Supplementary materials

Title: Effects of different types of additional fertilizers on root-associated microbes of napa cabbage grown in an Andosol field in Japan

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Materials and Methods

Soil chemical analyses

Soil samples (between 5 cm and 15 cm depth) prior to planting seedlings were collected from 5 places (center and 4 corners on the diagonal line drawn in the experimental field) by using an auger and were combined as a composite soil sample on June 29, 2020 after potato cultivation. Soil samples after sampling of heads and roots of napa cabbage were collected from 3 places of intra-row spaces and were combined as a composite soil sample for each of CO and CK plots on December 23, 2020. The analyses of chemical characteristics for field soils used in the present study were carried out by employing the following methods: pH (H2O) and pH (KCl); pH meter, EC; EC meter, NH4-N; indophenol method, NO3-N; hydrazine reduction method, P2O5; Murphy-Riley method, K2O; Flame photometry, MgO; Xylidil blue method, CaO; o-cresolphthalein complexon method, Phosphate absorption coefficient; Ammonium vanadomolybate absorption photometry (30 min buffer extraction instead of 24 hours buffer extraction), and CEC; Indophenol method (simplified method). All analyses,
except pH and EC, were carried out by using a Soil & Plant Analyzer Development SFP-4i (Fujihira Industry Co., Ltd, Tokyo, Japan).

**Sequencing and editing of sequence data prior to statistical analyses**

Briefly, PCR amplification of a partial sequence of bacterial 16S rRNA gene was conducted using the 1st-515f_MIX and 1st-806r_MIX primer set that amplified the V3-V4 region of the bacterial 16S rRNA gene (Caporaso et al. 2011). PCR amplification for bacterial 16S rRNA gene was performed as follows: 94°C for 2 min initially, 30 cycles of a program (94°C for 30 s, 50°C for 30 s, and 72°C for 30 s) and then final extension at 72°C for 5 min. PCR amplification for fungal ITS was conducted using the 1st-gITS and 1st-gITS4 primer set that amplified the internal transcribed sequence of the fungal 18S rRNA gene region (Ihrmark et al. 2012). PCR amplification for fungal ITS was performed as follows: 94°C for 2 min initially, 30 cycles of a program (94°C for 30 s, 50°C for 30 s, and 72°C for 60 s) and then final extension at 72°C for 5 min. PCR amplicons of bacterial 16S rRNA gene and fungal ITS were used as a template DNA for the second PCR and paired-end sequence (2 ×300 bp) on a MiSeq sequencer using a MiSeq Reagent Kit v3 (Illumina K.K., Tokyo, Japan) as described by Nakamura et al. (2020).

For bacterial 16S rRNA gene, extraction of raw reads containing a perfect primer sequence, removal of primer and 50 bases of 3’end sequences, and trimming (minimum length 40 bases; minimum average quality score 20) were carried out as described by Nakamura et al. (2020). Trimmed reads were merged (minimum overlap 10 bases; average merged length 250 bases; average length of individual reads 230 bases) using the FLASH software (Magoc and Salzberg, 2011). Taxonomic assignment for the merged sequences was carried out using a script of Qiime (Caporaso et al., 2010), and sequences classified into archaea, chloroplast, mitochondria, and unassigned sequences were removed. Then, removal of chimera and noise sequences and generation of ASVs and their representative sequences were carried out with data2Qiime2 plugin (Bolyen et al., 2019). Taxonomic assignment for the representative sequences was carried out with feature-classifier Qiime2 plugin by comparing with 97% OTUs of Greengene. Alpha-diversity measures were carried out with a script of Qiime.

