Temporal Hyperspectral Imaging of Corn Seeds During the Germination Process

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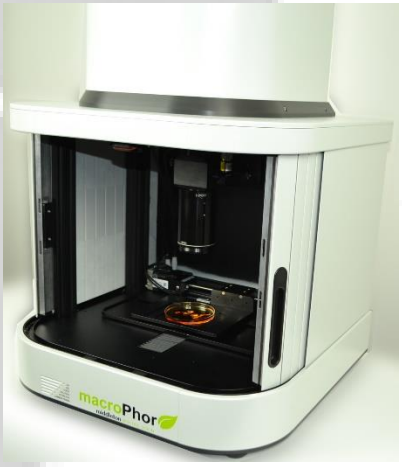
Introduction/Background

Information about seed quality can be determined by understanding the statistics of the seed germination process for a particular seed genotype. For example, the percentage of seeds that fail to germinate and the average length of time the seeds take to germinate are parameters of interest. Researchers are also interested in understanding how growing factors, such as amount of moisture, temperature, and light affect the germination process. Techniques that can improve the ability to study and understand the seed germination process will be important for plant physiologists trying to improve crop production. Fluorescence hyperspectral imaging may provide earlier and more reliable indications of seed germination prior to visible physical signs apparent once the seed begins to sprout.

We investigated the germination of corn seeds over a 4-day period with the Middleton Spectral Vision MacroPhor™ Fluorescence Hyperspectral Imaging System. The MacroPhor Hyperspectral Imaging System is well suited for this application, providing easy and fast image acquisition. The MacroPhor has powerful analysis tools that are used to extract out valuable information about the seed based upon the spectral diversity present in the collected hyperspectral Image. This study utilized six different genotypes of corn seeds (11 seeds in total). The hyperspectral images collected over the course of the study were analyzed using Multivariate Curve Resolution (MCR) to reveal the unique fluorescence signatures, their distribution, onset of their appearance and to quantify the relative intensities of those signatures. The analysis illustrates the type of information that can be extracted and applied to study the germination process. Larger studies with the use of control seeds (seeds that don't germinate) may also help determine the spectral signatures or combination of spectral signatures that provide the most information in regards to seed quality.



MacroPhor™ Fluorescence Imaging System

Methods

Fluorescence imaging has been a valuable tool for studying plant material because it can easily excite and detect the photosynthetic pigments present within the plant [1, 2, 3]. The MacroPhor Hyperspectral Imaging System is optimized for macro-scale scanning of plants or plant related material. Image collection begins by illuminating the specimen under investigation with line illumination from a laser oriented along the specimen's x axis (see Figure 1). The fluorescence emission from this line illumination is collected in 180degree geometry and is directed to an

imaging spectrograph equipped with a sensitive sCMOS spectral camera. The spectrograph disperses the fluorescence along the Y axis of the spectral camera sensor. Hence, a single collected fluorescence line sent to the imaging spectrograph gives information about the spectral diversity at each spatial point on the collected line. Successive image lines are collected and sent to the spectral camera to build up a full image of the specimen by translating the specimen in the y direction via an automated stage in a push broom manner. This push-broom line imaging system excites the sample with either of two available laser wavelengths, 405 or 488 nm, and collects the emission in the 430-800 nm or 460 – 800 nm region, respectively. For this study, a 488nm laser light was used. The end result of the image collection is the generation of a hypercube of data that contains information **related to spatial axies x,y, and the spectral axis** (wavelength). The detected laser line for this study was approximately 6 mm wide (typical scanning line is about 22-25 mm) with a pixel width of 7 microns in sample space. The spectral resolution for this system was approximately 2 nm (typical resolution is approximately 8 nm). Due to uncertainty in emission intensities as a function of germination time, the laser power and integration time was set at the beginning of the study to fill the 12 bit dynamic range of the sCMOS sensor (16 bit high dynamic range setting is also available) set to high sensitivity mode. In addition, image data was collected with and without an emission filter. A red emission filter was placed in front of the camera to reduce and remove the emission from the more intense non-chlorophyll wavelength region ($\lambda < 650$nm). With this filter in place, more laser light could be delivered to the sample without saturating the spectral camera at these wavelengths [4]. By delivering more laser light, we increased our sensitivity for the earliest signs of chlorophyll within the seed. This collection mode upweighted and concentrated the imaging on the chlorophyll spectral region (Figure 1).

