I – Overview of the application

The goal of this application is to segment images of plant leaves inoculated with a pathogen or mock inoculated. The procedure contains three independent parts.

The Expert-based thresholding approach consists in the segmentation of the images using fixed thresholds previously determined by trained raters.

The Model-based thresholding approach consists in the segmentation of the images modeling the pixelwise distribution as a mixture of Gaussian distributions. Each Gaussian distribution represents a stage of alteration of plant tissues.

The Probability-based thresholding approach consists in the segmentation of the images using thresholds defined on the control samples. First, a threshold corresponding to a quantile, i.e. the value splitting the pixelwise distribution of mock-inoculated samples in two parts is defined. Second a clustering approach is performed to delimit various stages of alteration of plant tissues.

The application calculates the percentages of diseased tissues and/or the number of pixels representing the diseased tissues using each approach. Images are then recolored according to the various areas segmented using the thresholds.

II – Prerequisites

The application is written in R language. R (http://www.r-project.org/) and several packages must be installed on your computer before running the application:
stringr (http://cran.r-project.org/web/packages/stringr/index.html)
mclust (http://cran.r-project.org/web/packages/mclust/index.html)
EBImage (http://www.bioconductor.org/packages/2.12/bioc/html/EBImage.html)
fpc (http://cran.r-project.org/web/packages/fpc/index.html)
reshape2 (http://cran.r-project.org/web/packages/reshape2/index.html)
tcltk2 (http://cran.r-project.org/web/packages/tcltk2/index.html)

This application was written to analyze Fv/Fm images but it also can treat other kind of images. However, if non-Fv/Fm images are treated, some functions of the application will not work. In this case, you can extract the functions written on the top of the script and adapt them to your experiment.

To quantify the symptomatic area, the application needs a pixelwise distribution of the intensity of each pixel. All the possible values taken by the intensity is indicated in the first column. The number of pixels taking each value is indicated in the second column (see example).

0	0
0.001	1
...	
0.999	0
1	3

To recolor the images, the application needs an image in grey levels and in *.tiff*. The grey levels must represent the measured parameter. For example, Fv/Fm can take values between 0 and 1. The associated grey levels image is take intensities between 0 and 1.

To use this application, respect the file tree as used in the directory “Example”. The names of the pixelwise distribution and of the associated image have to be the same. If a kinetic analysis is performed in order to quantify the shrinking of the leaves, the names of a leaf have to be the same for each measurement.

III – Run the application

1 – Run the script in R
2 – Answer the questions in the pop-ups
3 – The approach used and the file that is analyzed are written in the console

IV - Results

Expert-based thresholding: Once the calculations performed, you will find a file named “Results_Expertbasedthresholding.xls”. This file indicates the number and/or the percentage of pixels segmented for each image.
Column 0.25: percentage or number of pixels displaying Fv/Fm ≤0.25 – Column 0.45: percentage or number of pixels displaying Fv/Fm values >0.25 and ≤0.45 – Column 0.65: percentage or number of pixels displaying Fv/Fm >0.45 and ≤0.65. Diseased area: total diseased area (percentage or number of pixels displaying Fv/Fm ≤0.65).

Probability-based thresholding: Once the calculations performed, you will find files named “Results_Probabilitybasedthresholding.xls” with a number. The number corresponds to the various thresholds.
Column Threshold: the values of the thresholds used. Column Diseased area: percentage or number of pixels representing the total diseased area – Column Nb of clusters: number of clusters found by the clustering into the pixelwise distribution of the diseased area – Columns G: percentage or number of pixels representing each stage of alteration of plant tissues – Column Out of clustering: percentage or number of pixels that cannot be classified in a cluster – Column parameter: is “moy” if the row indicates the mean of the cluster, “prop” if the row indicates the percentage or number of pixel.

Model-based thresholding:
Column Nb of clusters: number of clusters found by the clustering - Columns G: percentage or number of pixels representing each stage of alteration of plant tissues – Column threshold : indicate the threshold used for the discrimination between clusters representing healthy tissues and clusters representing diseased tissues – Column parameter: is “moy” if the row indicates the mean of the cluster, “prop” if the row indicates the percentage or number of pixel, “var” if the row indicates is the cluster is “healthy” or “diseased”.

The .xls file(s) beginning by “Means_” indicate the mean number and/or percentage of pixels segmented for each condition.

The file “recoloration.pdf” gather all the recolored images using each approach.

V – Run the example

Bean leaflet cv. Flavert inoculated with *Xanthomonas fuscans* subsp. *fuscans* strain CFBP4834-R were imaged at 0 day after inoculation (dai), 7 dai and 11 dai.
Piwelwise Fv/Fm-distributions and Fv/Fm images are in the directory names "fluorescenceFv/Fm". If you want to compare the results of the thresholding with visual observation, you will find the scans of each leaf in the directory "conv_color_images".

1 – Select a directory: a window is open. Enter into the directory "fluorescenceFv/Fm". It should show several directories to analuze (0 dai, 7 dai and 11 dai). Click OK
2 – Which approach(es) do you want to run? : You can select all the approaches or not
3 – How many thresholds do you want to use? : Indicate the number of thresholds or clusters to use for each approach
In this example, as bean leaflet cv. Flavert inoculated with *Xanthomonas fuscans* subsp. *fuscans* strain CFBP4834-R, select 3 thresholds for the Expert-based thresholding.
4 – Which Expert-based thresholds do you want to use? : Indicate the thresholds
In this example, as bean leaflet cv. Flavert inoculated with *Xanthomonas fuscans* subsp. *fuscans* strain CFBP4834-R, select 0.25, 0.45 and 0.6.
5 – Which Probability-based thresholds do you want to use? : Indicate the thresholds
Example: with a threshold of 1/500, there is a probability of 0.002 to misclassify a healthy pixel
6 – Do you want to calculate the shrinking of the leaves? : Answer yes or no
In this example, a kinetic analysis was not performed, so click no
7 – You want the result in terms of: select percentage or number
8 – Do you want to recolore the images? : This option will create the "recoloration.pdf" file. This file will show the images colored according to the thresholds
9 – What is the name of the control? : Indicate the exact name of the control. Respect the case.
In this example, use h2o
10 - Did you extract the pixelwise Fv/Fm-distributions from FluorCam? : pixelwise Fv/Fm-distributions extracted from FluorCam are not readable by R. A function in included in the script to make it readable.

As each approach produces independent files, you can read the results of the end of the run.

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