Image Processing Method for Epidermal Cells Detection and Measurement in *Arabidopsis Thaliana* Leaves

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Abstract. *Arabidopsis thaliana* is the most important model specie employed for genetic analysis in plants. As it has been extensively proven, the first pair of extended leaves and its cellular and morphological changes during *Arabidopsis* development, is and accurate model to understand the molecular and physiological events that control cell cycle progression in plants. Nevertheless, cell analysis on leaves depends significantly on images acquired from a microscopy coupled to a drawing tube, where cells are traced by hand for posterior digitalization and analysis. This process is tedious, inaccurate and highly temporally inefficient. A new image processing method for cell detection in leaves of *Arabidopsis thaliana* is presented. Using complementary image processing techniques, we introduce a good way to obtain the original cell contour shapes, surpassing the limitations given by factors like noise, stomata, blurred edges, and non-uniform illumination. Results show the new methodology minimizes considerably the time of cell detection compared with the microscopy coupled tube method, and produces matching percentages over 80%.

Keywords: *Arabidopsis thaliana* · Cell drawings · Epidermal cells image detection · Image analysis method

1 Introduction

In the model plant *Arabidopsis thaliana*, the analysis of epidermal cells belonging to the first pair of extended leaves is a fairly widespread technique in the scientific community that uses this model plant to understand different biological phenomena, since it provides truthful information, easily quantifiable and comparable in relation to the phenotypic response of the plant to physiological stimuli, environmental alterations and to genetic modifications directly induced in the plant genome. Thus, *Arabidopsis* is

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an accurate model to understand the molecular and physiological events that control cell cycle progression in plants [1].

Given that a good percentage of the academic community that does research in plants uses *Arabidopsis thaliana* as a biological model of experimentation, and in addition, given that the analysis of epidermal cells in this model plant is a generalized technique for obtaining biological information that can support a particular biological phenomenon, actually the *Arabidopsis Thaliana* epidermal cells quantification and characterization is a highly laborious and temporarily inefficient process because this methods are based in free hand drawings performed using an optical microscopy coupled to a tube drawing where cells are traced by hand for posterior digitalization and analysis, which is clear from recent academic publications still describing the drawing-based methodology [2–7].

The generation of a specific application that can replace the drawings of epidermal cells made upon leaves structure, turns into a great technical facilitator for the rapid collection and analysis of images that can sustain or distort a specific hypothesis. Additionally, an automated tool for epidermal cells analysis will deliver trustable results about cell number and area in a reduced period of time and with more reproducible results.

In our knowledge, currently, there is not software or plug-in, available to the scientific community, that performs the cellular detection of *Arabidopsis* epidermal cells based on pictures. Some applications described in [8–12], shows detection strategies for different kind of cells; each one depends of image acquisition and image properties. However, no one of these methods consider images with similar properties of *Arabidopsis* epidermal cells images. The present research presents a new method to detect *Arabidopsis thaliana* epidermal cells improving precision, consumed time and reproducibility of the results compared with the manual technique.

2 Methodology

2.1 Samples Processing and Image Acquisition

For the acquisition of abaxial cell surface of epidermal tissue from *Arabidopsis thaliana*, plants with 21 days of post-germination growth (DAG) were used. Plants were processed by immersing the first real pair of leaves overnight in acetone and transferred to a 1:9 acetic acid: 100% ethanol solution afterwards. Subsequently, cleared leaves were stored in lactic acid for microscopy. Epidermal cells were photographed under an optical microscope Carl Zeiss AXIO-Lab-A1 with a 10X magnification coupled to a Carl Zeiss-AxioCam ERc 5s camera. Forty images with a resolution of 2560 $\times$ 1920 pixels were acquired (see Fig. 1) and employed for testing. Ten of them were randomly chosen to validate the generated method for epidermal cells detection. The ground-truth of two images was traced by hand by a specialist.

2.2 Algorithm Development

For the automatic detection of epidermal cells based on leaves pictures, here we propose a multistep technique that includes two filtering processes to eliminate the undesired structures of the leaf and the acquisition noise, a contrast enhancement procedure, an edge detection step and the implementation of some editing tools. As a final result an application written in Java, compatible with the freely available ImageJ software, was developed.

