Response to comments

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Words labeled with red color are the editor's and reviewers' comments, and labeled with blue color is the response.

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Comments from reviewer(s):

Referee #1:

General comments

The paper showed an experiment to study the fate of free amino acids in a paddy soil, considering the bacterial communities structure fluctuations during different plant growth stages. The study of free amino acids in soil to understand their impact on N dynamics in soil could be very interesting for soil scientists to have information about the microbial activities and organic input can alterate the N cycle processes and this information could help to achieve information on N dynamics in soil. Unfortunately the paper showed problems in the text, in particular many information about the methods used are absent or incomplete in Materials and methods, and the presentation of results and the discussion are poor. The authors in many cases did not support the methods proposed or their statements by references.

Thank you so much for your appreciation and the good comments and suggestions. We have revised according to the suggestion in the manuscript.

Q1:The materials and methods requires a deep revision. The application of Chinese milk vetch in soil and the fertilization are not clear and many information are absent: e.g. the soil sampling methods for 20-40 cm and 40-60cm were not described, the method for free amino acids extraction is not clearly described or any references were not reported. The free amino acids extraction and quantification is the core of the paper, therefore it's important to include the details about the method you used to quantify the FAA in soil.

A1: Thanks for such good comments. It is very helpful to improve our manuscript.

Firstly, the application of Chinese milk vetch and fertilizer in soil has been added to the paper. The content added was as follows: The fertilizers applied in the experiment were urea at 481.67 kg hm^{-2},

superphosphate at 900 kg hm^{-2} and potassium chloride at 300 kg hm^{-2}, within which 50% of urea and potassium chloride were used as base fertilizer on the 9th day after CMV overturning and 50% those fertilizers were applied at the tillering stage of rice growth on the 25th day after CMV overturning, and all off the superphosphate was applied as the base fertilizer. The variety of CMV was Minzi No.7, with 90% water content, 752.75 g $kg^{-1}$of total organic matter, 30.94 g $kg^{-1}$of total nitrogen, 5.91 g kg^{-1} of total phosphorus, 32.47 g $kg^{-1}$of total potassium, 82.35 mg kg^{-1} of acid-hydrolyzed amino acid (acid-hydrolyzed amino acid compositions shown in Fig. S1), 193.40 g kg-1 of protein. A certain amount of CMV was harvested at the flowering stage and immediately distributed evenly in the corresponding plots, then pressed into the topsoil. Please see lines 124-132 in the revised manuscript.

Secondly, the soil sampling methods for 20-40 cm and 40-60cm added in the Materials and methods were as follows: Soil samples at 0-20 cm, 20-40 cm and 40-60 cm were successively extracted with soil drill after PVC pipe isolated the surface water. (Fig.R.1). Please see lines 142-144 in the revised manuscript.

Fig.R1. Sampling diagram of bottom soil sample

Thirdly, the method for free amino acids extraction have been reported and some references have been added in lines 155, and the qualitative and quantitative methods using an automatic amino acid analyzer have been added to the paper. The content added was as follows: 1 ml filtrate was absorbed and determined by automatic amino acid analyzer (Biochrom 30+, Biochrom LTD., Cambridge, England). A mixture of amino acid at known concentration (Sigma Chemical Co., Milan, Italy) was used as the external standard (Standard spectra and concentrations are shown in Fig. S2 and Table S1), and quantitative and qualitative analysis were carried out according to the peak time and peak area after gradient elution. Please see lines 159-163 of the revised manuscript.

Q2: It's not clear if the authors performed the plasmid extraction, the protocol used is not well described and however the benefit to include this analysis is not explained.