For fungal ITS, extraction of raw reads containing a perfect primer sequence, removal of primer sequences, and trimming (minimum length 40 bases; minimum average quality score 20) were carried out as described by Nakamura et al. (2020). Trimmed reads were merged (minimum overlap 10 bases; average merged length 320 bases; average length of individual reads 280 bases) using the FLASH software. For
unmerged reads, 50 bases of 3’end were deleted and merged again as described above. These processes for unmerged reads were repeated another twice. A chimera check of these sequences was performed via the UCHIME algorithm in the USEARCH package (Edgar, 2010; Edgar et al., 2011). A database for chimera check was 97% OTU of UNITE. Taxonomic assignment for the non-chimera sequences was carried out using a script of Qiime, and sequences classified into unassigned sequences were removed. Then, generation of OTUs with 97% identity and taxonomic assignment for the representative sequences of OTUs were carried out with a workflow script of Qiime (no reference, default parameters). Alpha- and beta-diversity measures were carried out with a script of Qiime.

**Phylogenetic tree analyses**

Sequences were aligned using the CLUSTAL W program. The neighbor-joining method was used to build the trees (Saitou and Nei, 1987). The PHYLIP format tree output was applied using the bootstrapping procedure with 1,000 replicates (Felsenstein, 1985). The tree was constructed with TreeView software (Page, 1996).

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Table S1. Chemical characteristics of field soils used in the present study

| Analysis items          | Prior to planting (2020/6/26) | CO (2020/12/23) | CK (2020/12/23) |
|-------------------------|-------------------------------|-----------------|-----------------|
| pH (H$_2$O)             | 6.21                         | 5.38            | 5.64            |
| pH (KCl)                | 5.25                         | 4.92            | 4.99            |
| EC (1:5)                | 0.07                         | 0.94            | 1.292           |
| NH$_4$-N                | 23.3                         | 213             | 686             |
| NO$_3$-N                | 3.5                          | 210             | 192             |
| Available P$_2$O$_5$    | 418                          | 360             | 340             |
| Exchangeable K$_2$O     | 316                          | 525             | 716             |
| Exchangeable MgO        | 465                          | 567             | 596             |
| Exchangeable CaO        | 3128                         | 3290            | 3700            |
| Base saturation (%)     | 77.6                         | 86              | 97.1            |
| Phosphate absorption coefficient | 1900             | -               | -               |
| CEC (me kg$^{-1}$)      | 182                          | -               | -               |

aData for NH$_4$-N, NO$_3$-N, available P$_2$O$_5$, exchangeable K$_2$O, exchangeable MgO, and exchangeable CaO are expressed as mg kg$^{-1}$.

bCO and CK stand for a compound fertilizer and Chiyoda-kasei, respectively.

cSampling dates.
| Microbes/Fertilization | No. of singletons | No. of ASVs or OTUs | Chao1 | ACE | Shannon | Simpson |
|------------------------|------------------|---------------------|-------|-----|---------|---------|
| **Bacteria**           |                  |                     |       |     |         |         |
| CO                     | 1.8 ± 3.5        | 152.75 ± 8.3        | 153.5 ± 9.7 | 153.3 ± 9.3 | 6.7 ± 0.05 | 0.987 ± 0.0005 |
| CK                     | 3.5 ± 2.5        | 161.25 ± 8.8        | 162.1 ± 9.6 | 162.2 ± 9.5 | 6.8 ± 0.10 | 0.988 ± 0.0011 |
| **Fungi**              |                  |                     |       |     |         |         |
| CO                     | 65.3 ± 4.2       | 148.5 ± 18.7        | 246.0 ± 38.6 | 247.7 ± 18.5 | 3.3 ± 0.5 | 0.70 ± 0.05 |
| CK                     | 69.8 ± 13.9      | 149.8 ± 28.5        | 264.4 ± 73.3 | 264.7 ± 68.2 | 3.2 ± 0.6 | 0.71 ± 0.06 |

*CO and CK stand for a compound fertilizer and Chiyoda-kasei, respectively.

*Diversity indexes are calculated based on 2927 and 2835 reads per sample for bacteria and fungi, respectively, and the results of average and S.D. (n=4) are shown.

