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Potential of Nitrification Inhibition and Change of Soil Bacterial Community Structure by Biofumigation of *Brassica juncea* Green Manure in Succeeding Sweet Corn Cultivation under Gray Lowland Soil Conditions

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Abstract

This study investigated the biofumigation effects of *Brassica juncea* crop on soil nitrification and soil bacterial communities under gray lowland soil conditions. Treatments included incorporating *B. juncea* containing high levels of glucosinolate (GLS) or *Sinapis alba* containing low levels of GLS and unamended control. Nitrification activity was evaluated using soils collected at the sweet corn transplantation (8 days after incorporation) and tassel emergence stage (46 days after incorporation). Sweet corn growth, yield, and nitrogen status were compared among treatments. Additionally, soil bacterial community structure in initial soils and soils at maize transplantation were investigated using next-generation sequencing. The results showed that incorporating *B. juncea* plants did not inhibit soil nitrification at the transplanting and tassel emergence stages, and apparent differences in sweet corn yield and nitrogen uptake were not observed among treatments. Differences between treatments regarding the effects of the incorporation on the soil bacterial abundance were observed in some bacterial families, but the abundances of nitrifying bacteria were not statistically different. Our results showed that incorporating *B. juncea*, which has a high GLS content, into sweet corn cultivation soil changed the abundance of certain soil bacterial families; however, nitrification inhibition effect is not expected under gray lowland soil conditions.

Discipline: Agricultural Environment
Additional key words: Glucosinolate, Nitrosomonadaceae, Nitrospiraceae, *Sinapis alba*, soil mineral nitrogen

Introduction

Nitrification is an important biological process in the nitrogen (N) cycling of soil–plant systems. However, nitrification products, such as nitrite and nitrate, are vulnerable to leaching and denitrification, resulting in 45%-60% N loss in applied nitrogenous fertilizer (Jarvis 1996) and available N reduction for crops. Furthermore, the greenhouse gas N_2O can be emitted into the air during the nitrification process and through nitrifier denitrification (Tian et al. 2020, Wrage et al. 2001). Therefore, considerable effort has been invested in inhibiting nitrification or determining how to slow the process of nitrification (Jumadi et al. 2020, Lu et al. 2019, Torralbo et al. 2017). Plant-derived nitrification inhibitors have been identified in some plants (Fillery 2007, Subbarao et al. 2015). The nitrification inhibition of *Brachiaria humidicola*, a tropical pasture grass, has been well studied, and its potential utility in improving N use efficiency in crops is expected (Subbarao et al. 2006, 2009).

Glucosinolate (GLS)-containing plants in the Brassicaceae family represent a potential source of allelochemical control for various soil-borne pests as reviewed by dos Santos et al. (2021). Following tissue

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damage, GLSs stored in the cell vacuole come into contact with thioglucosidases and are hydrolyzed to a number of toxic hydrolysis products, including isothiocyanates (ITCs) and nitriles (Cole 1976). Angus et al. (1994) defined this process as "biofumigation."

During the biofumigation process, GLS-rich species or materials are mixed into the soil to release the volatile and toxic ITCs. Crops with high GLS content, such as *Brassica juncea* (Morra & Kirkegaard 2002), are usually chosen for biofumigation treatments. Recently, ITCs have been shown to inhibit microbial activity and have been implicated in both nitrification inhibition and nitrifying organisms (Bending & Lincoln 2000, Brown & Morra 2009). Brown & Morra (2009) indicated that Brassicaceae crops containing high GLS contents inhibit nitrification and increase plant-available N in soil incubation tests. However, GLS hydrolysis occurs rapidly, and ITCs and other hydrolysis products generally have a short life span in the soil, with a rapid decrease in their concentration within a few days and a mean soil persistence of up to 14 days (dos Santos et al. 2021). GLSs present in the plant materials added to the soil were detected 30 min after incorporation, and GLSs were detectable in soil for up to 8 days (Gimsing & Kirkegaard 2006). Morra and Kirkegaard (2002) investigated the pattern of ITC release in the soil following the incorporation of the biofumigant crop, *B. juncea*, and indicated that most of the ITC was released within the first 4 days after tissue incorporation. Therefore, as many of the products released during GLS hydrolysis are volatile, losses should be reduced by covering the soil with plastic mulching (van Bruggen et al. 2016, dos Santos et al. 2021). Although GLS hydrolysis products are considered to inhibit nitrification activity, most research is limited in laboratory tests. Little is known about their effects on the nitrification activity and N status of cultivated crops under field conditions with gray lowland soil.

In the present study, we cultivated commercially available *B. juncea*, known to contain high GLS levels, as the preceding green manure crop. We also evaluated the nitrification activity in the soil and the growth, yield, and N uptake of succeeding sweet corn by comparing the green manure crop, *Sinapis alba*, which is known to contain low GLS levels (Takehara 2016), and the conventional vegetable production system (*Brassica rapa*, containing low GLS levels). Knowledge of the effect of soil microbial communities is essential for developing sustainable biofumigation strategies. Therefore, as a complementary approach, next-generation sequencing (NGS) analysis of 16S rRNA gene fragments was used to evaluate whether biofumigation influences the soil bacterial community, including nitrifying bacteria, such as Nitrosomonadaceae and Nitrospiraceae.

