0.4) was added to Winogradsky's medium in the 30-mL gas chromatographic vials. After 7 days incubation at 20 °C in dark, the headspace gas was analyzed with GC. The initial medium containing 0.05% of sucrose and adjusted to pH 6.0, from which the N_2O emitters were selected for further incubation to analyze optimum pH and N_2O emission assay under relatively poor nutrient conditions.

A 100 µL portion of cell suspension of *Pseudomonas* sp. 05CFM15-6D was inoculated to the assay medium and vortexted for 1 min. After incubation at 20 °C for 7 days in dark, 1 mL of the headspace gas was analyzed with a gas chromatograph (Shimadzu GC-14B, Kyoto, Japan) equipped with an electron capture detector (ECD) (Shimadzu ECD-2014) using a 1 m Porapak N column (Waters, Milford, MS, USA) [18].

2.3 Assay conditions for N_2O production: *Pseudomonas* sp. 05CFM15-6D (accession no. LC007968.1) used for the culture-based N_2O emission assay was an incomplete denitrifier isolated from thawing soil of corn farms as a culturable and active N_2O emitter. Identification and characteristics of this isolate was described in another paper [19]. As this bacterium is likely a heterotrophic saprophyte, it grew well in potato-dextrose medium.

To evaluate effect of allyl isothiocyanate on N_2O emitters, a 100-µL portion of 2-30 mM allyl isothiocyanate in MeOH was added to 10 mL of Winogradsky's medium supplemented with 5 mg KNO_3. The final concentration of allyl isothiocyanate was 20-300 µM. In the same process, methyl isothiocyanates, a glucosinolate sinigrin hydrate, and cyanamide, an allelopathic principle of hairy vetch, were dissolved in MeOH and also tested at 150, 1000, and 300 µM respectively, while betanin dissolved in water was subjected to dilution into 500 µM for final concentration in the assay medium. For these phytochemicals initially dissolved in MeOH, the control medium contained 100 µL MeOH.

III. RESULTS

3.1 Effect of root extracts from seedlings of cover crops on bacterial N_2O emission: Using the culture-based N_2O emission assay to evaluate activity in regulation of N_2O emission by *Pseudomonas* sp. 05CFM15-6D, extracts from seedlings of some cover crops showed slight repression of N_2O emission, particularly the root extracts from the 6 seedlings of yellow flowering leaf mustard (*B. juncea*) and 12 seedlings of hairy vetch (*V. villosa*) (Table 1 and Fig. 1). However, the extracts precisely adjusted as equivalent to 10 seedlings showed less activity in the N_2O emission. As marked activities, only the root extracts from *B. juncea* and crimson clover (*T. incarnatum*) seedlings showed statistically significant inhibition of N_2O emission (P <0.001 and <0.01 respectively). Inhibition ratio of the root extract from *B. juncea* was more than 90%, while only 35% in the extract of *T. incarnatum* (Fig. 2).

TABLE 1
SEEDLINGS OF COVER CROPS USED FOR PRELIMINARY N_2O EMISSION ASSAY

Cover crop seedling	Root biomass (dry weight, g)	Seedling number	Metabolites from root	Amount (mg)
Brassica juncea	2.68	59	extract exudate	56 72
Raphanus sativus var. *sativus*	1.60	110	extract exudate	48 44
Trifolium incarnatum	1.24	187	extract exudate	46 45
Vicia villosa	4.85	122	extract exudate	41 39

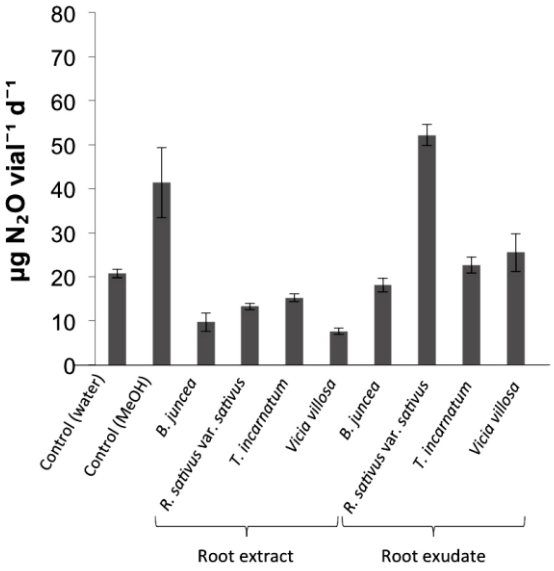

FIG. 1. EFFECT OF ROOT EXTRACT/EXUDATE FROM COVER CROPS ON N_2O EMISSION BY PSEUDOMONAS SP. 05CFM15-6D.

