DETECTION AND QUANTIFICATION OF THE HYPERSENSITIVE RESPONSE CELL DEATH IN *ARABIDOPSIS THALIANA*

Running head: Quantification of HR cell death in Arabidopsis

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Summary

In plants, the hypersensitive response (HR) is a programmed cell death modality that occurs upon recognition of harmful non-self. It occurs at the site of pathogen infection, thus preventing pathogens to live off plant tissue and proliferate. Shedding light on the molecular constituents underlying this process requires robust and quantitative methods that can determine whether plants lacking functional genes are defective in HR execution compared to wild-type controls. In this chapter, we provide two quantitative protocols in which we measure cell death from *Arabidopsis thaliana* leaves infected with avirulent HR-causing bacterial strains. Firstly, we use trypan blue staining to quantify the stained area of leaves upon bacterial infection using a personalised macro in the Image J (Fiji) software. Alternately, we incorporate an electrolyte leakage protocol in order to measure HR caused by different avirulent bacterial strains at different bacterial titers. We encourage users to perform a combination of both methods when assessing HR in different plant genotypes.

Key words

Arabidopsis thaliana, Hypersensitive response, *Pseudomonas syringae* pv *tomato* DC3000, Trypan Blue Staining, Cell death quantification by Image J, Electrolyte leakage.

1. Introduction

As a means of restricting pathogen growth, plants deploy a tightly regulated form of immune cell death at the attempted pathogen ingress site, traditionally known as the hypersensitive response (HR) [1,2]. Upon recognition of harmful non-self, host intracellular immune receptors of the nucleotide-binding leucine rich repeat (NLR) type recognize pathogen effector molecules triggering an amplified immune response named effector-triggered immunity (ETI), which usually culminates in HR cell death [3] . When plant cells undergo HR as a consequence of pathogenic infection, the following hallmarks are generally displayed: cytoplasmic shrinkage, mitochondrial swelling, chromatin condensation, chloroplast and plasma membrane disruption, and vacuolisation [4,5].

A thorough understanding of the molecular players and mechanisms regulating HR-cell death is still lacking. With the advent of the genomic era, numerous HR regulators have been reported [6]. Consequently, robust methods for quantitative quantification of HR cell death are of utter importance to effectively evaluate whether mutations in certain genes renders a plant unable to execute HR.

Trypan Blue staining of infected plant tissue has been extensively used as a qualitative method for visualization of dead cells [7-9]. Since live cells possess intact membranes, the Trypan Blue dye is excluded from the cells, whereas in dead cells the dye transverse the plasma membrane as a consequence of the loss of its integrity [10]. Hence, dead cells are stained and appear in a distinctive blue colour when imaged under a microscope. Subsequently, stained cells can be quantified in order to precisely determine whether differences exist between distinct plant genotypes in terms of HR cell death.

Loss of plasma membrane integrity in dying cells also results in the release of electrolytes to the extracellular milieu. The degree of electrolyte leakage from dying cells can also be used as a readout of the extent to which cell death is taking place in the infected tissue [11]. Currently available conductivity meters allow measurements of electrolyte leakage in relatively small volumes (2 ml), which facilitate accurate and rapid quantification of a larger number of samples.

On the one hand, we provide a detailed method for the quick and automated quantification of cell death using trypan blue staining. For this, we use *Arabidopsis thaliana* plants (Arabidopsis) belonging to the Columbia-0 ecotype (Col-0) inoculated with the HR-causing bacterial strain *Pseudomonas syringae* pv*. tomato* DC3000 carrying the effector *avrRpm1* (*Pto* DC3000 *avrRpm1*) using the syringe-infiltration method. In Col-0 HR is triggered upon recognition of avrRpm1 by the NLR receptor RPM1[12]. Upon trypan blue staining of leaves at different time points after infection, we quantify stained cells in the infiltrated leaves using the image processing package Fiji (built upon the ImageJ2 free software) [13], using a newly developed macro that allows automated quantification of the stained area.