*ASVs and OTUs were shown for bacteria and fungi, respectively.
Table S3. Results of Blast analyses with representative sequences of microbial ASVs or OTUs showing statistical difference for the relative abundance between a compound fertilizer and Chiyoda-kasei

| OTU | Length (base) | Closest known species (Accession No.) | Score (bit) | Expect | Identity (%) |
|-----|---------------|---------------------------------------|-------------|--------|--------------|
| **Bacteria** | | | | | |
| ASV-B1 (Chitinophagaceae) | 253 | Mucibacter soli (NR_165710) | 412 | 6E-111 | 96 |
| ASV-B2 (Chitinophaga) | 253 | Chitinophaga filiformis (JX280517) | 453 | 2E-123 | 100 |
| ASV-B3 (Niastella) | 253 | Niastella gongjuensis (NR_137250) | 453 | 7E-123 | 99 |
| ASV-B4 (Sphingobacteriales) | 253 | Soliidea koreensis (NR_044566) | 314 | 5E-81 | 88 |
| ASV-B5 (Comamonadaceae) | 253 | Variovorax boronicumulans (MN826549) | 457 | 4E-125 | 100 |
| ASV-B6 (Comamonadaceae) | 253 | Pelomonas saccharophila (NR_114189) | 425 | 2E-115 | 98 |
| ASV-B7 (Ellin6067) | 253 | Sulfuritortus calidifontis (NR_156937) | 385 | 2E-103 | 94 |
| ASV-B8 (Methylotenera mobilis) | 253 | Kofleria flava (NR_04198) | 358 | 3E-95 | 91 |
| ASV-B9 (Myxococcales) | 253 | Halicangium tepidum (NR_024781) | 358 | 3E-95 | 91 |
| ASV-B10 (Legionellales) | 253 | Marinobacterium zhoushanense (NR_151997) | 335 | 3E-88 | 89 |
| ASV-B11 (Dokdonella) | 253 | Dyella monticola (NR_171458) | 403 | 8E-109 | 95 |
| **Fungi** | | | | | |
| OTU-F1 (Bipolaris) | 238 | Aspergillus flavus (CP051057) | 283 | 7E-72 | 79 |
| OTU-F2 (Bipolaris) | 238 | Aspergillus flavus (CP051057) | 306 | 6E-79 | 81 |
| OTU-F3 (Bipolaris) | 273 | Aspergillus flavus (CP051057) | 372 | 6E-99 | 89 |
| OTU-F4 (Bipolaris) | 238 | Aspergillus flavus (CP051057) | 288 | 2E-73 | 80 |
| OTU-F5 (Bipolaris) | 282 | Aspergillus flavus (CP051057) | 469 | 1E-127 | 97 |
| OTU-F6 (Bipolaris) | 283 | Aspergillus flavus (CP051057) | 476 | 7E-130 | 98 |
| OTU-F7 (Bipolaris) | 238 | Aspergillus flavus (CP051057) | 283 | 7E-72 | 79 |
| OTU-F8 (Bipolaris) | 273 | Aspergillus flavus (CP051057) | 359 | 1E-94 | 88 |
| OTU-F9 (Bipolaris) | 282 | Aspergillus flavus (CP051057) | 455 | 7E-124 | 96 |
| OTU-F10 (Bipolaris) | 282 | Aspergillus flavus (CP051057) | 469 | 1E-127 | 97 |
| OTU-F11 (Bipolaris) | 282 | Aspergillus flavus (CP051057) | 473 | 2E-129 | 97 |
| OTU-F12 (Bipolaris) | 303 | Aspergillus flavus (CP051057) | 367 | 3E-97 | 87 |
| OTU-F13 (Bipolaris) | 282 | Aspergillus flavus (CP051057) | 505 | 1E-138 | 99 |
| OTU-F14 (Bipolaris) | 282 | Aspergillus flavus (CP051057) | 482 | 5E-132 | 98 |
| OTU-F15 (Bipolaris) | 238 | Aspergillus flavus (CP051057) | 290 | 5E-74 | 80 |
| OTU-F16 (Bipolaris) | 281 | Aspergillus flavus (CP051057) | 465 | 1E-126 | 97 |
| OTU-F17 (Bipolaris) | 282 | Aspergillus flavus (CP051057) | 464 | 1E-126 | 96 |
| OTU-F18 (Bipolaris) | 282 | Aspergillus flavus (CP051057) | 473 | 2E-129 | 97 |
| OTU-F19 (Podospora) | 246 | Podospora intestinacea (MN341351) | 408 | 7E-110 | 97 |
| OTU-F20 (Olpidium brassicae) | 345 | Olpidium brassicae (AB205212) | 623 | 3E-174 | 100 |