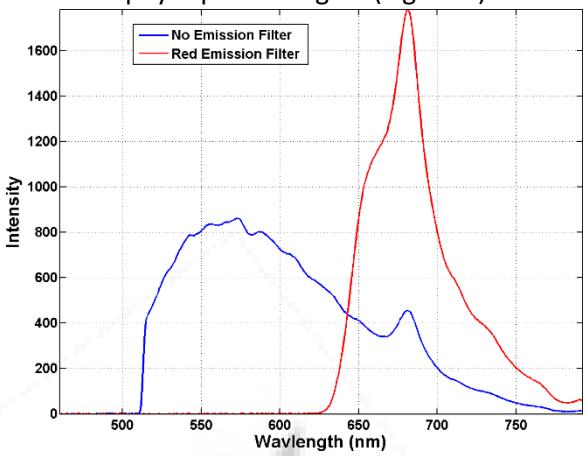
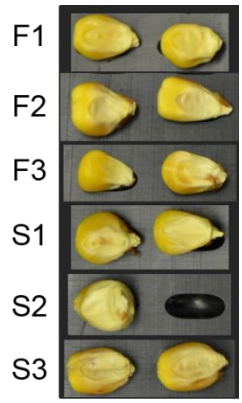


Figure 1: Mean spectrum of the same pixels with and without the use of a red emission filter in front of the spectral camera. Notice that a notch filter for both arrangements cuts out light below 510 nm to prevent laser light from reaching the sensor.

Although this method section describes the specific configuration of the system for this particular study, the system is also adaptable to other configurations, e.g., type of laser excitation, spatial resolution, etc.

Corn Seeds and Experiment



Corn seeds obtained from the Botany department at the University of Wisconsin. and included six different genotypes, three of which were known to germinate relatively fast (Fn) and three known to germinate slower (Sn).

Two seeds of each type were imaged, except for "S2" where only one seed was available. The seeds were kept on a loosely covered agar plate in an office environment (typically about 70 degrees F with mostly artificial fluorescent lighting and periods of darkness). For imaging activities, the seeds were transferred (using

forceps) to an anodized metal plate to avoid specular reflections due to the agar base.

Seeds were imaged twice during each session at times 0, 10, 27, 52 and 76 hours after placing the seeds onto agar base to begin the germination process. The first image was acquired with lower laser power and no emission filter, and then a second image with higher laser power with the red emission filter. Extra exposure of the seeds to the laser light source was minimized to avoid photo-bleaching as much as possible; focus and sample alignment activities were conducted with a broadband light source or with minimal laser power.

Line scan images were acquired in a stop-and stare manner such that the corn seed was stationary during the capture of each line of the image. Seeds were typically imaged in pairs (two seeds of each genotype). The image of one pair of seeds spanned about an inch of sample space; each such scan included about 230 image lines and were acquired in less than one minute.

Image Preprocessing and Data Analysis

Prior to the data analysis, the spectral data were preprocessed to remove any excess baseline offset present in the data. The spectral region between 460-480 nm is dark due to the light blocking notch filter and is used to quantify the offset to be removed. At each pixel, the mean intensity of the dark region is calculated and then removed from each wavelength in the spectrum [5]. In addition, saturated pixels (spectral intensities equal to or greater than 12 bits (4096 counts) are found and removed from the image prior to the Multivariate Curve Resolution (MCR) analysis step.

MCR is a multivariate spectral analysis technique that is capable of determining extracting all independently varying spectral signatures present in the hyperspectral image data and their corresponding intensities [6, 7, 8]. To ensure that MCR identified extracted the most representative emission signatures present in the corn seeds, all images collected from the entire study were combined and analyzed together. Since the red emission filter changed the shape of the spectral intensities, the data collected with and without the emission filter were analyzed separately.