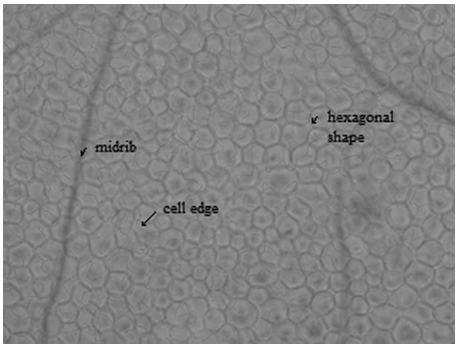


Fig. 1. Sample of microscopy *Arabidopsis Thaliana* image.

Figure 2 depicts the different steps that were implemented for the development of the method for automatic epidermal cells detection.

In the first step the acquired image is pre-processed to reduce noise, which is composed of undesired physical structures of the leaf epidermis and the inherent acquisition noise. In order to eliminate these undesired structures a morphological filtering is done. Given that these structures have a stronger signal than the wall cells, we look for eliminating the wall cells instead, so the undesired noise remains in the resulting images. Then, wall cells are obtained by just subtracting the filtered image from the original one. Given that cell walls are seen as thin dark lines (see Fig. 1), they are eliminated by applying a morphological opening [13], where the side of the structural element is wider than the thickness of the walls. Figure 3 shows the image obtained after filtering with a structural element of radius 2.

Once the undesired structures have been eliminated, it is observed than the cell intensity is quite low, therefore the image is treated in such a way that the contours of the cell walls can be accentuated, since, being a very weak signal, it must be conditioned for the detection process to ensure high detection performance. Therefore, a contrast adjustment is applied using the CLAHE (Contrast Limited Adaptive Histogram Equalization) technique [14], which equalizes the image by regions increasing the intensity difference between the background and the walls. Figure 4a illustrates the result of adjusting the contrast (CLAHE) on Fig. 3.

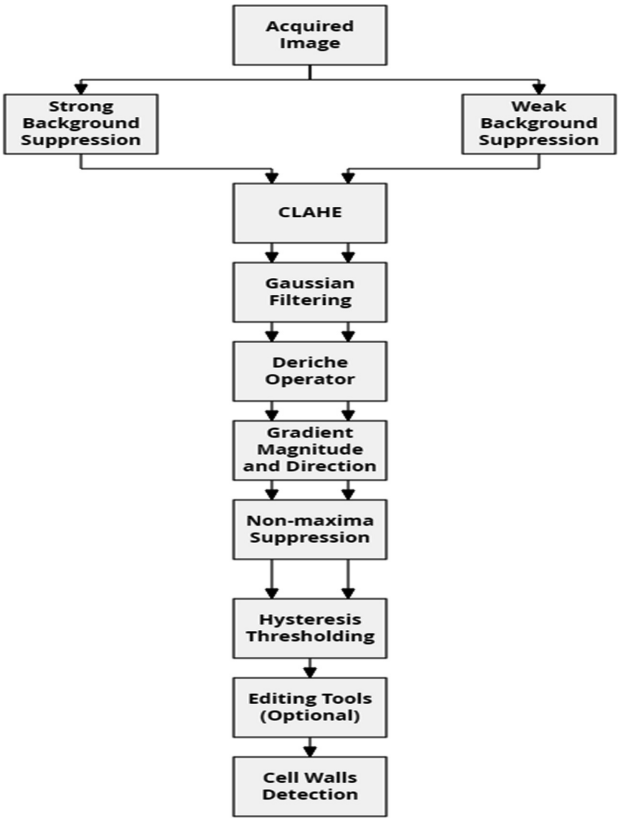


Fig. 2. Proposed algorithm.

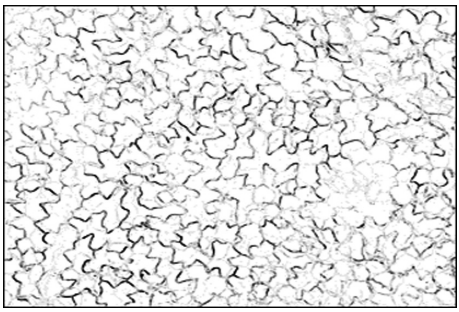


Fig. 3. *Arabidopsis* image after subtracting the background (Contrast was adjusted for visualization).

With the morphological filtering noise due to undesired structures is greatly attenuated, but the acquisition noise still remains. To eliminate it a Gaussian filter is used, given that it provides noise reduction without introducing ringing artefacts. The

standard deviation of the filter was 2; enough to eliminate the acquisition noise without notably blurring the cell walls. Figure 4b depicts the results after Gaussian filtering is applied.