Amount of extracts of these seedlings (*B. juncea*, *R. sativus* var. *sativus*, *T, incarnatum*, and *V. villosa*) applied to the N_2O emission assay is equivalent to 5.9, 11.0, 18.7, 12.2 seedlings respectively. Incubation was done at 25 °C, for 6 days. Bar is ± standard deviation. Replication, n=5.

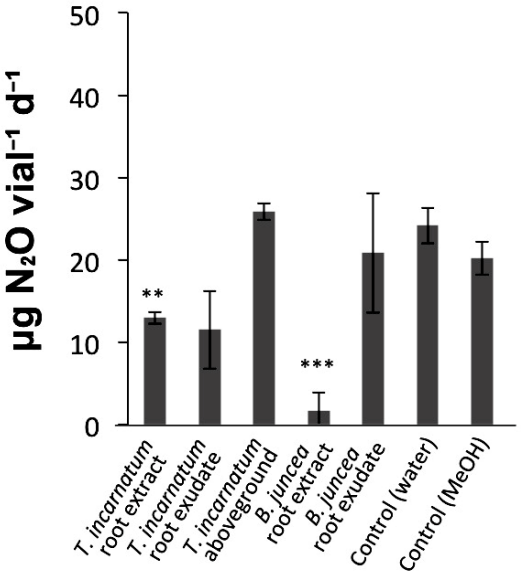

FIG. 2. EFFECT OF MEOH EXTRACTS FROM *Brassica juncea* AND *Trifolium incarnatum* ON N_2O EMISSION.

MeOH extracts of cover crops (*B. juncea* and *T. incarnatum*) were equivalent to 10 seedlings. Amounts of the extracts applied to the N_2O emission assay from root extract, root exudate, and aboveground of *T. incarnatum* seedlings are 3.8, 6.2, and 23.6 mg, while root extracts and root exudates from *B. juncea* root are 3.2 and 11.4 mg respectively. Incubation was done at 25 °C, for 3 days. Bar is ± standard deviation. Replication, n=3. $^{**}P<0.01$, $^{***}P<0.001$.

The root exudate equivalent to 4 seedlings cultivated in an approximately 10-times larger scale of the seedlings did not show any N_2O emission-suppressing activity. Only the aboveground of *B. juncea* extracted with MeOH showed the relatively clear inhibition of N_2O emission by *Pseudomonas* sp. strain 05CFM15-6D. Conversely, methanolic extract from the aboveground of European small radish (*R. sativus* var. *sativus*) showed statistically significant stimulating activity twice as large as

untreated control (Fig. 3). This activity was found only in water-soluble fraction, which had passed through a C_{18}-reverse phase silica gel with water.

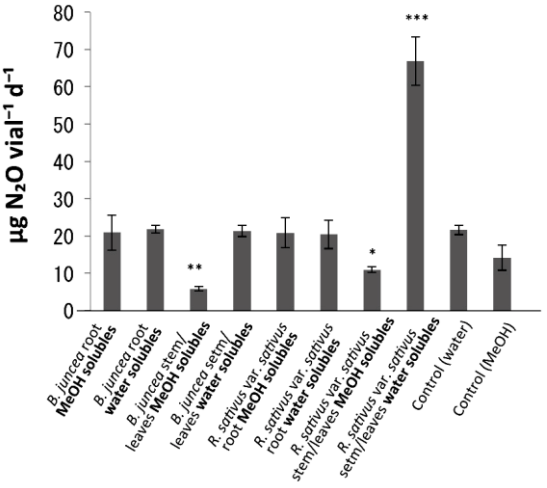

FIG. 3. EFFECT OF MEOH EXTRACTS EQUIVALENT TO FOUR SEEDLINGS OF *Brassica juncea* AND *Rahanus sativus* CV. *sativus* ON N_2O EMISSION BY *Pseudomonas* SP. 05CFM15-6D.

MeOH extracts of two cover crops (*B. juncea* and *R. sativus* var. *sativus*, as the root and aboveground parts respectively) were divided into MeOH soluble and insolubles. The latter was re-dissolved in water. All the extracts equivalent to 4 seedlings were subjected to N_2O emission assay. Incubation was done at 25 °C, for 4 days. Bar is ± standard deviation. Replication, n=5. **P*<0.05, ***P*<0.01, ****P*<0.001.