Results

No Emission Filter Results

The MCR analysis results for the hyperspectral corn seed images without the emission filter extracted 4 major fluorescence emission spectral signatures (Figure 2). The first is the signature of *Chlorophyll-a*. The other three signatures are related to fluorescence species emanating from other parts of the seed (e.g., endosperm, germ, seed-coat).

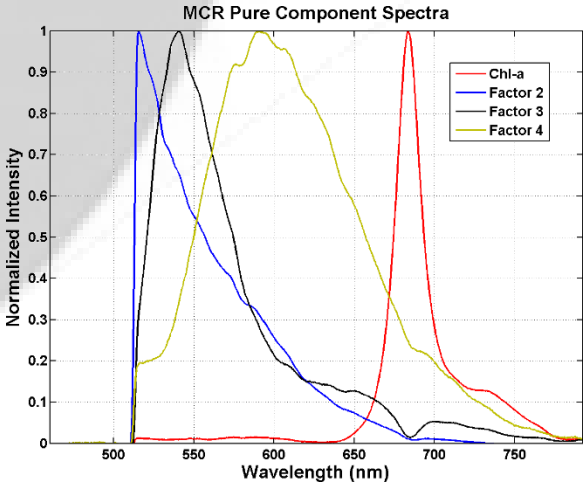


Figure 2: Extracted spectral signatures without using the emission filter.

Although it is difficult to fully assign factors 2-4 to specific areas of the seed, some factors are present in some areas of the seed more than other areas. For example, factor 2 shows up in the tip cap and the embryonic root. Factor 3 shows up more intensely in the endosperm of the corn seed. Factor 4 was present throughout

the seed, but was sometimes more concentrated in the germ.

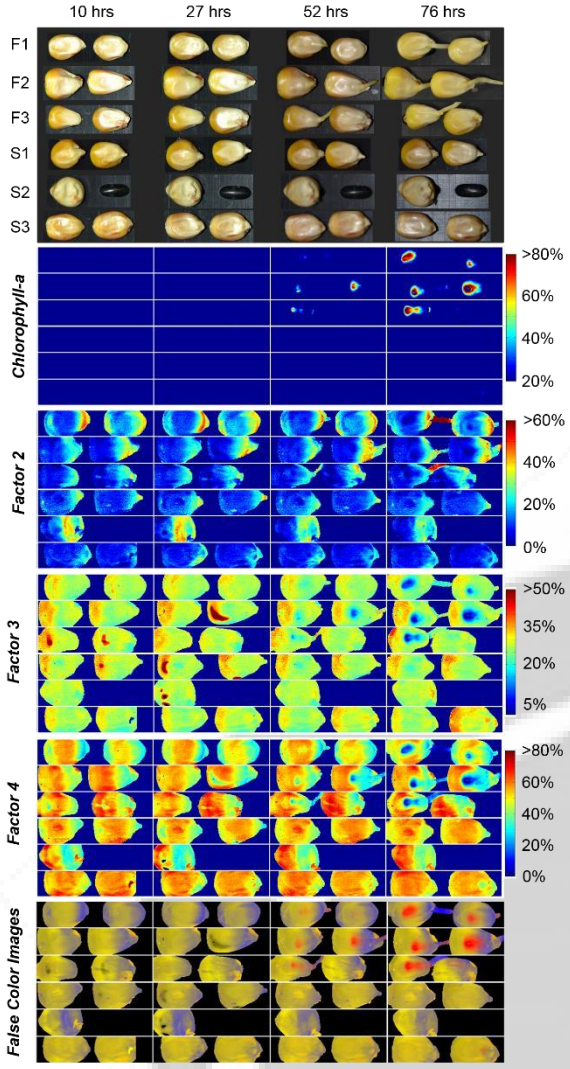


Figure 3: Top photo shows the seeds as a function of germination time and seed type followed by the corresponding percent intensity images for each factor. The final set of images are false color images, colored as follows: Red = Chl-a, Yellow = Factor 6?, Blue = Factor 4.