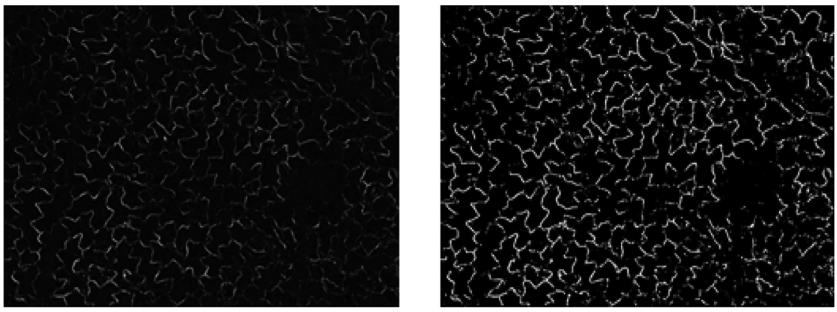


Fig. 4. (a) Enhanced contrast by using the CLAHE method, (b) Gaussian filter (r = 2).

Although a non-linear edge-preserving smoothing filter was tested [15], it did not improve the quality of the following segmentation. At this point the pre-processing stage ends.

Edge operators are appropriate to detect cell walls, given that they appear as thin lines in a clear dark background. Under this idea, the Deriche operator is used; which is still considered in the state of art [16]. This operator is based on the Canny principle, but has a better behavior in order to get image segmentation [17]. In this way, the derivatives for the x and y coordinates are obtained and shown in Fig. 5a, and b respectively.

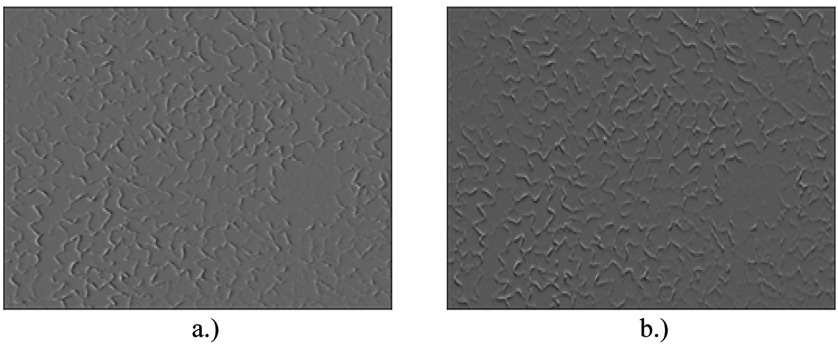


Fig. 5. (a) Derivate *x,* (b) Derivate *y.*

The Deriche operator provides the gradient of the image but does not deliver edge pixels as such. Thus, it is necessary to employ contour selection techniques to find the

cell walls. Then the selection of contours proposed by Canny is employed [18]. It consists of two stages: non maxima suppression and hysteresis thresholding.

The suppression of pixels that do not correspond to a local maximum makes possible to eliminate all those pixels that are not part of the true border. To do this, the magnitude and direction of the gradient are calculated and results are depicted on Fig. 6a and b.

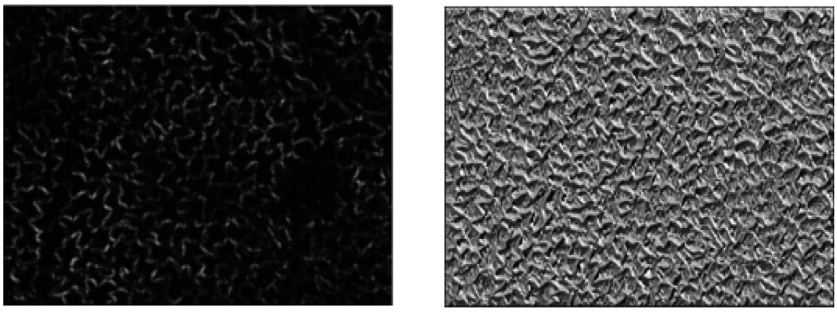


Fig. 6. (a) Gradient magnitude, (b) Gradient direction.

For each pixel, the magnitude of the gradient to both sides of each pixel to a distance d in the direction of the gradient of the pixel is evaluated, and compared against the magnitude of the pixel (see Fig. 6b). Those pixels identified as local maxima, that is, when the gradient magnitude is greater than both of its neighbors, are retained, eliminating those others.

