

Analyzing chlorophyll fluorescence images in PlantCV

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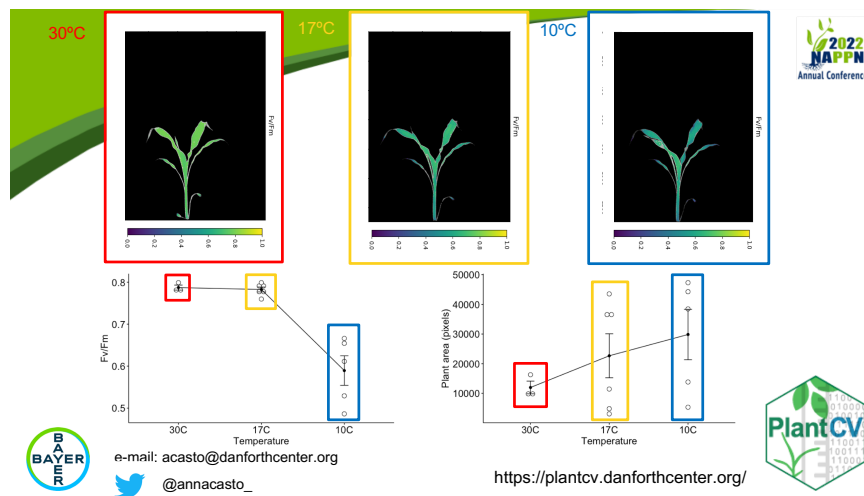
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November 30, 2022

Abstract

Whole plant chlorophyll fluorescence imaging is a powerful tool for non-destructive analysis of photosynthesis. Analysis of such images requires software that is able to process and calculate photosynthetic parameters per plant pixel. PlantCV is an open-source, Python-based library of image analysis tools for plant science. Previous versions of PlantCV included tools to analyze photosynthetic efficiency data, but recent developments to the photosynthesis subpackage have expanded to include more photosynthetic parameters based on chlorophyll fluorescence and spectral indices. This paper highlights the newest updates to the photosynthesis package of PlantCV and discusses applications of these tools on a sorghum dataset that was imaged with a PhenoVation CropReporter system.



Analyzing chlorophyll fluorescence images in PlantCV

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ABSTRACT

Whole plant chlorophyll fluorescence imaging is a powerful tool for non-destructive analysis of photosynthesis. Analysis of such images requires software that is able to process and calculate photosynthetic parameters per plant pixel. PlantCV is an open-source, Python-based library of image analysis tools for plant science. Previous versions of PlantCV included tools to analyze photosynthetic efficiency data, but recent developments to the photosynthesis subpackage have expanded to include more photosynthetic parameters based on chlorophyll fluorescence and spectral indices. This paper highlights the newest updates to the photosynthesis package of PlantCV and discusses applications of these tools on a sorghum dataset that was imaged with a PhenoVation CropReporter system.

Keywords: phenotyping, chlorophyll fluorescence, sorghum, computer vision, photosynthesis

1. INTRODUCTION

Chlorophyll fluorescence imaging is a useful tool in plant biology for non-destructive measurements of a plant's photosynthetic performance [1]. Parameters calculated from chlorophyll fluorescence states have been used as an indicator of plant health [1]. For example, dark-adapted measurements of photosystem II (PSII) efficiency (F_v/F_m) are generally consistent in healthy plants, usually falling around 0.8 [1]. Reduced F_v/F_m indicates possible damage to PSII subunits and reduced photosynthetic efficiency. Other parameters such as the operating efficiency of PSII (F_q'/F_m') and nonphotochemical quenching (NPQ) can also be measured to study PSII photochemistry and to better understand the effects of environmental stress on photosynthesis [1]. Chlorophyll fluorescence measurements have been used for many years at the leaf level, but whole plant fluorescence imaging gives spatial information that can show different responses of different parts of the plant, giving a fuller picture of stress response [2].

