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Consortia of anti-nematode fungi and bacteria in the rhizosphere of soya bean plants attacked by root-knot nematodes

Hirokazu Toju and Yu Tanaka

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Original submission: 10 October 2018
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2nd revised submission: 20 February 2019
Final acceptance: 21 February 2019

Note: Reports are unedited and appear as submitted by the referee. The review history appears in chronological order.

Review History

RSOS-181693.R0 (Original submission)

Review form: Reviewer 1

Is the manuscript scientifically sound in its present form?
Yes

Are the interpretations and conclusions justified by the results?
Yes

Is the language acceptable?
Yes

Is it clear how to access all supporting data?
Yes

Do you have any ethical concerns with this paper?
No

Have you any concerns about statistical analyses in this paper?
I do not feel qualified to assess the statistics

Recommendation?
Accept with minor revision (please list in comments)

Comments to the Author(s)
The study investigated bacterial and fungal communities in root and rhizosphere of healthy and diseased soybean plants, affected by root-knot nematodes, in two rows of a field. Several OTU were identified that preferentially occurred on healthy or affected plants, and their connections in microbe-microbe networks described. The experimental design was very good. The data are well presented and conclusions supported by the data. I suggest to better discuss the underlying mechanisms or consequences of preferential occurrence of OTU. Those OTU on healthy plants might indicate their role in protecting the plant from nematode attack. Preferential OTU on diseased plants might live on the nematodes (following nematode population dynamics but not controlling it), or simply profit from resource leakage of diseased roots.
Minor comments:
L. 31-37: add summary of OTU preferentially occurring on healthy plants; remove list of nativorous species.
L. Is "awaited" the right word?
L. 132 square
L. 226, 228 randomized
L. 273 better describe this OTU: SH group in UNITE?
L. 348 plays
L. 364 lilacinum
L. 379 Calonectria
L. 534, 547, 549, 620 (
L. 695, 704 volume, pages missing

Review form: Reviewer 2

Is the manuscript scientifically sound in its present form?
Yes

Are the interpretations and conclusions justified by the results?
Yes

Is the language acceptable?
Yes

Is it clear how to access all supporting data?
Yes

Do you have any ethical concerns with this paper?
No

Have you any concerns about statistical analyses in this paper?
I do not feel qualified to assess the statistics

Recommendation?
Major revision is needed (please make suggestions in comments)

Comments to the Author(s)
This paper dealt with relationship between microbial communities in rhizosphere and root of soybean plants and the infection by root-knot nematodes. Interestingly, authors sampling the soybean individual in the one field plot and separate the soybean individuals into three groups (normal, yellow and no leaf) corresponding to the infection of root-knot nematodes. Overall, the manuscript is well-written, methods and results are well presented and conclusions are fully justified.
However, the significantly weaknesses are that there were no any data on the nematode infections. It is quite easy to measure the root-knot index, which is quite necessary to explain how serious of the plants infected by nematode. Moreover, the infection of the nematodes (secondary-stage juveniles) occurred in the seedling stage of the plants. However, sampling was conducted at the late stage of the soybean. It is quite hard to correlate the microbes in the rhizosphere soil and roots to the nematode damaging. Although there are some relationships between some nematophagous microbes previously reported and nematode damaging, most of those microbes could survive as saprophytes in the soil. The other key issue is that root-knot nematode is kind of animals and many microbes can colonize on the different stages of root-knot nematode. However, there was no any examination on the microbial community on nematode.
Following are some recent papers that may have reference value to help promote the significance of this manuscript:
The analysis of rhizosphere microbial communities during an invasion by Ralstonia solanacearum (Wei et al., Ralstonia solanacearum pathogen disrupts bacterial rhizosphere microbiome during an invasion, 2018), besides, the results on rhizosphere, root and cyst microbial communities in disease suppressive soil provide more insights into consortia of anti-nematode bacteria (Hussain et al., Bacterial community assemblages in the rhizosphere soil, root endosphere and cyst of soybean cyst nematode-suppressive soil challenged with nematodes, 2018).

Review form: Reviewer 3 (Paul Orwin)

Is the manuscript scientifically sound in its present form?
Yes

Are the interpretations and conclusions justified by the results?
Yes

Is the language acceptable?
No

Is it clear how to access all supporting data?
Yes

Do you have any ethical concerns with this paper?
No

Have you any concerns about statistical analyses in this paper?
I do not feel qualified to assess the statistics

Recommendation?
Accept with minor revision (please list in comments)

Comments to the Author(s)
I thought the overall science was sound, and the paper provides an interesting insight into the correlation between plant health, root-knot nematode colonization, and corresponding recruitment of nematophagous fungi and nematocidal bacteria to the root and rhizosphere. The paper makes a sound contribution to the literature on these complex interactions, and the authors are careful not to overinterpret their results. The only issue I found with the article was some minor formatting issues and some substantive grammatical concerns especially in the Summary, Introduction, and Discussion sections. These concerns are described in the attached review file (Appendix A).

Decision letter (RSOS-181693.R0)

09-Jan-2019

Dear Dr Toju,

The editors assigned to your paper ("Consortia of anti-nematode fungi and bacteria in the rhizosphere of soybean plants attacked by root-knot nematodes") have now received comments from reviewers. We would like you to revise your paper in accordance with the referee and Associate Editor suggestions which can be found below (not including confidential reports to the Editor). Please note this decision does not guarantee eventual acceptance.

Please submit a copy of your revised paper before 01-Feb-2019. Please note that the revision deadline will expire at 00.00am on this date. If we do not hear from you within this time then it will be assumed that the paper has been withdrawn. In exceptional circumstances, extensions may be possible if agreed with the Editorial Office in advance. We do not allow multiple rounds of revision so we urge you to make every effort to fully address all of the comments at this stage. If deemed necessary by the Editors, your manuscript will be sent back to one or more of the original reviewers for assessment. If the original reviewers are not available, we may invite new reviewers.

To revise your manuscript, log into http://mc.manuscriptcentral.com/rsos and enter your Author Centre, where you will find your manuscript title listed under "Manuscripts with Decisions." Under "Actions," click on "Create a Revision." Your manuscript number has been appended to denote a revision. Revise your manuscript and upload a new version through your Author Centre.

When submitting your revised manuscript, you must respond to the comments made by the referees and upload a file "Response to Referees" in "Section 6 - File Upload". Please use this to document how you have responded to the comments, and the adjustments you have made. In order to expedite the processing of the revised manuscript, please be as specific as possible in your response.

In addition to addressing all of the reviewers' and editor's comments please also ensure that your revised manuscript contains the following sections as appropriate before the reference list:

• Ethics statement (if applicable)
If your study uses humans or animals please include details of the ethical approval received, including the name of the committee that granted approval. For human studies please also detail

whether informed consent was obtained. For field studies on animals please include details of all permissions, licences and/or approvals granted to carry out the fieldwork.

• Data accessibility
It is a condition of publication that all supporting data are made available either as supplementary information or preferably in a suitable permanent repository. The data accessibility section should state where the article's supporting data can be accessed. This section should also include details, where possible of where to access other relevant research materials such as statistical tools, protocols, software etc can be accessed. If the data have been deposited in an external repository this section should list the database, accession number and link to the DOI for all data from the article that have been made publicly available. Data sets that have been deposited in an external repository and have a DOI should also be appropriately cited in the manuscript and included in the reference list.

If you wish to submit your supporting data or code to Dryad (http://datadryad.org/), or modify your current submission to dryad, please use the following link:
http://datadryad.org/submit?journalID=RSOS&manu=RSOS-181693

• Competing interests
Please declare any financial or non-financial competing interests, or state that you have no competing interests.

• Authors' contributions
All submissions, other than those with a single author, must include an Authors' Contributions section which individually lists the specific contribution of each author. The list of Authors should meet all of the following criteria; 1) substantial contributions to conception and design, or acquisition of data, or analysis and interpretation of data; 2) drafting the article or revising it critically for important intellectual content; and 3) final approval of the version to be published.

All contributors who do not meet all of these criteria should be included in the acknowledgements.

We suggest the following format:
AB carried out the molecular lab work, participated in data analysis, carried out sequence alignments, participated in the design of the study and drafted the manuscript; CD carried out the statistical analyses; EF collected field data; GH conceived of the study, designed the study, coordinated the study and helped draft the manuscript. All authors gave final approval for publication.

• Acknowledgements
Please acknowledge anyone who contributed to the study but did not meet the authorship criteria.

• Funding statement
Please list the source of funding for each author.

Once again, thank you for submitting your manuscript to Royal Society Open Science and I look forward to receiving your revision. If you have any questions at all, please do not hesitate to get in touch.

Kind regards,
Royal Society Open Science Editorial Office
Royal Society Open Science

openscience@royalsociety.org

on behalf of Dr Berat Haznedaroglu (Associate Editor) and Professor Kevin Padian (Subject Editor)
openscience@royalsociety.org

Editor's comments:
Please consider carefully all the comments of the reviewers, who are largely positive about the manuscript but do have some substantial issues that need to be addressed.

Additionally, please have a native speaker of English edit the manuscript; we will not be able to accept it with extensive grammatical errors. Thanks for your submission and best of luck with your revision.

