Title of Paper: Analyzing chlorophyll fluorescence images in PlantCV

Reviewer Comments:

This paper presents a novel update on the PlantCV that can be used for other interesting purposes. PlantCV is a fabulous tool and the add-ons to PlantCV for quantification of photosynthetic parameters have potential to be widely used. Given that PlantCV is an open-source software package, it is an accessible tool for quantifying plant growth parameters. The add ons provide a relatively easy combination of photosynthetic parameters with plant growth. The data described shows robust quantification of NPQ, Fv/Fm and Fq'/Fm' in two sorghum genotypes using a commercially available PAM fluorescence imaging system.
While the study is well written and data is sound, I have the following questions and concerns that could be addressed in the text. For point 4 I do not think this needs to be addressed in the text, but is more of a broader point for consideration.

1. Does the software analysis package work with other PAM imaging systems, or only CropReporter? How transferable is the analysis package for those that do not have this equipment?
2. How transferable is the package in other plant species? It would be beneficial to test the package in other species before selling it as a blanket tool for analyzing chlorophyll fluorescence images? What would users need to consider when working in other species, particularly species with contrasting morphology to sorghum with (easily defined large leaves) that are harder to quantify with image analysis such as soybean or rice (more, smaller leaves).
3. The abstract could make clear exactly what parameters are being made available rather than just listing 'photosynthetic parameters based on chlorophyll fluorescence and spectral indices'.
4. With regards to reproducibility, I am not sure if the package can be used with other PAM imaging systems. Further with all analysis packages, they assume the user has a good understanding of data capture, which is not always the case. I understand it is challenging to ensure users are correctly using any open source package and software package creators can not be responsible for ensuring the user has correctly applied fluorescence principles to data capture in this case, but it would be useful to know if there are quality control thresholds in place to ensure fluorescence data capture protocols are followed and the data being input is sound.

The paper details and add on for PlantCV software that offers derivation of plant photosynthetic fluorescence parameters from fluorescence and spectral imaging analysis. There is great need for high throughput and non destructive quantification of photosynthetic parameters in order to meet goals for improved crop photosynthetic efficiency for increased global food production. The ability to quantify whole plant productivity linked with plant growth parameters in this way is

sought after. Bringing together crop growth and photosynthetic parameters is a challenge and the PlantCV software streamlines this process with an open source tool. PlantCV and the add-ons described in this work could be widely used and beneficial to the plant research community. An expanded paper could be a fit for a special issue of TPPJ.

Response to reviewers

We thank the reviewers for their comments and suggestions. Below we address the questions that the reviewer raised:

1. **Does the software analysis package work with other PAM imaging systems, or only CropReporter? How transferable is the analysis package for those that do not have this equipment?**
   The chlorophyll fluorescence analysis part of the subpackage is compatible other PAM imaging systems. One addition that is missing to fully bridge the gap for additional image file formats from other systems is that if they differ from the Phenovation format, they would need a helper function to read in and stack data into x-array formats. Full support for this is coming soon (in PlantCV version 4).

2. **How transferable is the package in other plant species? It would be beneficial to test the package in other species before selling it as a blanket tool for analyzing chlorophyll fluorescence images? What would users need to consider when working in other species, particularly species with contrasting morphology to sorghum with (easily defined large leaves) that are harder to quantify with image analysis such as soybean or rice (more, smaller leaves).**
   The PlantCV tools are transferable to other plant species. We have analyzed both top-down and side-view images of tobacco and maize in addition to sorghum using this package. Others have also validated CropReporter data analyzed with the photosynthesis subpackage using more established photosynthesis meters such as LICOR. Adding this additional data would be beneficial for clarifying the broad applicability of these new tools. Our updates provide better segmentation and allow users to do segmentation on whichever frame gives the best contrast in their unique dataset. As long as the contrast from the background is sufficient (which is usually the case in chlorophyll fluorescence), a good segmentation will allow for analysis regardless of leaf size or shape. Individual species differences that arise due to plant morphology such as camera distance and resolution are certainly issues to take into consideration but have more to do with imaging practices than software.

3. **The abstract could make clear exactly what parameters are being made available rather than just listing 'photosynthetic parameters based on chlorophyll fluorescence and spectral indices'.**
   We thank the reviewer for their suggestion and will update the abstract accordingly.

4. **With regards to reproducibility, I am not sure if the package can be used with other PAM imaging systems. Further with all analysis packages, they assume the user has a good understanding of data capture, which is not always the case. I understand it is challenging to ensure users are correctly using any open source package and**

software package creators cannot be responsible for ensuring the user has correctly applied fluorescence principles to data capture in this case, but it would be useful to know if there are quality control thresholds in place to ensure fluorescence data capture protocols are followed and the data being input is sound.
Informative error messages are generally implemented as we come across specific examples of where users may make mistakes. However, it is true that the analysis of these specific signals expects the images to conform to qualities (such as range of values, matching size of frames, metadata about frames including labels) which can be incorrectly collected. As part of the analysis, the package does output some quality control plots, such as fluorescence induction curves (if multiple measurement frames are provided); however, we do acknowledge that this type of imaging requires more base knowledge compared to other types. We will certainly consider the addition of more quality control steps to ensure appropriate image capture.