Figure 3 shows the percent relative intensity images for each spectral signature at each image pixel of each seed. The rows of Figure 3 represent the seed types (F1 – F3, S1 – S3). The columns represent the different imaging sessions (times). Although the seeds were imaged at time zero (prior to the seeds being placed onto the agar base) no significant differences were observed no additional information was seen in the results compared to the 10 hour images, therefore these images are not shown. Due to the high emission intensity of chlorophyll, some of the image pixels saturated, especially after 76 hours of germination. These pixels were removed from the analysis and this is the reason why the percent intensity is shown as zero inside some of the brightest chlorophyll regions of the images. These pixels were added back in at 100% to create the false colored images. Notice that chlorophyll is seen in almost all of the fast germinating seeds after 52 hours. Only a small amount of chlorophyll can be seen in one slow germinating seed after 76 hours (seed 2 of S3). It should be noted that all 11 seeds from both the fast and slow germinating seed genotypes did finally germinate. Unfortunately, no specific trends were identified with the spatial or temporal distribution of the non-chlorophyll spectral signatures (factors 2-4). The use of controls (or non-germinating seeds) would have helped elucidate the spectral signatures associated with non-viable seeds.

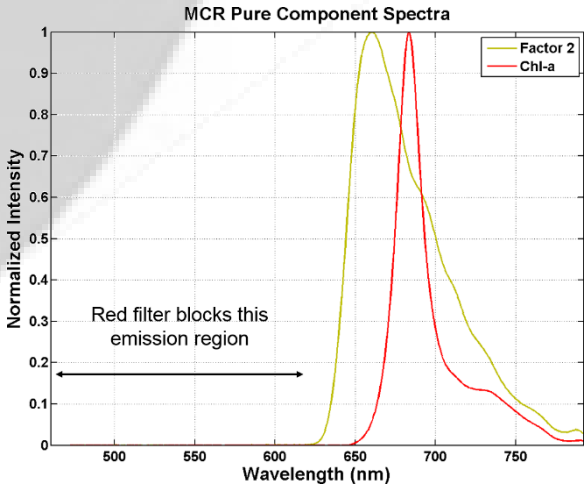


Figure 4: Spectral signatures with the red emission filter in place.

Red Emission Filter Results

The MCR analysis for the hyperspectral corn seed images with the red emission filter in place extracted 2 major emission spectral signatures (Figure 4). Recall, the red emission filter was

inserted to improve the sensitivity for chlorophyll detection by preventing the other more intense fluorescence species from saturating the CCD when additional laser power was delivered to the seed. Based on this relationship, we can view factor 2 as a composite signature of the tails of the other fluorescence species (factors 2-4) in figure 2.

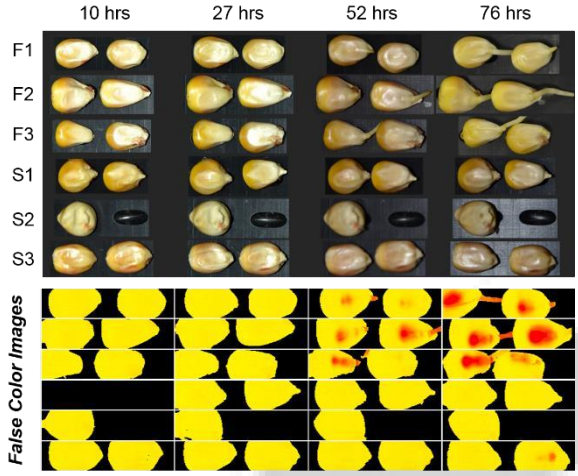


Figure 5: Top: Seed photos, Bottom: false color images, colored as follows: Red = Chl-a, Yellow = Factor 2.