Materials and methods

1. Field design, plant materials, and cultivation

The field experiment was performed from 2018 to 2019 at Osaka Prefecture University in Japan (34.5°N, 135.5°E). The soil of the experimental field was categorized as a humic gray lowland soil (Gleysol). Barley was grown prior to the experiment. The experiment involved a randomized block design with three replications. The size of each plot was 12.5 m^2 (2.5 m × 5.0 m). Three crop rotation systems were established: 1) the control treatment, *B. rapa* (Komatsuna, Tohoku Seed Co., Ltd., Tochigi, Japan)–maize (*Zea mays* L. "Gold rush 86"; Sakata Seed Corp., Yokohama, Japan); 2) the *S. alba* treatment, *S. alba* ("Kikarashi"; Snow Brand Seed Co., Ltd., Chiba, Japan)–maize; and 3) the *B. juncea* treatment, *B. juncea* ("Karajin"; Snow Brand Seed Co., Ltd. Chiba, Japan)–maize. *Sinapis alba* and *B. juncea* shoots were incorporated into the soil, whereas *B. rapa* was harvested without incorporation. The maize was cultivated after incorporating green manure crops or the harvest of *B. rapa* aboveground.

Brassica rapa was cultivated twice from October to November 2018 and from December 2018 to February 2019, with eight rows per plot (inter-row: 20 cm; intra-row: 10 cm). Basal chemical fertilizer (N, P_2O_5, K_2O, and magnesium lime = 20, 10, 10, and 100 g m^{-2}, respectively) was applied before the first cultivation. In the second cultivation, N, P_2O_5, K_2O, and magnesium lime (10, 10, 10, and 100 g m^{-2}, respectively) were applied to the soil. The green manure plants, *S. alba* and *B. juncea*, were cultivated from October 2018, with eight rows per plot (inter-row: 20 cm). Basal chemical fertilizer (N, P_2O_5, K_2O, and magnesium lime = 10, 10, 10, and 100 g m^{-2}, respectively) was applied before sowing. On April 19, 2019, the chopped shoots (< 10 cm) of the green manure plants were incorporated into the soil. Before incorporation, subsamples (two plants per plot) were taken after fresh weight (FW) measurements to determine the N concentration and C/N ratio. The subsamples were dried at 70°C for 48 h, weighed, and ground. The N and C concentrations of each plant were analyzed using Vario Max (Elementar, Germany). The total N supplied into each plot was calculated by multiplying the total FW per plot by the N concentration of the subsamples.

All plots were rotary-tilled with basal chemical fertilizer (N, P_2O_5, K_2O, and magnesium lime = 8, 12, 12, and 180 g m^{-2}, respectively) on the same day as the incorporation of green manure plants. Three rows were established in each plot, and black plastic mulch was

deposited over the rows in all treatments. Mulching has been recommended to obtain a fumigation effect (van Bruggen et al. 2016). The sweet corn plants (about 3-4 leaf stage) were transplanted with inter-row and intra-row spacing of 90 cm and 30 cm, respectively, 8 days after the incorporation. The central row was used for data collection, and each side of the row was left for border effect. N top dressing (4 kg 10 a^{-1}) with ammonia sulfate was applied during the tassel emergence stage (June 2019). Growth parameters, such as plant length, leaf number, and soil plant analysis development (SPAD) value, were measured during cultivation. At harvest (July 2), five continuous plants in the central row were harvested to evaluate the growth, yield, and quality parameters. The N contents of the stover and ear were also determined as mentioned above.

2. Soil survey

During the sowing of each plant, the initial field soils (approximately 2 cm-10 cm from the soil surface) after the fertilization were sampled using a small trowel from five points in each plot. The soils collected from the five points were combined to give a composite sample. The soils were also sampled immediately before the transplantation (April 26) and tassel emergence stage (June 4) of the sweet corn. Then, 2 mm of sieved soils were used to analyze soil pH and electrical conductivity. To evaluate soil physical characteristics in each plot, soil penetration resistance immediately after the harvest of sweet corn was measured at three points per plot using a digital cone penetrometer (DIK-5532, Daiki Rika Kogyo Co., Ltd., Saitama, Japan).