*Mcrobial groups showing higher abundance in CK plot (Chiyodakasei) than in CO plot (a compound fertilizer) are shown as bold font.

*Closest taxon to a representative sequence of an ASV or OTU is shown in parentheses.
Table S4. *Bipolaris*-related OTUs showing significant negative correlations with OTU-F20 (*Olpidium*) and their relative abundances in a compound fertilizer and Chiyoda-kasei plots

| OTU ID | CO         | CK         | $R^2$ |
|--------|------------|------------|-------|
| OTU-F1 | 1.60 ± 0.29 | 0.97 ± 0.35* | 0.70 ** |
| OTU-F3 | 0.63 ± 0.10 | 0.26 ± 0.05*** | 0.82 ** |
| OTU-F4 | 0.82 ± 0.24 | 0.43 ± 0.18* | 0.74 ** |
| OTU-F6 | 0.04 ± 0    | 0.01 ± 0.02* | 0.87 ***|
| OTU-F7 | 2.74 ± 0.05 | 1.82 ± 0.35** | 0.85 ** |
| OTU-F8 | 0.04 ± 0.03 | 0 ± 0*      | 0.57 *  |
| OTU-F9 | 0.64 ± 0.17 | 0.34 ± 0.13* | 0.80 ** |
| OTU-F10| 0.13 ± 0.07 | 0.03 ± 0.03* | 0.77 ** |
| OTU-F11| 0.07 ± 0.03 | 0.01 ± 0.02** | 0.88 ***|
| OTU-F12| 0.29 ± 0.08 | 0.11 ± 0.06* | 0.73 ** |
| OTU-F13| 50.14 ± 5.24 | 30.25 ± 7.36** | 0.96 ***|
| OTU-F14| 0.18 ± 0.06 | 0.07 ± 0.04* | 0.80 ** |
| denovo8| 0.48 ± 0.12 | 0.30 ± 0.23 | 0.58 *  |
| denovo13| 0.10 ± 0.04 | 0.04 ± 0.07 | 0.49 ** |
| denovo72| 0.28 ± 0.09 | 0.18 ± 0.04 | 0.58 *  |
| denovo19| 0.04 ± 0.03 | 0 ± 0    | 0.53 *  |
| denovo128| 2.72 ± 0.61 | 1.23 ± 0.54 | 0.87 ***|
| denovo158| 0.10 ± 0.08 | 0.04 ± 0.04 | 0.47 ** |
| denovo166| 0.72 ± 0.18 | 0.34 ± 0.25 | 0.89 ***|
| denovo174| 0.03 ± 0.02 | 0 ± 0    | 0.69 *  |
| denovo181| 0.07 ± 0.05 | 0.01 ± 0.02 | 0.58 *  |
| denovo193| 0.17 ± 0.07 | 0.07 ± 0   | 0.69 *  |
| denovo195| 0.23 ± 0.12 | 0.14 ± 0.10 | 0.62 *  |
| denovo215| 0.90 ± 0.16 | 0.56 ± 0.22 | 0.80 ** |
| denovo260| 0.21 ± 0.13 | 0.11 ± 0.03 | 0.61 *  |
| denovo261| 0.66 ± 0.16 | 0.38 ± 0.25 | 0.58 *  |
| denovo280| 0.30 ± 0.18 | 0.14 ± 0.08 | 0.52 *  |
| denovo284| 0.10 ± 0.04 | 0.05 ± 0.05 | 0.65 *  |
| denovo327| 0.22 ± 0.12 | 0.12 ± 0.06 | 0.54 *  |
| denovo337| 0.10 ± 0.03 | 0.04 ± 0.07 | 0.74 ** |
| denovo340| 6.09 ± 0.87 | 4.11 ± 1.66 | 0.86 ***|
| denovo359| 0.56 ± 0.14 | 0.36 ± 0.17 | 0.72 ** |
| denovo369| 0.07 ± 0.06 | 0.04 ± 0.03 | 0.53 *  |
| denovo390| 0.23 ± 0.15 | 0.06 ± 0.05 | 0.62 *  |
| denovo394| 0.50 ± 0.14 | 0.33 ± 0.19 | 0.69 *  |

