

Quantification of Callose Deposition in Plant Leaves

Loredana Scalschi¹, Eugenio Llorens¹, Gemma Camañes¹, Victoria Pastor², Emma Fernández-Crespo¹, Victor Flors^{2*}, Pilar García-Agustín¹ and Begonya Vicedo^{1*}

¹Grup de Bioquímica i Biotecnologia, Area de Fisiologia Vegetal, Departament de Ciències Agràries i del Medi Natural, ESTCE, Universitat Jaume I, Castellón, Spain; ²Metabolic and Cell Signaling Group, Area de Fisiologia Vegetal, Departament de Ciències Agràries i del Medi Natural, ESTCE, Universitat Jaume I, Castellón, Spain

*For correspondence: bvicedo@uji.es and flors@uji.es

[Abstract] Callose is an amorphous homopolymer, composed of β -1, 3-glucan, which is widespread in higher plants. Callose is involved in multiple aspects of plant growth and development. It is synthesized in plants at the cell plate during cytokinesis, in several stages during pollen development and is deposited at plasmodesmata to regulate the cell-to-cell movement of molecules. Moreover, it is produced in response to multiple biotic and abiotic stresses (Chen and Kim, 2009). Callose is considered to act as a physical barrier by strengthening the plant cell wall to slow pathogen infection and to contribute to the plant's innate immunity. Thus the callose staining method is useful to quantify activity of plant immunity. In addition, this staining can be used to visualize structures in plant tissue, where the callose may be implied whether during the development of plants or response against pathogen infection. This method is based on the use of methyl blue which reacts with (1 \rightarrow 3)- β -glucans to give a brilliant yellow fluorescence in UV light. Moreover, calcofluor stains chitin present in fungal cell membranes and also binds to cellulose at locations where the cuticle is damaged.

Materials and Reagents

1. Plant materials: *Arabidopsis thaliana* cotyledons and leaves, *Solanum lycopersicum* leaves or Citrus leaves
2. 96% ethanol
3. Na₂HPO₄·2H₂O
4. NaH₂PO₄·2H₂O
5. Methyl blue (Sigma-Aldrich, catalog number: M6900)
6. Sodium phosphate buffer (see Recipes)
7. Methyl blue solution 0.5% and 0.05% (see Recipes)

Optional

1. Trizma[®] base (Sigma-Aldrich, catalog number: T1503)

2. Fluorescent Brightener 28 (synonym Calcofluor white) (Sigma-Aldrich, catalog number: F3543)
3. HCl
4. Tris:HCl buffer (see Recipes)
5. Fluorescent brightener solution 0.01% (see Recipes)

Equipment

1. Leica IRB epifluorescence microscope with UV filter (BP 340 to 380 nm, LP 425 nm) equipped with a Leica DC300F camera (Leica Microsystems, model: Leica IRB)

Software

1. Digital photographs analysis software like GIMP (<http://www.gimp.org>) or Adobe Photoshop (adobe.com) are recommended

Procedure

Note: Staining with fluorescent Brightener is optional. This will stain the chitin present in the sample, and can be useful to stain the fungus. However, the use of this staining can mask the fluorescence emitted by callose. For this reason, if the goal of the experiment is to quantify callose deposition, the use of fluorescent Brightener is not recommended.

A. Staining procedure

1. This procedure can be used for staining *Arabidopsis thaliana* cotyledons and leaves, *Solanum lycopersicum* leaves or Citrus leaves with little variation according to tissue toughness.
2. Sample the entire leaves or the cotyledons from the plant and place them in 50 ml tubes containing 96% ethanol for chlorophyll removal. Distain the samples in 96% ethanol until they appear completely white (time can vary between 1 day for small leaves like the ones of *Arabidopsis* till one week for citrus leaves). The saturated distaining 96% ethanol can be replaced, if necessary.
3. Rehydrate the samples in sodium phosphate buffer for 30 min.
4. Discard the phosphate buffer and cover the sample with 0.05% Methyl blue solution.
Optional: Add 1/5 of fluorescent brightener solution (1 ml per each 4 ml of methyl blue solution used in the previous step).
5. Incubate for 30 min and discard the solution.
6. For *Arabidopsis* cotyledons go to step A8. Callose can be observed after 3-4 h of incubation with 0.05% Methyl blue as previously described by Luna *et al.* (2011).

7. Cover the sample with 0.5% Methyl blue solution and incubate for 24 h in darkness. (Time may vary depending on the sample. Thought samples, such as citrus leaves, may require a vacuum stroke of 2 min and up to 7 days of staining.)
8. For microscope observation, mount the samples on slides with the adaxial surface up using freshly prepared 0.05% Methyl blue.
9. Observe the samples with an epifluorescence microscope with UV filter. Stained callose appears as fluorescent yellow spots (Figure 1).

Note: Be aware to use all the time the same augmentation.