Reviewers' Comments to Author:
Reviewer: 1

Comments to the Author(s)
The study investigated bacterial and fungal communities in root and rhizosphere of healthy and diseased soybean plants, affected by root-knot nematodes, in two rows of a field. Several OTU were identified that preferentially occurred on healthy or affected plants, and their connections in microbe-microbe networks described. The experimental design was very good. The data are well presented and conclusions supported by the data. I suggest to better discuss the underlying mechanisms or consequences of preferential occurrence of OTU. Those OTU on healthy plants might indicate their role in protecting the plant from nematode attack. Preferential OTU on diseased plants might live on the nematodes (following nematode population dynamics but not controlling it), or simply profit from resource leakage of diseased roots.
Minor comments:
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L. Is "awaited" the right word?
L. 132 square
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L. 379 Calonectria
L. 534, 547, 549, 620 (
L. 695, 704 volume, pages missing

Reviewer: 2

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However, the significantly weaknesses are that there were no any data on the nematode infections. It is quite easy to measure the root-knot index, which is quite necessary to explain how serious of the plants infected by nematode. Moreover, the infection of the nematodes (secondary-stage juveniles) occurred in the seedling stage of the plants. However, sampling was conducted at the late stage of the soybean. It is quite hard to correlate the microbes in the rhizosphere soil and roots to the nematode damaging. Although there are some relationships between some nematophagous microbes previously reported and nematode damaging, most of those microbes could survive as saprophytes in the soil. The other key issue is that root-knot nematode is kind of animals and many microbes can colonize on the different stages of root-knot nematode. However, there was no any examination on the microbial community on nematode.
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The analysis of rhizosphere microbial communities during an invasion by Ralstonia solanacearum (Wei et al., Ralstonia solanacearum pathogen disrupts bacterial rhizosphere microbiome during an invasion, 2018), besides, the results on rhizosphere, root and cyst microbial communities in disease suppressive soil provide more insights into consortia of anti-nematode bacteria (Hussain et al., Bacterial community assemblages in the rhizosphere soil, root endosphere and cyst of soybean cyst nematode-suppressive soil challenged with nematodes, 2018).

Reviewer: 3

Comments to the Author(s)
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Author's Response to Decision Letter for (RSOS-181693.R0)

See Appendix B.

RSOS-181693.R1 (Revision)

Review form: Reviewer 2

Is the manuscript scientifically sound in its present form?
Yes

Are the interpretations and conclusions justified by the results?
Yes

Is the language acceptable?
Yes

Is it clear how to access all supporting data?
Yes

Do you have any ethical concerns with this paper?
No

Have you any concerns about statistical analyses in this paper?
I do not feel qualified to assess the statistics

Recommendation?
Accept with minor revision (please list in comments)

Comments to the Author(s)
The manuscript is acceptable except a minor comment. Actually Dactylellina is trapping fungus and can capture secondary-stage juveniles and Clonostachys, Pochonia and Purpureocillium can parasitize on nematode eggs. Those fungi associated with no-leaf individuals of soybean, that means high nematode densities in no-leaf individuals can stimulate the multiply of those fungi. Authors may discuss this point a little bit.

Decision letter (RSOS-181693.R1)

07-Feb-2019

Dear Dr Toju:

On behalf of the Editors, I am pleased to inform you that your Manuscript RSOS-181693.R1 entitled "Consortia of anti-nematode fungi and bacteria in the rhizosphere of soybean plants attacked by root-knot nematodes" has been accepted for publication in Royal Society Open Science subject to minor revision in accordance with the referee suggestions. Please find the referees' comments at the end of this email.

The reviewers and Subject Editor have recommended publication, but also suggest some minor revisions to your manuscript. Therefore, I invite you to respond to the comments and revise your manuscript.

• Ethics statement
If your study uses humans or animals please include details of the ethical approval received, including the name of the committee that granted approval. For human studies please also detail whether informed consent was obtained. For field studies on animals please include details of all permissions, licences and/or approvals granted to carry out the fieldwork.

• Data accessibility
It is a condition of publication that all supporting data are made available either as supplementary information or preferably in a suitable permanent repository. The data accessibility section should state where the article's supporting data can be accessed. This section should also include details, where possible of where to access other relevant research materials such as statistical tools, protocols, software etc can be accessed. If the data has been deposited in

an external repository this section should list the database, accession number and link to the DOI for all data from the article that has been made publicly available. Data sets that have been deposited in an external repository and have a DOI should also be appropriately cited in the manuscript and included in the reference list.

If you wish to submit your supporting data or code to Dryad (http://datadryad.org/), or modify your current submission to dryad, please use the following link:
http://datadryad.org/submit?journalID=RSOS&manu=RSOS-181693.R1

• Competing interests
Please declare any financial or non-financial competing interests, or state that you have no competing interests.

• Authors' contributions
All submissions, other than those with a single author, must include an Authors' Contributions section which individually lists the specific contribution of each author. The list of Authors should meet all of the following criteria; 1) substantial contributions to conception and design, or acquisition of data, or analysis and interpretation of data; 2) drafting the article or revising it critically for important intellectual content; and 3) final approval of the version to be published.

All contributors who do not meet all of these criteria should be included in the acknowledgements.

We suggest the following format:
AB carried out the molecular lab work, participated in data analysis, carried out sequence alignments, participated in the design of the study and drafted the manuscript; CD carried out the statistical analyses; EF collected field data; GH conceived of the study, designed the study, coordinated the study and helped draft the manuscript. All authors gave final approval for publication.

• Acknowledgements
Please acknowledge anyone who contributed to the study but did not meet the authorship criteria.

• Funding statement
Please list the source of funding for each author.

Please note that we cannot publish your manuscript without these end statements included. We have included a screenshot example of the end statements for reference. If you feel that a given heading is not relevant to your paper, please nevertheless include the heading and explicitly state that it is not relevant to your work.

Because the schedule for publication is very tight, it is a condition of publication that you submit the revised version of your manuscript before 16-Feb-2019. Please note that the revision deadline will expire at 00.00am on this date. If you do not think you will be able to meet this date please let me know immediately.

To revise your manuscript, log into https://mc.manuscriptcentral.com/rsos and enter your Author Centre, where you will find your manuscript title listed under "Manuscripts with Decisions". Under "Actions," click on "Create a Revision." You will be unable to make your revisions on the originally submitted version of the manuscript. Instead, revise your manuscript and upload a new version through your Author Centre.

When submitting your revised manuscript, you will be able to respond to the comments made by the referees and upload a file "Response to Referees" in "Section 6 - File Upload". You can use this to document any changes you make to the original manuscript. In order to expedite the processing of the revised manuscript, please be as specific as possible in your response to the referees.

When uploading your revised files please make sure that you have:

1) A text file of the manuscript (tex, txt, rtf, docx or doc), references, tables (including captions) and figure captions. Do not upload a PDF as your "Main Document".
2) A separate electronic file of each figure (EPS or print-quality PDF preferred (either format should be produced directly from original creation package), or original software format)
3) Included a 100 word media summary of your paper when requested at submission. Please ensure you have entered correct contact details (email, institution and telephone) in your user account
4) Included the raw data to support the claims made in your paper. You can either include your data as electronic supplementary material or upload to a repository and include the relevant doi within your manuscript
5) All supplementary materials accompanying an accepted article will be treated as in their final form. Note that the Royal Society will neither edit nor typeset supplementary material and it will be hosted as provided. Please ensure that the supplementary material includes the paper details where possible (authors, article title, journal name).

Supplementary files will be published alongside the paper on the journal website and posted on the online figshare repository (https://figshare.com). The heading and legend provided for each supplementary file during the submission process will be used to create the figshare page, so please ensure these are accurate and informative so that your files can be found in searches. Files on figshare will be made available approximately one week before the accompanying article so that the supplementary material can be attributed a unique DOI.

Once again, thank you for submitting your manuscript to Royal Society Open Science and I look forward to receiving your revision. If you have any questions at all, please do not hesitate to get in touch.

Kind regards,
Royal Society Open Science Editorial Office
Royal Society Open Science
openscience@royalsociety.org

on behalf of Dr Berat Haznedaroglu (Associate Editor) and Professor Kevin Padian (Subject Editor)
openscience@royalsociety.org

Reviewer comments to Author:
Reviewer: 2

Comments to the Author(s)
The manuscript is acceptable except a minor comment. Actually Dactylellina is trapping fungus and can capture secondary-stage juveniles and Clonostachys, Pochonia and Purpureocillium can parasitize on nematode eggs. Those fungi associated with no-leaf individuals of soybean, that

means high nematode densities in no-leaf individuals can stimulate the multiply of those fungi. Authors may discuss this point a little bit.

Author's Response to Decision Letter for (RSOS-181693.R1)

See Appendix C.

Decision letter (RSOS-181693.R2)

21-Feb-2019

Dear Dr Toju,

I am pleased to inform you that your manuscript entitled "Consortia of anti-nematode fungi and bacteria in the rhizosphere of soybean plants attacked by root-knot nematodes" is now accepted for publication in Royal Society Open Science.

You can expect to receive a proof of your article in the near future. Please contact the editorial office (openscience_proofs@royalsociety.org and openscience@royalsociety.org) to let us know if you are likely to be away from e-mail contact. Due to rapid publication and an extremely tight schedule, if comments are not received, your paper may experience a delay in publication.