On the other hand, we describe a robust method for quantification of electrolyte leakage of dying cells from Arabidopsis Col-0 leaves infiltrated with both *Pto* DC3000 (*avrRpm1*) and *Pto* DC3000 (*avrRpt2*) using different bacterial titers adapted from a previously described protocol [11]. avrRpt2 also causes HR in Col-0, as this effector is recognized by the NLR RPS2[14]. As a negative control for our experiments, we use the Arabidopsis Col-0 *rpm1-3* and *rps2* mutants, which do not display HR triggered by *Pto* DC3000 (*avrRpm1*) and

Pto DC3000 (*avrRpt2*), respectively, since they are defective in the cognate NLRs RPM1 and RPS2 [12,14]

2. Materials

2.1. Plant material and growth conditions

1. Arabidopsis Col-0 seeds from the following phenotypes: wild-type, *rpm1-3* (N68739) and *rps2* (N6196) from the Nottingham Arabidopsis Stock Centre (NASC) based in the University of Nottingham, UK (*see* **Note 1**).
2. Soil mix: 5 parts peat soil + 2 parts vermiculite + 1 part perlite.
3. A growth chamber with controlled temperature (22^{o}C),-photoperiod (9 h light, 15 h dark), humidity (70% relative humidity) and white LED light intensity of 150 µmoles/m²/s.
4. Small size plastic pots.
5. Flat polypropene trays.

2.2. Bacterial strains, preparation of inoculum and infection.

1. *Pto* DC3000 (*avrRpm1*) and *Pto* DC3000 (*avrRpt2*) avirulent strains (*see* **Note 2**).
2. Solid King's Broth medium (KB medium): For 500 ml: 10 g peptone from meat, 0.75 g K_2HPO_4, 0.75 g $MgSO_4 \bullet 7H_2O$, 5 ml glycerol, 7.5 g bacteriological agar and Milli Q sterilised water.
3. Antibiotics for selection of avirulent *Pto* DC3000 strains (*see* **Note 3**).
4. Microwave.
5. Water bath with adjustable temperature.
6. Resuspension buffer: Autoclaved 10 mM magnesium chloride ($MgCl_2$)
7. 50 and 15-ml centrifuge tubes.
8. Petri dishes.

9. Polystyrene disposable cuvettes.

10. 1 ml needleless syringes.

8. Spectrophotometer.

9. Laminar flow hood.

10. Plastic wrapping paper/plastic dome.

11. Marker pen (black).

2.3. Trypan blue staining and microscopy slide preparation.

1. Stock of trypan blue staining solution: 100 mg phenol (solid), 100 ml lactic acid, 100 ml glycerol, 100 ml Milli Q sterilized water.

2. Trypan blue staining working solution: 1 part trypan blue staining solution + 3 parts 96% ethanol.

3. Destaining solution: 1 kg chloral hydrate dissolved in 400 ml Milli Q sterilized water.

4. Magnetic stirrer with adjustable temperature.

5. Grid cloth mesh.

6. Tilt shaker.

7. Fume hood.

8. Slide preparation: 50% glycerol, fine painting brush, microscopy glass slides and coverslips.

9. Optivisor lenses 3,5x .

10. Clear glue.

11. Microdissection microscope.

2.4. Electrolyte leakage.

1. Scissors.

2. Cork borer.

3. Forceps.

4. Milli Q sterilised water.

5. Sterile 12-well plate.

6. LAQUAtwin EC-11 Conductivity meter (HORIBA Advanced Techno Co., Ltd).

2.5. Quantification of cell death by trypan blue staining and electrolyte leakage.

1. Image J (Fiji) software for trypan blue staining quantification [13].

2. R software for graph plotting of conductivity measurements and statistical analysis.

3. Methods

3.1. Sowing of Arabidopsis seeds and plant growth.

1. Fill small plastic pots with soil peat, vermiculite and perlite mix ($5/1/_{1/2}$). Compress the mix without exerting too much pressure into the pot, place the pots on a middle size flat polypropene tray and wet the mixture to field capacity with tap water.

2. Sow 4-5 seeds in each pot. Fill 6-8 pots per genotype for electrolyte leakage experiments and 8 pots per genotype for a time course of infected leaves (2 plants per time point) stained with trypan blue staining (*see* **Note 4**).

3. Randomize the previously labelled pots on the tray.

4. Cover the tray with plastic wrapping paper or a plastic dome in order to maintain humidity required for germination.

5. Stratify the seeds by placing the tray on a cold room/refrigerator at 4 ^{o}C for 2 days.

6. Transfer the tray to a growth chamber with a photoperiod of short-day conditions: 9 h light/15 h dark (*see* **Note 5**), 22 ^{o} C, 70% relative humidity and light intensity of 150 µmoles/m^2/s.