The false color images (assigning red to chlorophyll and yellow to MCR factor 2 image pixels) in Figure 5 were generated from the percent intensity images of the two factors. Since there are only two factors, where there is no chlorophyll, factor 2 intensities are represented as 100%. These images truly focus on where and how much chlorophyll is present in each seed. (Note, image data with red filter was unavailable for the S1 sample at 10 hours.) Although the same conclusions can be drawn with respect to when each seed first presents chlorophyll, an indication that the seed has successfully germinated, the higher sensitivity afforded by using the red emission filter greatly aids in the early detection of chlorophyll. Chlorophyll can be seen in several seeds prior to any visual sprouting signs: F1 seed 2 at 52 hours, F3 seed 2 at 52 and 76 hours, and S3 seed 2 at 76 hours.

Conclusions

The use of hyperspectral imaging can aid researchers studying different genotypes of seeds and factors which may affect the germination process of these seeds. We were able to identify four unique fluorescence signatures found in these corn seeds. These signatures highlight specific areas of the seed and may allow greater understandings of the germination process overall. The use of the red emission filter effectively concentrated the imaging directly on chlorophyll emission. Although this study was focused on seed germination, fluorescence hyperspectral imaging can be used to understand other seed and plant processes and imaging applications.

References

1. Pedroso, M. C.; Sinclair, M. B.; Jones, H. D. T.; Haaland, D. M., Hyperspectral Confocal Fluorescence Microscope: A New Look into the Cell. Microscopy Today 2010, 18, (05), 14-18.
2. W.F.J. Vermaas, J.A. Timlin, H.D.T. Jones, M.B. Sinclair, L.T. Nieman, S. Hamad, D.K. Melgaard, and D.M. Haaland, "In vivo Hyperspectral Confocal Fluorescence Imaging to Determine Pigment Localization and Distribution in Cyanobacterial Cells," Proceedings of the National Academies of Sciences, 105, 4050-4055 (2008).
3. A.M. Ruffing, H.D.T. Jones, Physiological effects of free fatty acid production in genetically engineered Synechococcus elongatus PCC 7942, Biotechnology and Bioengineering, 109 (2012) 2190-2199.
4. R.W. Davis, H.D.T. Jones, A.M. Collins, J.B. Ricken, M.B. Sinclair, J.A. Timlin, S. Singh, Labelfree measurement of algal triacylglyceride production using fluorescence hyperspectral imaging, Algal Research, Volume 5, July 2014, Pages 181-189, ISSN 2211-9264, http://dx.doi.org/10.1016/j.algal.2013.11.010.
5. H.D.T. Jones, D.M. Haaland, M.B. Sinclair, D.K. Melgaard, A.M. Collins, and J.A. Timlin, "Preprocessing Strategies to Improve MCR Analyses of Hyperspectral Images", Chemometrics and Intelligent Laboratory Systems, 117 (2012) 149-158.
6. D.M. Haaland, H.D.T. Jones, M.H. Van Benthem, M.B. Sinclair, D.K. Melgaard, C.L. Stork, M.C. Pedroso, P. Liu, A.R. Brasier, and N.L. Andrews, "Hyperspectral

Confocal Fluorescence Imaging: Exploring Alternative Multivariate Curve Resolution Approaches," Applied Spectroscopy, 2009; 63: 271-279.

7. D.M. Haaland, J.A. Timlin, M.B. Sinclair, M.H. Van Benthem, M.J. Martinez, A.D. Aragon, M. Werner-Washburne. Multivariate curve resolution for hyperspectral image analysis: Applications to microarray technology. In: Levenson RM, Bearman GH, Mahadevan-Jansen A, editors. Spectral imaging: Instrumentation, applications, nd analysis. San Jose, CA: 2003 SPIE. p 55–66.
8. H.D.T. Jones, D.M. Haaland, M.B. Sinclair, D.K. Melgaard, M.H. Van Benthem, and M.C. Pedroso, "Weighting hyperspectral image data for improved multivariate curve resolution results," Journal of Chemometrics, 2008; 22: 482-490.