Royal Society Open Science operates under a continuous publication model (http://bit.ly/cpFAQ). Your article will be published straight into the next open issue and this will be the final version of the paper. As such, it can be cited immediately by other researchers. As the issue version of your paper will be the only version to be published I would advise you to check your proofs thoroughly as changes cannot be made once the paper is published.

On behalf of the Editors of Royal Society Open Science, we look forward to your continued contributions to the Journal.

Kind regards,
Royal Society Open Science Editorial Office
Royal Society Open Science
openscience@royalsociety.org

on behalf of Dr Berat Haznedaroglu (Associate Editor) and Professor Kevin Padian (Subject Editor)
openscience@royalsociety.org

Appendix A

Review for RSOS root-knot nematode paper

Formatting concerns.

1) Figure and Table notations should be consistent throughout – I would suggest Bold, written out. For supplementary data/figures, these can just be labeled “Figure Sx” rather than redundantly labeling them “electronic supplementary material, Figure Sx”
2) The sections labeled Ethics, Data Accessibility, etc. These should be bolded
3) Tables should be rotated so the text can be big enough to read. Tables 2-4. Alternatively just use the most specific classification for the identified OTU (perhaps labeled with (P/O/F/C/G to clarify), to make the table easier to read. Also separate tables for Green and No Leaf associated OTUs would be clearer
4) The primers used for sequencing and amplification should be put in a Table, and the Tables in the paper should be renumbered.

Writing concerns.

The writing is generally clear, but there are a few bad habits that make for difficult reading. The most pervasive is the use of words like “However”, “Overall”, and “Therefore” to start sentences. In most cases these words can be removed without changing the meaning of the text. There are also several instances of run on sentences using several commas to delineate clauses that could be broken up into separate sentences. This is largely in the Summary, Introduction, and to a lesser extent in the discussion. Almost everywhere in the text where a sentence starts with a short clause followed by a comma, this clause can be removed or place at the end of the sentence to make the writing clearer.

A specific concern in the discussion is that the preference analysis used to suggest that certain microbes are preferentially found on the roots of the diseased plants, but no quantitative data is provided (in other words, how much more prevalent is *Pseudomonas* in the “no leaf” rhizosphere?). Similar analysis on each of the OTUs found to have a preference could shed additional light on the magnitude of the effect of disease.

The discussion is pretty good,but a little long. The network analysis is discussed a lot, and I think lengthy discussion of the nematicidal properties of various organisms can be cut, considering that you don’t know if these specific organisms are present (only organisms in the same genus).

Appendix B

Consortia of anti-nematode fungi and bacteria in the rhizosphere of soybean plants attacked by root-knot nematodes

Hirokazu Toju[1,2] and Yu Tanaka[2,3]

[1]Center for Ecological Research, Kyoto University, Otsu, Shiga 520-2133, Japan

[2]Precursory Research for Embryonic Science and Technology (PRESTO), Japan Science and Technology Agency, Kawaguchi, Saitama 332-0012, Japan

[3]Graduate School of Agriculture, Kyoto University, Kitashirakawa-oiwake-cho, Sakyo, Kyoto, 606-8502, Japan

This article includes 5 figures, 4 tables, 5 supplementary figures, and 5 supplementary data.

Keywords: disease suppressive soil; *Glycine max*; *Meloidogyne*; nematophagous fungi; phytopathogenic pathogens and pests; sustainable agriculture

Author for correspondence: Hirokazu Toju (toju.hirokazu.4c@kyoto-u.ac.jp).

bioRxiv accession: https://doi.org/10.1101/332403

Summary.

Cyst and root-knot nematodes are major risk factors of agroecosystem management, often causing devastating impacts on crop production. The use of microbes that parasitize or prey on nematodes has been considered as a promising approach for suppressing phytopathogenic nematode populations. However, ~~as~~ effects and persistence of those biological control agents often vary substantially depending on regions, soil characteristics, and agricultural practices~~,~~: m~~more~~ ore insights into microbial community processes are required to develop reproducible control of nematode populations. By performing high-throughput sequencing profiling of bacteria and fungi, we examined how root and soil microbiomes differ between benign and nematode-infected plant individuals in a soybean field in Japan. Results indicated that various taxonomic groups of bacteria and fungi occurred preferentially on the soybean individuals infected by root-knot nematodes or those uninfected by nematodes. Based on a network analysis of potential microbe–microbe associations, we further found that several fungal taxa potentially preying on nematodes [*Dactylellina* (Orbiliales), *Rhizophydium* (Rhizophydiales), *Clonostachys* (Hypocreales), *Pochonia* (Hypocreales), and *Purpureocillium* (Hypocreales)] co-occurred in the soybean rhizosphere at a small spatial scale. ~~Overall, t~~This study suggests how "consortia" of anti-nematode microbes can derive from indigenous (resident) microbiomes, ~~thereby~~ providing basic information for managing anti-nematode microbial communities in agroecosystems.

1. Introduction

Plant pathogenic nematodes, such as cyst and root-knot nematodes, are major threats to crop production worldwide [1, 2]. Soybean fields, in particular, are often damaged by such phytopathogenic nematodes, resulting in substantial yield loss [3, 4]. A number of chemical nematicides and biological control agents (e.g., nematophagous fungi in the genera *Purpureocillium* and *Clonostachys*) have been used to suppress nematode populations in farmlands [5, 6]. However, once cyst and root-knot nematodes appear in a farmland, they often persist in the soil for a long time [7], causing high financial costs in agricultural management. ~~Therefore, f~~Finding ways to suppress pathogenic nematode populations in agroecosystems is a key to reducing risk and management costs in production of soybean and other crop plants.

To reduce damage by cyst and root-knot nematodes, a number of studies have evaluated effects of crop varieties/species, crop rotations, fertilizer inputs, and tillage intensity on nematode density in farmland soil [1, 8-10]. However, the results of those studies varied considerably depending on regions, soil characteristics, and complicated interactions among multiple factors (e.g., interactions between organic matter inputs and tillage frequency) [11]. ~~Therefore~~Thus, it remains an important challenge to understand the mechanisms by which phytopathogenic nematode populations are suppressed in some farmland soils but not in others [12]. New lines of information are required for building general schemes for making agroecosystems robust to the emergence of pest nematodes.

Based on the technological advances in high-throughput DNA sequencing, more and more studies have examined structures of microbial communities (microbiomes) in order to evaluate biotic environmental conditions in the endosphere and rhizosphere of plants [13-16]. ~~Indeed, r~~Recent studies have uncovered microbiome compositions of "disease suppressive soils", in which pests and pathogens damaging crop plants have been suppressed for long periods of time [17-19]. Some studies have further discussed how some microbes within such disease-suppressive microbiomes contribute to health and growth of crop plant species [17, 20, 21]. In one of the studies, soil microbiome compositions were compared among soybean

fields that differed in the density of cyst nematodes [12]. The study then revealed that bacteria and fungi potentially having negative impacts on nematode populations (e.g., *Purpureocillium* and *Pochonia*) were more abundant in long-term than in short-term monoculture fields of soybeans [12]. ~~While s~~Such among-farmland comparisons have provided invaluable insights into ecosystem functions of indigenous (native) microbiomes. Nonetheless, potential relationship between cropping system management and community processes of anti-nematode microbes remains obscured because the farmlands compared in those studies could vary in climatic and edaphic factors~~, obscuring potential relationship between cropping system management and community processes of anti-nematode microbes~~. Moreover, because incidence of cyst and root-knot nematodes generally varies at small spatial scales [22], there can be spatial heterogeneity in abundance and community compositions of anti-nematode bacteria and fungi within a farmland. ~~Thus, s~~Studies focusing on fine-scale assembly of anti-nematode microbes ~~are~~ are required~~awaited~~ for developing agroecosystem management protocols for controlling phytopathogenic nematodes.

We herein conducted ~~By~~ an Illumina sequencing analysis of bacteria and fungi in a soybean (*Glycine max*) field~~, we~~ and then examined how root and rhizosphere microbiome structures varied among host plant individuals that differed in damage by root-knot nematodes (*Meloidogyne* sp.). Based on the data of microbiomes at a small spatial scale, we statistically explored microbial species/taxa that occurred preferentially in the roots or rhizosphere soil of nematode-infected soybean individuals. We further investigated the structure of networks depicting co-abundance patterns of microbial species/taxa within the soybean field, thereby examining whether multiple anti-nematode bacteria and fungi form consortia (assemblages) on/around the plant individuals infected by root-knot nematodes. ~~Overall, this study~~Our results suggest~~s~~ that various taxonomic groups of anti-nematode bacteria and fungi are present within indigenous microbiomes. ~~Our results~~This study also suggests that microbiome assembly at fine spatial scales is a key to manage populations and communities of such functional microbes.

