

Microbial and volatile profiling of soils suppressive to *Fusarium culmorum* of wheat

Adam Ossowicki, Vittorio Tracanna, Marloes L. C. Petrus, Gilles van Wezel, Jos M. Raaijmakers, Marnix H. Medema and Paolina Garbeva

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Review timeline

Original submission: 26 June 2019
1st revised submission: 29 October 2019
2nd revised submission: 21 January 2020
Final acceptance: 21 January 2020

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

Review History

RSPB-2019-1469.R0 (Original submission)

Review form: Reviewer 1

Recommendation

Accept with minor revision (please list in comments)

Scientific importance: Is the manuscript an original and important contribution to its field?

Excellent

General interest: Is the paper of sufficient general interest?

Good

Quality of the paper: Is the overall quality of the paper suitable?

Excellent

Is the length of the paper justified?

Yes

Should the paper be seen by a specialist statistical reviewer?

No

Do you have any concerns about statistical analyses in this paper? If so, please specify them explicitly in your report.

No

It is a condition of publication that authors make their supporting data, code and materials available - either as supplementary material or hosted in an external repository. Please rate, if applicable, the supporting data on the following criteria.

Is it accessible?

N/A

Is it clear?

N/A

Is it adequate?

N/A

Do you have any ethical concerns with this paper?

No

Comments to the Author

This paper was well organized and written. Only one issue I am concerning about the experiment designation. In the soil transplantation experiment, authors used the sterilized suppressive soil as conducive soil. In my research, we uses the conducive soil which collected from the nearby field of the suppressive soil because the conducive soil has its own microbial community. We can compare the differences of microbial communities among suppressive soil, conducive soil and the conducive soil mixed with 10% suppressive soil. I think this experiment designation is more easy to find the specific microbes that involve in the suppressiveness. If the authors used the conducive soils that collected from the nearby corresponding fields of S01, S03, S11 and S28, It might be found some specific microbes responding to suppressiveness.

Review form: Reviewer 2

Recommendation

Major revision is needed (please make suggestions in comments)

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work done with volatiles and high-throughput amplicon sequencing. Introduce the Supplementary Material in the order found in the text (in the current version this is not done). Also, the authors need to indicate in the main text the accession number of the project where the raw amplicon sequencing data is deposited and add properly the metadata. Someone has to check it prior the acceptance of a future version of the manuscript.

Supplementary material part I (Materials and Methods)

Add a supplementary table with the number of reads per sample. This information is confuse in the current version of the manuscript.

$p < 10e-10$ - change that for the correct mathematical notation is it 10 to the power of -10 or e to the power of -10.

(major) The authors need to indicate how betweenness and centrality scores were used to indicate keystone taxa. I also believe keystone taxa is a 'strong' notation usually supported by multiple analysis. The authors carried out several analyses and only this was able to indicate relevant species towards suppressiveness. That should be made clear and better explained in the text. I am not a specialist in GC-Q-TOF or MS analysis, a specialist in those should judge this part.

Results

Line 156: 'Table S1' seems to be a print screen of a table. I suggest the authors to write the table in a word document or to submit the xls file.

Line 159: 'figure' or Figure? Check in the guidelines.

Line 162: 'S01, S03, S11 and S28' the colors are not color-blind friendly and not very easy to distinct. I suggest the authors to change that.

Line 174: 'figure S2', no figure S1. renumber the supplementary figures and the corresponding text.

Line 186: You have two different controls in figure 2 (A and B). I suggest the authors to improve the description of figure 2 to indicate what exactly are the controls for A and B.

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Line 196: No figure 3 after figure 2. Renumber and reorder the figures.

Lines 207-208: 'see Supplementary material part I' the supplementary material should be better described in the materials and methods session. A brief indication of the overall approach and experimental design and where the materials and methods should be found should be included there.

Line 212: 'unweighted Unifrac' this is one of the standards outputs of QIIME 2. Why did the authors chose unweighted and not weighted? Also, no statistics was done. The authors should perform anova with permutation analysis (PERMANOVA) there are several statistical packages that can do that analysis.

Supplementary Figure 5 has a better color coding than figure 3B. I suggest the authors to use it also in figure 3B.

Lines 220-221: 'Again...diversity'. Diversity can be divided in evenness and richness. Although this is a vague statement. The authors need to improve the flow of the sentences before.

Line 227: 'random forest classifier' this analysis was not described in the methods. To understand if the analysis was performed properly it is necessary the authors describe them. They could also add this data as supplementary material. How did they analyze this data?

Line 229: 'OTU-level clustering'. First, the analysis were made with ASVs not OTUs. Also, DADA2 checks potential sequencing errors and exclude the necessity of clustering ASVs. It is important the same analysis are made here without clustering and with weighed unifrac.

Line 229-233: (major) If the authors accept the my suggestions to add a hypothesis, they can change the descriptive character of the current version of the manuscript for a hypothesis driven one. The analysis might be biased due to the clustering at 97% and the use of unweighed unifrac. Second, even if this is not the case. This part of the text is discussion, not results. There is also a third option: 'iii) we were not able to resolve bacterial groups involved with suppressiveness due

to the fact that the length of the amplicons only allow phylogenetic analysis to genus level'. Species variation within the same level could allow the answer of such question.

