Covering Letter for a Revised Manuscript

Manuscript reference number: 23184

Associate Editor Evaluations:  
Recommendation: Accept Revision Required  
  
Associate Editor (Comments for the Author (Required)):  
  
Dear authors,  
  
My apologies for the delay in returning this manuscript, as you can understand it can be difficult to find reviewers at this time of year. We have now received two comprehensive reviews of your manuscript. Both reviewers found the science to be interesting and thought provoking. I agree, it was a very interesting read. However, reviewer 1 found it difficult to understand some of the terminology and methods and reviewer 2 has requested clarity on some of the assumptions in your modeling. Both have provided detailed comments for you to address. Could you please revise the manuscript and address their comments before resubmission to AoBP.  
  
Kind regards,  
William

Dear William,

Thank you so much for organizing a review of this manuscript. The reviewer’s comments have been extremely useful in clearing up the details of the ms for publication in AoBP. We appreciate your help in getting this science in print. We summarize our changes here for an easy review; details on specific comments can be found below.

We have clarified our terminology, adding in definitions where needed and standardizing all mentions of specific terms. We’ve clarified our methodology by being more explicit about which data are real and which are synthetic. We have added all the references and relevant discussion of them as suggested by reviewer 1. We’ve clarified the wording of our hypotheses following the suggestion of reviewer 1. And, as was addressed by both reviewers 1 and 2, we’ve clarified the limitations of our model assumptions and extensively addressed them in the introduction and discussion. To accommodate some of these changes, we’ve made some minor changes elsewhere in the text; however, the largest changes are all reflected in this document. We hope that, with these significant clarifications, changes, and additions of important caveats, our ms will be considered for publication in AoBP. Thank you for your time and consideration.

Sincerely,

The authors  
  
Referee #1 Evaluations:  
Recommendation: Major changes needed  
  
Referee #1 (Comments for the Author (Required)):  
  
The MS explore stomatal patterning on both adaxial and abaxial leaf surfaces in relative to CO2 diffusion and photosynthesis using both observations and modelling approaches. This is a topical area at the moment and some of the findings interesting and valuable to research in this area. However several sections of the MS were difficult to follow and further clarification of several aspects is required to widen the readership.  
There are several descriptions used that are not entirely clear. What exactly is meant by "over dispersal" in the context of stomatal patterning. Does this mean there are too many stomata on the leaf or their spacing is not optimal but the correct number? This is not common terminology when referring to stomatal patterning. The assessment of distribution relative to a uniform random distribution needs further explanation as it is well established that stomata are randomly distributed.

Dear Reviewer 1,

Thank you for taking the time to read and comment on our manuscript. You have brought up important points of clarification that must be addressed before the ms is ready to be published.

Regarding your concerns about our term overdispersed and our assessment of stomatal patterning, we have clarified and standardized our terminology, adding explanations when needed, and it has long been known that stomata are non-randomly distributed, tending to be more uniformly distribution than random (see (Croxdale 2000) for a nice review of historical evidence of stomatal patterning). In Croxdale (2000), there is also confusion over terminology in the published literature, so we are not sure that there are field-accepted terms. So, we have adopted our own set of terminology which mirrors that of the field and related fields which discuss the spatial patterns of discrete objects. Thanks for pointing out these inconsistencies in our work.

In the material and methods section it is often not clear which measurements/data are real and which are synthesized.

Thank you for noting this. Upon re-reading the ms, we can see how this confusion may arise, we have edited the methods section to be more explicit about which type of data are which. We’ve also added a sentence in the beginning of the methods describing how synthetic data fit into our analyses. It reads: “A number of synthetic leaf surface datasets were also prepared (details below) so as to be mathematically explicit when defining null distributions against which to test our hypotheses and to avoid any methodological influence on our results (e.g. stomatal density uncertainty, boundary effects when calculating patterning metrics: uniform random, non-random overdispersion, and uniform). All synthetic leaf surfaces were simulated based on the size of the real leaf images and stomatal densities matched those of real leaf images.” This can be found at the end of the data preparation section of the methods.

There are several references missing I am surprised not to see included in the introduction and/or discussion- see specifics below.

Thanks for your unique insights into the literature. Our reference list is certainly not complete and we thank you for allowing us to give credit where it is due. We have added all the references that you mention here and the appropriate discussion.

