RC1&2

Review of the manuscript egusphere-2025-2584

This is a valuable study investigating the effects of plant diversity on soil carbon accrual and biopore formation. The topic is novel and highly relevant to the journal's scope. The title is well-formulated, the experimental design is robust, and the dataset is comprehensive. However, there are a few minor issues, such as formatting errors, that should be addressed. Additionally, some methodological details in the Materials and Methods section need to be clarified or expanded. Overall, I believe the manuscript is suitable for publication after minor revisions.

Please review and feedback the general and specific comments as follows:

Specific remarks

Line 30: Please delete the extra space between *including* and *grasslands*.

Line 57: Please remove the extra space between *Root-originated* and *biopores*.

We apologize for the typographical errors, and have removed the extraneous spaces throughout the manuscript.

Lines 84–88: Could you clarify the rationale behind the assignment of treatment (CE) numbers? For instance, why is the bare system labeled as CE1, while the monoculture is labeled CE7?

➔ Treatment identifiers (CE1–CE12) were assigned by the Kellogg Biological Station. We selected only the treatments relevant to our objectives, which explains the non-sequential numbering. We have revised the manuscript for clarification as follows:

"The experiment consists of twelve plant systems representing a 12-point gradient of plant diversity (CE1-CE12), six of which were used in this study." (Lines 105-106 in the revised manuscript)

Additional details for all treatments are available at: https://lter.kbs.msu.edu/research/long-term-experiments/cellulosic-biofuels-experiment/ which is also provided in lines 112-113 in the revised manuscript. Lastly, we provided the description of each plot with their treatments in each caption of related figures (Fig. 1-4).

Line 125: Please provide a citation to a published study that supports the biopore identification method used.

➔ We have provided a reference as follows:
"Detailed procedures for biopore segmentation are publicly available (https://github.com/Maik-Lu/Roots_and_Biopores) (Lucas et al., 2022)." (Line 155 in the revised manuscript)

Line 129: Could you clarify what is meant by "distance average distance to biopore"? The phrasing is unclear.

➔ We apologize for the confusion. We have changed the term to "mean distance of soil matrix to biopores" throughout the manuscript. (Line 157, 224, 248, 250, 282, and Figure 4c and d in the revised manuscript)

Line 161: It would be helpful to include a brief summary sentence at the end of each paragraph or subsection to reinforce key points.

We have added a dedicated summary paragraph to each section in Discussion, as follows:
"These findings emphasize the currently underestimated importance of formation, abundance, and surface properties of biopores in affecting soil biochemical status,

specifically, influencing plant-derived inputs and their potential protection within the soil matrix. Our study suggests that this role might be particularly pronounced in ecosystems with high plant species diversity." (Lines 287-291 in the revised manuscript)

"We see the need for further investigation into the role that individual members of grassland communities may play in stimulating soil pore structure development and SOC accumulation. Identifying keystone species enabling more efficient C accumulation can guide plant restoration and SOC accrual efforts." (Lines 318-321 in the revised manuscript)

Line 181: Please format the R^2 correctly by placing the 2 as a superscript.

➔ We have updated all instances to "R^2" throughout the manuscript.

Line 255–256: This sentence is unclear—"accumulated for 12 years" appears abrupt and could be better integrated.

➔ According to reviewer's comment, we deleted those sentence from discussion part.

Lines 255–262: These sentences are more appropriate for the Introduction section and would fit better there.