6. Remove the plastic wrapping paper or dome after 3 days and let the seedlings grow for 5-6 more days.

7. With the help of thin forceps, remove unwanted seedlings from each pot and leave only one seedling growing.

9. Water plants 2-3 times per week without overwatering to avoid stress on the plants.

10. On the second to third week of growth, use a marker pen to mark leaf 8th of the Arabidopsis plant, which will be the one infected (*see* **Note 6**).

11. Four to five-week-old plants grown in these conditions are ideal for bacterial infection by syringe infiltration.

3.2. Preparation of bacterial inoculum and syringe infiltration.

Growth of bacteria in KB medium plates

1. Sterilize a laminar flow cabin by cleaning surfaces with 70% ethanol and switch on the UV light for 5 minutes.

4. Prepare the KB medium and add appropriate antibiotics for selection of avirulent bacterial strains.

5. Pour 25 ml of KB + antibiotics into each plate.

6. Three days before infecting Arabidopsis, streak avirulent bacteria from a -80ºC glycerol stock with a sterile tip. Place the plate on a still 28 ^{o} C incubator. Bacteria will grow after 2 days of incubation.

7. One day before infecting Arabidopsis, collect all bacteria grown on the initial plate and re-streak them on the surface of a new KB plate using a sterile inoculating loop.

Preparation of bacterial inoculum

1. On the day of the infection, add 10 ml of autoclaved 10 mM Mg_2Cl inside the plate and wait 10 minutes in order for the bacteria to detach from the surface of the plate.

2. Re-suspend bacteria with the help of a 10 ml pasteur pipette by gently pipetting up and down in order to detach as much bacteria as possible from the plate.

3. Take 1 ml of bacteria from the plate and mix it with 9 ml of 10 mM $MgCl_2$ in a 15 ml tube.

4. Make a 1:10 dilution in 10 mM $MgCl_2$ and measure bacterial optical density at 600 nm (OD_{600}) using a spectrophotometer. Calculate the volume needed from undiluted bacteria in the previous step and dilute it in 10 mM $MgCl_2$ in order to reach the OD_{600} desired for infection (*see* **Note 7**).

3.3. Trypan blue staining.

1. Label the time point after infection at which each plant leaf will be collected on each pot.

2. Pressure infiltrate the 8th leaf of Arabidopsis plant with avirulent bacteria using a needleless syringe (*see* **Note 8**).

3. After infiltration, gently dry the excess of liquid on the surface of the leaf and collect the leaves corresponding to each time point by cutting through the petiole with the aid of small scissors.

4. Place the leaves in a 50 ml tube containing 15 ml of trypan blue staining working solution. Always work in a fume hood when handling trypan blue staining solution and destaining solution.

5. Pour boiling water into a plastic box and submerge the sealed tubes inside the water for 5 minutes or until the leaves turn blue.

6. Pour the trypan working solution along with the leaves onto a sieve and transfer the leaves carefully with tweezers to a new 50 ml tube.containing 20 ml of destaining solution (*see* **Note 9**). From this step onwards, the leaves will stay in the same tube in order to avoid damage caused by transferring leaves from one tube to another.

7. Let the tubes rotate on a tilt shaker at 80 rpm for 1 hour.

8. Use a mesh grid in order to sieve and discard the destaining solution and replace it with fresh 20 ml destaining solution. (*see* **Note 10**) Let the 50 ml tubes rotate overnight.

9. The following day, sieve the destaining solution using a new mesh grid and add 20 ml of 50% glycerol. Leaves can be stored for prolonged periods in this solution.

3.4. Mounting microscopy slides.

1. Pour the 20 ml of 50% glycerol containing 8 leaves into a petri dish.

2. With the aid of a fine painting brush, gently transfer a single leave onto a microscopy glass slide.

3. Place 500 µL of 50% glycerol on top of the glass slide.

4. Gently expand the leaf on the surface of the glass slide with fine touches using a paint brush (*see* **Note 11**).

5. Once the leaf is correctly expanded on the glass slide, place a coverslip on top of the leaf by gently dropping the coverslip from the top of the leaf to the bottom. Try to avoid bubbles forming in between the leaf and the coverslip (*see* **Note 12**).