Line 19 - should include earlier references that examined Gopp - McElwain et al., 2016  
Line 24 - should include the work of De Boar et al.  
Line 59 - there are several other papers that have examined lateral flux and the impact of stomata and photosynthetic consumption, including Morison et al 2007.  
The work of Wall et al., 2021 is missing and should be included.  
The authors have not mentioned or considered the published references that have explored heterogeneity in stomatal distribution over the leaf surface and between surfaces (see work by Weyers et al.).  
How do these findings for Arabidopsis translate to crops. Arabidopsis grows very close the ground and therefore boundary layers esp for the abaxial surface will be very different to key C3 crops.

The goal of our study is not necessarily to translate the findings to crops; however, these results may prove to be relevant to crops upon further investigation. We agree that the boundary layer dynamics may differ drastically between the short *A. thaliana* stems and those of Maize. This does not nullify our results nor our discussion of them.

Hypothesis 3 - it is well established that high light alters stomatal density.

We are aware that this hypothesis has been tested and validated many times; however, stomatal density is still an important metric to consider and illustrate in this study as stomatal density may interact strongly with stomatal patterning.

The wording of hypothesis 5 is particularly unclear. "5) stomatal length will be positively correlated with the area of the leaf surface to which it is closest". What is the area of the leaf surface to which is it closest?

Thanks for bringing up this potential ambiguity in our hypothesis. It now reads: “Stomatal length (and hence its area) will be positively correlated with the area of the leaf surface to which it is spatial closest as defined by Voronoi tessellation techniques. We refer to this as the 'stomatal zone', the leaf area surrounding a focal stomate closest to that stomate and therefore the zone it supplies with CO2). This way, each stomate may be optimally sized relative to the mesophyll volume it supplies.”

The high light used in the experiment is quite low at 200 umol m-2 s-1

This concern can be related to your comment on hypothesis 3. 200 umol m-2 s-1 is relatively low; however, it was high enough to drastically increase stomatal density, suggesting a change in many other unmeasured leaf parameters. This was convincing enough for us to define 200 umol m-2 s-1 as high light, especially when compared to 50 umol m-2 s-1, our low light treatment. It would make for an interesting study to repeat these measurements across multiple crops (to address the boundary layer differences between *A. thaliana* and crops) and a wider range of light environments.

Line 52 - provide more explanation as to why the equilateral triangular grid is the ideal distribution.

We have done so. The text now reads: “An equilateral triangular grid is ideal because it standardizes the distance between stomata (for a given stomatal density) and thereby also standardizes the mesophyll volume with which each stomate is spatially closest to, ultimately reducing redundancy associated with clustered stomata and allowing fewer stomata to supply the same leaf area (or the same number of stomata to adequately supply a larger leaf area).”

Line 94 - details of the size of the image need to be included.

Thanks for catching this! We’ve now added the text in the appropriate spot in the data preparation section of the methods. It now reads: Images were square with an area of 0.386 mm2.

Line 95 - sample image - is this real or synthetic? Terminology used needs to be clear and consistent.

Sample image is our standard term for real leaf surface. Synthetic leaf surface is our standard term for simulated leaf surfaces against which the real leaves were compared.

Line 105 - please provide further clarification on why the ideal dispersal is an equilateral triangle.

We’ve provided clarification as to why this is the ideal dispersal in the introduction as you requested. We feel it would be redundant to also include it here.

Line 106 - wording of this sentence is difficult to follow.

We agree, this sentence is a little vague. We’ve changed it be more clear following your suggestion: “To account for uncertainty in the stomatal density of each sample image with *n* stomata, we integrated over plausible stomatal densities and then conditioned on synthetic leaf surfaces with exactly *n* stomata.”

Line 129 - each pixel of what?

It now reads: “pixel of the surface.”

Line 157 - ideal pattern is "hexagonal"?

Thanks for catching this. We’ve now standardized this term to be the same as every other mention of the uniform, equilateral triangular grid.

Line 168 - "All parameters....exceeded 103" - please clarify what you mean by this statement.

Thank you for all of your comments. We’ve clarified this statement by adding the name of the model for which parameters converged.  
  
  
  
  
  
Referee #2 Evaluations:  
Recommendation: Major changes needed  
  
Referee #2 (Comments for the Author (Required)):  
  
Dear authors,  
The authors made a theoretical analysis of the optimal distribution of stomata on each leaf surface and then compared it with actual data. I only have a few comments, most of them concerning the assumptions. I think the consequences of their assumptions could be better addressed and explained with further details, especially the concept of "optimal distribution".  
  