3.2 Effect of phytochemicals to characterize family and species of cover crops tested: At 30 μM or higher, allyl isothiocyanate, a major isothocyanate responsible for the pungent taste of brassicaceous mustard, radish, horseradish, and wasabi [20,21], completely inhibited N_2O emission (Fig. 4). Up to concentration of 150 μM, allyl isothiocyanate visibly allowed cell growth of *Pseudomonas* sp. 05CFM15-6D (data not shown). As the N_2O emitter used for this assay is a highly motile bacterium, this bacterium generally forms haze-like colonies in the soft gel medium. However, bacterial cells cultured upon exposure to 30-150 μM at pH 6.0, the bacterial cells grown in the soft gel medium had lost their swimming ability to form particle-like colonies [15].

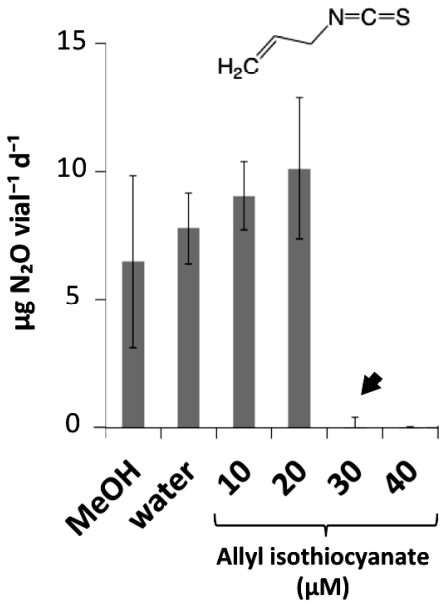

FIG. 4. EFFECT OF ALLYL ISOTHIOCYANATE ON N_2O EMISSION.

A 100-µL portion of ally isothiocyanate dissolved in medium. To the control (MeOH), 100 µL MeOH was added, while another control (water) contained no organic solvent. Filled arrow shows the threshold concentration of allyl isothiocyanate for complete inhibition of N_2O emission by *Pseudomonas* sp. 05CFM15-6D. Incubation was done at 25 °C, for 6 days. Bar is ± standard deviation. Replication, n=5.

In contrast, a glucosinolate sinigrin that is a precursor of allyl isothiocyanate did not suppress N_2O emission and cell growth performance of *Pseudomonas* sp. 05CFM15-6D even at 1000 µM (Fig. 5A). Methyl isothiocyanate showed activity at 300 µM, but not at 150 µM. Similarly, both cyanamide and betanin were inactive at 300 µM and 500 µM respectively (Fig. 5B, C, and D).

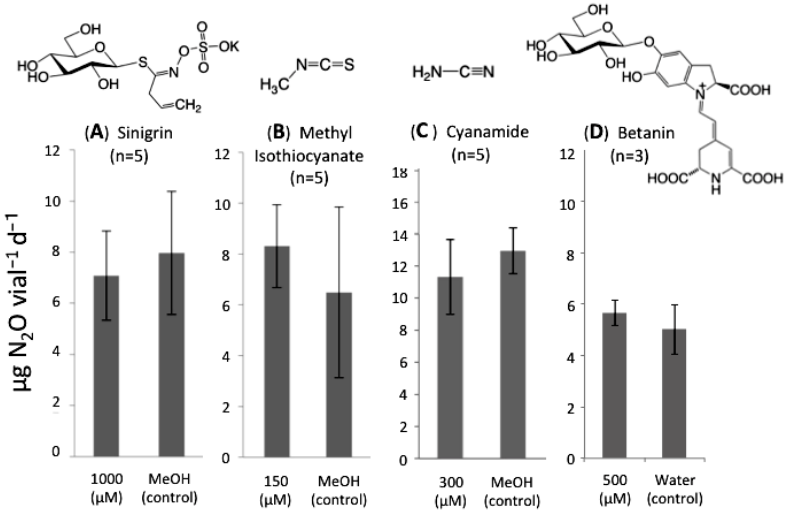

FIG. 5. EFFECT OF SOME OTHER PHYTOCHEMICALS ON N_2O EMISSION.

Incubation was done at 25 °C, for 3-6 days. Bar is ± standard deviation.