Line 241: (major) 'highlighted' no where in the manuscript this was described (see comment on supplementary materials and methods).

Lines 251-253: (major) Acidobacteria as a group. Acidobacteria is as phylogenetically (and functionally) diverse as Proteobacteria. Fig 5 only indicates a few Acidobacteria as relevant for suppressive soils. Which groups do these Acidobacteria belong to? I suggest a figure on the phylogenetic analysis of the acidobacteria relevant ASVs would be made to improve the description of the results.

Discussion

Line 259: change '14%' by '14% of X screened soils'.

Line 273: (major) 'microbiome activation step'. The authors added seeds that became plants and then removed before the beginning of the study. They do not show data of what would have happened if they did not do this. I believe that this is important to describe, but it should not be made as a major point of discussion to avoid future misinterpretation of the data. This is mentioned only a few times in the manuscript the authors can actually indicate what was done instead of create a slang that can be misinterpreted later.

Line 286: diversity is the sum of richness and evenness. Discuss these terms separately.

Lines 290-291: 'However,...networks'. The current study is analyzes correlation between volatiles, ASVs (they could have been done in combination, I don't know why the authors decided not to) and a classification based system of suppressive soils. There was no functional analysis and the authors do not have the data to discuss that. If they would like to do so. They need to indicate that that is not only an speculation but also which part of their data could give any support for that.

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Line 301: 'suppressive core'. According to the current version of the manuscripts, I understood the authors could only define bacterial groups correlated to suppression in a few cases. It is very important that the authors state that these are only based in correlation otherwise these type of statements pass along stronger than it should be.

Line 305: change 'fungi and protists' by 'fungi, protists and viruses'.

Line 312: Remove 'Interestingly'.

Lines 313-314: 'Volatile...Soils'. This very statement support the fact that different bacteria species (even within the same genera) might be involved with the production of volatiles and therefore might be responsible for disease suppression.

Line 323: (major) 'Acidobacteria' see previous comments.

Figure 1:

Colo codes are not color-blind friendly. Add soil samples abbreviations to the circles in A.

Remove C from the figure and transform it in a supplementary figure that would be bigger and with high resolution (specialists in the field may need these pictures to be able to reproduce or to further such study in the future).

Decision letter (RSPB-2019-1469.R0)

30-Aug-2019

Dear Dr Garbeva:

I am writing to inform you that your manuscript RSPB-2019-1469 entitled "Microbial and volatile

profiling of soils suppressive to *Fusarium culmorum* of wheat" has, in its current form, been rejected for publication in Proceedings B.

This action has been taken on the advice of referees, who have recommended that substantial revisions are necessary. With this in mind we would be happy to consider a resubmission, provided the comments of the referees are fully addressed. However please note that this is not a provisional acceptance.

The resubmission will be treated as a new manuscript. However, we will approach the same reviewers if they are available and it is deemed appropriate to do so by the Editor. Please note that resubmissions must be submitted within six months of the date of this email. In exceptional circumstances, extensions may be possible if agreed with the Editorial Office. Manuscripts submitted after this date will be automatically rejected.

Please find below the comments made by the referees, not including confidential reports to the Editor, which I hope you will find useful. If you do choose to resubmit your manuscript, please upload the following:

- 1) A 'response to referees' document including details of how you have responded to the comments, and the adjustments you have made.
- 2) A clean copy of the manuscript and one with 'tracked changes' indicating your 'response to referees' comments document.
- 3) Line numbers in your main document.

To upload a resubmitted manuscript, log into <http://mc.manuscriptcentral.com/prsb> and enter your Author Centre, where you will find your manuscript title listed under "Manuscripts with Decisions." Under "Actions," click on "Create a Resubmission." Please be sure to indicate in your cover letter that it is a resubmission, and supply the previous reference number.

Sincerely,
 Professor Hans Heesterbeek
 mailto: proceedingsb@royalsociety.org

Associate Editor
 Board Member: 1
 Comments to Author:

This is an interesting and comprehensive study focussing on the role of soils in the suppression of disease by soil-borne plant pathogens. A key strength is the rigour of the experimental design, and inclusion of 28 soil sites, rather than testing a single soil type, which is common in previous studies. By comparing soil physiochemical and biological properties, the authors demonstrate that biological rather than physicochemical properties are responsible for the suppressiveness. Overall, the reviewers are positive about the manuscript, but they suggest some revisions to improve the narrative; I agree that the last section of the introduction should be modified to better frame the rationale, research questions and hypotheses to be tested. It would also be important to address the point raised by reviewer 2 on data accessibility.

Reviewer(s)' Comments to Author:

Referee: 1

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Author's Response to Decision Letter for (RSPB-2019-1469.R0)

See Appendix A.

RSPB-2019-2527.R0

Review form: Reviewer 2 (Ulissess Rocha)

Recommendation

Accept with minor revision (please list in comments)

Scientific importance: Is the manuscript an original and important contribution to its field?
Excellent

General interest: Is the paper of sufficient general interest?
Good

Quality of the paper: Is the overall quality of the paper suitable?
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Yes

Should the paper be seen by a specialist statistical reviewer?
No

Do you have any concerns about statistical analyses in this paper? If so, please specify them explicitly in your report.
No

It is a condition of publication that authors make their supporting data, code and materials available - either as supplementary material or hosted in an external repository. Please rate, if applicable, the supporting data on the following criteria.