Please see my comments below.

Dear reviewer 2,

We really appreciate your comments as they are most insightful and will certainly make this manuscript better and more useful for its readership. If we’ve interpreted your concerns correctly, your main issue with the ms in its current state is that the limitations brought upon by our model assumptions, particularly concerning uniform mesophyll tortuosity/diffusion resistance and its consequences for our definition of “ideal, uniform” stomatal patterning, are not adequately explained in the introduction and discussion. We agree that these caveats are not discussed in great enough detail. We hope we can convince you that we have indeed thought about this and that it does not nullify our analyses, but simply puts them into context of the larger discussion about how each variable of the leaf may be modulated to optimize photosynthesis, especially diffusion limitations.  
  
L64-65. The impact of varying tortuosity (horizontal vs. vertical) on stomatal distribution needs clearer exploration. Specifically, how do differences in adaxial and abaxial tortuosity affect your analysis? I guess this must be in coordination with different horizontal and vertical tortuosity, as the authors mentioned: if the horizontal tortuosity is higher than the vertical tortuosity, this consideration should vary, especially if the tortuosity in the adaxial and abaxial half are different.

We have addressed this extensively in the discussion. See our text from the discussion which now reads:

“First, our predictions are wrong because they are based on overly simplistic assumptions about epidermal and mesophyll anatomy. In real leaves, diffusion through the mesophyll depends on the position of each cell and resulting IAS geometry. If, for example, horizontal diffusion was modeled to be much slower than vertical diffusion, we likely would have seen an increased benefit of ab- adaxial stomatal coordination across a greater range of feasible trait values. However, further information about the three-dimensional structure of the leaves would need to be incorporated into the model to address any further questions about how mesophyll anatomy may influence stomatal anatomy.”  
  
L64-66 (hypotesises 1 and 2). Stomata distribution and tortuosity of the mesophyll also contribute to modulating transpiration, not only CO2 distribution. Did the authors account for this?

Yes, the model and our discussion of it both account for the affects of transpiration.  
  
L69-71. Clarification is needed on what is meant by "the surface closest to." Does this imply an assumption of homogeneous tortuosity, leading to a triangular distribution as the only optimisation? Doesn't this already assume that tortuosity is homogeneous and, therefore, the only optimisation possible is the triangular distribution?

Thanks for noticing this. Reviewer 1 also noticed this, so see our response to their comments on these lines for how we’ve addressed this issue. And regarding the homogeneous tortuosity, we’ve addressed the limitations of this assumption thoroughly in the text. And we were aware that defining the conditions of the model this way would lead to such an optimization, but we then compared this optimization to real leaf surface data and provided alternative explanations as to why such an optimization is not reached in nature. In other words, the question of the paper is not: what is the optimal distribution of stomatal given these parameters? Rather it is, why is the optimal distribution (as suggested by our simplistic modeling and geometric assumptions) not reached? What are these other mechanisms which are potentially more important than uniform stomatal spacing?  
  
L140-143 and 194-198. I apologise if I have missed something. There seems to be a presupposition of homogeneous internal resistance to CO2 diffusion or tortuosity. How does this assumption define the optimal arrangement and affect the photosynthesis modelling based solely on stomatal distribution? It seems to me that the authors already define the optimal arrangement by assuming homogeneous internal resistance to CO2 diffusion or homogeneous tortuosity. So, photosynthesis modelling for given photosynthetic parameters will be a function of the distribution of the stomata only, isn't it?

We agree with your sentiments in this comment and in your over-arching concernings with the entire ms. We have since modified the introduction text accordingly to account for your comments. It now reads:

“Assuming uniform mesophyll diffusion resistance in all directions (porous medium), an ideal stomatal anatomy can be predicted. To maximize CO2 supply from the stomatal pore to chloroplasts, stomata should be uniformly distributed in an equilateral triangular grid on the leaf surface so as to minimize stomatal number and CO2 diffusion path length (Parkhurst, 1994). With this assumption, an equilateral triangular grid is ideal because it standardizes the distance between stomata (for a given stomatal density) and thereby also standardizes the mesophyll volume with which each stomate is spatially closest to, ultimately reducing redundancy associated with clustered stomata and allowing fewer stomata to supply the same leaf area (or the same number of stomata to adequately supply a larger leaf area).