2. Methods

2.1. Sampling

Fieldwork was conducted at the soybean field on the Hokubu Campus of Kyoto University, Japan (35.033 ºN, 135.784 ºE). In the field, the soybean strain "Sachiyutaka" was sown at 15 cm intervals in two lines (electric supplementary material, figure S1) on July 4, 2016 [basal fertilizer, $N:P_2O_5:K_2O = 3:10:10$ g/m^2]. In the lines, 69 and 62 individuals ("set 1" and "set 2", respectively), respectively, were sampled every other position~~s~~ (i.e., 30 cm intervals) (~~Fig~~figure~~.~~ 1) on October 7, 2016. The sampled soybean individuals were classified into three categories: normal individuals with green leaves ("green"), individuals with yellow leaves ("yellow"), and those with no leaves ("no leaf") (~~Fig.~~figure 1*~~A C~~a-c*). Among them, "green" individuals exhibited normal growth, while "no leaf" individuals were heavily infected by root-knot nematodes: "yellow" individuals showed intermediate characters. In total, 97 "green", 19 "yellow", and 15 "no leaf" individuals were sampled (~~Fig.~~figure ~~1D~~1*d*).

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For each individual, two segments of 5-cm terminal roots and rhizosphere soil were collected from ca. 10-cm below the soil surface. The root and soil samples were transferred into a cool box in the field and then stored at -80ºC until DNA extraction in the laboratory. The ~~whole~~ above-ground bodies ~~bodies~~ of the individuals were placed in drying ovens at 80 ºC for 72 hours to measure dry mass. The dry mass data indicated that "green", "yellow", and "no leaf" soybean individuals significantly differed in their biomass (~~Fig.~~figure ~~1C~~1*c*).

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2.2. DNA extraction, PCR, and sequencing

The root segments of each individual were transferred to a 15 mL tube and washed in 70% ethanol by vortexing for 10 s. The samples were then transferred to a new 15 mL tube and then washed again in 70% ethanol by sonication (42 Hz) for 5 min. After an additional sonication wash in a new tube, one of the two root segments were dried and placed in a 1.2 mL tube for each soybean individual. DNA extraction was then performed with a cetyltrimethylammonium bromide (CTAB) method [23] after pulverizing the roots with 4 mm

zirconium balls at 25 Hz for 3 min using a TissueLyser II (Qiagen).

For DNA extraction from the rhizosphere soil, the ISOIL for Beads Beating kit (Nippon Gene) was used as instructed by the manufacturer. For each sample, 0.5 g of soil was placed into a 2 mL microtubes of the ISOIL kit. To increase the yield of DNA, 10 mg of skim milk powder (Wako, 198-10605) was added to each sample [24].

For each of the root and soil samples, the 16S rRNA V4 region of the prokaryotes and the internal transcribed spacer 1 (ITS1) region of fungi were amplified. The PCR of the 16S rRNA region was performed with the forward primer 515f [25] fused with 3–6-mer Ns for improved Illumina sequencing quality [26] and the forward Illumina sequencing primer (5'- TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG- [3–6-mer Ns] – [515f] -3') and the reverse primer 806rB [27] fused with 3–6-mer Ns and the reverse sequencing primer (5'- GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA G [3–6-mer Ns] - [806rB] -3') (0.2 ~~–~~ µM each). To prevent the amplification of mitochondrial and chloroplast 16S rRNA sequences, specific peptide nucleic acids ~~{~~(mPNA and pPNA; ~~Lundberg, Yourstone [26]}~~ Lundberg, Yourstone [26]) (0.25 µM each) were added to the reaction mix of KOD FX Neo (Toyobo). The temperature profile of the PCR was 94 ºC for 2 min, followed by 35 cycles at 98 ºC for 10 s, 78 ºC for 10 s, 50 ºC for 30 s, 68 ºC for 50 s, and a final extension at 68 ºC for 5 min. To prevent generation of chimeric sequences, the ramp rate through the thermal cycles was set to 1 ºC/sec [28]. Illumina sequencing adaptors were then added to respective samples in the supplemental PCR using the forward fusion primers consisting of the P5 Illumina adaptor, 8-mer indexes for sample identification [29] and a partial sequence of the sequencing primer (5'- AAT GAT ACG GCG ACC ACC GAG ATC TAC AC - [8-mer index] - TCG TCG GCA GCG TC -3') and the reverse fusion primers consisting of the P7 adaptor, 8-mer indexes, and a partial sequence of the sequencing primer (5'- CAA GCA GAA GAC GGC ATA CGA GAT - [8-mer index] - GTC TCG TGG GCT CGG -3'). KOD FX Neo was used with a temperature profile of 94 ºC for 2 min, followed by 8 cycles at 98 ºC for 10 s, 55 ºC for 30 s, 68 ºC for 50 s (ramp rate = 1 ºC/s), and a final extension at 68 ºC for 5 min. The PCR amplicons of the 131 soybean individuals were then pooled after a purification/equalization process with the AMPureXP Kit (Beckman Coulter). Primer dimers, which were shorter than

200 bp, were removed from the pooled library by supplemental purification with AMpureXP: the ratio of AMPureXP reagent to the pooled library was set to 0.6 (v/v) in this process.

The PCR of fungal ITS1 region was performed with the forward primer ITS1F_KYO1 [30] fused with 3–6-mer Ns for improved Illumina sequencing quality [26] and the forward Illumina sequencing primer (5'- TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG- [3–6-mer Ns] – [ITS1F_KYO1] -3') and the reverse primer ITS2_KYO2 [30] fused with 3–6-mer Ns and the reverse sequencing primer (5'- GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA G [3–6-mer Ns] - [ITS2_KYO2] -3'). The buffer and polymerase system of KOD FX Neo was used with a temperature profile of 94 ºC for 2 min, followed by 35 cycles at 98 ºC for 10 s, 50 ºC for 30 s, 68 ºC for 50 s, and a final extension at 68 ºC for 5 min. Illumina sequencing adaptors and 8-mer index sequences were then added in the second PCR as described above. The amplicons were purified and pooled as described above.

The sequencing libraries of the prokaryote 16S and fungal ITS regions were processed in an Illumina MiSeq sequencer (run center: KYOTO-HE; 15% PhiX spike-in). Because the quality of forward sequences is generally higher than that of reverse sequences in Illumina sequencing, we optimized the MiSeq run setting in order to use only forward sequences. Specifically, the run length was set 271 forward (R1) and 31 reverse (R4) cycles in order to enhance forward sequencing data: the reverse sequences were used only for discriminating between 16S and ITS1 sequences based on the sequences of primer positions.

2.3. Bioinformatics

The raw sequencing data were converted into FASTQ files using the program bcl2fastq 1.8.4 distributed by Illumina. The output FASTQ files were demultiplexed with the program Claident v0.2.2017.05.22 [31, 32], by which sequencing reads whose 8-mer index positions included nucleotides with low (< 30) quality scores were removed. The sequencing data were deposited to DNA Data Bank of Japan (DDBJ) (DRA006845). Only forward sequences were used in the following analyses after removing low-quality 3'-ends using Claident. Noisy reads [31] were subsequently discarded and then denoised dataset consisting of 2,041,573 16S and

1,325,199 ITS1 reads were obtained.

For each dataset of 16S and ITS1 regions, filtered reads were clustered with a cut-off sequencing similarity of 97% using the program VSEARCH [33] as implemented in Claident. The operational taxonomic units (OTUs) representing less than 10 sequencing reads were subsequently discarded. The molecular identification of the remaining OTUs was performed based on the combination of the query-centric auto-*k*-nearest neighbor (QCauto) method [32] and the lowest common ancestor (LCA) algorithm [34] as implemented in Claident. Note that taxonomic identification results based on the combination of the QCauto search and the LCA taxonomic assignment are comparable to, or sometimes more accurate than, those with the alternative approaches [32, 35, 36]. In total, 5,351 prokatyote (bacterial or archaeal) OTUs and 1,039 fungal OTUs were obtained for the 16S and ITS1 regions, respectively (electric supplementary material, data S1). The UNIX codes used in the above bioinformatic pipeline are available as electric supplementary material, data S2.

For each combination of target region (16S or ITS1) and sample type (root or soil), we obtained a sample × OTU matrix, in which a cell entry depicted the number of sequencing reads of an OTU in a sample (electric supplementary material, data S3). The cell entries whose read counts represented less than 0.1% of the total read count of each sample were removed to minimize effects of PCR/sequencing errors [37]. The filtered matrix was then rarefied to 1,000 reads per sample using the "rrarefy" function of the vegan 2.4-1 package [38] of R 3.4.3 [39]. Samples with less than 1,000 reads were discarded in this process: the numbers of samples in the rarefied sample × OTU matrices were 119, 128, 117, and 128 for root prokaryote, root fungal, soil prokaryote, and soil fungal matrices, respectively (electric supplementary material, data S4).

2.4. Prokaryote and fungal community structure

Relationship between the number of sequencing reads and that of detected OTUs was examined for each dataset (root prokaryote, root fungal, soil prokaryote, or soil fungal dataset) with the "rarecurve" function of the R vegan package. Likewise, relationship between

the number of samples and that of OTUs was examined with the vegan "specaccum" function. For each dataset, difference in OTU compositions among "green", "yellow", and "no leaf" soybean individuals was examined by the permutational analysis of variance (PERMANOVA; Anderson [40]) with the vegan "adonis" function (10,000 permutations). To control effects of sampling positions (lines) on the community structure, the information of sampling sets (set 1 or set 2) was included as an explanatory variable in the PERMANOVA. The variation in OTU compositions was visualized with nonmetric multidimensional scaling (NMDS) using the vegan "metaMDS" function. To examine potential relationship between root/soil microbial community structure and plant biomass, an additional PERMANOVA was performed for each dataset. The information of sampling sets was included in the models. To explore signs of spatial autocorrelation in the community data, a Mantel's correlogram analysis was performed with the vegan "mantel.correlog" function. The "Bray-Curtis" metric of β-diversity was used in the PERMANOVA, NMDS, and Mantel's correlogram analyses.