Is it accessible?
No

Is it clear?
No

Is it adequate?
No

Do you have any ethical concerns with this paper?
No

Comments to the Author

The manuscript entitled 'Microbial and volatile profiling of soils suppressive to *Fusarium culmorum* of wheat' by Ossowicki and collaborators describes a hypothesis driven study with multiple soils sites tackling volatiles as a potential mechanism for disease suppression. The fact that the manuscript is draw around strong hypothesis, high scientific standards and great experimental design made the review of the manuscript a delightful experience. Potentially, the key strength of the manuscript is the fact that the authors studies multiple soils sites (28) what is not common in studies involving disease suppression. The authors made use of their hypothesis to construct a strong experimental design that allowed an in depth discussion of the potential mechanisms involved with disease suppression. More important in their discussion, the authors raised several hypothesis that now can be tested by the larger community studying the broad topic of disease suppression. I believe the current version of the manuscript is of high scientific standards. Nevertheless, I have minor comments (in particular the one involving the accession number and metadata of the raw sequence data) that need to be addressed prior the manuscript can be published.

Abstract:

The abstract is well written and sufficiently describes the depth of the work.

Introduction:

Lines 51-63: 'For many... *Paraburkholderia graminis* [10, 17, 18].' The authors describe several mechanisms of disease suppression and the organisms involved with it. In the current manuscript. The focus of the work is on amplicon sequencing and analysis of volatiles. I would focus on it and reduce (or remove) this part of the introduction. As it is Lines 51-63 read more like a review than the introduction of a research article.

Line 66: 'This work', I would suggest the authors to indicate in a more direct manner that they are referring to reference 23. In the current version of the manuscript, 'This work' may confuse the reader.

Line 78-85: The last paragraph indicates the work is hypothesis driven and help the reader with what to expect from the manuscript.

Materials and Methods:

This session is very well described. I have one important point. The authors need to make the raw sequence data available prior to publication. It is important that the reviewers are able to check the metadata to determine if the link between the publication and the raw data have sufficient quality. I am yet to check the supplementary material but the publication cannot be released prior the raw data is publicly released and the metadata is available to peer review (e.g., ENA or NCBI). That information should be available in the main text of the publication.

Results:

All the points that I made regarding the old version of the manuscript were addressed. I have no suggestions for the current version of the manuscript.

Discussion:

The strength of the manuscript lies in the number of soils analyzed in this study. That allows the authors to tackle important questions regarding soils suppressiveness. For example the fact that both soils with high and low diversity exhibit suppressive phenotypes. This also allow the authors to hypothesize that the different mechanisms may be involved with disease suppression in different soils even if attacked by the same pathogen. This is ground breaking for the field and indeed opens many new doors for research in this field.

Line 313: The paragraph preceding this line is very well written and address the points why machine learning was not able to define bioindicators for disease suppression. I would add a sentence or two on the fact that the same technique could be used to test if different ecosystem processes or if functional redundancy in different taxa are responsible for this phenomena when using metagenomes or multi-omics data as input for the analysis.

Figures:

All figures and legends were in accordance with good scientific standards in the field.

Supplementary Material:

Besides the small comments made here, all my previous suggestions were addressed in the supplementary material.

Decision letter (RSPB-2019-2527.R0)

10-Jan-2020

Dear Dr Garbeva

I am pleased to inform you that your manuscript RSPB-2019-2527 entitled "Microbial and volatile profiling of soils suppressive to *Fusarium culmorum* of wheat" has been accepted for publication in Proceedings B.

The referee and the Associate Editor have recommended publication, but also request some minor revisions to your manuscript. Therefore, I invite you to respond to the comments and revise your manuscript. Because the schedule for publication is very tight, it is a condition of publication that you submit the revised version of your manuscript within 7 days. If you do not think you will be able to meet this date please let us know.

To revise your manuscript, log into <https://mc.manuscriptcentral.com/prsb> 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. 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 referee(s) and upload a file "Response to Referees". You can use this to document any changes you make to the original manuscript. We require a copy of the manuscript with revisions made since the previous version marked as 'tracked changes' to be included in the 'response to referees' document.

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

- 1) A text file of the manuscript (doc, txt, rtf or tex), including the references, tables (including captions) and figure captions. Please remove any tracked changes from the text before submission. PDF files are not an accepted format for the "Main Document".
- 2) A separate electronic file of each figure (tiff, EPS or print-quality PDF preferred). The format should be produced directly from original creation package, or original software format. PowerPoint files are not accepted.
- 3) Electronic supplementary material: this should be contained in a separate file and where possible, all ESM should be combined into a single file. All supplementary materials accompanying an accepted article will be treated as in their final form. They will be published alongside the paper on the journal website and posted on the online figshare repository. 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.

Online supplementary material will also carry the title and description provided during submission, so please ensure these are accurate and informative. Note that the Royal Society will not edit or typeset supplementary material and it will be hosted as provided. Please ensure that the supplementary material includes the paper details (authors, title, journal name, article DOI). Your article DOI will be 10.1098/rspb.[paper ID in form xxxx.xxxx e.g. 10.1098/rspb.2016.0049].