The initial field soils and soils at maize transplantation (8 days after incorporation) were collected to investigate the effect of biofumigation on the bacterial community structure. The sieved fresh soils were immediately preserved at −20°C until DNA extraction. The soils were removed at the tassel emergence stage (46 days after incorporation) because the biofumigation effect duration is relatively short (Gimsing & Kirkegaard 2009). The 16S rRNA-based NGS approach was used to evaluate bacterial community structures. Soil microbial genomic DNA was extracted using soil DNA extraction kits (ISOIL, Nippon Gene Co., Ltd., Toyama, Japan). The extracted soil genomic DNA was used as a template to amplify 16S rRNA genes. The V3-V4 regions of the 16S rRNA gene were amplified using 2X KAPA HotStart Ready Mix with the forward (5′-TGCCAGCMGCCGCGGTAA-3′) and reverse (5′-GGACTACHVGGGTWTCTAAT-3′) primers (Klindworth et al. 2013) using a polymerase chain reaction (PCR) Thermal Cycler Dice (Takara Bio). The PCR mixtures consisted of 12.5 μL of KAPA HotStart Ready Mix, 5 μL of 1-μM primers, and 2.5 μL of genomic DNA in a final volume of 25 μL. The reaction conditions were as follows: initial denaturation for 3 min at 95°C, followed by 25 cycles of 30 s at 95°C, 30 s at 55°C, and 30 s at 72°C, with a final extension of 5 min at 72°C. For Illumina library preparation, the barcodes were added using the 2X KAPA HiFi HotStart Ready Mix and Nextera XT v2 Index Primers 1/2 with eight cycles. Before sequencing, the amplicons were purified using the AMPure XP Beads. The amplicons were sequenced for 300 bp of both ends on the Illumina MiSeq Platform (Illumina Inc., USA).

3. Incubation tests for the evaluation of nitrification activity

Incubation tests were conducted to investigate the nitrification activity of the soil at transplantation (8 days after incorporation) and the tassel emergence stage (46 days after incorporation) of sweet corn. Then, 4 g of the FW samples of soil were dispensed into 50-mL polystyrene tubes, and a solution that included 1.2-mg NH_4^+-N of $(NH_4)_2SO_4$ was added to each tube. A small amount of deionized water was also added to maintain 60% soil moisture. After thoroughly mixing the soil, the tubes were incubated in the dark at 25°C. Because it is reported that the effects of GLS on nitrification inhibition are kept for 24-35 days after incubation (Bending & Lincoln, 2000, Brown & Morra 2009), the incubation period was set to 30 days in this study. During the incubation, deionized water was added to the tubes once every 3-4 days after measuring the weight of the tubes to maintain 60% moisture. After 3 h (zero time) and 30 days, five replicate tubes from each treatment were taken for the determination of NH_4^+-N and NO_3^--N. The NH_4^+-N and NO_3^--N in soil were extracted from the same tube as follows. First, tubes containing 4 g of soil were shaken with 20 mL of deionized water at 120 rpm for 1 h; then, 1 mL of suspension was collected to determine the concentrations of NO_3^--N and water-soluble NH_4^+-N. After adding 1 mL of deionized water to each tube, 20 mL of 4 M KCl was added to the tubes (final concentration: 2 M KCl). Then, the tubes were shaken again at 120 rpm for 1 h, at which point 1 mL of the suspension was collected to determine the NH_4^+-N concentration extracted with 2 M KCl. The NO_3^--N and NH_4^+-N concentrations were determined using the colorimetric assays (Arakawa et al. 2003, Cataldo et al. 1975). The sum of water- and KCl-extracted NH_4^+-N was used as NH_4^+-N concentration in the soils.

The nitrification activity under plant tissue–amended conditions was investigated using the method of

Brown & Morra (2009). *Sinapis alba* and *B. juncea* subsamples were freeze-dried and powdered. The same soils obtained from the sweet corn field were used in this experiment. Four grams of soil were dispensed into the 50-mL polystyrene tubes, and a solution containing 280 µg of NH_4^+-N of $(NH_4)_2SO_4$ was added to all tubes. A small amount of deionized water was also added to maintain 60% soil moisture. In the *S. alba* and *B. juncea* treatment, 40 mg of powdered tissue was further added to the soil. The NH_4^+-N and NO_3^--N concentrations were determined with five replicate tubes at 3 h (zero time) and 30 days after incubation.

4. Statistical analysis

The data of mean growth, yield, and N status values (five plants per plot) were used for the statistical analysis. The treatment effects were evaluated using analysis of variance, followed by Tukey's test, when significant differences were found ($P < 0.05$). Statistical analyses among treatments were performed using SPSS (IBM SPSS Statistics version 26). In the NGS data analysis, raw sequence reads were processed using the QIIME2 pipeline. The forward and reverse reads were joined, denoised, and checked for chimeras using the software package deblur, and the low-quality reads were removed. For microbial diversity analysis, the QIIME2 software was also used to assess the relative abundance of operational taxonomic units (OTUs). For taxonomy assignment, SILVA was used as a reference database. OTU clusters were defined by a 97% identity threshold. The relative abundance of each OTU was normalized by dividing the readings of individual OTU by the total readings in a sample. Data from a total of 243 OTUs were analyzed using principal coordinate analysis (PCoA) with the R package. Because the Nitrosomonadaceae and Nitrospiraceae families are responsible for soil nitrification (Amoo & Babalola 2017, Shen et al. 2012), their abundances in the initial soil and soils at maize transplantation (8 days after the incorporation of green manure crops) were compared between treatments. The abundances of other bacterial families were also compared among treatments, and only the bacterial families with significant differences in the abundances among treatments were shown in this study.