As displayed in Fig. 7a, the edges corresponding to the cell walls are better defined; however not all pixels correspond to the true edge. Therefore, a hysteresis threshold is applied, in which two thresholds are defined, one high, and one low. Thus, when the image is evaluated, the pixels that exceed the high threshold are classified immediately as true edge pixels, and those connected to them, which magnitude gradient exceeds the low threshold and are located in the perpendicular direction to the direction of the gradient at that point, are also classified as true, the remaining ones are eliminated. To ensure good classification, the chosen high and low thresholds were 20 and 250 respectively. These values were obtained experimentally by exploring in the histogram of the images the average gray level ranges of the edge pixels. Figure 7b illustrates the result of the hysteresis thresholding with low value of 1 and high value of 30.

As shown in Fig. 7b, most of the cellular contours were detected; however some edges are still missing. Some of the missing borders are caused by the presence of the undesired physical structures at the leaf that are overlapped to the cell walls. Therefore, in the first step where these structures were removed, the overlapped cell walls were also removed. In order to recover as many missing edges as possible, the following strategy is proposed: considering the original image that contains the whole information about the edges, it is possible to start from it again in order to recover the missing information connected to the borders already found. Therefore, it is necessary to use

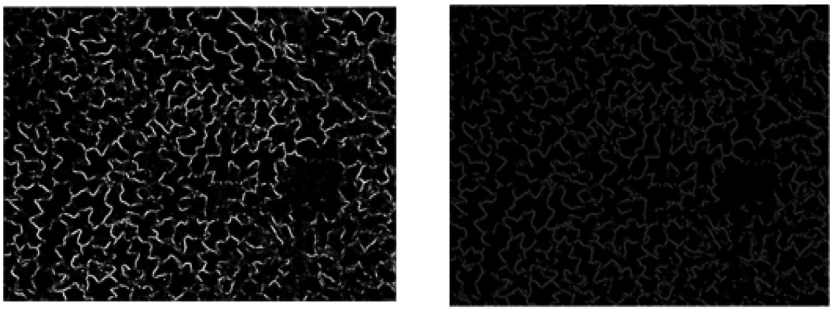


Fig. 7. (a) Non-maxima suppression, (b) Hysteresis thresholding.

different pre-processing parameters to get weak noise reduction, i.e., more noise is present, but also more edges are retained.

Therefore, the original image is smoothed again but using a weaker filter, i.e., a bigger structural element, allowing having more information about the cell walls, but also more noise. Figure 8a illustrates the result of subtracting the background from the original figure with a radius value of 10. Then, the same steps previously proposed after filtering are applied until the non-maxima suppression.

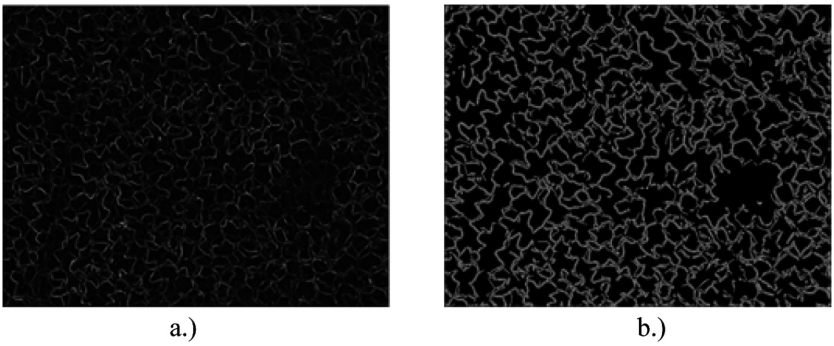


Fig. 8. (a) Weak background subtraction, (b) Final hysteresis thresholding result

As it can be seen in Fig. 8a, comparing with Fig. 4a, many edges, previously missing, appears now in the image, but also some objects that are not part of the cell contours. The edges detected in Fig. 7b are now used to extract the missing ones from Fig. 8a, while rejecting the undesired leaf structures. This is done, based on the functional principle of the hysteresis thresholding, because the magnitude and direction of the true edge pixels, found in Fig. 7b are now used to select from Fig. 8a those pixels connected to the true edge pixels, perpendicular to the gradient direction and similar magnitude, while rejecting false cell edges. Thus, the result obtained recovers

edge pixels lost during the first process. Figure 8b illustrates the final hysteresis thresholding of the process with a low value of 15 and high value of 250.