- 4) A media summary: a short non-technical summary (up to 100 words) of the key findings/importance of your manuscript.

- 5) Data accessibility section and data citation

It is a condition of publication that data supporting your paper are made available either in the electronic supplementary material or through an appropriate repository.

In order to ensure effective and robust dissemination and appropriate credit to authors the dataset(s) used should be fully cited. To ensure archived data are available to readers, authors should include a 'data accessibility' section immediately after the acknowledgements section. This should list the database and accession number for all data from the article that has been made publicly available, for instance:

- DNA sequences: Genbank accessions F234391-F234402

- Phylogenetic data: TreeBASE accession number S9123
- Final DNA sequence assembly uploaded as online supplemental material
- Climate data and MaxEnt input files: Dryad doi:10.5521/dryad.12311

NB. From April 1 2013, peer reviewed articles based on research funded wholly or partly by RCUK must include, if applicable, a statement on how the underlying research materials – such as data, samples or models – can be accessed. This statement should be included in the data accessibility section.

If you wish to submit your data to Dryad (<http://datadryad.org/>) and have not already done so you can submit your data via this link

[http://datadryad.org/submit?journalID=RSPB&manu=\(Document not available\)](http://datadryad.org/submit?journalID=RSPB&manu=(Document+not+available)) which will take you to your unique entry in the Dryad repository. If you have already submitted your data to dryad you can make any necessary revisions to your dataset by following the above link. Please see <https://royalsociety.org/journals/ethics-policies/data-sharing-mining/> for more details.

6) For more information on our Licence to Publish, Open Access, Cover images and Media summaries, please visit <https://royalsociety.org/journals/authors/author-guidelines/>.

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

Sincerely,
Professor Hans Heesterbeek
mailto:proceedingsb@royalsociety.org

Associate Editor
Board Member
Comments to Author:

The revised manuscript is much improved and has comprehensively addressed the reviewers initial comments. The reviewer has recommended several further minor modifications to the manuscript, and I would also recommend two further minor modifications.

Line 259 – change ‘alfa’ to ‘alpha’

Lines 210-220 – the pathogen is not in direct contact with suppressive microbiota in either of the approaches tested here, and it is therefore possible that the lack of effect of volatiles observed could be due to a lack of direct interaction between the pathogen and microbiota, to induce a response. It may be worth adding a sentence to the discussion to explain this.

Overall, this is a very nice study and I believe that it will be of great interest to the broad readership of Proceedings B.

Reviewer(s)' Comments to Author:

Referee: 2

Comments to the Author(s).

The manuscript entitled ‘Microbial and volatile profiling of soils suppressive to *Fusarium culmorum* of wheat’ by Ossowicki and collaborators describes a hypothesis driven study with multiple soils sites tackling volatiles as a potential mechanism for disease suppression. The fact that the manuscript is draw around strong hypothesis, high scientific standards and great experimental design made the review of the manuscript a delightful experience. Potentially, the key strength of the manuscript is the fact that the authors studies multiple soils sites (28) what is not common in studies involving disease suppression. The authors made use of their hypothesis to construct a strong experimental design that allowed an in depth discussion of the potential

mechanisms involved with disease suppression. More important in their discussion, the authors raised several hypothesis that now can be tested by the larger community studying the broad topic of disease suppression. I believe the current version of the manuscript is of high scientific standards. Nevertheless, I have minor comments (in particular the one involving the accession number and metadata of the raw sequence data) that need to be addressed prior the manuscript can be published.

Abstract:

The abstract is well written and sufficiently describes the depth of the work.

Introduction:

Lines 51-63: 'For many... Paraburkholderia graminis [10, 17, 18].' The authors describe several mechanisms of disease suppression and the organisms involved with it. In the current manuscript. The focus of the work is on amplicon sequencing and analysis of volatiles. I would focus on it and reduce (or remove) this part of the introduction. As it is Lines 51-63 read more like a review than the introduction of a research article.

Line 66: 'This work', I would suggest the authors to indicate in a more direct manner that they are referring to reference 23. In the current version of the manuscript, 'This work' may confuse the reader.

Line 78-85: The last paragraph indicates the work is hypothesis driven and help the reader with what to expect from the manuscript.

Materials and Methods:

This session is very well described. I have one important point. The authors need to make the raw sequence data available prior to publication. It is important that the reviewers are able to check the metadata to determine if the link between the publication and the raw data have sufficient quality. I am yet to check the supplementary material but the publication cannot be released prior the raw data is publicly released and the metadata is available to peer review (e.g., ENA or NCBI). That information should be available in the main text of the publication.

Results:

All the points that I made regarding the old version of the manuscript were addressed. I have no suggestions for the current version of the manuscript.

Discussion:

The strength of the manuscript lies in the number of soils analyzed in this study. That allows the authors to tackle important questions regarding soils suppressiveness. For example the fact that both soils with high and low diversity exhibit suppressive phenotypes. This also allow the authors to hypothesize that the different mechanisms may be involved with disease suppression in different soils even if attacked by the same pathogen. This is ground breaking for the field and indeed opens many new doors for research in this field.