2.5. Screening of host-state-specific OTUs

To explore prokaryote/fungal OTUs that preferentially occurred on/around "green", "yellow", or "no leaf" soybean individuals, a randomization test was performed by shuffling the plant state labels in each of the root prokaryote, root fungal, soil prokaryote, and soil fungal data matrices (100,000 permutations). We then evaluated preference of a prokaryote/fungal OTU (i) for a plant state (j) ("green", "yellow", or "no leaf") as follows:

$$Preference\ (i, j) = [N_{observed}\ (i, j) - \text{Mean}\ (N_{randomized}\ (i, j))] / \text{SD}\ (N_{randomized}\ (i, j)),$$

where $N_{observed}\ (i, j)$ denoted the mean number of the sequencing reads of OTU i among state j soybean samples in the original data, and the Mean ($N_{randomized}\ (i, j)$) and SD ($N_{randomized}\ (i, j)$) were the mean and standard deviation of the number of sequencing reads for the focal OTU–plant state combination across randomized matrices. Regarding this standardized preference index, values larger than three generally represent strong preferences (false discovery rate (FDR) < 0.05; see results of a previous study [35]): hence, we listed OTUs whose preference values exceeded three.

2.6. Microbe–microbe networks

To examine how prokaryote and fungal OTUs co-occurred in root or soil samples, a co-abundance network analysis was performed based on the sparse inverse covariance estimation for ecological association inference (Spiec-Easi) method [41]. In each of the root and soil sample analyses, the input data matrix was prepared by merging the sample × OTU matrices of prokaryotes and fungi. As inferences of co-abundance patterns were unavailable for rare OTUs, only the OTUs detected from 30 or more samples were retained in the input matrices. For each of the root and soil data matrices, a co-abundance analysis was performed with the "spiec.easi" function of the R "SpiecEasi" package [41]. The networks depicting the co-abundance patterns were drawn using the R "igraph" package [42].

3. Results

3.1. Prokaryotes and fungal community structure

On average, 107.9 (SD = 18.0), 25.4 (SD = 8.9), 172.5 (SD = 17.3), and 78.3 (SD = 10.5) OTUs per sample were observed, respectively, from the root prokaryote, root fungal, soil prokaryote, and soil fungal dataset after filtering and rarefaction steps (electric supplementary material, figure S2). The total number of OTUs observed was 1387, 346, 1191, and 769 for the root prokaryote, root fungal, soil prokaryote, and soil fungal datasets, respectively (electric supplementary material, figure S3).

In the soybean field, the prokaryote community on roots was dominated by the bacterial classes Proteobacteria, Actinobacteria, Chloroflexi, and Bacteroidetes, while that of rhizosphere soil consisted mainly of Proteobacteria, Actinobacteria, and Acidobacteria, and the archaeal lineage Thaumarchaeota (~~Fig.~~figure ~~2A~~2*a*). The fungal community of roots was dominated by the fungal orders Hypocreales, Sordariales, Plesporales, while that of soil consisted mainly of Hypocreales, Agaricales, Eurotiales, Mortierellales, and Filobasidiales (~~Fig.~~figure ~~2B~~2*b*). Regarding the order level compositions of fungi in the rhizosphere soil, the

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proportion of Orbiliales reads was much higher in “yellow” (3.62 %) and “no leaf” (4.82 %) soybean individuals than in “green” ones (0.89 %) (~~Fig.~~figure 2). The genus level compositions of the samples are shown in electric supplementary material, figure S4.

In each dataset (i.e., root prokaryote, root fungal, soil prokaryote, or soil fungal data), microbial community structure varied among “green”, “yellow”, or “no leaf” soybean individuals, although the effects of sampling sets on the community structure were much stronger (~~Fig.~~figure 3). Even within each sampling set, spatial autocorrelations of bacterial/fungal community structure were observed (electric supplementary material, ~~figure S4~~figure S5). Significant relationships between microbial community structure and soybean biomass were observed in the soil prokaryote and soil fungal datasets but not in the root prokaryote and root fungal datasets (~~Table~~ table 1).

3.2. Screening of host-state-specific OTUs

In the root microbiome, only an unidentified fungal OTU showed a strong preference for “green” soybean individuals, while 18 bacterial and 4 fungal OTUs occurred preferentially on “no leaf” host individuals (~~Table~~ table 2; electric supplementary material, ~~figure S5~~figure S6). The list of the bacteria showing preferences for “no leaf” soybean individuals included OTUs whose 16S rRNA sequences were allied to those of *Dyella*, *Herbaspirillum*, *Labrys*, *Phenylobacterium*, *Gemmata*, *Chitinophaga*, *Pedobacter*, *Niastella*, and *Streptomyces* (~~Table~~ table 2). The four fungal OTUs showing preferences for “no leaf” hosts were unidentified basidiomycetes (~~Table~~ table 2).

In the rhizosphere soil microbiome, seven prokaryote OTUs, including those belonging to Chloroflexi (e.g., *Sphaerobacteraceae* sp.) and Proteobacteria (*Kofleriaceae* sp.), occurred preferentially on “green” host individuals (~~Table~~ table 3). Likewise, five fungal OTUs, including those allied to basidiomycete yeasts in the genera *Solicoccozyma* and *Saitozyma*, showed preferences for “green” soybean individuals (~~Table~~ table 3). Results also revealed that 26 bacterial and 11 fungal OTUs had biased distributions in the rhizosphere of “no leaf” soybean individuals (~~Table~~ table 3). The list of microbes showing preferences for “no leaf”

hosts included OTUs allied to bacteria in the genera *Pesudomonas*, *Nevskia*, *Cellvibrio*, *Massilia*, *Duganella*, *Novosphingobium*, *Mucilaginibacter*, and *Flavobacterium* and OTUs allied to fungi in the genera *Burgoa*, *Clonostachys*, *Plectosphaerella*, *Xylaria*, *Dactylellina*, *Talaromyces*, *Cladosporium*, *Alternaria*, and *Peniophora* (~~Table~~ table 3). The list of microbes that preferentially occurred on "no leaf" hosts involved OTUs with high sequence similarity to the nematophagous fungi, *Clonostachys rosea* (Hypocreales) and *Dactylellina* sp. (Orbiliales) (~~Table~~ table 3). The reads of the *Clonostachys* (F_0257) and *Dactylellina* (F_0163) OTUs, respectively, represented 9.5% and 3.5% of the sequencing reads of "no leaf" samples (electric supplementary material, data S5). The indices of preferences for "yellow" soybean individuals are shown in electric supplementary material, data S5.

3.3. Microbe–microbe networks

The structure of microbe–microbe networks (~~Fig.~~figure 4) were more complicated in the soil microbiome data (~~Fig.~~figure 4~~C-D~~*c-d*) than in the root microbiome data (~~Fig.~~figure 4~~A-B~~*a-b*). Within the network representing co-abundance of microbes across root samples, the *Clonostachys* OTU (F_0257) had a significant link with a *Streptomyces* OTU, while *Dactylellina* was absent from the root microbiome network data (~~Fig.~~figure ~~4A~~4*a*). Within the positive co-abundance network of the rhizosphere soil microbiome (~~Fig.~~figure ~~4C~~4*c*), the *Clonostachys* (F_0257) and *Dactylellina* (F_0163) nematophagous fungal OTUs were connected with each other (~~Table~~ table 4). In addition, the *Clonostachys* OTU was linked with two bacterial OTUs (*Ralstonia* and Rhizobiales) and fungal OTUs in the genera *Calonectria* and *Purpureocillium* (~~Table~~ table 4). Likewise, the *Dactylellina* OTU was connected also with two Alphaproteobacterial OTUs and a bacterial OTU allied to *Nitrospira japonica* as well as fungal OTUs in the genera *Rhizophydium*, *Pochonia*, *Purpureocillium* (~~Table~~ table 4).

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4. Discussion

Based on Illumina sequencing, we herein compared root-associated/rhizosphere microbial

communities between soybean individuals infected by root-knot nematodes and those showing no symptoms. The results indicated that, in both soybean roots and rhizosphere soil, prokaryote and fungal community structures significantly varied depending on host plant states (~~Figs.~~figures 2 and 3). We further performed statistical analyses for screening prokaryote and fungal OTUs preferentially associated with infected and benign soybean host individuals (~~Tables~~ tables 2-3; ~~Fig.~~figure 4). The results are based on purely descriptive data and hence they, in principle, are not direct evidences of interactions among plants, nematodes, and microbiomes~~.~~: i.e., causal relationship among those agents remains unknown. ~~Moreover~~A~~, a~~s this study provided only "snap-shot" information of microbiome structure at the end of a growing season, we need to conduct further studies uncovering temporal microbiome dynamics throughout the growing season of soybeans ~~are awaited~~. Nonetheless, as detailed below, the statistical analyses suggest assembly of diverse anti-nematode bacteria and fungi from indigenous microbial communities in the soybean field, providing a basis for exploring ways to reduce damage by root-knot nematodes with those indigenous functional microbes.