Although the proposed method is quite efficient, some contours can still be missing because some edges are diffused due to poor focus or presence of stomata. Thus, in order to give to the user the possibility of manually editing the results of the detection of epidermal cells in *Arabidopsis thaliana* leaves, the developed software *Arabidopsis* Leaf Cell Detection (ALCD), includes some editing tools for the modification of results after detection, as well as a cell characterization function that allows to obtain the number of cells and their area and label each cell with a different color according to its label or area.

In order to edit the images the following steps are suggested: first the walls are thinned. Before edge thinning an iterative dilation process is repeatedly applied until the double contour of the cell walls is connected. Once the dilatation is done, a thinning or skeletonization technique is applied to the image, in which each edge contour get one pixel width. This tool facilitates the process of cells labeling.

The next step consists in debugging the detected borders with the objective of completing those contours of cells that were impossible to recover by the proposed method, or where the image presents a focus or illumination problem and removing those lines or small branches connected to edges that resulted from the thinning process. Once the image is prepared, the cell analysis tool from the user interface is applied, so each cell is represented with a unique label value that identifies it, and assigned a color that differentiates it from the rest in order to facilitate visualization. Subsequently a table is obtained including the corresponding tag number for each cell and its corresponding area in pixels. The number of tags relates the approximate number of cells in the image. Figure 9 illustrates the result obtained by operating the cell analysis tool.

Fig. 9. Labeled image. (Color figure online)

Table 1 specifies the output format of the interface data, where the column on the left represents the label of the cell and the one on the right its area in pixels.

Table 1. Label and cell area specification

Label	Area (pixels)
0	93788
1	202371
2	6014
3	3945
4	4830

3 Method Validation

To validate the method ten *Arabidopsis thaliana* leaves images were chosen with different physical characteristics, belonging to the group of samples acquired. As is shown in Fig. 10, each of the images taken for evaluation has different physical characteristics allowing us to demonstrate the performance of the proposed method. The detection of the cell contours provided satisfactory results even in presence of non-uniform illumination or blurry regions resulting from the acquisition. The edges that cannot be recovered by the development of the method correspond to those in which undesired structures appear quite accentuated, where there is not presence of undesired objects, are completed and differentiable, showing that better acquisition conditions of the samples, means better edge segmentation. On average the necessary time to detect epidermal cells under our approach is 7 ± 1.5 min.

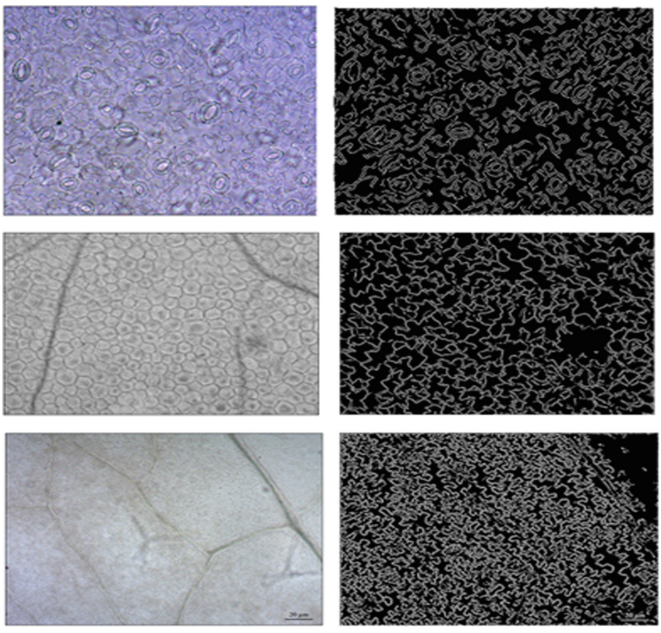


Fig. 10. Experimental result for different acquired images.

Two measures were taken into account to validate the method: percentage of time optimization used in detection and percentage of edge matching of cell detected with corresponding ground truth images. The ground truth images were generated by tracing manually the pixels that corresponded to *Arabidopsis Thaliana* cell walls from two acquired images. With the percentage of optimization, it is possible to describe a quantitative notion of the total time improvement that is used to get a satisfactory detection of the epidermal cells, between the proposed method in relation to the time demanded for the same purpose but through the classic freehand drawing method.