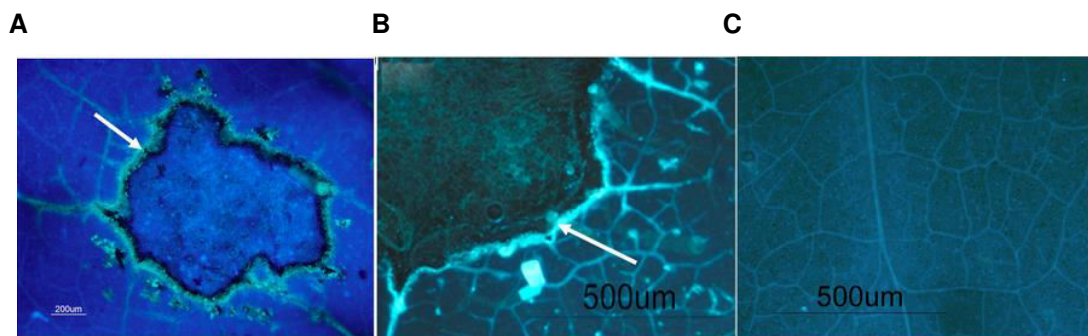
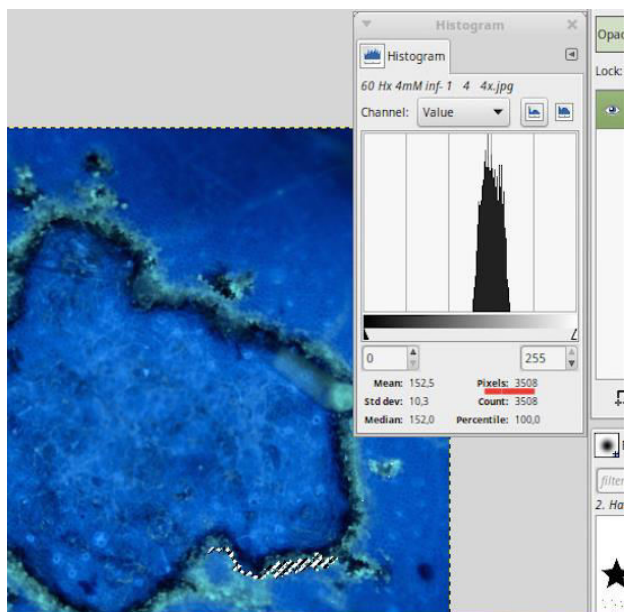


Figure 1. Representative picture of callose deposition in tomato leaves upon *Botrytis cinerea* infection with fluorescent brightener solution (A) and without fluorescent brightener solution (B). The arrow highlights callose. Representative picture of control leaves (C).

B. Image processing

1. Open the images with the photography software (Figure 2).

A



B

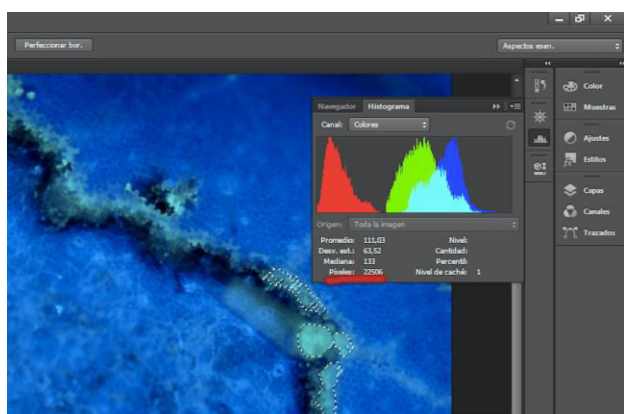
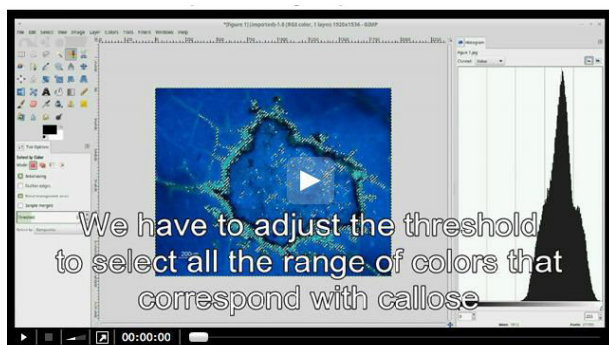


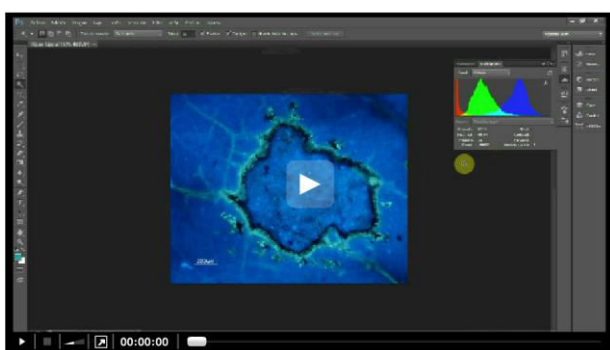
Figure 2. Yellow spots corresponding to stained callose analyzed for the number of pixels using A. GIMP (GNU Image Manipulation Program) or B. Adobe Photoshop

2. Select the brilliant yellow pixels that correspond to callose using a Magic wand tool or select by color range.
3. Use the histogram window to determine the number of pixels.
4. Represent callose intensity as average number of bright pixels/number of total pixels. In the case of cotyledons it is possible to calculate the yellow pixels per leaf area. See Videos for more details.

Video 1. Quantification process using Gimp



Video 2. Quantification process with photoshop



Recipes

1. Sodium phosphate buffer (0.07 M, pH=9)
 - Dissolve 12.46 g of $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ in 1 L of distilled water
 - Dissolve 0.966 g of $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ in 100 ml of distilled water
 - Adjust the pH of $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ solution to 9, using the solution of $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$
2. Methyl blue solution freshly prepared
 - For 0.5% solution: Dissolve 0.5 g of Methyl Blue in 100 ml of sodium phosphate buffer
 - For 0.05% solution: Dissolve 0.05 g of Methyl Blue in 100 ml of sodium phosphate buffer

Optional solutions

3. Tris·HCl buffer (0.1 M, pH =8.5)
 - Dissolve 0.6057 g of Tris base in 50 ml of distilled water and adjust the pH at 8.5 with HCl
4. Fluorescent brightener solution
 - Dissolve 0.0125 g of Fluorescent Brightener 28 in 50 ml of Tris buffer
 - Stored the solution in dark at room temperature

Acknowledgments

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References

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