Line 313: The paragraph preceding this line is very well written and address the points why machine learning was not able to define bioindicators for disease suppression. I would add a sentence or two on the fact that the same technique could be used to test if different ecosystem processes or if functional redundancy in different taxa are responsible for this phenomena when using metagenomes or multi-omics data as input for the analysis.

Figures:

All figures and legends were in accordance with good scientific standards in the field.

Supplementary Material:

Besides the small comments made here, all my previous suggestions were addressed in the supplementary material.

Author's Response to Decision Letter for (RSPB-2019-2527.R0)

See Appendix B.

Decision letter (RSPB-2019-2527.R1)

21-Jan-2020

Dear Dr Garbeva

I am pleased to inform you that your manuscript entitled "Microbial and volatile profiling of soils suppressive to *Fusarium culmorum* of wheat" has been accepted for publication in Proceedings B.

You can expect to receive a proof of your article from our Production office in due course, please check your spam filter if you do not receive it. PLEASE NOTE: you will be given the exact page length of your paper which may be different from the estimation from Editorial and you may be asked to reduce your paper if it goes over the 10 page limit.

If you are likely to be away from e-mail contact please let us know. Due to rapid publication and an extremely tight schedule, if comments are not received, we may publish the paper as it stands.

If you have any queries regarding the production of your final article or the publication date please contact procb_proofs@royalsociety.org

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Paper charges

An e-mail request for payment of any related charges will be sent out after proof stage (within approximately 2-6 weeks). The preferred payment method is by credit card; however, other payment options are available

Electronic supplementary material:

All supplementary materials accompanying an accepted article will be treated as in their final form. They will be published alongside the paper on the journal website and posted on the online figshare repository. 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.

Thank you for your fine contribution. On behalf of the Editors of the Proceedings B, we look forward to your continued contributions to the Journal.

Sincerely,

Professor Hans Heesterbeek

Editor, Proceedings B

<mailto:proceedingsb@royalsociety.org>

Associate Editor:
Board Member
Comments to Author:
(There are no comments.)

Appendix A

Dear Editors,

We would like to submit our revised manuscript 'Microbial and volatile profiling of soils suppressive to *Fusarium culmorum* of wheat' for consideration for publication in the special issue of Proceedings of the Royal Society B entitled 'Application of ecological and evolutionary theory to microbiome community dynamics across systems'.

We thank you and the reviewers for the positive comments and constructive suggestions. These comments and suggestions are addressed in the revised manuscript. A point-by-point rebuttal is given below.

We hope that we have addressed the suggestions satisfactorily and look forward to your final decision.

Sincerely, on behalf of all authors,

Paolina Garbeva and Marnix Medema

Point by point reply

Comments to the Author

This paper was well organized and written. Only one issue I am concerning about the experiment designation. In the soil transplantation experiment, authors used the sterilized suppressive soil as conducive soil. In my research, we uses the conducive soil which collected from the nearby field of the suppressive soil because the conducive soil has its own microbial community. We can compare the differences of microbial communities among suppressive soil, conducive soil and the conducive soil mixed with 10% suppressive soil. I think this experiment designation is more easy to find the specific microbes that involve in the suppressiveness. If the authors used the conducive soils that collected from the nearby corresponding fields of S01, S03, S11 and S28, It might be found some specific microbes responding to suppressiveness.

Reply:

Good suggestion. The primary goal of our study was to screen a wide range of soils for disease suppressiveness and determine if suppressiveness of selected soils was (micro)biological in nature . To that end, we adopted γ -sterilization and transplantation. Indeed, some of our sampling sites were close to each other, for example S10 and S17, and could have been used for the transplant experiments. However, for this large-scale survey we first wanted to know if the transplanted microbial community was able to confer the same suppressive phenotype in the same physical-chemical background from which they originated. Also, we were interested to see whether soils from diverse geographic sites that are suppressive against the same pathogen have a common taxonomic denominator. Whether the transplanted community can also provide this phenotype in physicochemically different soils against a background of other indigenous microorganisms will be addressed in future experiments. Whether the latter approach facilitates or complicates finding the specific microbes associated with suppressiveness will also be addressed in these future studies.

Referee: 2

The manuscript 'Microbial and volatile profiling of soils suppressive to *Fusarium culmorum* of wheat' by Ossowicki and collaborators describe screening of 28 sites for suppressive soils against *F. culmorum*. I follow studies involving suppressive soils for more than 13 years and very few studies have been published in such scale. The authors also indicate that the suppressive character found was biological. Further, they attempt to indicate if the suppressive could be correlated to the presence of specific volatile compounds or with specific groups of Bacteria. The experimental design and statistical analyses are sufficient to explore the potential questions and hypothesis that could have been asked within the scope of the study. I particularly believe the manuscript has good merit and it would be attractive for the audience of the Proc. R. Soc. B.

Reply:

We thank the reviewer for this positive appraisal.

Nevertheless, I believe that the text needs major revision for the following reasons: i) the authors can make it more direct and clear in the introduction what is their hypothesis and research questions associated with it; (ii) the Materials and Methods needs to be reorganized to make it more clear to the reader what was done; (iii) accession number of the high-throughput sequencing data is lacking (or very difficult to find, I did not); and, (iv) I believe that their study points to a denial of a big hypothesis made in soil suppressive studies and that should be better interpreted and discussed.