PlantCV is an open-source Python library of image analysis tools that is focused on enabling flexible user-defined analysis workflows, parallelizable image processing for fast and high-throughput analysis, and implementation that lowers the barrier to community participation [3], [4]. Many fluorescence imaging platforms have built-in software for analysis of chlorophyll fluorescence images, but PlantCV can be used to pull out additional information about plant morphology (size, shape, etc.) from the same image dataset and image workflows can analyze image data in parallel. Here, we describe the updates to the photosynthesis subpackage of PlantCV and the added tools for analysis of chlorophyll fluorescence images, and we illustrate the functionality of these new features in a dataset of sorghum images.

2. MATERIALS AND METHODS

Sorghum genotypes Nui Sheng Zi (NSZ, PI 568016) and BTx623 (PI#) have been demonstrated to differ in chilling tolerance, with NSZ being the more tolerant genotype [5]. Both genotypes were planted in a 50/50 mixture of C/V Pro-Line (Jolly Gardener) and Turface (Turface Athletics) and were grown at 30°C with 400 $\mu\text{mol}/\text{m}^2/\text{s}$ light and 60% humidity for 2 weeks then transferred to 12°C with 400 $\mu\text{mol}/\text{m}^2/\text{s}$ light and 60% humidity for 2 weeks.

Chlorophyll fluorescence images were taken of three replicate plants of each genotype before and after chilling treatment using the Pulse-Amplitude-Modulation technique [1] on a CropReporter (Phenovation Life Sciences). Images were taken from the side view. Plants were dark adapted for 20 minutes prior to imaging. The CropReporter first takes dark-adapted images then light adapts the plants for 10 minutes before taking light-adapted images. The “Save All Frames” option was selected since this is essential for downstream analysis with PlantCV. Analysis of all images was done in PlantCV v4-beta. Plotting was done in R using `ggribes` (<https://cran.r-project.org/web/packages/ggribes/>) and `ggpubr` (<https://cran.r-project.org/web/packages/ggpubr/>), and statistical analyses were done in R (version 4.0.3). Histogram distributions were statistically compared using the two-sample Kolmogorov-Smirnov test.

3. RESULTS AND DISCUSSION

Recent developments to the PlantCV photosynthesis subpackage include new functions to extract additional photosynthetic parameters from chlorophyll fluorescence image series and updates to existing functions to be more consistent with current terminology.

3.1 Read_croreporter Function

The updated ‘`read_croreporter`’ function from PlantCV version 4 returns just one data object which contains all the frames found by having users point the function to the .INF file (a text file with metadata regarding the imaging protocol and available image frames) rather than reading in data files directly. The output data are stored within X-Array (<http://xarray.pydata.org/en/stable/>) DataArrays, which are labeled arrays so that frames are identifiable by downstream PlantCV photosynthesis or spectral functions. The edits that came with this update include compatibility with photosynthetic imaging and the existing spectral index functionality.

3.2 Reassign_frame_labels Function

Chlorophyll fluorescence analyses are performed on dark-adapted or light-adapted plants to obtain measures of photosynthetic efficiency [1]. After a saturating pulse of light in the dark-adapted state or actinic light in the light-adapted state, sequential images are taken to capture the fluorescence induction curve. The ‘`reassign_frame_labels`’ function plots the fluorescence induction curve from all imaging frames and assigns labels to the maximum fluorescence (F_m or F_m') frames.

3.3 Analyze_yii Function

The ‘`analyze_yii`’ function calculates and plots histograms of F_v/F_m and F_q'/F_m' from dark- and light-adapted images, respectively. The function was renamed from `analyze_fvfm` to this more general name since the functionality was extended to handle image data from both protocols. The additional input parameter called “`measurement_labels`” ensures that users can differentiate between the dark- and light-adapted protocol after analysis.

3.4 Analyze_npq Function

Analysis of NPQ of PSI is enabled with the ‘`analyze_npq`’ function. This calculates $(F_m/F_m') - 1$ from a defined area of the image where F_m is the maximum fluorescence level in the dark before the actinic light pulse and F_m' is the maximum fluorescence during the light pulse. This function, as with others from the photosynthesis subpackage, are dependent on the `PSII_Data` instance file structure that is created while reading in data with the `read_croreporter` function that is described in section 3.1.