Results and discussion

Plant tissues contain a great variety of secondary metabolites, which mainly act as defenses against herbivores, pests, or pathogens (Bennett & Wallsgrove 1994). The present study explored the possibility of nitrification inhibition induced by the biofumigation of brassicaceous amendments to increase the N availability of succeeding sweet corn. Two green manure plants, *S. alba* and *B. juncea*, showed almost the same properties: the average aboveground biomass of *S. alba* and *B. juncea* were 3.45 kg and 3.59 kg FW m^{-2} with an N concentration of 1.99% and 1.95% and C/N ratio of 20.8 and 20.9, respectively (Table 1). Contrastingly, the yield of *B. rapa* was 1.52 kg m^{-2} in the first cultivation and 1.49 kg m^{-2} in the second cultivation.

At the time of maize transplantation, soil pH was almost the same among treatments. The electrical conductivity value was slightly higher in the control than in the green manure treatments (Table 2). The leaf number

Table 2. Soil pH and electrical conductivity (EC) in sweet corn cultivation

Sampling period	Preceding crop	pH [a]	EC [a] ($mScm^{-1}$)
Initial soil	*B. rapa*	6.2±0.3	1.50±0.05
	S. alba	5.6±0.5	0.81±0.10
	B. juncea	6.4±0.3	0.82±0.21
Transplanting	*B. rapa*	5.9±0.3	0.39±0.04
	S. alba	5.8±0.3	0.33±0.03
	B. juncea	6.0±0.2	0.29±0.04
Tassel emergence	*B. rapa*	6.1±0.3	0.54±0.06
	S. alba	5.7±0.4	0.60±0.14
	B. juncea	5.6±0.2	0.74±0.15

[a] Means ± standard error (N = 3)

Table 1. The aboveground biomass of *Brassica rapa* and green manure properties of *Sinapis alba* and *B. juncea*

	Aboveground biomass [a] (kg FW m^{-2})	N concentration [a] (%)	N content [a] (g m^{-2})	C/N ratio [a]
B. rapa (First)	1.52±0.27	-	-	-
B. rapa (Second)	1.49±0.43	-	-	-
S. alba	3.45±0.30	1.99±0.09	14.3±1.2	20.8±1.1
B. juncea	3.59±0.41	1.95±0.12	12.2±2.0	20.9±1.2

[a] Means ± standard error (N = 3)

and SPAD value of the sweet corn in the *B. juncea* treatment tended to be greater than those of other treatments (Fig. 1). However, after top dressing (June 4), the differences in the growth parameters were not observed. Finally, there were no significant differences in the aboveground biomass, yield, ear quality parameters, and N content of the stover and ear among treatments (Tables 3, 4, 5). Since the C/N ratio was approximately 20, the green manures were supposed to be relatively decomposable. Unexpectedly, there were no differences in the N contents of the stovers and ears among treatments, despite the addition of N via green manures (Table 5). In the present study, although plastic mulch was applied to the soil to obtain the biofumigation effect, mulching might have also helped protect the surface soil from erosion and reduce fertilizer losses, as reported by Pedda Ghouse Peera et al. (2020). Therefore, the maize in the control treatment could uptake enough nutrients, resulting in no significant differences in yield and N content among treatments. Furthermore, the differences in soil penetration resistance might have influenced maize growth and N uptake. It is suggested that soil hardness is alleviated by the cultivation and incorporation of green manure plants (Latif et al. 1992). However, the present study showed that soil penetration resistance up to 20-cm depth was lower in the control soil than that in the *B. juncea* treatment (Fig. 2). Twice plowing for *B. rapa* cultivation in the control plots could explain the alleviation of soil compaction, resulting in the improvement of root development and N uptake.