Reply: *Thank you so much for the constructive comments and suggestions. Our hypothesis has now been clearly added at the end of the introduction (see Ln 91-94). The Materials and Methods section is reorganized and more elaborate, although part has been moved to the supplementary information due to word limits. The accession number of the high-throughput sequencing data is now provided and the discussion section is revised with a more extensive and balanced interpretation of the obtained results. For more specifics, please see our answers below.*

Abstract:

Lines 21-22: change 'the microbiota' by 'this microbiota' to indicate link to between this and the previous statement. Line 29: change 'were' by was

Reply: *changed as suggested.*

Line 31: (major) 'Acidobacteria'. Acidobacteria is a group as diverse as Proteobacteria. Only a few ASVs belonging to Acidobacteria were found exclusively in suppressive soils. This has many implications. But here the authors need to indicate which groups of Acidobacteria they are referring to.

Reply: *The extended list of Acidobacteria has been added at line 427.*

Line 35: change 'of disease suppressive microbiomes' by 'how microbiomes enable suppression of diseases'. This change will help to improve the clarity of the text.

Reply: *Changes were made as suggested.*

Lines 39-42: 'The phenomenon...development.' is a very long sentence. I suggest to divide it in 2-3 to make the text more clear.

Reply: *The sentence has been divided in two, as suggested.*

Line 40: delete 'in 1983' as the reference can indicate that and it would be confusing to compare this date with the century suppressive soils are known.

Reply: *Changes have been made as suggested.*

Line 41: 'only little' is a dangling noun. A few more words need to be added here so the sentence make sense.

Reply: *The sentence has been revised.*

Lines 47-48: (major) 'specific microbial genera'. I believe the authors can create their hypothesis from here. The belief that whole genera of microbes could be sole responsible for a phenomenon as complex as soil suppressiveness is at least weak. Correlations as such may have raised from studies where a limited number of samples was used or when specific strains of a specific genus was added to soil. In microbial ecology is well know that genera may contain species with a wide range of physiological traits and phenotypes. Therefore, the authors can indicate that 'based on previous studies in the field, we hypothesize that specific genera and volatiles will correlate with disease suppression in soils against *F. culmorum*'. This is a good hypothesis that is denied by their data and it will lead to a rich discussion.

Reply: *Changes were made as suggested; the hypothesis is included and the discussion is extended*

Lines 69-72: 'Most... suppressive soils.' is a very long sentence and it takes effort to be understood. I suggest the authors to divide it in 2-3 sentences.

Reply: *The sentence has been revised.*

Lines 72-73: 'In addition... host plant'. Just because something is little known is not a direct indication that should be studied. The authors indicate earlier that volatiles can inhibit fungal growth. I suggest the authors to use this rational to indicate why they are studying it, not only because it is little known.

Reply: *The sentence has been revised. Considering the studies cited [19-23], volatile compounds may play a role in soil disease suppression (hypothesis 3). In this revised sentence, we indicate the knowledge gap that leads us to the hypothesis.*

Line 74-79: (major) In this paragraph, the authors indicate the subject of study. This is a very well designed experiment but in the current version of the manuscript they indicate that this will be a plain descriptive study. Together with the hypothesis I stated earlier, this paragraph can be transformed in research questions that would be used to test the hypothesis. I believe that if the authors adapt the manuscript for this, it would be much richer and more interesting for a broader audience in the Proc. R. Soc. B.

Reply: *The paragraph has been revised and the hypothesis included.*

Materials and Methods

The materials and methods in general is well written. But it needs to be reorganized. When I finished reading the materials and methods I thought the authors forgot to add the part of the manuscript

regarding the volatiles and the high-throughput amplicon sequencing. Also, I could not find where the authors deposited their sequencing data. I need to look into the metadata to see if it was deposited correctly. If the authors have yet to submit the data, I suggest to use the ENA.

Reply: *The material and methods section has been reorganized and partially rewritten to improve clarity, see Supplementary material. In the process, short sections regarding the volatiles and amplicon sequencing have been added, which refer to the corresponding supplementary material sections for more details. Sequencing data has been submitted to ENA [accessions ERX3618593- ERX3618704].*

Line 113: (major) 'activated'. I tried to understand why the authors would like to indicate the addition of plants to the soil for a week prior to the study as activation. This word can be misinterpreted and lead to many mistakes in the future. This word is not used a lot in the text and I would suggest the authors to describe in 4-5 words what they did instead of saying that the soil was 'activated'.

Reply: *The term activation is described in the revised manuscript in lines 122-124 and later used in line and in 138 line 320. At these points, a brief explanation has been added.*

Line 128: 'dune soil', was this soil sterilized as the other 2?

Reply: *In our experiments, we used only one method of soil sterilization that is described in lines 109-112. For clarification, we have now made changes in line 129.*

(major) As said earlier, I suggest the authors to add a few lines here that would briefly describe the work done with volatiles and high-throughput amplicon sequencing. Introduce the Supplementary Material in the order found in the text (in the current version this is not done). Also, the authors need to indicate in the main text the accession number of the project where the raw amplicon sequencing data is deposited and add properly the metadata. Someone has to check it prior the acceptance of a future version of the manuscript.