➔ We moved the mentioned sentences to Introduction part, and reorganized paragraph as follows:
"The positive association between SOC and bioporosity was another result consistent with our expectations (Fig. 5b). The correlation between plant richness and SOC was largely driven by the highest species richness of 30, which was a high leverage point in our regression analysis (Fig. 1b and d). Thus, even though the R2 for bioporosity-SOC regression was lower than that from species richness-SOC (0.36 vs. 0.42), bioporosity could be viewed as a better predictor of SOC. Moreover, the CE8 system with its unexpectedly high SOC turned out to have higher bioporosity and higher volumes of biopores of all sizes than the other studied systems (Figs. 2b and 3b. This observation is consistent with previous reports identifying biopores as sites of high inputs of labile substrates (Pierret et al., 1999; Xiong et al., 2022), increased microbial abundance and activity (Wendel et al., 2022), and enhanced necromass accumulation (Banfield et al., 2018), all of which highlight the role of biopores in contributing to SOC accrual.
The biopores in CE8 had the greatest surface area, followed by the most diverse system (Fig. 4a). The mean distance of soil matrix to biopores was the shortest in CE8, followed by diverse systems (CE10 and CE12) (Fig. 4c). The surface area of biopores is critical as it represents the specific portion of the soil matrix that directly intercepts root-derived C inputs, i.e., rhizodeposits (Keller et al., 2021) and decomposed root residues processed by soil microbes (Kim et al., 2022). A shorter mean distance from soil matrix to biopores indicates that root-derived C was accessible to a large community of soil microorganisms, probably facilitating efficient transfer and sequestration of microbially processed C into the soil matrix (Kravchenko et al., 2019). These findings emphasize the currently underestimated importance of formation, abundance, and surface properties of biopores in affecting soil biochemical status, specifically, influencing plant-derived inputs and their potential protection within the soil matrix. Our study suggests that this role might be particularly pronounced in ecosystems with high plant species diversity." (Lines 271-291 in the revised manuscript)

L284 It would be reasonable to include a clear conclusion section at the end of the main text.

➔ Following reviewer's suggestion, we have provided Conclusion as follows:
"The 12-year grassland experiment demonstrates that SOC accumulation is governed by plant diversity, with the benefits of high plant diversity being, in part, exhibited via development of biopores. Yet, certain species combinations may lead to biopore formation

and SOC accumulation benefits disproportional to the actual level of the plant system diversity. Specifically, the two-species mixture of C4 switchgrass and C3 Canadian rye created the greatest bioporosity, shortest mean soil-to-pore distance, and the largest biopore surface area, thereby accelerating microbial processing and stabilization of root-derived C in the surrounding matrix. These findings highlight bioporosity as a more reliable proxy for SOC gains than plant species number alone, and point to potential existence of specific "keystone" species combinations that may disproportionately enhance soil structure and C sequestration. Identifying and deploying such functionally complementary plant species can offer a targeted pathway for optimizing grassland restoration and long-term SOC storage." (Lines 323-332 in the revised manuscript)

RC3

The manuscript egusphere-2025-2584 titled "Soil carbon accrual and biopore formation across a plant diversity gradient" utilized a plant diversity gradient experiment (1-30 species) and employed X-ray computed micro-tomography scanning to quantitatively analyze soil pore structures (particularly biopores) under different plant systems, and examined their relationship with soil organic carbon (SOC) accumulation.

➔ We appreciate the reviewer's thoughtful assessment and the opportunity to clarify our study's scope and interpretation.

The study suggests that plant diversity can enhance SOC content through biopore-mediated mechanisms. However, the conclusions lack generalizability due to the limited SOC data from only one year of measurement.

➔ Our SOC sampling was conducted in 2019, but it was intended to capture the cumulative outcome of 12 years of experimentally maintained plant-diversity treatments (established in 2010). Thus, the SOC values reflect long-term biodiversity effects rather than a single-year snapshot.

More importantly, the interrelationships among plant diversity, biopores, and SOC remain unclear. The correlations among the three do not represent a causal relationship, and the driving process of diversity is also not deep enough.

➔ We agree that correlations do not, by themselves, establish causation. We present the biopore pathway as a mechanistic hypothesis supported by pattern concordance (plant diversity / specific plant combinations→ bioporosity → SOC) rather than as a demonstrated causal chain. In the revised manuscript, we completely revised the introduction to clarify this point. (Lines 38-87 in the revised manuscript)

Although the authors repeatedly emphasize the importance of microorganisms in biopores, direct

microbial measurements are lacking. It would be valuable to incorporate such data to substantiate the claims.