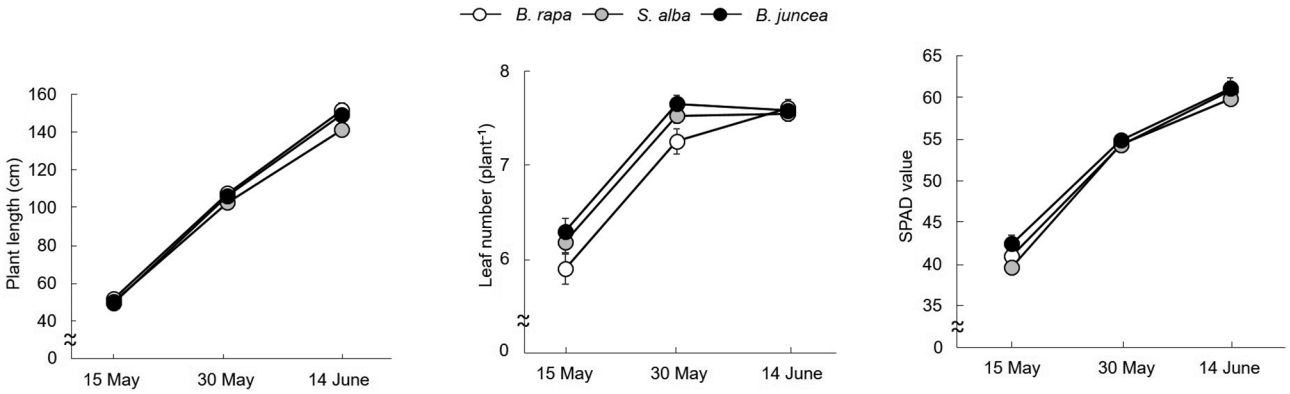

Fig. 1. Changes in the plant length, leaf number, and soil plant analysis development (SPAD) value of sweet corn rotated with different preceding crops

Table 3. Dry weights of the stover and ear and harvest index (HI) of sweet corn rotated with different preceding crops

Preceding crop	Stover[a] (g plant^{-1})	Ear[a] (g plant^{-1})	HI (%)
B. rapa	134.0±11.8	99.6±5.6	43
S. alba	106.0±10.6	90.8±9.9	48
B. juncea	153.8±17.6	92.5±1.9	39

[a] Means ± standard error (N = 3)

Table 5. Nitrogen content of the stover and ear of sweet corn rotated with different preceding crops

Preceding crop	Stover[a] (g m^{-2})	Ear[a] (g m^{-2})
B. rapa	7.4±1.0	5.5±0.2
S. alba	5.6±0.3	4.3±0.1
B. juncea	7.3±0.9	4.5±0.4

[a] Means ± standard error (N = 3)

Table 4. Yield and ear qualities of sweet corn rotated with different preceding crops

Preceding crop	Yield[a] (kg m^{-2})	Length[a] (cm)	Width[a] (mm)	Row number[a] (ear^{-1})	Kernel number[a] (row^{-1})
B. rapa	1.2±0.1	20.1±0.5	50.3±1.5	15.9±0.7	39.5±2.2
S. alba	1.2±0.1	19.5±0.6	51.2±0.6	15.5±0.5	39.3±1.6
B. juncea	1.2±0.0	19.5±0.3	51.2±0.4	14.9±0.3	38.8±1.1

[a] Means ± standard error (N = 3)

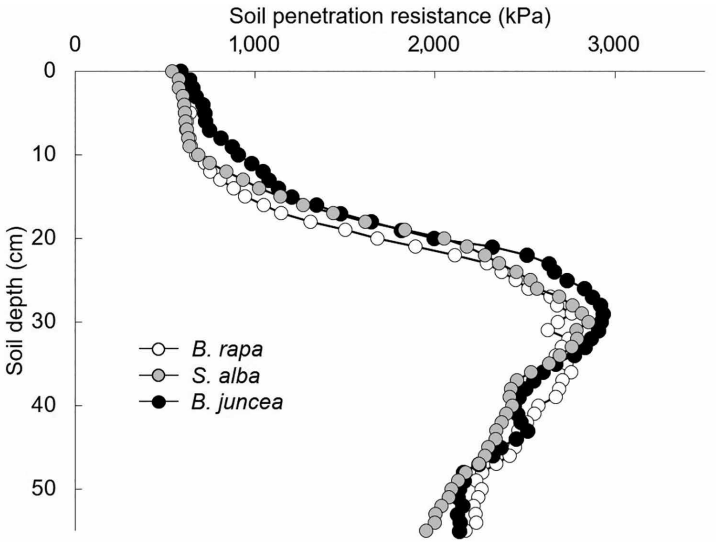

Fig. 2. Soil penetration resistance up to 55-cm depth measured after the harvest of sweet corn rotated with different preceding crops
Average values were plotted (N = 3).

Incubation tests were conducted to compare the nitrification activity among treatments. In the soil collected at the time of transplanting (8 days after incorporation), nitrification inhibition was not observed in the *B. juncea* treatment; NH_4-N was reduced as in the control and *S. alba* treatments, and nearly the same amount of NO_3^--N was detected with the other treatments after 30 days (Fig. 3 a, b). The initial concentration of NH_4^+-N (3 h) in the *B. juncea* soil tended to be higher than that in the soils of the control and *S. alba* treatments (Fig. 3 a). One reason would be the mineralized NH_4^+-N by the decomposition of *B. juncea* plants. Another reason might be the N release from the killed microbiota by the biofumigation effect of *B. juncea*, as Brown & Morra (2009) suggested that the NH_4^+-N accumulation in soils amended with GLS-containing tissues is the result of N release from any killed biomass by biofumigation. In the soil collected at the time of top dressing, the reduction rate of the applied NH_4^+-N was rapid compared with the soil at the time of transplanting; NH_4^+-N was not detected in either treatment (Fig. 3 c, d). Namely, nitrification inhibition in the *B. juncea* treatment was not observed even in the soil collected at the top dressing period. Higher nitrification in the top dressing soil compared with that in the transplantation soil would be due to the difference in the population of nitrifying bacteria.