Reply: *The part material and methods has been revised accordingly.*

Supplementary material part I (Materials and Methods)

Add a supplementary table with the number of reads per sample. This information is confuse in the current version of the manuscript. $p < 10e-10$ - change that for the correct mathematical notation is it 10 to the power of -10 or e to the power of -10.

Reply: *A supplementary table with reads per sample has been added as Supplementary table S2.*

(major) The authors need to indicate how betweenness and centrality scores were used to indicate keystone taxa. I also believe keystone taxa is a 'strong' notation usually supported by multiple analysis. The authors carried out several analyses and only this was able to indicate relevant species towards suppressiveness. That should be made clear and better explained in the text.

Reply: *We agree that the keystone notation used in the article is usually supported by multiple analyses including time series data, which is not available for this study. Accordingly, we do not reference to this bacterial group as 'keystone' anymore in the revised text. Still, we provide multiple proofs that associate this taxonomic group to the plant phenotype which warrant our remaining statements in the text: ASVs enriched in suppressive samples, a strong Spearman*

correlation and exclusivity to suppressive samples for a subgroup within this network component.

Results

Line 156: 'Table S1' seems to be a print screen of a table. I suggest the authors to write the table in a word document or to submit the xls file.

Reply: *This has been corrected.*

Line 159: 'figure' or Figure? Check in the guidelines.

Reply: *We checked, and it was correct as written.*

Line 162: 'S01, S03, S11 and S28' the colors are not color-blind friendly and not very easy to distinct. I suggest the authors to change that.

Reply: *We modified the color scheme to be more color-blind friendly.*

Line 174: 'figure S2', no figure S1. renumber the supplementary figures and the corresponding text.

Reply: *This has been corrected.*

Line 186: You have two different controls in figure 2 (A and B). I suggest the authors to improve the description of figure 2 to indicate what exactly are the controls for A and B.

Reply: *This has been corrected as follows: "Disease symptoms observed in wheat inoculated with Fusarium culmorum grown in eight prioritized soils. (A) Natural and gamma-sterilized soil with sterile BS soil/vermiculite mix as a control (B) 10% and 30% in volume of natural soil mixed with standardized sterile substrate or with sterile BS soil as a control." (lines 696-700, Figure 2 legend)*

Line 191: Only here that I realized the MM for volatiles was not written in the main text. It took me a while to find it. The readers of the manuscript should not have to go through this problem.

Reply: *A basic description of the volatile analysis methods has now been included in the main text, with a clear reference to more details that can be found in the supplement.*

Line 196: No figure 3 after figure 2. Renumber and reorder the figures.

Reply: *This has been corrected.*

Lines 207-208: 'see Supplementary material part I' the supplementary material should be better described in the materials and methods session. A brief indication of the overall approach and experimental design and where the materials and methods should be found should be included there.

Reply: *We appreciate the suggestion. The material and methods section (and Supplementary material part I) has been revised accordingly.*

Line 212: 'unweighted Unifrac' this is one of the standard outputs of QIIME 2. Why did the authors choose unweighted and not weighted? Also, no statistics were done. The authors should perform ANOVA with permutation analysis (PERMANOVA) there are several statistical packages that can do that analysis.

Reply: *We performed both weighted and unweighted unifrac and they revealed similar results. We also consciously refrained from performing a PERMANOVA test, as this test is not reliable in unbalanced designs. Notably, the samples being unbalanced does not represent an experimental flaw but is a consequence of the "rare" nature of the suppressive phenotype. The visual results from our PCA analysis and the inability of the random forest classifier to differentiate suppressive from non-suppressive soils constitute a strong indication that there is no simple taxonomic explanation for the difference in phenotype.*

Lines 220-221: 'Again...diversity'. Diversity can be divided into evenness and richness. Although this is a vague statement. The authors need to improve the flow of the sentences before.

Reply: *We revised the manuscript and now address both richness and evenness separately.*

Line 227: 'random forest classifier' this analysis was not described in the methods. To understand if the analysis was performed properly it is necessary the authors describe them. They could also add this data as supplementary material. How did they analyze this data?

Reply: *The description of the method has now been included in Supplementary material part I.*

Line 229: 'OTU-level clustering'. First, the analysis were made with ASVs not OTUs. Also, DADA2 checks potential sequencing errors and excludes the necessity of clustering ASVs. It is important the same analysis are made here without clustering and with weighted unifrac.

Reply: *We now clarify this in the text. All analysis were performed with ASVs.*

Line 229-233: (major) If the authors accept my suggestions to add a hypothesis, they can change the descriptive character of the current version of the manuscript for a hypothesis driven one. The analysis might be biased due to the clustering at 97% and the use of unweighted unifrac. Second, even if this is not the case. This part of the text is discussion, not results. There is also a third option: 'iii) we were not able to resolve bacterial groups involved with suppressiveness due to the fact that the length of the amplicons only allow phylogenetic analysis to genus level'. Species variation within the same level could allow the answer of such question.