A2: Many thanks for such good comments. Of course, **plasmids were extracted in this study and used as standards to determine the gene copy number of samples**. Plasmid extraction methods have been added to the Materials and Methods as follows: The standard gene was extracted as the standard for the preparation of the standard curve and the quantitative standard curve was established. These gene standards were generated from synthetic gene plasmid cloning vectors (Integrated DNA Technologies, Inc., Coralville, IA, United States) transformed into One ShotTM TOP10 Competent *Escherichia coli* (Life Technologies, Carlsbad, CA, United States) with the TOPO-TA cloning kit (Invitrogen, Karlsruhe, Germany) which uses the pCRTM4-TOPO® TA vector. The cloned plasmids were subjected to amplification with primers and product sizes were verified with gel electrophoresis. The PCR products were purified using the Qiagen PCR Purification Kit (Qiagen, Hilden, Germany) and quantified using a Qubit fluorometer (Thermo Fisher Scientific, Waltham, MA, United States) (Beach., 2009). Please see lines 173-181 of the revised manuscript.

Q3: In results section the authors reports some soil parameters (e.g. pH, SOM, urease, protease, bacterial biomass), but the protocols they used are not reported in materials and methods.

A3: Thanks for the good suggestion. It is very helpful to improve our manuscript. The protocols for measuring soil physical and chemical properties have been added in the paper as follows: Soil pH was determined by a pH meter (PHS-3E, INESA Scientifific Instrument Co., Ltd, Shanghai,, China) in a 1:2.5 soil/water suspension. Soil organic matter (SOM) was measured by the potassium dichromate oxidation method (Lu, 2000). Soil urease and protease activities were estimated by indophenol blue colorimetry and Folin colorimetry, respectively (Guan, 1986). Please see lines 148-152 of the revised manuscript.

Reference

Guan, S.Y., 1986. Soil enzyme and research methods for soil enzyme. Agricultural Press, Beijing. (in Chinese)

Lu R.K., 2000. Methods of soil agricultural chemical analysis. China Agricultural Science and Technology Press, Beijing. (in Chinese)

Q4: In Statistical analysis the authors could support the structural equation model analysis by appropriate references and I suggest including a description of the method to permit the reproducibility.

A4: Thanks for the good suggestion. It is very helpful to improve our manuscript. The SEM description methods and references have been added in the paper as follows: Structural equation modeling (SEM)

includes confirmatory factor analysis and regression or path analysis (Liu et al., 2016; Soliman et al., 2016), which allows for both the direct and indirect theoretical causal relationships between inter-correlated variables to be tested, and for potential multivariate relationships to be identified (Grace, 2006). SEM analysis was used to analyze the main impact factors and their pathways affecting FAAs in paddy soil. In our study, six observable variables (pH, SOM, protease, urease, bacterial biomass, bacterial community), and FAAs dynamics in paddy soil after CMV application were used to construct the SEM (The detailed construction process of SEM is shown in S3). In the model, the Maximum Likelihood method was used to estimate the parameters, and *P*-values and chi-square test ($\chi 2$) were used to assess the general model fit, because the respective *P*-values (*P*-values > 0.05) associated with the model chi-square are used to judge the fit between model and data (Eisenhauer et al., 2015). Several indices are also used to evaluate the ideal model, including the relative fit index (RFI), root mean square error of approximation (RMSEA), normed fit index (NFI), tucker-lewis index (TLI); comparative fit Index (CFI) and incremental fit index (IFI). Except for RMSEA which is less than 0.05, the value of these indices close to 1 indicates a good fit (Grace, 2006, Yang et al; 2020). In addition, the construction process and analysis of the SEM in this study have been described in detail in the supplementary material.

Reference

Eisenhauer, N., Bowker, M.A., Grace, J.B., Powell, J.R., 2015. From patterns to causal understanding: Structural equation modeling (SEM) in soil ecology. Pedobiologia. 58: 65-72. https:// doi.org/ 10.1016/j.pedobi.2015.03.002

Grace, J.B., 2006. Structural equation modeling and natural systems. Cambridge University Press, New York.

Liu, Y., Pan, X.Z., Wang, C.K., Li, Y.L., Shi, R.J., 2016. Can subsurface soil salinity be predicted from surface spectral information? -From the perspective of structural equation modelling. Biosyst Eng. 152: 1-10. https://doi.org/10.1016/j.biosystemseng.2016.06.008.