The results of the incubation test with the amendment of plant tissues were different from that of the field-collected soil (Fig. 4). As in the field-collected soil, the applied NH_4^+ was completely metabolized after 30 days, indicating that nitrification was not inhibited by the amendment with *B. juncea* tissues. However, the NO_3^--N concentration in the *B. juncea* treatment was significantly lower than that in the control. Because the *S. alba* treatment also showed a significantly lower value in NO_3^--N than in the control, this could be due to the immobilization of soil mineral N by soil microbiota.

Hanschen et al. (2015) investigated the effect of GLS on bacteria by denaturing gradient gel electrophoresis and revealed that the soil bacterial community composition was significantly affected by the addition of GLS to the soil. In the present study, the PCoA results showed that the samples were roughly divided into two groups, samples of initial soil and soil collected at the time of transplantation. The *B. juncea* soil at transplantation showed the near position with the control, although the *S. alba* treatment positioned away from the control to some extent (Fig. 5). This result indicates that seasonal changes in the bacterial community were observed, but 8 days after incorporation, the green manure amendment had little influence.

Relative abundances of nitrifying bacteria, Nitrosomonadaceae and Nitrospiraceae, obtained from the soils at transplantation were compared among treatments (Fig. 6). Bending & Lincoln (2000) reported that ITCs reduced the populations of NH_4^+-N-oxidizing bacteria and inhibited their growth. This report contrasts with our findings; there were no significant differences in the abundances of Nitrosomonadaceae and Nitrospiraceae among treatments, although lower abundances were observed in green manure treatments. Individual analysis showed that significant differences in

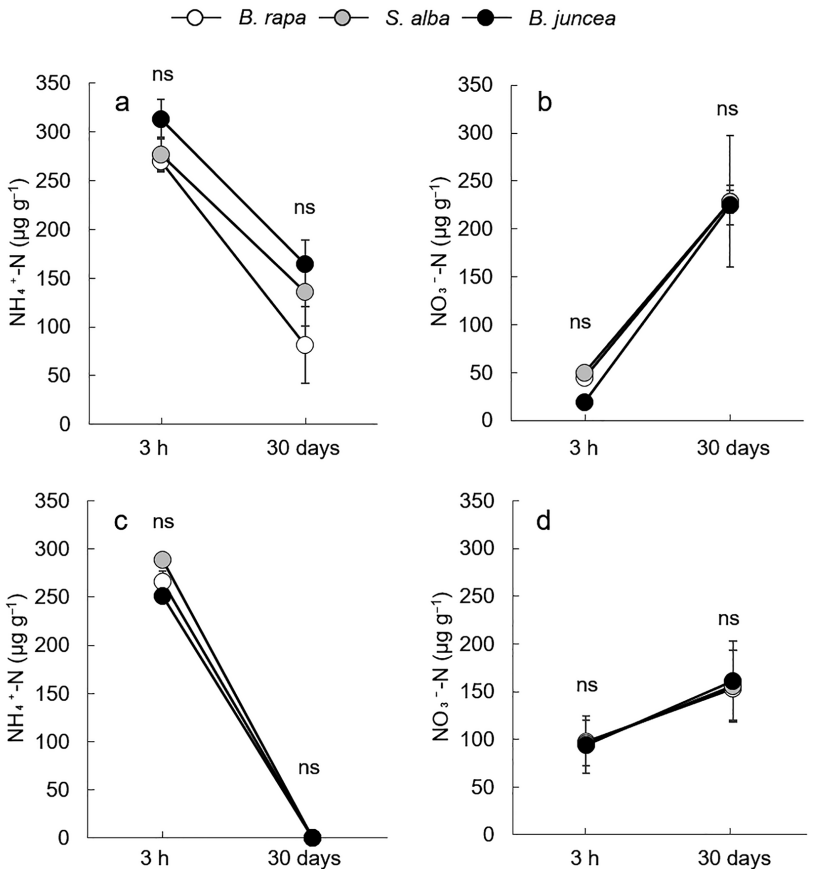

Fig. 3. Nitrification activity in soil collected at transplantation (a, b) and the tassel emergence stage (c, d) of sweet corn
Error bars indicate standard error (N = 3).