6. Gently brush clear glue at the edges of the coverslip so that it adheres to the glass slide and coverslips do not detach.

3.5. Microscopy imaging.

1. Image individual leaves with a microdissection microscope at 5x magnification. Always use the same settings for all samples.

3.6 Quantification of cell death using Image J.

1. Open the image files obtained in the microscope using the Fiji software (image J distribution).

2. Install the cell death quantification macro (See **Note 13**).

3. Select process image for cell death macro and follow the instructions for quantification.

4. Plot the percentage of stained leaf as a function of time (Fig.1)

3.7. Electrolyte leakage assay.

1. Pressure infiltrate the 7th and 8th leaf of an Arabidopsis plant with avirulent bacteria. Four plants per genotype are required for the experiment.

2. After infiltration, gently dry the excess of liquid on the surface of the leaf.

3. Collect the leaves by cutting through the petiole with the aid of small scissors.

4. Place the infiltrated leaves on top of a flat surface and punch out discs (one disc per leaf) using a cork-borer (size 4, diameter = 7.5 mm) (*see* **Note 14**).

5. Immediately after punching out leaf discs, place two leaf discs from a single plant into one well of a 12-well plate containing 2 ml Milli Q sterilised water.

6. Use as many 12-well plates as required depending on the number of genotypes included in the experiment.

7. Cover the plate with the lid and place it on a tilt shaker at 90 rpm for 1 hour (*see* **Note 15**).

8. In the meantime, perform a one-point calibration of the LAQUAtwin EC-11 Conductivity meter (HORIBA Advanced Techno Co., Ltd) using the conductivity standard solution to 1.41 mS/cm.

9. Replace the 2 ml water from the wells with new 2 ml Milli Q sterilised water. Once the water is replaced, a time series of measurements of water conductivity start (*see* **Note 16**)

10. Record water conductivity by pipetting 100 µL of water per well into the conductivity meter. Ions released from dying cells during the course of HR correlate with the conductivity of the solution. The unit used to measure conductivity is microSiemens per centimetre (µS/cm) where cm denotes the distance between the two electrodes sensors of the conductivity meter.

11. Return the water from the device to the well in order to maintain the same volume of water in the wells throughout the experiment (see **Note 17**).

12. Record conductivity at each time point. Meanwhile leave the 12-well plate rotating on the tilt shaker.

3.8. Data representation and statistical analysis.

1. Plot conductivity in µS/cm as a function of time (Fig. 2).

2. For statistical analysis, compare the conductivity (in µS/cm) of two genotypes at a given time point by a two tailed Student's t-test. For comparison of more than one genotype, use a one-way analysis of variance (ANOVA).

4. Notes

1. Whilst NASC distributes seeds to Europe, the Biological Resource Center (ABRC) based at Ohio State University (USA) delivers seeds to North and South America. Laboratories located in other parts of the world may order stocks from either of both stock centres. Arabidopsis Col-0 accessions carry the resistance (R) genes *RPM1* and *RPS2,* which encode for the NLRs RPM1 and RPS2, respectively. In contrast, *rpm1-3* and *rps2* mutants are not equipped with functional RPM1 and RPS2, respectively[12,14].

2. *Pto* DC3000 (*avrRpm1*) and *Pto* DC3000 (*avrRpt2*) avirulent strains overexpress the effector molecules avrRpm1 and avrRpt2, respectively. Plant NLRs RPM1 and RPS2 recognize perturbations in the host cell caused by the aforementioned effectors eliciting an ETI response that is accompanied by HR.

3. For selection of *Pto* DC3000 (*avrRpm1*) and *Pto* DC3000 (*avrRpt2*) in KB media. Kanamycin is added for selection of the construct that carries the *avrRpm1* and *avrRpt2*, whereas resistance to rifampicin comes inherently in *Pto* DC3000. Working concentrations for kanamycin and rifampicin are 50 µg/ml.

4. We recommend including at least 8 pots per time point and genotype in the trypan blue experiment to have robust and consistent results when comparing genotypes that show mild differences.