IV. DISCUSSION

As oxidation of ammonia in soil known as nitrification is leads to nitrogen loss due to provision of nitrate, substrate for denitrification process in soil. Hence, some synthetic nitrification inhibitors, such as dicyandiamide, nitrapyrin, and thiourea, have long been studied [22-24]. In current needs for sustainable food production, however, eco-friendly nitrification inhibitor is awaited. One milestone is a discovery of a naturally-occurring nitrification inhibitor, brachialactone, from the root-exudate of the poaceous grass *Brachiaria humidicola* [25]. Another important discovery is nitrification inhibitors from a cropping plant, sorghum. Sakuranetin (4',5-dihydroxy-7-methoxyflavanone) and sorgoleone (2-hydroxy-5-methoxy-[(8*Z*,11*Z*)-8,11,14-pentadecatriene]-*p*-benzoquinone) produced by the roots of sorghum selectively inhibited ammonia monooxygenase of *Nitrosomonas* (ED_{80} 0.6 and 13.0 µM respectively) [26].

As our previous research revealed that 2 µM paraquat suppressed N_2O emission by *Pseudomonas* denitrifiers, biological resources for denitrification inhibitors to repress N_2O emission was also predicted as the next target for the screening of naturally occurring substances [16]. Thus, main purpose of this study is to demonstrate some possibilities of secondary metabolites uniquely contained in cover crops cultivable for biofumigation or green manure. Some cover crops are important candidates to relieve soil microbial community in the fertilized farm soil where N_2O is actively emitted, because some cover crops are known to change soil microbial community structures [27].

Both isothiocyanate and cyanamide possessed a ketene-like 1,2-double bond in the molecule, and these chemical structures are similar with N_2O (Fig. 5). We therefore tested isothiocyanates and cyanamide as candidates for repressing agent against N_2O emission. Allyl isothiocyanate showed a clear repressive activity in N_2O emission by the *Pseudomonas* denitrifier (Fig. 4). However, methyl isothiocyanate did not show any inhibitory effect on the N_2O emitter at 150 µM (Fig. 5B). The inactive action of methyl isothiocyanate implied that isothiocyanate moiety is not fundamental in allyl isothiocyanate as the inhibiting agent against N_2O emission. In the same assay system, we also tested cyanamide, obtainable as allelopathic substances from

hairy vetch [13]. Although some biological activities of cyanamide have been reported, this compound did not affect N_2O emission at 300 µM (Fig. 5C).

As betanin that is richly contained in red beetroot is an outstanding antioxidant phytochemical, we estimated that betanin could inhibit N_2O emission by denitrifiers because nitrate respiration is a series of redox-reaction on inorganic nitrogen molecules and electron donors [6]. However, neither repressing nor accelerating effect of betanin was observed at 500 µM in our culture-based N_2O emission assay (Fig. 5D).

Not only seedlings but also mature plants of *B. juncea* are rich in sinigrin, a precursor of allyl isothiocyanate [28]. Therefore, after the tillage of this cover crop with topsoil, a large amount of allyl isothiocyanate would be released effectively to the tilled farm soil du to mechanical damages. Our culture-based N_2O emission assay using *Pseudomonas* sp. 05CFM15-6D implied that allyl isothiocyanate selectively represses nitrate respiration-associating enzymes. Together with the effect of *B. juncea* as cover crop that stimulates soil microbial diversity and population density, allyl isothiocyanate may practically suppress N_2O emission from cropland soils.

As 1000 µM sinigrin did not repress N_2O production of *Pseudomonas* sp. 05CFM15-6D in the culture-based N_2O assay (Fig. 5A), it is obvious that *Pseudomonas* sp. 05CFM15-6D does not possess bacterial myrosinase. This speculation is acceptable because not many proteobacteria possess bacterial myrosinase that degrades sinigrin to release allyl isothiocyanate from the substrate [29]. Hence, effect of *B. juncea* on biofumigation for the tilled soil is probably due to plant myrosinase activated after the mechanical damage by the tillage to provide allyl isothiocyanate in micro/macro-pore space of soil.

V. CONCLUSION

The N_2O emitter *Pseudomonas* sp. 05CFM15-6D is a facultative anaerobe, and its N_2O emission progresses under anaerobic conditions with an excessive organic matter utilizable for the copiotrophic and saprophytic denitrifier [19]. Therefore, green manure as a possible accelerator for N_2O emission from the tilled soil is great concern. Indeed, we found that MeOH extract from the aboveground of *R. sativum* var. *sativum* seedlings accelerated N_2O emission by the incomplete denitrifiers in this study (Fig. 3), while allyl isothiocyanate from *B. juncea* showed marked suppression of N_2O emission by an incomplete denitrifier in the culture-based bioassay (Fig. 4). We therefore imply necessity of risk management for such tilling the green manure and pre-harvest vegetable crops.

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