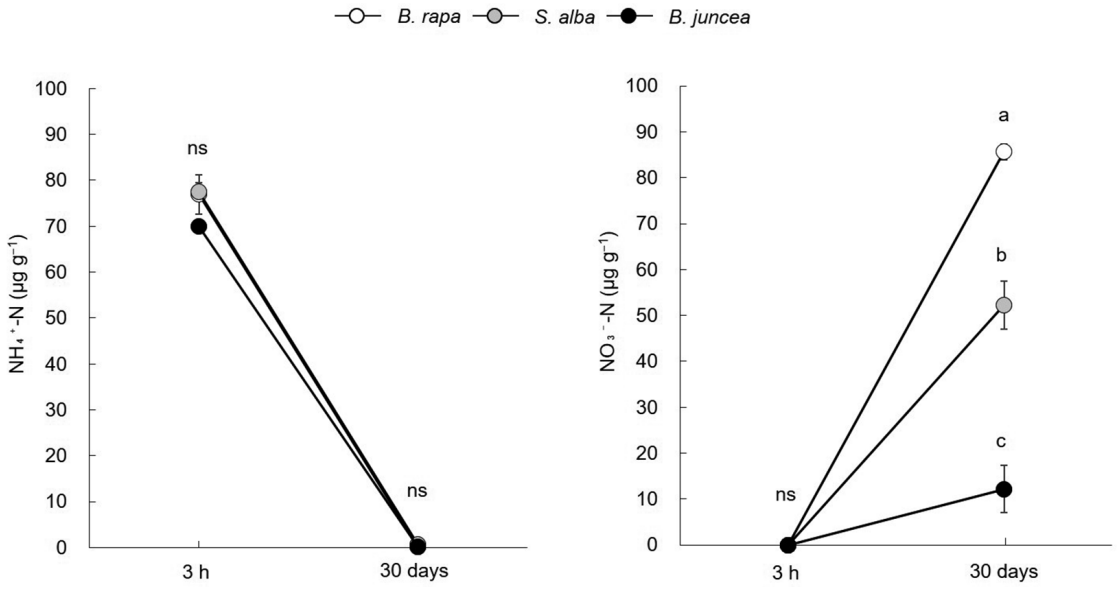

Fig. 4. Nitrification activity in soil with or without plant tissues of *Sinapis alba* and *Brassica juncea*
Error bars indicate standard error (N = 5). Different letters indicate significant difference using Tukey's test ($P < 0.05$).

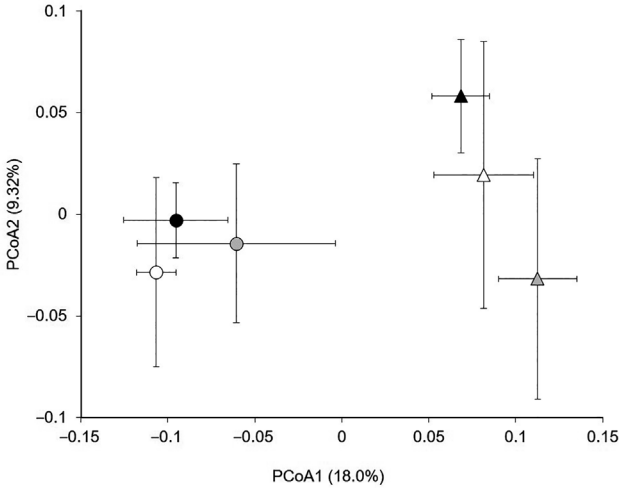

Fig. 5. Principal coordinate analysis (PCoA) of relative abundances of 16S rRNA gene fragment data using next-generation sequencing (NGS) in sweet corn field soils
White symbols: control treatment; gray symbols: *Sinapis alba*; black symbols: *Brassica juncea*; circle symbols: initial soils; triangle symbols: soils at sweet corn transplantation. Error bars indicate the standard error of PCoA scores (N = 3).

relative abundance were observed in the families Erythrobacteraceae, Gaiellaceae, Nocardiaceae, Paenibacillaceae, Sphingomonadaceae, Spirobacillales, and Thermoactinomycetaceae (Fig. 7). The *B. juncea* treatment showed higher abundances in Nocardiaceae, Paenibacillaceae, and Thermoactinomycetaceae. Thermoactinomycetaceae are commonly found in compost and manures at thermophilic growing temperature (Sathya et al. 2017). Although *B. juncea* and *S. alba* were incorporated as the green manure, there could be a difference in decomposability between these plants. The family Nocardiaceae, as well as the genera *Rhodococcus* and *Nocardia*, have been reported as siderophore and phytohormone producers (Sathya et al. 2017). Paenibacillaceae, the majority of which are found in soil, are often associated with plant roots; these rhizobacteria promote the growth of plants, including maize (Grady et al. 2016). Therefore, the incorporation of *B. juncea* would increase some beneficial bacteria, promoting plant growth under gray lowland soil conditions. In the present study, we targeted only the bacterial community. Zuluaga et al. (2015) reported that plants containing high GLS showed toxicity to springtail, which is responsible for organic matter decomposition but not to earthworm. Therefore, community analysis targeting a wide range of soil organisms is needed in further studies.