5. We recommend avoiding walk-in chambers for pathogenesis-related experiments in order to avoid stresses from other pathogens (i.e., insect infestations) that can be present in a chamber where other plants are growing or where users come in and out on a regular basis. We suggest a photoperiod of 9h light/15h dark that resembles short day conditions (8h light/16h dark) but adds an extra hour of light, allowing plants to be at their optimal stage for infiltration earlier than the classical short-day photoperiod (in between the 4^{th} and 5^{th} week of growth). This extended short-day cycle is used by many laboratories working on molecular plant pathology.

6. In order to have comparable results between different plants, we always infiltrate the 7^{th} and 8^{th} leaf of the plant[15]. In this way leaves of comparable developmental stages that may respond similarly to the pathogen are chosen for infiltration.

7. The CFUs/O.D.$_{600}$ (OD_{600}=1.0 correlate to $3.55*10^8$ CFU ml^{-1} determined by serial dilutions and plating) of the bacterial inoculum may be adapted depending on the genotype being infected[16], bacterial strain used in the experiment or time points at which samples are collected after infection.

8. Gently exert pressure on the abaxial side of the leaf with a needleless syringe and infiltrate the leaf thoroughly. If users are not experienced, we recommend practicing beforehand with water on plants that will not be used in the experiment. Besides including mutants impaired in pathogen effector recognition as negative controls (i.e., *rpm1-3* and *rps2*) when available, we encourage users to include leaves infiltrated with 10 mM $MgCl_2$ as an additional negative control.

9. Pouring trypan blue working solution along with the already stained leaves onto a sieve will allow you to grab the leaves from the petiole and transfer them easily to a new 50 ml tube containing destaining solution.

10. Once leaves are incubated in destaining solution; they need to be handled very carefully to avoid damage. Furthermore, since leaves will lose the green colour due to the loss of chlorophyll, it will become harder to identify where the petiole is. As a result, we recommend working always in the same tube, once the destaining solution has been added to the leaves.

11. The abaxial side of the leaf faces the coverslip. Use Optivisor lenses in order to aid vision when handling the leaves.

12. Gently drop the coverslip on top of the leaf very slowly from top to bottom of the leaf by sliding a 1000 µL pipette tip below the coverslip really slowly. Avoiding as many bubbles as possible at this step is critical so that they do not appear in the images and do not affect quantification.

13. Follow the instruction guide for running the cell death processing macro located in the GitHub platform: https://github.com/Celldeathquantification/Cell-death-quantification.

14. When punching out leaf discs, we recommend users to excise the leaf disc from the center part of the leaf. Exerting strong pressure towards a flat surface covered with a fine layer of tissue paper allows neat excision of discs.

15. The first hour of incubation of leaf discs under constant rotation is intended to remove electrolytes leaked from damaged cells on the edges of the leaf discs as a consequence of the excision caused by the cork-borer.

16. Time points selected for conductivity measurements can vary depending on the bacterial inoculum used. We recommend a time series of measurements from 0h to 10h once the water from step 9 has been replaced, with measurements being taken every two hours.

17. Always clean the sensor of the conductivity meter with Milli Q sterilised water in between samples.

5. Acknowledgements

Research was funded by the Ministry of Science and Innovation / Spanish State Research Agency PID2019-108595RB-I00 / AEI / 10.13039/501100011033, and through the "Severo Ochoa Programme for Centres of Excellence in R&D" (SEV-2015-0533). This work was also supported by the CERCA Programme / Generalitat de Catalunya.

6. Figure legends

Fig 1. Quantification of trypan blue stained area of Arabidopsis leaves infected with an HR-causing avirulent bacterial strain. (A) Four to five weeks-old Arabidopsis leaves of either Col-0 or *rpm1-3* were syringe infiltrated with *Pto* DC3000 (*avrRpm1*) at 2.5x10^7 CFUs/O.D_{600}=0.05. Two independent leaves were stained in trypan blue at different time points after infiltration (0, 2, 4 and 8 hours) and subsequently imaged under the microscope. (B) Image J software was used for quantification of stained area which is represented as a percentage (*see* Note 12).