➔ We also recognize the value of direct microbial measurements within biopores. Unfortunately, *this long-term ecological research site has since been decommissioned*, which precludes additional field sampling to add microbial assays at this location. In the revised manuscript we emphasized the need for more microbial analyses to be done in similar studies in the future. "We also suggest that future studies incorporate more detailed analyses of microbial biomass, composition, and diversity to better elucidate microbial C processes occurring in biopores. Combining X-ray μCT with pore-targeted microbial analyses, as demonstrated by Li et al. (2024), is expected to provide valuable insights into SOC accumulation driven by soil microorganisms in biopores." (Lines 301-311 in the revised manuscript)

Additionally, introduction lacks sufficient background and theoretical foundation, and results are not adequately contextualized within existing literature or mechanistic frameworks.

➔ We have expanded the Introduction to provide a clearer theoretical foundation for how plant diversity can reorganize soil structure and thereby influence SOC dynamics. This includes a brief synthesis of the biopore-as-hotspot framework and its implications for substrate inputs, microbial processing, and stabilization pathways. We also clarify why examining pore architecture offers complementary insight beyond biomass-based explanations.

It is recommended to strengthen the theoretical discussion and improve the scientific interpretation of the findings. Please see the specific comments below.

➔ Although we acknowledge limitations, we believe the manuscript makes a useful contribution by elevating pore architecture as an integrative lens for understanding biodiversity and SOC linkages. Given the diversity of mechanisms currently discussed in the literature, we consider it valuable to foreground the biopore-mediated pathway and to provide a transparent framework and dataset that others can build upon. We hope these clarifications and revisions address the reviewer's concerns and improve the theoretical and interpretive strength of the manuscript. Nevertheless we addressed all the specific comments the Reviewer made towards improvement in the Introduction as we detail our responses below.

Introduction

The Introduction fails to clearly articulate the research gap or motivate the study's focus on plant

diversity effects on pore structure and SOC. The logic between paragraphs is relatively weak, and the importance of biopores in mediating plant-soil- carbon interactions is not sufficiently established.

➔ We have revised the Introduction part thoroughly, to connect the logic between paragraphs.

Lines 34-37 and 41: The repeated emphasis on microbial processes is misleading since no microbial data were collected. This section should be refocused on mechanisms more directly related to the study

➔ We would like to point out that the first paragraph (in which Lines 34-47 and 41 are located) is reviewing known-mechanisms of how plant diversity increases SOC, rather than emphasizing the importance of soil microbial process for SOC accumulation. Lines 34-37 includes most frequently discussed mechanisms on how high plant diversity can gain more soil organic carbons. Line 41 includes sentences emphasizing how C process can be affected by "root growth" and changes in "volume of soil matrix".
Following the reviewer's general comments, we clarified the logic that biopores are formed by roots and soil organisms, and that these structures serve as sites where they deposit carbon (From Line 38 in the revised manuscript).

Lines 44-45: The claim that pore structure affects root-soil interactions and plant-derived carbon requires supporting references or clearer conceptual linkage.

➔ We have provided clearer conceptual linkage in the expanded introduction (Lines 38-55 in the revised manuscript).

Lines 46-50: Lack of references.

➔ This sentence objectively outlines well-recognized challenges in soil research, namely the opacity of soil and the difficulty of conducting long-term experiments. As it does not pertain to specific research findings or the claims of individual scholars, a reference is not deemed strictly necessary.

Lines 61-64: How are biopores and SOC related?

➔ We described the biopores as the specific location within the soil matrix where SOC is processed. Also, please note that our hypothesis on biopore-SOC relationship, that higher plant diversity promotes the abundance and formation of root biopores and that this enhanced biopore development is associated with greater gains in SOC, is supported by the results.

Lines 74-77: The research objective is the relationship between biopores and C. However, the

significance of this part of the research is not elaborated in the Introduction. Instead, it has been constantly describing the spatial distribution and morphological characteristics of the root system. Here, no data on the morphology and distribution of the root system were measured in this study.

➔ We thoroughly revised the introduction part, following the reviewer's comment (Lines 29-99 in the revised manuscript).

Materials and Methods

Lines 80-90: Key agronomic details are missing. i.e., sowing dates and frequency for *switchgrass* and *Canadian rye*.