Within the root microbiome analyzed, various taxonomic groups of bacteria preferentially occurred on "no leaf" soybean samples (~~Table~~ table 2). Among them, the genus *Streptomyces* is known to involve some species that suppress nematode populations, potentially used as biological control agents for root-knot nematodes [43-46]. In contrast, *Herbaspirillum*, *Rickettsia*, *Chitinophaga*, and *Pedobacter* have been reported as symbionts of nematodes, potentially playing beneficial roles for host nematodes [47-49]. ~~Thus, r~~Results of these statistical analyses should be interpreted with caution, as they are likely to highlight not only prospective microbes potentially parasitizing on pests/patho~~n~~gens but also microbes that can form mutualistic interactions with disease agents.

Within the soybean rhizosphere soil microbiome, diverse taxonomic groups of not only bacteria but also fungi preferentially occurred around "no leaf" soybean individuals (~~Table~~ table 3). Among them, *Pseudomonas* has been known to suppress root-knot nematode populations [50, 51] potentially by producing hydrogen cyanide [52] or extracellular protease [53], but interactions with root-knot nematodes have not yet been examined for other bacteria

preferentially found in the rhizosphere of "no leaf" soybean individuals. Meanwhile, the list of the fungal OTUs frequently observed in the rhizosphere of "no leaf" soybeans included some fungi whose ability to ~~suppressing~~ suppress nematode populations had been well documented (~~T~~table 3). *Clonostachys rosea*, for example, has been known as a prospective biological control agent of plant- and animal-pathogenic nematodes [54, 55]. An observational study based on green fluorescent protein imaging has indicated that the conidia of the fungus adhere to nematode cuticle and their germ tubes penetrate nematode bodies, eventually killing the invertebrate hosts [56]. The fungus is also known to produce a subtilisin-like extracellular protease, which plays an important role during the penetration of nematode cuticles [57]. ~~In addition to *Clonostachys*, o~~Our analysis also highlighted a ~~another~~ nematophagous fungus in the genus *Dactylellina* (teleomorph = *Orbilia*). Species in the genus and many other fungi in the order Orbiliales produce characteristic trap structures with their hyphae to prey on nematodes [58-60], often nominated as prospective biological control agents [61-63].

An additional analysis focusing on *Clonostachys* and *Dactylellina* highlighted bacteria and fungi that frequently co-occurred with the nematophagous fungi (~~Fig.~~figure 4). In the root microbiome, *Clonostachys* and a *Streptomyces* OTU showed positively correlated distributions across soybean samples (~~Table~~ table 4). In the rhizosphere microbiome, *Clonostachys* and *Dactylellina* showed significant co-abundance patterns (~~Table~~ table 4). Moreover, in the soil, the two nematophagous fungi co-occurred frequently with other taxonomic groups of nematophagous fungi such as *Purpureocillium*, *Pochonia*, and *Rhizophydium* (~~Table~~ table 4; ~~Fig.~~figure 5). Among them, fungi in the genus *Purpureocillium* (Hypocreales: Ophiocordycipitaceae) have been known to suppress plant parasitic nematodes, insect pests, and oomycete phytopathogens [64-67]~~ and their genome sequences have been analyzed for understanding the physiological mechanisms of the pest/pathogen suppression [64, 68, 69]. As one of *Purpureocillium* species (*P. liacinum*) can form symbiotic interactions with plants as endophytes [67, 70], it has been recognized as promising biological control agents for commercial use [64].~~. Another Hypocreales genus, *Pochonia* (previously placed in the genus *Verticillium*; teleomorph = *Metacordyceps*; Clavicipitaceae) has been known as

nematophagous as well and they can kill eggs and females of root-knot (*Meloidogyne* spp.) and cyst (*Globodera* spp.) nematodes [68-71]. ~~*Pochonia* fungi, especially *P. chlamydosporia*, are also endophytic and hence they have been used in agriculture [75-78].~~ Species in the chytrid genus *Rhizophydium* involve species that utilize nematodes as parasites or saprophytes [72, 73]. They are known to explore host nematodes in the form of zoospores [72]. ~~Overall, our~~All these results suggest that indigenous anti-nematode or nematophagous microbes can form consortia in soil ecosystems of soybean fields. It is important to note that the members of the consortia do not necessarily interact with each other directly: i.e., they may merely share habitat preferences [36, 37, 74]. However, the inferred structure of microbe–microbe networks helps us understand overall consequences of ecological processes in microbiomes [15].

Along with the consortia of anti-nematode microbes, an OTU in the genus *Calonectria*, which causes leaf blight, wilt, and root rot of various plant species [75, 76], was frequently observed (~~Table~~ table 4). The phytopathogenic fungus might have attacked soybean individuals weakened by root-knot nematodes. Alternatively, *Calonectria* may have infected host soybeans earlier than root-knot nematodes, followed by the emergence of nematodes and their exploiters (i.e., anti-nematode microbes). Given that fungi can interact with each other both antagonistically and mutualistically in the soil [77, 78], direct interactions between *Calonectria* and nematophagous fungi in the genera *Clonostachys*, *Dactylellina*, *Purpureocillium*, *Pochonia*, and *Rhizophydium* are of particular interest. Studies examining potential interactions involving soybeans, root-knot nematodes, anti-nematode bacteria/fungi, and *Calonectria* will help us understand ecological processes that structure consortia of nematophagous fungi.

Although this study did not evaluate potential effects of background environmental conditions (e.g., soil pH and inorganic nitrogen concentration) on microbiome structure, management of edaphic conditions are expected to have great impacts on dynamics of anti-nematode microbiomes. A number of studies have explored ways to suppress nematode populations by optimizing cropping systems [1]. Crop rotation, in which planting of a crop variety and that of nematode-resistant varieties/species are rotated, has been recognized as an

effective technique for regulating root-knot and cyst nematode populations [8, 79, 80]. In contrast, long-term continual cropping in soybean monoculture fields can increase anti-nematode bacteria and fungi (e.g., *Pseudomonas*, *Purpureocillium*, and *Pochonia*), potentially resulting in lowered densities of cyst nematodes [12]. Tillage regimes [9-11] and introduction of organic matter (e.g., alfalfa leaves or crop residue) [81-83] have great impacts on nematode densities in farmlands, but their effects vary considerably among studies [1]. In addition, because nematode-infected plant individuals ~~infected by nematodes~~ can show highly aggregated distributions at a small spatial scale within a farmland (~~Fig.~~figure ~~1D~~1*d*), tillage can promote the spread of plant damaging nematodes [22]. Frequent tillage may have negative impacts on populations of nematophagous fungi as a consequence of hyphal fragmentation (cf. [84]), but such destructive effects on fungal communities have not yet been tested intensively. Given that microbiome structures were not taken into account in most previous studies evaluating effects of cropping systems on nematode suppression (but see -[12, 21]), more insights into relationship between agroecosystem management and indigenous (native) microbiome dynamics are required for building reproducible ways ~~for developing~~to develop disease-suppressive soil.

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We herein found that consortia of anti-nematode bacteria and fungi could develop at a small spatial scale within a field of soybeans infected by root-knot nematodes. ~~Taking into account~~Given the diversity of those anti-nematode microbes observed in this study, multiple biological control agents are potentially available *in situ* without introducing exogenous ones depending on base compositions and conditions of indigenous microbiomes ~~within and around a focal farmland~~. In this respect, design of cropping systems (e.g., crop rotations, tillage frequencies, and inputs of fertilizer or organic matter) is of particular importance in activating and maximizing ecosystem functions that stem from resident microbial diversity [15]. Because those indigenous microbes, in general, have adapted to local biotic and abiotic environments, their populations are expected to persist more stably than exogenous microbes artificially introduced to a target agroecosystem (see [19] for reviews of the success/failure of microbial introduction). Elucidating relationship between cropping systems and microbiome processes is the key to design disease-suppressive agroecosystems.

Ethics. The fieldwork and sampling of materials were permitted by Crop Science Laboratory, Graduate School of Agriculture, Kyoto University. No ethical assessment was required prior to conducting this research. As this research does not target humans and animals, neither informed consent nor animal ethical investigations were required.

Data accessibility. Data are available from the electric supplementary material, data S1-S5 and DNA Data Bank of Japan (DDBJ) (DRA006845).

Authors' contributions. H.T conceived and designed the work. H.T. and Y.T. performed fieldwork. H.T. conducted molecular experiment and analyzed the data. H.T. wrote the manuscript with Y.T.. All authors gave final approval for publication.

Competing interests. The authors declare no competing interests.

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716 **Table 1.** Relationship between prokaryote/fungal community structure and the biomass of
717 soybean individuals. For each dataset (i.e., root prokaryote, root fungal, soil prokaryote, or
718 soil fungal data), a PEMANOVA model of community structure was constructed. The
719 information of the sampling set ("set 1" or "set 2") and the dry mass of host soybean
720 individuals were included as explanatory variables.
721

Variable	df	F_{model}	*P*
Root prokaryotes			
Sampling set	1	10.4	< 0.0001
Dry mass	1	1.3	0.1139
Root fungi			
Sampling set	1	14.0	< 0.0001
Dry mass	1	0.6	0.8267
Soil prokaryotes			
Sampling set	1	15.4	< 0.0001
Dry mass	1	3.1	0.002
Soil fungi			
Sampling set	1	36.7	< 0.0001
Dry mass	1	2.2	0.0145

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Table 2. Prokaryote and fungal OTUs showing strong preferences for host states in the root microbiome datasets. The prokaryote/fungal OTUs that showed strong preferences for "green" or "no leaf" soybean individuals (preference value ≥ 3) are shown. The taxonomic assignment results based on the QCauto–LCA pipeline are shown with the top-hit results of NCBI BLAST searches. The OTU code starting with P (P_xxxx) and F (F_xxxx) are prokaryotes and fungi, respectively.