According to the records studied from specialized personnel in this area, the average time taken to draw a significant number (100–200 cells) of epidermal cells, varies from one hour to two hours. The calculation of the percentage of time optimization follows the next expression:

$$t_{op}(\%) = \frac{t_m - t_{prom}}{tprom} \times 10 \tag{1}$$

Where t_m corresponds to the total employed time in the proposed method, including both the processing time and editing time, and t_{prom} is the average time spent in the freehand detection methods.

Complementarily, the matching percentage is determined by the number of pixels that correspond between the resulting images of the developed method and the ground truth. The ground truth image refers to the result of manually tracing the cellular contours of the same processed image, in which it is ensured that the drawn edges are correct. The matching percentage is calculated as follows:

$$m(\%) = p/c \times 100 \tag{2}$$

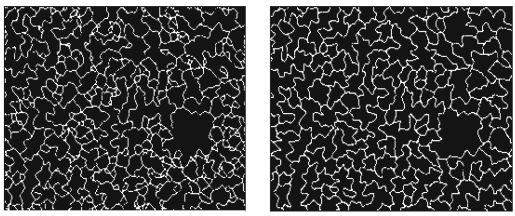
Where p is the number of pixels in the processed image that correspond spatially to those in the ground truth, and c refers to the total number of edge pixels of the ground truth image.

For validation purposes, two samples from the previously analyzed images were evaluated measuring the time demanded during the detection of the cellular contours, and their correspondence of edges with each ground truth. Table 2 summarizes the percentages obtained for each case. The validated samples are illustrated in Fig. 11; the image on the right corresponds to the result of the proposed method, and the one on the left to its corresponding ground truth.

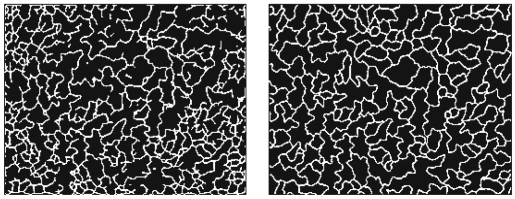
Table 2. Percent detection time optimization and matching of true edges with ground truth

Validation	$t_{op}(\%)$	$m(\%)$
1	91.66	86.11
2	90	80.4

As it is shown in Table 2, the percentages of optimization in time and matching are quite high, so it is verified that the proposed method does offer improvements in the



a.) Image validation 1.



b.) Image Validation 2

Fig. 11. Validation images.

time consumption for detection of cellular contours in microscopic images of *Arabidopsis thaliana*, compared to the tedious and inefficient freehand tracing techniques commonly used for these purposes.

4 Conclusions

For cell cycle analysis in *Arabidopsis thaliana*, characterization of epidermal cells in number and size is one of the most important inputs to take into account in order to properly describe a specific phenomenon. For this reason, this depiction implies that all cells must be detected from the acquired microscopy image. The protocol of acquisition and preparation of the proposed method ensures that each sample is taken under the same conditions, thus reducing the probability of variation from one type of sample to another.

The main problem to carry out this kind of analysis in plants is based on the tedious process of being able to detect all the cells, because it is necessary to use a drawing tube device coupled to the microscope, to trace by freehand each one of the cellular contours. This takes a long time, between one or two hours, so the whole process is inefficient in time and resources. The techniques of digital image processing help to get a good solution to this problem. The proposed method of detecting epidermal cell borders in *Arabidopsis Thaliana* leaves uses complementary techniques such as background subtraction, noise filtering, contrast enhancement, edge detection, non-maximal suppression and hysteresis thresholding, in a strategic way to detect the contours of the cells in just a few minutes, making this process faster than the classical form with freehand tracing, improving the efficiency of cellular analyzes.

Experimental results show that the proposed method detects most of the cell borders, even in the presence of undesired factors such as noise, blurring, non-uniform

illumination, presence of undesired morphological structures as trichomes. It is possible to modify any result and adjust it to the needs of the researcher, thanks to the graphical user interface developed, which provides all the necessary tools for this purpose, making it possible to complement the analysis processes with approximate information of the number of cells and their area.

These results will facilitate the cell cycle associated research, where the methods of tracing cells contours by tubes coupled to the microscope can be replaced, thus facilitating the process of accurately quantifying phenotypic characteristics such as leaf area, number of cells per leaf, leaf cells area and number of stomata, basic but sufficient measures to analyze the phenotypic effect of a particular gene on the control of the cell cycle in this model plant.

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