Soliman, A., Bellaj, T., Khelifa, M., 2016. An integrative psychological model for radicalism: Evidence from structural equation modeling. Pers Indiv Differ. 95: 127-133. https://doi.org/10.1016/j.paid.2016.02.039.

Yang, J., Yang, W.H., Wang, F., Zhang, L.M., Zhou, B.Q., Sarfraz, R., Xing, S.H., 2020. Driving factors of soluble organic nitrogen dynamics in paddy soils: Structure equation modeling analysis. Pedosphere 30(6), 801-809. https://doi.org/10.1016/S1002-0160(18)60032-3.

Q5: The description of FAA result is not sufficiently clear. The authors should indicate the statistical significant differences (by ANOVA) between CK and each different treatment to highlight the effect of different treatment. The description of the trend of FAA under different treatment during the different plant growth stages could help the reader to understand the information provided by the experiment. Morever, I suggest in legend of figure 1 including the description of different treatment acronyms.

A5: Thanks for the good suggestion. It is very helpful to improve our manuscript.

Firstly, FAAs results have been restated according to the suggestion, as follows: The temporal variations in concentration of FAAs displayed a similar pattern under different fertilization treatments (Fig. 1). The concentration of FAAs increased rapidly under different fertilization treatments and reached a peak at seedling stage. The FAA concentrations of CK, CL, CM and CH treatments at the seedling stage were increased by 28.77%, 11.34%, 31.64% and 38.58%, respectively, compared to the background soil. The concentration of FAAs of CK, CL, CM and CH treatments decreased rapidly from seedling stage to tillering stage, and tillering stage only accounted for 22.94%, 50.57%, 49.95% and 47.76% of the seedling stage, respectively. The concentration of FAAs under CK, CL, CM and CH treatment gradually increased after the tillering stage, and reached the second peak in the flowering stage, and increased by 185.06%, 44.21%, 21.71% and 26.92% respectively compared with the tillering stage. After the flowering stage, the concentration of FAAs decreased gradually, and the concentration of FAA in the treatments of CK, CL, CM and CH at maturity stage only accounted for 55.54%, 62.61%, 66.45% and 65.82% of the flowering stage. The application of CMV increased soil FAAs concentrations, but excessive application has a certain inhibitory effect. In both background soil and seedling stage, only CM treatment was significantly increased compared with CK treatment (P <0.05). Compared to CK treatment, the FAAs concentration under CL, CM and CH treatments were significantly increased by 131.42%, 182.02% and 133.30% at tillering stage (P <0.05) , and increased by 31.99%, 44.06% and 23.11% at maturity stage (P <0.05). However, there was no

significant difference among different treatments at flowering stage. Please see lines 220-236 of the revised manuscript.

Secondly, the descriptions of different treatment acronyms have been added in the legend was as follows: CK: chemical fertilizer, CL:low amount of CMV, CM:medium amount of CMV, CH: high amount of CMV. Please see line 240 of the revised manuscript.

Q6: The authors should better describe the results reported in PCA plot. Moreover, the authors should explain why in the PCA plot are reported 8 dots: how many replicates in the experiment? The authors should consider the fertilization effect, too.

A6: Thanks for the good suggestion. It is very helpful to improve our manuscript.

Firstly, the contribution rates of the two axes of PCA have been added in the paper as follows: PCA analysis showed that the contribution rate of the first axes and the second axes was 13.20% and 8.59% respectively (Fig. 5b). Please see lines 304-306 of the revised manuscript. In this study, the PCA mainly explained the differences in each growth period of rice based on the distribution of the dispersion and aggregation of the samples, so there was no more detailed description. However, the distribution of samples under different fertilization treatments was relatively scattered, and the differences between groups were smaller than those within groups during the rice growth period. Therefore, this paper did not describe the differences.

Secondly, in this study, 3 replicates were performed, and for the reliability of the data in the microbiological assay, 2 parallel samples were made for each replicate. In the PCA plot, the mean of 3 replicates was taken, so each sample had 2 points and 8 points per period.