OTU	Phylum	Class	Order	Family	Genus	NCBI top hit	Accession	Cover	Identity
Green									
F_0437	Ascomycota	-	-	-	-	*Knufia* sp.	KP235641.1	83%	98%
No leaf									
P_3453	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Rhodanobacteraceae	-	*Dyella marensis*	LN890104.1	100%	99%
P_3207	Proteobacteria	Gammaproteobacteria	Legionellales	Coxiellaceae	*Aquicella*	*Aquicella siphonis*	NR_025764.1	100%	94%
P_2827	Proteobacteria	Betaproteobacteria	-	-	-	*Duganella zoogloeoides*	KT983992.1	100%	100%
P_2733	Proteobacteria	Betaproteobacteria	Burkholderiales	Oxalobacteraceae	*Herbaspirillum*	*Herbaspirillum chlorophenolicum*	MG571754.1	100%	100%
P_2590	Proteobacteria	Alphaproteobacteria	-	-	-	*Croceicoccus mobilis*	NR_152701.1	100%	88%
P_2481	Proteobacteria	Alphaproteobacteria	Rickettsiales	Rickettsiaceae	-	*Rickettsia japonica*	KU586263.1	100%	91%
P_2279	Proteobacteria	Alphaproteobacteria	Rhizobiales	Xanthobacteraceae	*Labrys*	*Labrys monachus*	KT694157.1	100%	100%
P_2042	Proteobacteria	Alphaproteobacteria	Caulobacterales	Caulobacteraceae	*Phenylobacterium*	*Phenylobacterium* sp.	JX458410.1	100%	99%
P_3664	Proteobacteria	-	-	-	-	*Desulfofrigus oceanense*	AB568590.1	97%	93%
P_3658	Proteobacteria	-	-	-	-	*Rudaea* sp.	KM253197.1	100%	85%
P_1748	Planctomycetes	Planctomycetia	Planctomycetales	Gemmataceae	*Gemmata*	*Gemmata* sp.	GQ889445.1	100%	99%

P_1278	Chloroflexi	Thermomicrobia	-	-	-	*Sphaerobacter thermophilus*	AJ871227.1	100%	92%
P_1058	Bacteroidetes	-	-	-	-	*Chitinophaga polysaccharea*	MG322237.1	100%	92%
P_1049	Bacteroidetes	-	-	-	-	*Pedobacter terrae*	MG819444.1	100%	98%
P_0994	Bacteroidetes	-	-	-	-	*Chitinophaga terrae*	LN890054.1	100%	95%
P_0887	Bacteroidetes	Chitinophagia	Chitinophagales	Chitinophagaceae	*Niastella*	*Niastella koreensis*	NR_074595.1	100%	100%
P_0498	Actinobacteria	Actinobacteria	Streptomycetales	Streptomycetaceae	-	*Streptomyces albiaxialis*	KP170480.1	100%	98%
P_0444	Actinobacteria	Actinobacteria	Streptomycetales	Streptomycetaceae	*Streptomyces*	*Streptomyces olivaceoviridis*	KP823723.1	100%	98%
F_0796	Basidiomycota	-	-	-	-	*Classiculaceae* sp.	KY548838.1	92%	84%
F_0792	Basidiomycota	-	-	-	-	*Classiculaceae* sp.	KY548838.1	92%	83%
F_0790	Basidiomycota	-	-	-	-	*Classiculaceae* sp.	KY548838.1	91%	83%
F_0786	Basidiomycota	-	-	-	-	*Classiculaceae* sp.	KY548838.1	90%	84%

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Table 3. Prokaryote and fungal OTUs showing strong preferences for host states in the soil microbiome datasets. The prokaryote/fungal OTUs that showed strong preferences for "green" or "no leaf" soybean individuals (preference value ≥ 3) are shown. The taxonomic assignment results based on the QCauto–LCA pipeline are shown with the top-hit results of NCBI BLAST searches. The OTU code starting with P (P_xxxx) and F (F_xxxx) are prokaryotes and fungi, respectively.

OTU	Phylum	Class	Order	Family	Genus	NCBI top hit	Accession	Cover	Identity
Green									
P_0697	Actinobacteria	-	-	-	-	*Gaiella occulta*	NR_118138.1	100%	91%
P_1264	Chloroflexi	Thermomicrobia	Sphaerobacterales	Sphaerobacteraceae	*Sphaerobacter*	*Shewanella fodinae*	FM887036.1	98%	84%
P_1281	Chloroflexi	Thermomicrobia	-	-	-	*Thermomicrobium carboxidum*	NR_134218.1	100%	87%
P_2949	Proteobacteria	Deltaproteobacteria	Myxococcales	Kofleriaceae	*Haliangium*	*Kofleria flava*	HF937255.1	100%	91%
P_3762	-	-	-	-	-	*Planctomycetales bacterium*	AY673390.1	98%	94%
P_3715	-	-	-	-	-	*Brochothrix thermosphacta*	MG807446.1	99%	86%
P_0032	-	-	-	-	-	*Nitrosocosmicus exaquare*	CP017922.1	100%	99%
F_0477	Ascomycota	-	-	-	-	No significant match	-	-	-
F_0141	Ascomycota	Eurotiomycetes	-	-	-	*Penicillium clavigerum*	NR_121317.1	100%	81%
F_0700	Basidiomycota	Tremellomycetes	Filobasidiales	Piskurozymaceae	*Solicoccozyma*	*Solicoccozyma terreus*	KY102958.1	100%	100%
F_0734	Basidiomycota	Tremellomycetes	Tremellales	Trimorphomycetaceae	*Saitozyma*	*Saitozyma podzolica*	KY102943.1	82%	99%
F_0738	Basidiomycota	Tremellomycetes	Tremellales	Trimorphomycetaceae	*Saitozyma*	*Saitozyma podzolica*	KY102943.1	84%	99%
No leaf									
P_3294	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	*Pseudomonas*	*Pseudomonas psychrotolerans*	KY623077.1	100%	100%

P_3256	Proteobacteria	Gammaproteobacteria	Nevskiales	Sinobacteraceae	*Nevskia*	*Nevskia persephonica*	JQ710442.1	97%	99%
P_3189	Proteobacteria	Gammaproteobacteria	Cellvibrionales	Cellvibrionaceae	*Cellvibrio*	*Cellvibrio mixtus*	KC329916.1	100%	100%
P_3308	Proteobacteria	Gammaproteobacteria	-	-	-	*Steroidobacter* sp.	KP185148.1	100%	95%
P_3093	Proteobacteria	Deltaproteobacteria	Myxococcales	-	-	*Sorangiineae bacterium*	JF719608.1	100%	94%
P_3004	Proteobacteria	Deltaproteobacteria	Myxococcales	Polyangiaceae	*Byssovorax*	*Polyangium spumosum*	KX572839.2	100%	97%
P_3114	Proteobacteria	Deltaproteobacteria	-	-	-	*Stigmatella hybrida*	KX572784.2	100%	91%
P_2747	Proteobacteria	Betaproteobacteria	Burkholderiales	Oxalobacteraceae	-	*Massilia kyonggiensis*	NR_126273.1	100%	100%
P_2827	Proteobacteria	Betaproteobacteria	-	-	-	*Duganella radicis*	LC191531.1	100%	100%
P_2552	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	-	*Novosphingobium sediminicola*	KX987160.1	100%	100%
P_1637	Gemmatimonadetes	Gemmatimonadetes	Gemmatimonadales	Gemmatimonadaceae	*Gemmatimonas*	*Gemmatimonas aurantiaca*	KF228166.1	100%	93%
P_1544	Gemmatimonadetes	Gemmatimonadetes	Gemmatimonadales	Gemmatimonadaceae	*Gemmatimonas*	*Gemmatimonas* sp.	LN876485.1	100%	89%
P_0962	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Sphingobacteriaceae	*Mucilaginibacter*	*Mucilaginibacter gotjawali*	AP017313.1	100%	99%
P_0892	Bacteroidetes	Chitinophagia	Chitinophagales	Chitinophagaceae	-	*Ferruginibacter profundus*	NR_148259.1	100%	88%
P_1095	Bacteroidetes	-	-	-	-	*Flavisolibacter ginsengisoli*	NR_041500.1	100%	95%
P_1051	Bacteroidetes	-	-	-	-	*Flavobacterium lindanitolerans*	KP875419.1	100%	100%
P_1008	Bacteroidetes	-	-	-	-	*Solitalea canadensis*	CP003349.1	100%	88%
P_0652	Actinobacteria	Thermoleophilia	Solirubrobacterales	Solirubrobacteraceae	*Solirubrobacter*	*Solirubrobacter phytolaccae*	NR_133858.1	99%	92%
P_5169	-	-	-	-	-	*Desulfotomaculum nigrificans*	NR_074579.1	97%	85%
P_5087	-	-	-	-	-	*Stenotrophobacter roseus*	NR_146022.1	99%	97%
P_4649	-	-	-	-	-	*Alkalilimnicola ehrlichii*	NR_074775.1	99%	81%
P_4607	-	-	-	-	-	*Verrucomicrobia*	JF488114.1	100%	92%
P_4606	-	-	-	-	-	*Ruminococcus flavefaciens*	KX155563.1	99%	83%

