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.

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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 µmol/m^2/s light and 60% humidity for 2 weeks then transferred to 12°C with 400 µmol/m^2/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 ggridges (https://cran.r-project.org/web/packages/ggridges/) 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_cropreporter Function

The updated 'read_cropreporter' 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_cropreporter function that is described in section 3.1.

3.5 Optional spectral indices

In addition to chlorophyll fluorescence imaging, the CropReproter 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_lables' 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.

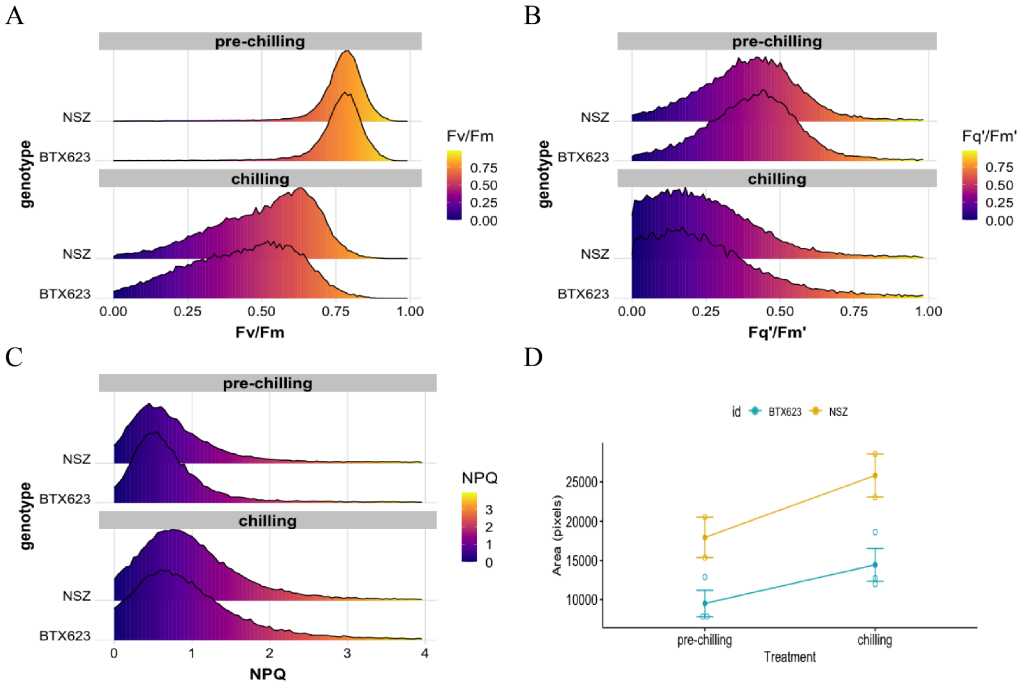

Figure 1. Histograms of (A) F_v/F_m, (B) F_q'/F_m', and (C) NPQ values of all plant pixels of BTx623 and NSZ before chilling treatment (pre-chilling) and after 2 weeks at 12 °C (chilling). (D) Plant area of BTx623 (blue) and NSZ (yellow) before and after 2 weeks of chilling treatment. Images were taken 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/e700a628159ffbf11660).

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