a Relative abundance (%) is calculated based on 2835 reads per sample and the results of average and S.D. (n=4) for each of a compound fertilizer (CO) and Chiyoda-kasei (CK) plots are shown.

b *, *, and *** indicate statistical significance between CO and CK plots at $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively.

$R^2$ stands for decision coefficient. *, *, and *** indicate statistical significance of negative correlations between *Bipolaris*-related OTUs and OTU-F20 (*Olpidium*) at $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively.
Fig. S1-A. Distribution of macronutrients on a particle of Chiyoda-kasei. Distribution of macronutrients of N, P, and K on the surface of Chiyoda-kasei particle was visualized by Energy Dispersive X-ray spectroscopy (EDX) (HITACHI, Miniscope TM3000 equipped with Swift ED 3000) (acceleration voltage: 15.0 kV and resolution: 256 x 192 pixels). Prior to the observation, the particle of Chiyoda-kasei was not sliced due to its hollow structure, but an intact particle was used for the analyses. The high similarities among nitrogen, phosphate, and potassium images suggest that each of macronutrients evenly distribute in a particle of Chiyoda-kasei.
Fig. S1-B. Distribution of macronutrients in a particle of a compound fertilizer. A particle of a compound fertilizer was sliced and distribution of macronutrients of N, P, and K on the surface of slice was visualized by Energy Dispersive X-ray spectroscopy (EDX) (HITACHI, Miniscope TM3000 equipped with Swift ED 3000) (acceleration voltage: 15.0 kV and resolution: 256 x 192 pixels). The drastic dissimilarities among nitrogen, phosphate, and potassium images suggest that each of macronutrients unevenly distribute in a particle of a general compound fertilizer.
Fig. S2. Phylogenetic tree analysis of representative sequences for 18 OTUs belonging to *Bipolaris* in Table 3. The tree was constructed using the neighbor-joining method with the sequences of the closest known species. The sequence of *Talaromyces* sp. NWU was used as an outgroup. The accession numbers for the sequences of closest known species and outgroup are given in parentheses. The numbers at the nodes are the proportions of 1000 bootstrap replicates, and values exceeding 500 are shown.
Fig. S3-A. Correlation plot of all above-ground tissue weight of napa cabbage and relative abundance of napa cabbage root-associated microbes showing statistical difference between a compound fertilizer and Chiyoda-kasei plots.
Fig. S3-B. Correlation plot of head weight of napa cabbage and relative abundance of napa cabbage root-associated microbes showing statistical difference between a compound fertilizer and Chiyoda-kasei plots.
Fig. S3-C. Correlation plot of ratio of head weight in above ground tissue of napa cabbage and relative abundance of napa cabbage root-associated microbes showing statistical difference between a compound fertilizer and Chiyoda-kasei plots.