➔ All plant communities were planted in 2008. There were no any further sawing events.

Line 95: The 7-12 cm sampling depth may not capture the full root profile, especially for deep-rooted species like *switchgrass*. Justification for this depth is needed.

➔ We have planned to characterize pore architecture across depth, particularly given the deep-rooting habit of species such as switchgrass. Unfortunately, high-resolution X-ray CT profiling at multiple depths is resource-intensive, and our funding did not permit comprehensive vertical imaging. We therefore focused on the topsoil interval (7–12 cm), which captures topsoil where fine roots are most abundant and dynamic, and where root-derived C inputs and associated microbial activity are known to be highest. This interval also falls within standard grassland sampling practices (e.g., 0–10 or 10–30 cm coring), facilitating comparability with prior studies while targeting the layer most sensitive to changes in root activity and expectedly, biopore formation.

We agree that deeper horizons may host additional biopore development in deep-rooted taxa, and we note this as an important avenue for future work when resources allow. We added a short sentence for justification as follows:

"This depth encompasses the zone of greatest fine-root abundance and turnover, where root-derived C inputs and microbial activity are most pronounced (Halli et al., 2022; Roosendaal et al., 2016)." (Lines 115-117 in the revised manuscript)

Line 104: The term "soil total C" is used in Methods, but "SOC" is used elsewhere. Ensure consistency.

➔ We revised the inconsistency in this sentence and throughout the manuscript.

"SOC and total soil N were measured by combustion…" (Line 130 in the revised manuscript)

Line 106: Is the water content of all treatments the same? Or should the original moisture content of each treatment be maintained? Has the evaporation of soil moisture been taken into account? Is it necessary to weigh and replenish water daily?

➔ As described in the manuscript, we maintained the same soil moisture across all treatments to standardize the incubation environment. Our objective was not to reproduce field mineralization dynamics (which would be more appropriately addressed using *in situ* chambers), but rather to compare relative C availability and microbial activity among soils under controlled conditions. For this reason, holding moisture constant across treatments was essential. We believe it is widely accepted method to evaluate treatment effects.

To address potential evaporation, we placed approximately 5 mL of purified water at the bottom of each incubation vessel to maintain a humid microenvironment and minimize moisture loss from the samples (Lines 104-106 in the revised manuscript). Under these conditions, evaporation was negligible, and it was not necessary to weigh and replenish water daily.

Lines 138-141: The functional richness calculation method claims support from Mangan et al. (2011) and Spiesman et al. (2018), but these studies do not explicitly use the same method. Please provide a clearer rationale or additional citations.

➔ We argue that our categorization for plant functional groups is well supported by reference literature. Spiesman et al. (2018) uses grass functional groups as C3 and C4 grass. Mangan et al. (2011) used grasses, legumes, and forbs. Since our experiment does not include legumes in plant species, it is reasonable to use grasses (C3 and C4) and forbs. We provided an additional reference from Tilman et al. (2006) in which plant functional groups were categorized as C3 grasses, C4 grasses, legumes or non-legume forbs.

Results

Line 166: The lack of correlation between aboveground biomass and SOC may be due to a temporal mismatch. SOC was measured in 2019, while biomass data came from 2018-2019. Aligning the data temporally or discussing this discrepancy is important.

➔ Thank you for your valuable comment. We provided a rationale for using biomass data for 2018 and 2019 as follows:

"For the present analysis, we used the 2018 and 2019 biomass datasets (Fig. S1b). Soil sampling for this study occurred in July 2019; thus, the 2019 fall biomass provides the most temporally proximate estimate of aboveground production for the soils analyzed, while the

2018 biomass offers the preceding-year context and helps mitigate interannual variability."
(Lines 120-123 in the revised manuscript)

Please note that restricting the analysis to the 2019 biomass dataset did not alter the results obtained using the combined 2018–2019 datasets.

Lines 185-190: Figure 2 lacks the statistical difference test between treatments. I suggest dividing the pore size into different size intervals and then comparing the differences between treatments.

Thank you for pointing this out. We performed formal tests at each pore-size class and updated Figure 2 accordingly. Pairwise comparisons among plant systems within each pore-size class were conducted and the resulting significances are now indicated in figure 2.