Here, we expected that incorporating *B. juncea* could inhibit the nitrification activities of soil microbiota, as previously reported. Since ITCs are easy to volatilize, the hills were covered with plastic mulch soon after the incorporation. However, in the nitrification potential and NGS analyses, nitrification inhibition with the incorporation of *B. juncea* was not observed. Bending & Lincoln (2000) compared the durability of nitrification inhibition between sandy loam soil and clay loam soil and indicated that its effect was longer in the sandy loam soil compared with the clay loam soil because sandy loam soil is superior to the fumigant effect. Since the experimental

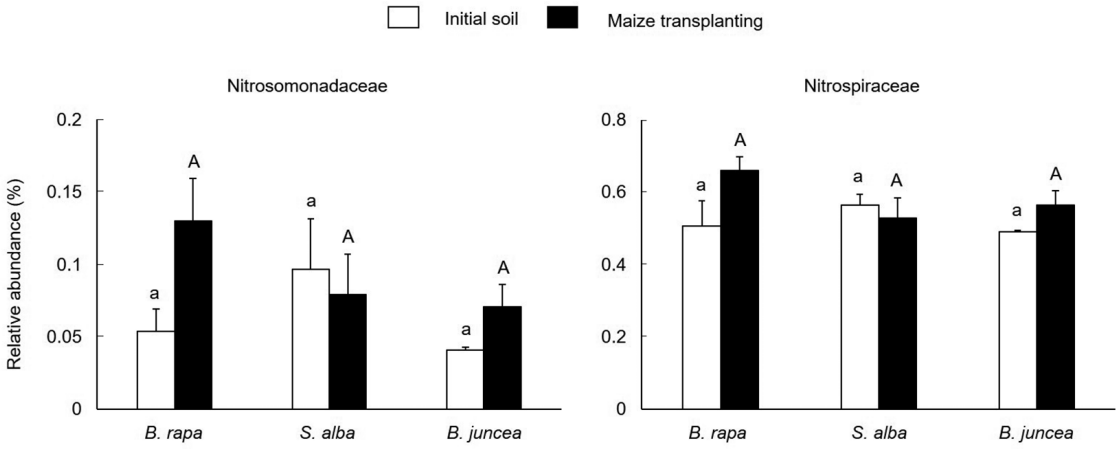

Fig. 6. Comparisons of relative abundance of nitrifying bacteria, Nitrosomonadaceae and Nitrospiraceae, identified using next-generation sequencing (NGS) of 16S rRNA gene
Error bars indicate standard error (N = 3). Same letters indicate no significant difference using Tukey's test ($P < 0.05$).

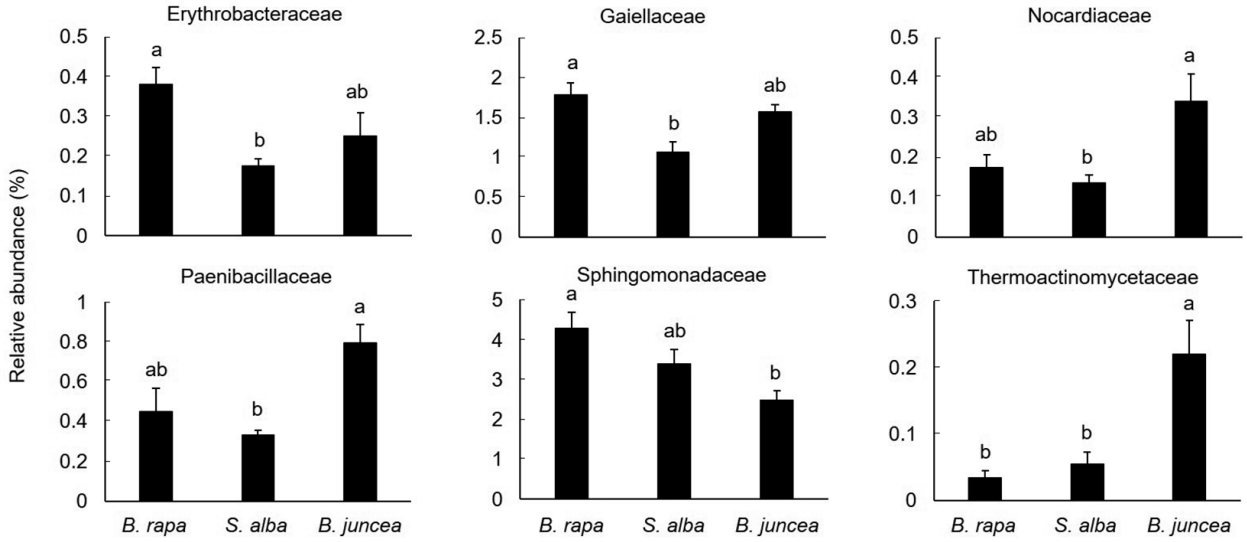

Fig. 7. Comparisons of relative abundance of bacteria identified using next-generation sequencing (NGS) of 16S rRNA gene

Error bars indicate standard error (N = 3). Different letters indicate significant difference using Tukey's test ($P < 0.05$).

field soil possessed high clay content, it is possible that ITCs were physically difficult to diffuse because of small pore space. Therefore, we think that the nitrification inhibition by biofumigation might be less effective in the field of lowland clay soil.

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