3.5 Optional spectral indices

In addition to chlorophyll fluorescence imaging, the CropReporter also optionally supports measurement protocols for spectral imaging, and these data are used to create a multispectral dataset in PlantCV using existing hyperspectral tools. The hyperspectral and `spectral_index` sub-packages were integrated into PlantCV prior to the photosynthesis package overhaul, and finding another method by which these

functions can prove useful aligns with the PlantCV mission of interoperability. The number of indices that are possible to calculate within PlantCV are theoretically limitless since the flexibility of the software allows for users to define and analyze their own indices and other custom measurements within a parallelized workflow. In the case of datasets from the CropReporter imaging system it is possible to examine NDVI (Normalized Difference Vegetation Index) [6], GDVI (Green Difference Vegetation Index) [7], ARI (Anthocyanin Reflectance Index) [8], CI red-edge (Chlorophyll Index Rededge) [9]. Different photosynthesis imaging protocols have different numbers of frames output, so this approach allows analysis functions to more intuitively select relevant frames from a combined stack of image data.

3.6 Photosynthetic parameters of sorghum under chilling temperatures

Photosynthesis is negatively affected by chilling temperatures in sorghum [10]. The sorghum genotype Nui Sheng Zi (NSZ) has previously been characterized as chilling tolerant based on measures of biomass, germination, emergence, seedling vigor at 10 - 15 °C [5], [11]. This genotype was contrasted with BTx623, which has been characterized as chilling sensitive. The seedlings were dark adapted before imaging. Both dark- and light-adapted images were taken with the CropReporter before and after 2 weeks at 12°C.

All frames were read in using the ‘read_cropreporter’ function. The F_0 frame was selected to construct a mask because it minimized measurement errors along the leaf edges caused by leaf movement that was visible in the later frames. A binary mask was constructed by setting a threshold on the greyscale F_0 image. Following several mask clean-up steps, the binary mask was used in all subsequent analysis steps. The ‘reassign_frame_labels’ function was used to visualize the fluorescence induction curves and select the dark- and light-adapted frames with maximum fluorescence. Finally, the ‘analyze_yii’ and ‘analyze_npq’ functions were used to calculate F_v/F_m , F_q'/F_m' , and NPQ. In addition to chlorophyll fluorescence measurements, this image analysis workflow also included functions to analyze plant shape and size (analyze_shape).

Before treatment at 12°C, F_v/F_m histograms of both genotypes center around 0.8, which is typical of healthy plants [1]. After 2 weeks at 12°C, F_v/F_m values of most of the plant pixels are significantly reduced and the spread of the histogram is much wider. This indicates that photosynthetic efficiency is reduced in nearly all plant pixels in both genotypes. NSZ appears to maintain slightly higher F_v/F_m values compared to BTx623, but the distributions of F_v/F_m values after the chilling treatment was not significantly different between BTx623 and NSZ ($p = 0.281$; Figure 1A). F_q'/F_m' was equally reduced in both genotypes (Figure 1B). Histograms of NPQ values across all plant pixels before chilling treatment were quite low, and after the chilling treatment, the spread of the histograms increased slightly (Figure 1C). Again, no differences were observed between the two genotypes after chilling treatment ($p = 0.581$). This indicates that under chilling temperatures, there is a slight increase in NPQ, which can be a protective measure against damage to photosystems from excess light in low temperatures when photochemistry is reduced [1]. The histogram mode values for F_v/F_m , F_q'/F_m' , and NPQ of BTx623 and NSZ in chilling temperatures are typical of other genotypes of sorghum in similar conditions [12]–[14]. The modularity of PlantCV workflows allowed us to add existing functions into this photosynthesis workflow to analyze additional characteristics such as plant shape and size. Both genotypes maintained their relative differences in size throughout the chilling treatment, though NSZ may have grown at a slightly higher rate (Figure 1D). In this small dataset, few statistically significant differences were observed between the two genotypes; however, measurement of much larger datasets is possible with whole plant fluorescence imaging. The addition of these tools to PlantCV enables flexible analysis pipelines that can extract multiple types of data from plant images.