"The statistical model for pore and biopore size distribution data included the pore size class and its interaction with plant system as fixed effects, and soil core nested within the plant system and experimental plot as the random effect. When the ANOVA was significant, all pairwise mean comparisons among plant systems were conducted within each pore size class."
(Lines 177-180 in the revised manuscript)
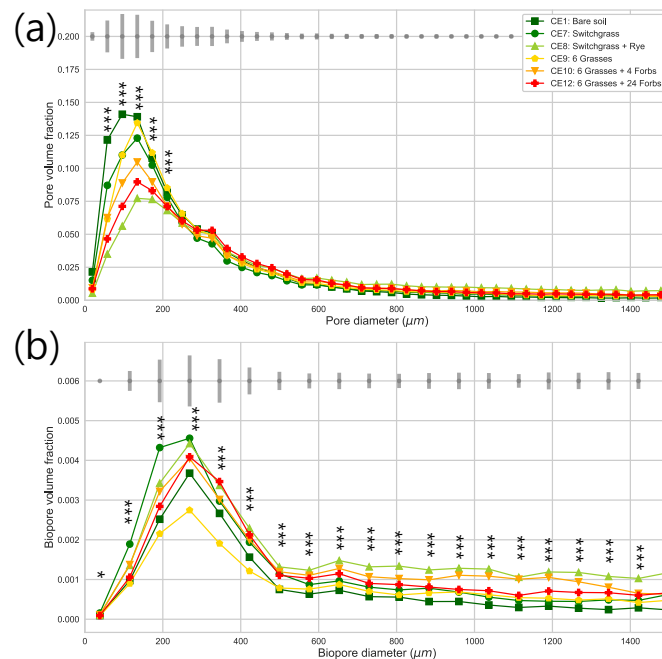
Figure 2: Pore size distribution (a) and biopore size distribution (b) in the studied plant diversity systems (CE1: Bare soil, CE7: Switchgrass, CE8: Switchgrass + Rye, CE9: 6 grasses, CE10: 6 grasses + 4 forbs. CE12: 6 grasses + 10 forbs). Gray bars indicate the least significant difference (LSD) in each (Bio)pore diameter group. Asterisks *** and * mark significant

differences among the plant diversity treatments at each (Bio)pore diameter at p < 0.01 and p< 0.10 significance.

Lines 208-216: The author needs to summarize the important correlation results instead of studying the correlation for each indicator.

➔ Following the reviewer's comment, we have revised section 3.3 as follows:

"Plant species richness and plant functional richness were both positively associated with SOC, with species richness explaining 42% of the variance in SOC (p < 0.01; Fig. 1b) and functional richness explaining 36% (p < 0.01; Fig. 1d). Within the pore metrics, biopore surface area was positively related to bioporosity (R2= 0.50, p < 0.01; Fig. 4b), whereas the mean distance from the soil matrix to the nearest biopore was negatively related to bioporosity (R2= 0.46, p < 0.01; Fig. 4d). Bioporosity increased with plant diversity. Species richness explained 10% of the variance in bioporosity (p < 0.01; Fig. S4a), and functional richness explained 29% (p < 0.05; Fig. 5a). Consistent with these patterns, bioporosity was positively correlated with SOC (R2 = 0.36, p < 0.01; Fig. 5b)." (Lines 239-245 in the revised manuscript)

Discussion

The Discussion should avoid referencing each figure individually. The authors are expected to focus on interpreting key findings and substantiating their arguments with relevant literature.

➔ While we agree with the reviewer that the discussion session should focus on interpreting key findings and providing supporting evidence from relevant literature, we do not agree that the discussion should avoid referencing each figure individually. To support the key arguments and their interpretation, it is essential to reference the results obtained in this study. Our response to reviewer's comment on Lines 237-240 is in line with this argument.

Lines 233-240: The variation in C mineralization across systems is not discussed.