Such an assumption, though an oversimplification, is a powerful tool for photosynthesis modelling, and may provide insight into how real leaves diverge from this. In real leaves, as the diffusion rate of CO2 though liquid is approximately 104 X slower than CO2 diffusion through air, mesophyll resistance is generally thought to be primarily limited by liquid diffusion (Aalto, 2002; Evans, 2009), but diffusion through the IAS has also been shown to be a rate limiting process because the tortuous, disjunct nature of the IAS can greatly increase diffusion path lengths (harwood, 2021). Additionally, tortuosity is higher in horizontal directions (parallel to leaf surface) than vertical directions (perpendicular to leaf surface) because of the cylindrical shape and vertical arrangement of pallisade mesophyll cells (Earles, 2018; Harwood, 2021). However, the ratio of lateral to vertical diffusion rate is still largely unknown and may be a highly pliable trait in leaves (Morison, 2005; Pieruschka, 2005; Pieruschka, 2006; Morison, 2007). Depending on the thickness of the leaf, porosity of the leaf mesophyll, tortuosity of the IAS, and lateral to vertical diffusion rate ratio, minimizing diffusion path length for CO2 via optimally distributed stomata may yield significant increases in CO2 supply for photosynthesis and higher Amax. Or plants may simply coordinate the development of stomata and mesophyll IAS to reach another optimal solution which does not rely on uniformly distributed stomata (Baillie, 2020).

We hypothesized that, in the absence of any constraint and assuming uniform mesophyll diffusion resistance, natural selection will favor stomatal patterning and distribution to minimize the diffusion path length…”  
  
L156-157 and 192-193. I don't see how a hexagon can be, in reality, an ideal distribution when there are other leaf structures in between and below the stomata that it seems are not being accounted for in this analysis. I don't think the authors need to account for them, but please clarify the limitations of not doing so.

Thanks for bringing this up. We indeed do not account for other leaf structures in this set of analyses, but we feel that we do a good job of clarifying the limitations of not doing so in the discussion and provide interesting pathways forward. Here is the related text:

“Our model also assumes an idealized leaf epidermal and mesophyll structure that is homogenous and unconstrained by other tradeoffs. Real leaves not only provide pathways for CO2 diffusion, but must supply water, intercept light, and deter herbivores and pathogens. All of these competing processes also happen on different time scales, and can be observed as heterogeneity in stomatal density, aperature, and internal leaf conditions across the leaf at any given moment (Lawson, 1998; Lawson, 1999). These competing interests result in non-uniform epidermal and mesophyll structure that could alter predictions about optimal stomatal spacing. In order to maintain consistent leaf water potential across the lamina, stomatal density must be coordinated with vein density (Fiorin, 2016). Thus, stomatal spacing may be optimized not at the interstomatal level, but at a higher level, coordinating water transport and water loss… Future gas exchange models should include multiple parameter sets for different strata of mesophyll tissue and water supply structures such as veins, though these are beyond the scope of this study.”  
  
L205-206. Shouldn't this be stated in the introductions instead of the discussion? This allows for a more in-depth discussion in the relevant section because it seems to be the most likely option, so I expected a more detailed discussion of this issue.

Thank you for noticing this. We agree, the issue does deserve more in-depth discussion. We’ve added the relevant introduction materials (see our earlier responses to your comments) and we’ve added a more in depth discussion here. The beginning of the Discussion introduces this caveat with the following text: “However, reducing this distance to its absolute minimum may be constrained by developmental processes, the photosynthetic benefit may be too small to be 'seen' by natural selection (i.e. the selection coefficient is less than drift barrier *sensu* Sung (2012), or alternative peaks in the adaptive landscape may be reached as the adaptive peak investigated in this study is based on oversimplistic assumptions of a porous medium mesophyll.”  
  
L252-253. Have alternative ideal distributions been tested, considering leaf structures like vein density, trichomes and substomatal cavity volume? Excluding these factors in estimations seems to be a significant omission. It seems problematic that other important leaf features, such as tortuosity, are not considered in the estimations but are discussed in the text. Again, I don't think the authors need to account for them, but please clarify the limitations of not doing so.

Thanks again for noticing this. A sentence in the last paragraph of the ms sums up these ideas nicely:

“To understand why stomata are not ideally dispersed, more modeling (with a more complex set of assumptions including vein density and IAS structure) should be done to estimate the fitness gain of stomatal dispersion.”