Reply: *The analysis was performed with and without OTU-level clustering and yielded the same result in both cases. Unweighted unifrac was chosen as it does a better job at grouping replicates than its weighted variant and it attenuates the PCR bias as it does not use counts. While the ASV-level analysis we performed is definitely below the genus level, it could indeed be that a shared taxonomic signal is present at the strain or subspecies level that is undetectable by 16S, and for which whole genomes of these strains would be required. We agree with the reviewer that the sentence describing the interpretation should be part of the discussion section, and have moved it accordingly.*

Line 241: (major) 'highlighted' nowhere in the manuscript this was described (see comment on supplementary materials and methods).

Reply: The word “highlighted” was changed to “indicated by squares with black border” in the text and in figure legend to clarify.

Lines 251-253: (major) Acidobacteria as a group. Acidobacteria is as phylogenetically (and functionally) diverse as Proteobacteria. Fig 5 only indicates a few Acidobacteria as relevant for suppressive soils. Which groups do these Acidobacteria belong to? I suggest a figure on the phylogenetic analysis of the acidobacteria relevant ASVs would be made to improve the description of the results.

Reply: The groups of the Acidobacteria have now been indicated specifically in the text (see above).

Discussion

Line 259: change '14%' by '14% of X screened soils'.

Reply: corrected

Line 273: (major) 'microbiome activation step'. The authors added seeds that became plants and then removed before the beginning of the study. They do not show data of what would have happened if they did not do this. I believe that this is important to describe, but it should not be made as a major point of discussion to avoid future misinterpretation of the data. This is mentioned only a few times in the manuscript the authors can actually indicate what was done instead of create a slang that can be misinterpreted later.

Reply: The term activation is now explained in the revised manuscript in lines 113-116 and later used in line 127 and in line 273

Line 286: diversity is the sum of richness and evenness. Discuss these terms separately.

Reply: We revised the manuscript and now address both richness and evenness separately. In the revised analysis, we still lack a clear indication that either of them are strongly correlated with the suppressive phenotype.

Lines 290-291: 'However,...networks'. The current study is analyzes correlation between volatiles, ASVs (they could have been done in combination, I don't know why the authors decided not to) and a classification based system of suppressive soils. There was no functional analysis and the authors do not have the data to discuss that. If they would like to do so, they need to indicate that that is not only an speculation but also which part of their data could give any support for that.

Reply: This part of the discussion was referring to previous literature by means of introduction to the rationale behind the co-occurrence network analysis; we are not drawing any conclusions here. We have clarified this textually by indicating that ‘various previous studies indicate that...’.

We also revised the following sentence as follows to avoid the appearance of functional studies having been performed: “The correlation-based network analysis performed here revealed complex inter-sample connections between individual bacterial taxa that are likely interacting either directly or indirectly, based on their observed co-occurrence”.

Line 297: (major) 'Acidobacteria'. As mentioned earlier, further analysis have to be made to define which groups of Acidobacteria were present in the data and correlated to suppressive soils.

Reply: *This has been corrected, see above.*

Line 301: 'suppressive core'. According to the current version of the manuscripts, I understood the authors could only define bacterial groups correlated to suppression in a few cases. It is very important that the authors state that these are only based in correlation otherwise these type of statements pass along stronger than it should be.

Reply: *We agree with the reviewer that it is important to avoid confusion, and make very clear in our text that this suppressive core is only 'core' to two of the four suppressive soils.*

Line 305: change 'fungi and protists' by 'fungi, protists and viruses'.

Reply: *corrected*

Line 312: Remove 'Interestingly'.

Reply: *corrected*

Lines 313-314: 'Volatile...Soils'. This very statement support the fact that different bacteria species (even within the same genera) might be involved with the production of volatiles and therefore might be responsible for disease suppression.

Reply: *We agree and have added a sentence to highlight this: “Alternatively, it might be that suppressiveness across the four soils is mediated by different mechanisms, involving volatiles in some cases but not in others; this could also explain the lack of commonly enriched taxa across the four suppressive soils.”*

Figure 1:

Color codes are not color-blind friendly. Add soil samples abbreviations to the circles in A. Remove C from the figure and transform it in a supplementary figure that would be bigger and with high resolution (specialists in the field may need these pictures to be able to reproduce or to further such study in the future).

Reply: *We modified the color scheme to be more color-blind friendly and moved panel C to supplementary material as suggested.*

Appendix B

Dear Editors,

We would like to thank you and the reviewers for the positive comments about the manuscript 'Microbial and volatile profiling of soils suppressive to *Fusarium culmorum* of wheat'.

We thank you and the reviewers for minor constructive suggestions to further improve the manuscript before the publication in the special issue of Proceedings of the Royal Society B entitled 'Application of ecological and evolutionary theory to microbiome community dynamics across systems'.

We hope that we have addressed the suggestions satisfactorily.

Sincerely, on behalf of all authors,

Paolina Garbeva and Marnix Medema

Point by point

Line 259 – change 'alfa' to 'alpha'- **corrected**

Line 66- 'This work', I would suggest the authors to indicate in a more direct manner that they are referring to reference 23. In the current version of the manuscript, 'This work' may confuse the reader- **corrected**

The authors need to make the raw sequence data available prior to publication. That information should be available in the main text of the publication- **the data is available and information is added to the main text**

As it is Lines 51-63 read more like a review than the introduction of a research article- **all authors would like to keep line 51-63 as it is. We think that this is important for our introduction**