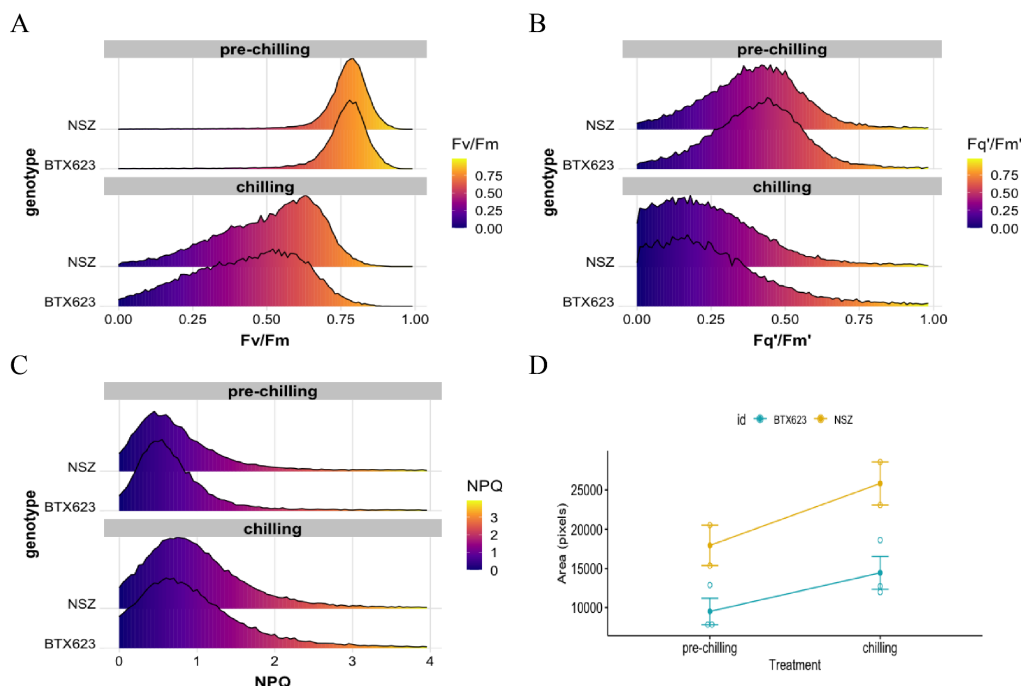


Figure 1. Histograms of (A) F_v/F_m , (B) F_q'/F_m' , and (C) NPQ values of all plant pixels of BTx623 and NSZ before chilling treatment (pre-chilling) and after 2 weeks at 12 °C (chilling). (D) Plant area of BTx623 (blue) and NSZ (yellow) before and after 2 weeks of chilling treatment. Images were taken of 3 biological replicates for each genotype. Histograms are normalized.

4. CONCLUSIONS

In conclusion, these updates to the PlantCV photosynthesis subpackage expand the number of photosynthetic parameters that are possible to extract from fluorescence images using PlantCV. Additionally, functions to calculate spectral indices commonly captured by fluorescence imaging systems have been integrated into the photosynthesis subpackage. The example photosynthetic workflow shown in sorghum seedlings is one of the more straightforward potential applications of these software tools. Modularity of PlantCV workflows includes the ability to label and record multiple observations per image stack means that it is possible to analyze signals from discrete plant organs. For example, the top down imaging of rosette plants shows that photosynthetic efficiency is not consistent across leaves [2]. Single leaf analysis is possible with PlantCV with both automated tools that can identify clusters and region of interest tools, which take more customization but allow for full control over analyzed regions. The integration of these updates with existing PlantCV capabilities provide additional tools and flexibility for the analysis of many kinds of plant image data.

DATA AVAILABILITY STATEMENT

The Python code for the PlantCV photosynthesis subpackage is available on Github (<https://github.com/danforthcenter/plantcv/tree/4.x>). A tutorial of the photosynthesis workflow is available here: https://plantcv.readthedocs.io/en/4.x/tutorials/psII_tutorial/. The image dataset of *Sorghum bicolor* seedlings is publicly available on figshare (<https://figshare.com/s/e700a628159ffb11660>).

ACKNOWLEDGMENTS

This work was supported by the Donald Danforth Plant Science Center ERAC Institute Fund and grants from the United States Department of Agriculture National Institute Food and Agriculture (2020-67034-31901 to A.C., 2021-67013-33778 to M.A.G., and 2019-67021-29926 to M.A.G. and N.F.).

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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.