P_4595	-	-	-	-	-	*Moorella thermoacetica*	NR_043076.1	97%	84%
P_3783	-	-	-	-	-	*Fimbriimonas ginsengisoli*	CP007139.1	100%	88%
P_3739	-	-	-	-	-	*Solibacter usitatus*	GQ287461.1	100%	88%
F_0866	Mucoromycota	Glomeromycetes	-	-	-	*Acaulospora delicata*	JF439203.1	45%	95%
F_0620	Basidiomycota	Agaricomycetes	Polyporales	-	*Burgoa*	*Burgoa anomala*	AB972783.1	100%	100%
F_0785	Basidiomycota	-	-	-	-	*Radulomyces copelandii*	MG722738.1	87%	99%
F_0257	Ascomycota	Sordariomycetes	Hypocreales	Bionectriaceae	*Clonostachys*	*Clonostachys rosea*	KY320599.1	100%	100%
F_0237	Ascomycota	Sordariomycetes	Glomerellales	Plectosphaerellaceae	-	*Plectosphaerella plurivora*	KU204617.1	98%	99%
F_0413	Ascomycota	Sordariomycetes	-	-	-	*Xylariales* sp.	KY031690.1	100%	100%
F_0163	Ascomycota	Orbiliomycetes	Orbiliales	Orbiliaceae	*Dactylellina*	*Dactylellina* aff. *ellipsospora*	KT215204.1	100%	99%
F_0131	Ascomycota	Eurotiomycetes	Eurotiales	-	-	*Talaromyces verruculosus*	KC937053.1	100%	98%
F_0003	Ascomycota	Dothideomycetes	Capnodiales	Cladosporiaceae	*Cladosporium*	*Cladosporium cladosporioides*	MG946764.1	100%	100%
F_0482	Ascomycota	-	-	-	-	*Alternaria alternata*	KY367499.2	100%	100%
F_0973	-	-	-	-	-	*Peniophora incarnata*	EU918698.1	100%	98%

Table 4. Prokaryote/fungal OTUs linked to nematophagous fungi in the microbe–microbe networks. For each of the microbe–microbe co-abundance networks (~~Fig.~~figure 4A, C), the prokaryote/fungal OTUs that showed positive co-abundance patterns with *Clonostachys* (F_0257) and *Dactylellina* (F_0163) nematophagous fungal OTUs are listed. The taxonomic assignment results based on the QCauto–LCA pipeline are shown with the top-hit results of NCBI BLAST searches. The OTU code starting with P (P_xxxx) and F (F_xxxx) are prokaryotes and fungi, respectively.

OTU	Phylum	Class	Order	Family	Genus	NCBI top hit	Accession	Cover	Identity
Root: OTUs linked to *Clonostachys rosea* (F_0257)									
P_0510	Actinobacteria	Actinobacteria	Streptomycetales	Streptomycetaceae	-	*Streptomyces nigrogriseolus*	MG984076.1	100%	98%
Soil: OTUs linked to *Clonostachys rosea* (F_0257)									
P_2689	Proteobacteria	Betaproteobacteria	Burkholderiales	Burkholderiaceae	*Ralstonia*	*Ralstonia pickettii*	MF179868.1	100%	100%
P_2243	Proteobacteria	Alphaproteobacteria	Rhizobiales	-	-	*Pedomicrobium americanum*	NR_104908.1	100%	90%
F_0163	Ascomycota	Orbiliomycetes	Orbiliales	Orbiliaceae	*Dactylellina*	*Dactylellina* aff. *ellipsospora*	KT215204.1	100%	99%
F_0278	Ascomycota	Sordariomycetes	Hypocreales	Nectriaceae	*Calonectria*	*Calonectria zuluensis*	NR_137728.1	97%	100%
F_0310	Ascomycota	Sordariomycetes	Hypocreales	Ophiocordycipitaceae	-	*Purpureocillium lilacinum*	KP691502.1	100%	100%
Soil: OTUs linked to *Dactylellina sp.* (F_0163)									
P_2443	Proteobacteria	Alphaproteobacteria	Rhodospirillales	-	-	*Azospirillum brasilense*	KY010284.1	100%	92%
P_2589	Proteobacteria	Alphaproteobacteria	-	-	-	*Elstera litoralis*	KR856497.1	100%	92%
P_3774	-	-	-	-	-	*Nitrospira japonica*	LT828648.1	100%	100%
F_0812	Chytridiomycota	Chytridiomycetes	Rhizophydiales	Rhizophydiaceae	*Rhizophydium*	*Rhizophydium* sp.	AY349124.1	99%	100%

F_0278	Ascomycota	Sordariomycetes	Hypocreales	Nectriaceae	*Calonectria*	*Calonectria zuluensis*	NR_137728.1	97%	100%
F_0265	Ascomycota	Sordariomycetes	Hypocreales	Clavicipitaceae	*Pochonia*	*Pochonia chlamydosporia*	KY977543.1	100%	100%
F_0257	Ascomycota	Sordariomycetes	Hypocreales	Bionectriaceae	*Clonostachys*	*Clonostachys rosea*	KY320599.1	100%	100%
F_0310	Ascomycota	Sordariomycetes	Hypocreales	Ophiocordycipitaceae	-	*Purpureocillium lilacinum*	KP691502.1	100%	100%

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Figure legends

Figure 1. Study site and soybeans. (*a*) Soybean field in which sampling was conducted. (*b*) Soybean states. Soybean individuals were classified into three categories: those heavily attacked by root-knot nematodes ("no leaf"; left), those exhibited normal growth ("green"; right), and those showing intermediate characters ("yellow"; middle). (*c*) Relationship between soybean states and biomass. Dry mass significantly differed among "no leaf", "yellow", and "green" soybean individuals (ANOVA; $F_2 = 20.5$, $P < 00001$). (*d*) Spatial distribution of "no leaf", "yellow", and "green" soybean individuals. Sampling sets 1 and 2 are shown separately.

Figure 2. Prokaryote and fungal community structure. (*a*) Phylum-level compositions of prokaryotes in the root and soil datasets. Mean proportions of sequencing reads are shown for ~~each~~ respective taxa. The numbers of the samples from which sequencing data were successfully obtained are shown in the parentheses. (*b*) Order-level compositions of fungi in the root and soil datasets.

Figure 3. Diversity of microbiome structures among samples. (*a*) NMDS of the root prokaryote dataset. The results of the PERMANOVA, in which sampling set ("set 1" or "set 2") and plant state ("green", "yellow", or "no leaf") were included as explanatory variables, are shown. (*b*) NMDS of the root fungal dataset. (*c*) NMDS of the soil prokaryote dataset. (*d*) NMDS of the soil fungal dataset.

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Figure 4. Microbe–microbe co-abundance networks. (*a*) Positive co-abundance network of the root microbiome data. A pairs of OTUs linked by a blue line frequently co-occurred in the same soybean samples. (*b*) Negative co-abundance network of the root microbiome data. A

769 pairs of OTUs linked by a red line rarely co-occurred in the same soybean samples. (*c*)
770 Positive co-abundance network of the soil microbiome data. (*d*) Negative co-abundance
771 network of the soil microbiome data.

772

773 **Figure 5**. Spatial distribution of nematophagous fungal OTUs. (*a*) Sampling set 1. For each
774 soybean individual, the proportions of sequencing reads representing nematophagous fungal
775 OTUs are shown. (*b*) Sampling set 2.

Appendix C

Dear Dr. Haznedaroglu and Prof. Padian,

We would like to re-submit our manuscript entitled “Consortia of anti-nematode fungi and bacteria in the rhizosphere of soybean plants attacked by root-knot nematodes” (RSOS-181693.R1; bioRxiv accession, http://biorxiv.org/cgi/content/short/365023v1) for possible publication in *Royal Society Open Science*.

We appreciate the reviewer for his/her constructive comments. Responses to the comments are shown below

This manuscript has never been published before and is not currently being considered for publication elsewhere. The manuscript has been deposited on the bioRxiv preprint server (doi: https://doi.org/10.1101/332403). We confirm that the manuscript has been read and approved by all authors.

We hope that we have addressed reviewer comments adequately and constructively.

Sincerely,

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Reviewer: 2

Comments to the Author(s)

The manuscript is acceptable except a minor comment. Actually Dactylellina is trapping fungus and can capture secondary-stage juveniles and Clonostachys, Pochonia and Purpureocillium can parasitize on nematode eggs. Those fungi associated with no-leaf individuals of soybean, that means high nematode densities in no-leaf individuals can stimulate the multiply of those fungi. Authors may discuss this point a little bit.

Response:

The suggested information of *Dactylellina* and other anti-nematode fungi has been included in the revised manuscript (l.353-355, 359-378).