➔ As the reviewer suggested, we primarily focused on interpreting the key findings. In our study, C mineralization did not exhibit a clear trend in relation to SOC or plant diversity. Nevertheless, we addressed the discrepancy between our results and previous reports and highlighted the need for further investigations into microbial processes within biopores to better explain these patterns.

"Our C mineralization results (Fig. 1c) did not much correspond with previous reports indicating that higher plant diversity enhances microbial C use efficiency (Eisenhauer et al., 2009), likely driven by greater chemical heterogeneity and enhanced substrate accessibility to

soil microbes (Mellado-Vázquez et al., 2016; Domeignoz-Horta et al., 2024). We suggest that future studies incorporate more detailed assessments of microbial biomass, community composition, and diversity to better elucidate carbon processes occurring in biopores. These inconsistencies highlight the need for further investigation of microbial activities and net C balance to identify the specific microbial pathways operating in biopores. In particular, the integration of X-ray μCT with pore-scale microbial analyses, as recently demonstrated by Li et al. (2024), represents a promising approach to advance our understanding of SOC accumulation mediated by soil microorganisms in biopores structures." (Lines 292-300 in the revised manuscript)

Line 237: The non-significant relationships between aboveground biomass and SOC may be related to C mineralization rates.

➔ We appreciate the comment, but this does not appear to be the case in our dataset. The relationship between aboveground biomass and SOC (Fig. S2c) shows no consistent trend. Specifically, the CE7 plots (dark green) would require an SOC concentration of approximately ~1.2% to align with a positive correlation. However, observed SOC in CE7 is lower than expected given its aboveground biomass. As pointed out by reviewer, one possible explanation would be unusually high C mineralization at CE7, leading to disproportionate C losses. However, our incubation data (Fig. 1c) indicate that CE7 exhibited the lowest mineralization rates among the diverse plant systems, arguing against elevated mineralization as the driver of the weak aboveground biomass–SOC relationship.

Lines 237-240: Lack of explanation.

➔ We view these lines as a concise recap that directly supports the preceding point that *plant biomass did not significantly contribute to SOC increases* in our dataset. The comparison between CE7 (monoculture) and CE8 (two-species system) highlights this lack of impact: despite CE8 having the lowest aboveground biomass, its SOC was comparatively high. We therefore believe no additional explanation is required beyond this clarifying contrast.

Lines 244-248: The results of the CE8 system were not explained. There is no logical connection between the two sentences.

➔ We have revised the sentence as follows:
"Moreover, the CE8 system with its unexpectedly high SOC turned out to have higher bioporosity and higher volumes of biopores of all sizes than the other studied systems (Figs. 2b and 3b. This observation is consistent with previous reports identifying biopores as sites of

high inputs of labile substrates (Pierret et al., 1999; Xiong et al., 2022), increased microbial abundance and activity (Wendel et al., 2022), and enhanced necromass accumulation (Banfield et al., 2018), all of which highlight the role of biopores in contributing to SOC accrual." (Lines 275-280 in the revised manuscript)

Lines 273-276: Using legumes as an example is not convincing, as *switchgrass* and *Canadian rye* do not belong to the leguminous family.

➔ Thank you for the comment. We cited legumes only to illustrate the general ecological concept of a "keystone effect" (i.e., a species exerting a disproportionately large functional influence), not to imply that legumes were part of our species set or that our results rely on legume-specific mechanisms. If there were published reports showing that the switchgrass–Canadian wildrye combination synergistically enhances SOC, we would have used those examples instead; to our knowledge, such evidence is currently unavailable. In the revision, we divided the paragraph between Line 309 and Line 310, to avoid such misunderstandings.

Also, if the reviewer can point us to studies demonstrating synergy for this particular species pair, we will gladly incorporate and discuss them.

Lines 276-278: Lack of literature.

➔ We carefully argue that these sentences connect directly to the following sentence, which cites evidence that the composition of switchgrass root exudates can vary with neighboring C3 species. We intentionally did not cite that study in the sentence highlighted by the reviewer to avoid potential misinterpretation, as the species used in the referenced work differ from those in our study.

Is a Conclusion section missing from the manuscript?

➔ We have provided a conclusion section in the revised manuscript (Lines 323-332 in the revised manuscript).