**Protocol for ImageJ scanning of Caracoles plant images.**

These are instructions for measuring the amount of diseased tissue on photos of whole plants collected at the Caracoles site in the Parque Nacional de Doñana, Spain, in 2016, in a collaborative project with Oscar Godoy, Ignasi Bartomeus, Ingrid Parker, and Gregory Gilbert.

We use the open-access software ImageJ, available for download from the National Institutes of Health at <https://imagej.nih.gov/ij/index.html>. The instructions below are for use on the Macintosh OS X, but the software is available on numerous platforms.

The goal is the measure the total number of pixels in the image that are leaf and stem material of the plant, and then measure the number of pixels that are disease tissue in the plant. Disease tissue may be yellow (chlorosis), brown/black (necrosis), or sometimes red/maroon (anthocyanin response to the pathogen). In this last case, care must be taken not to measure the red (anthocyanin) color that some plants show normally on their stems or underside of the leaves as diseased tissue. For this reason, it requires human judgement in the process to get the best data.

These methods are a work in progress, and we might find simplifying tweaks while using them.

On the very last page of this document is a space for you to add your comments and thoughts about improvements or needed clarification.

Any questions, please contact Greg Gilbert at [ggilbert@ucsc.edu](mailto:ggilbert@ucsc.edu) +1-831-459-5002.

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Gregory S. Gilbert, Ph.D.

Professor and Chair, Department of Environmental Studies

439 ISB, 1156 High St., University of California, Santa Cruz, CA 95064, USA

greggilbertlab.sites.ucsc.edu ferp.ucsc.edu scwibles.ucsc.edu +1.831.459.5002

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Si tú no usas la cabeza, otro por ti la va a usar. Rubén Blades

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| Open ImageJ, and then open the jpg file you want to analyze using File>Open or Command-O  Screen Shot 2016-12-13 at 3.22.05 PM.pngOr, to open from the Google Drive, double click on the image so it appears in the browser. Right-click and select Copy Image. In ImageJ, Select File>New> System Clipboard (or Shift-Command-V). |  |
| Use the select Rectangular tool to create a box around the plant leaves and stem, but do not include roots, flowers, or fruits.  Screen Shot 2016-12-13 at 3.36.51 PM.png  Alternatively, use the Polygon selection or Freehand selection tool if you need finer control than the rectangle. It is not a problem to include the blue background.  Screen Shot 2016-12-13 at 3.38.19 PM.png | Screen Shot 2016-12-13 at 3.35.51 PM.png |
| Select Image>Crop or shift-command-x to crop the image to only the desired part.  Screen Shot 2016-12-13 at 3.39.39 PM.png | Screen Shot 2016-12-13 at 3.40.48 PM.png |
| Open the Threshold Color toolbox, and set Threshold color to White and Color Space to RGB  Screen Shot 2016-12-13 at 3.42.53 PM.png | Screen Shot 2016-12-13 at 3.44.07 PM.png |
| Adjust the Red sliders to 0 and 255.  Adjust the Green sliders to 0 and 255  Adjust the top Blue slider to 0.  Adjust the bottom blue slider to where the entire plant, but not the background, turns white. Probably 130-170. | Screen Shot 2016-12-13 at 3.46.51 PM.png |
| Click on the Select button, to create an outline of the entire plant area. | Screen Shot 2016-12-13 at 3.47.56 PM.png |
| Click back on the Image window or the toolbar so that the menu bar appears again. Choose Analyze>Measure (or command-m) to measure the area of the entire plant.  Screen Shot 2016-12-13 at 3.50.05 PM.png  The measured values will appear in the Results window.  Transfer this Area value to the CaracolesScanData spreadsheet for the appropriate plant under Total Pixels. | Screen Shot 2016-12-13 at 3.51.31 PM.png  Only the Area measurement is useful; this is the number of pixels covered by the entire plant tissue. Because the images are not all size registered, we use pixels to calculate area diseased.  Screen Shot 2016-12-13 at 3.55.28 PM.png |
| To measure diseased tissue, go back to the Threshold Color toolbox. Look at the image of the plant, and see where there is diseased tissue. Adjust the sliders so that only those diseased areas are highlighted in white.  This is a trial and error process, but start by changing the Color Space to HSB, and adjusting the Hue and Brightness sliders. Be careful not to include normally red parts of plants (like the stem here) as diseased tissue. You can also try the other Color spaces - depending on the symptoms sometimes RBG or Lab work well.  This simple approach works on about half the plants, and depending on the quality of the image. See below for what to do when it isn’t that easy. | Screen Shot 2016-12-13 at 4.02.43 PM.png |
| Once it looks like you have highlighted all the diseased tissue and not the healthy tissue, click the Select button on the Threshold Color toolbox, and then Analyze>Measure (command-m) to measure the number of pixels of diseased tissue. | Screen Shot 2016-12-13 at 4.05.56 PM.png |
| Transfer that Area measurement for diseased tissue to the CaracolesScanData sheet under Diseased Pixels. Add your Name. | Screen Shot 2016-12-13 at 4.09.08 PM.pngYou can calculate the percent of tissue diseased. |
| **When it isn’t that easy…** About half the time is not possible to get very different diseased tones to appear in the same selection -- say yellow chlorosis and black necrosis. In this case it is easier to measure different colors of diseased tissue independently, and then add them up.  To do so, use the Oval tool to draw a small circle over diseased tissue of a particular color, including only that color. Then click on the Sample button on the Threshold Color panel. All the areas like that should turn white, but not other areas. If it looks appropriate, click Select, then command-m to measure it and send that area to the Results. Repeat for other diseased tissues.  Add up those Area values and put that value into the DiseasedPixels column in CaracolesScanData | |  |  | | --- | --- | | Screen Shot 2016-12-14 at 11.04.59 AM.png | Screen Shot 2016-12-14 at 11.05.06 AM.png | | Screen Shot 2016-12-14 at 11.04.38 AM.png | Screen Shot 2016-12-14 at 11.04.44 AM.png | |
| **If the color sliders fail you…** you can trace the area you want to measure. Use the Freehand tool to trace around the area you want to measure. Screen Shot 2016-12-15 at 12.42.35 PM.png. Then click command-m to measure that area. Add that to the diseased area in the spreadsheet. | Screen Shot 2016-12-15 at 12.43.51 PM.png |
| Close the image. **Be sure to select Don’t Save!**  Otherwise, it will overwrite the image with your various color filters. | Screen Shot 2016-12-13 at 4.12.51 PM.png |

**ADD YOUR COMMENTS, OBSERVATIONS, AND SUGGESTIONS HERE**

When attempting to analyze the diseased tissue: select desired diseased tissue and use that as a “sample.” This should highlight most of the diseased tissue for you, but you will still have to fine tune the selected tissue to ensure correct selection. In color threshold, use brightness, hue first and use the saturation last. If all desired tissue is not not selected or some undesired tissue is selected, begin to play around with the sliders to get the best match. But make sure to go in that order, I found this to work best.