Q7: A correlation between soil parameters and free amino acids by a structural equation model analysis was measured but the model and the coefficients they used are not clearly described and not supported by references.

A7: Thanks for the good suggestion. It is very helpful to improve our manuscript. The SEM descriptions have been added in Materials and Methods, and SEM fit indices have been added in Results as follows: The reliability coefficients and the overall fit indices of the second fitting fall within an acceptable range, with χ^2/ df = 0.500, RFI = 0.971, RMSEA = 0, NFI = 0.996, TLI = 1.031, CFI = 1.000, IFI = 1.004, indicating that the model was successful. Please see lines 333-335 of the revised manuscript.

Q8: The discussion section is not well supported by appropriate references in particular regarding the fate of free amino acids in soil. Moreover, the link between the presence of amino acids and bacterial communities is not well discussed and the potential impact of plant roots in the dynamics (degradation, transport, rhizodeposition and so on) are neglected. The bacterial communities (by 16sRNA) but not the fungal communities are analyzed, but the authors declared that the degradation and production of free amino acids are affected by microbial communities, including the fungal community. The authors could justify their choice in discussion section.

A8: Thanks for the good suggestion. It is very helpful to improve our manuscript.

Firstly, relevant references have now been added to the discussion section. The effect of microbial community on amino acids has been discussed, and the specific microbial communities with FAAs dynamics have been discussed in lines 555-576 of the manuscript.

Secondly, plant roots have important roles in FAA dynamics, both apparent and potential. Studies have shown that absorption and excretion are common ways that plant roots influence the dynamics of FAAs (Ma et al., 2018; Rae & Castro, 1976). L63-L65, L387-389, L407-L408, L440, L465 and L469-471 described the effects of roots on FAAs secretion and absorption in this paper. However, the extent of the potential effects of plant roots on the dynamics of FAAs has not been reported so far, so the potential impact of plant roots on the dynamics of FAAs needs to be further explored. We will also continue to research in this area.

Reference

Ma, Q.X., Wu, L.H., Wang, J., Ma, J.Z., Zheng, N.G., Hill, P.W., Chadwick, D.R., Jones, D.L., 2018. Fertilizer regime changes the competitive uptake of organic nitrogen by wheat and soil microorganisms: An in-situ uptake test using 13C, 15N labelling, and 13C-PLFA analysis. Soil Biol. Biochem. 125, 319-327. https://doi.org/10.1016/j.soilbio.2018.08.009.

Rae,I. C. M., Castro, T.F., 1976. Root exudates of the rice plant in relation to akagare, a physiological disorder of rice. Plant Soil 26: 317-323. https://doi.org/10.1007/BF01880181.

Thirdly, soil microorganisms play a critical role in nitrogen cycling by breaking down organic matter to amino acids and other small molecules of organic nitrogen through the mineralization processes. However, the relatively large proportion of bacteria in paddy soil, fungi only account for a small part. Previous studies have shown that bacteria accounted for more than 90% of the total microorganism in paddy soil, while fungi accounted for less than 1% (Kumar et al., 2019; Tan et al., 2014). Therefore, the effect of bacteria on free amino acids was studied in this paper, and the microbial communities was modified as bacterial communities.

Reference

Kumar, A., Kushwaha, K.K., Singh, S., Shivay, Y.S., Meena, M.C., Nain, L., 2019. Effect of paddy straw burning on soil microbial dynamics in sandy loam soil of Indo-Gangetic plains. Environ. Technol. Inno. 16: 1-10. https://doi.org/10.1016/j.eti.2019.100469.

Tan, Q., Song, T., Peng, W., Zeng, F, Du, H. , Zhang, H., Fan, F., 2014. Characteristics of soil microbial populations and biomass under different ecosystems in a canyon Karst region. Acta Ecol. Sinica 34:3302-3310. https://doi.org/10.5846/stxb201310252573.