Fig 2. Electrolyte leakage from Col-0, *rpm1-3* and *rps2* leaf discs after bacterial inoculation. Four to five weeks-old Arabidopsis leaves were syringe-infiltrated with either *Pto* DC3000 (*avrRpm1*) or *Pto* DC3000 (*avrRpt2*) with two independent bacterial titers: 2.5x10^7 CFUs/O.D_{600}=0.05 (triangles) or 5x10^6/O.D_{600}=0.01 (circles). Conductivity measurements of electrolyte leakage from dying cells were recorded from 2 to 10h after inoculation. Standard error bars represent four biological replicates. Asterisks denote significant differences

(**, P value < 0.01 or *, P value<0.05, NS, P value >0.05) from independent student t-tests for comparisons between two genotypes at each time point and $O.D_{600}$. NS= non-significant.

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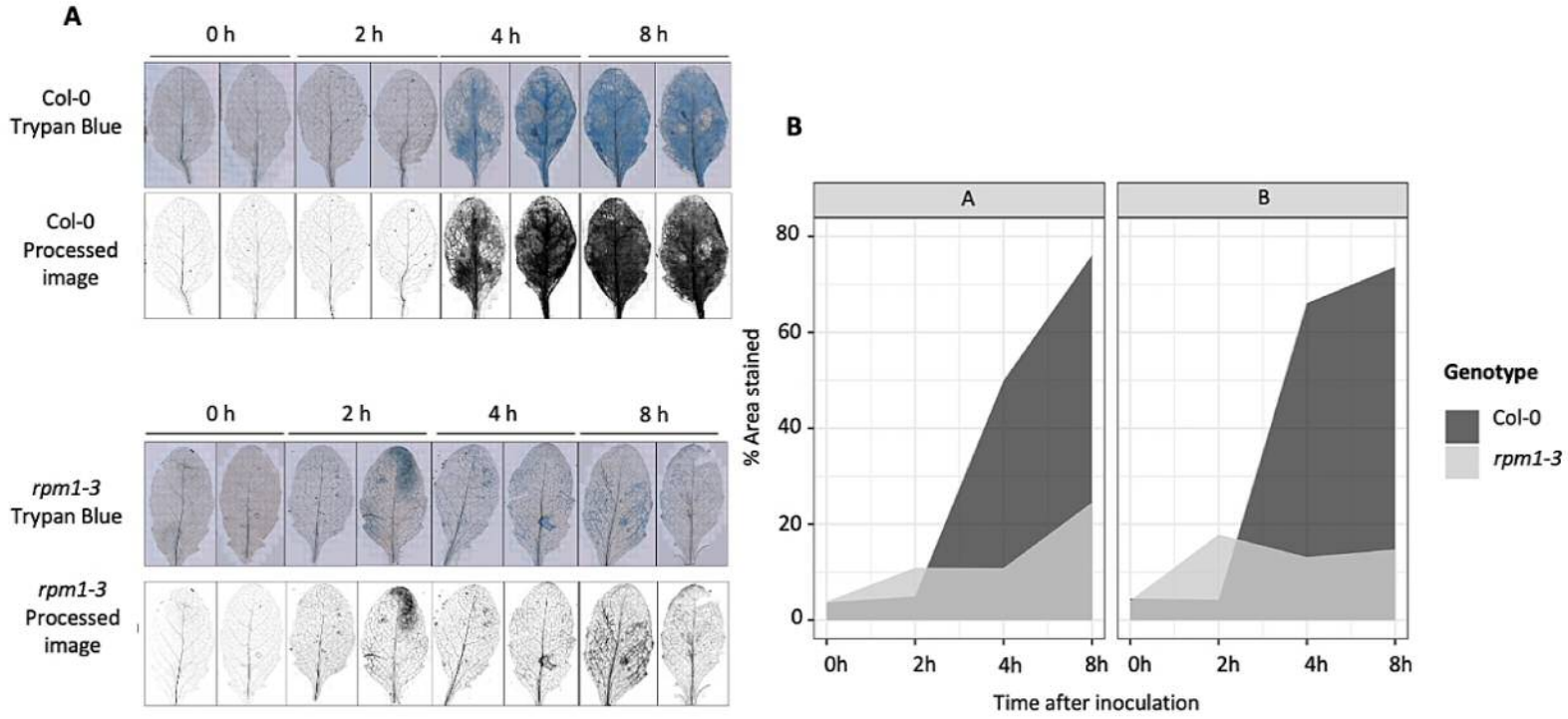
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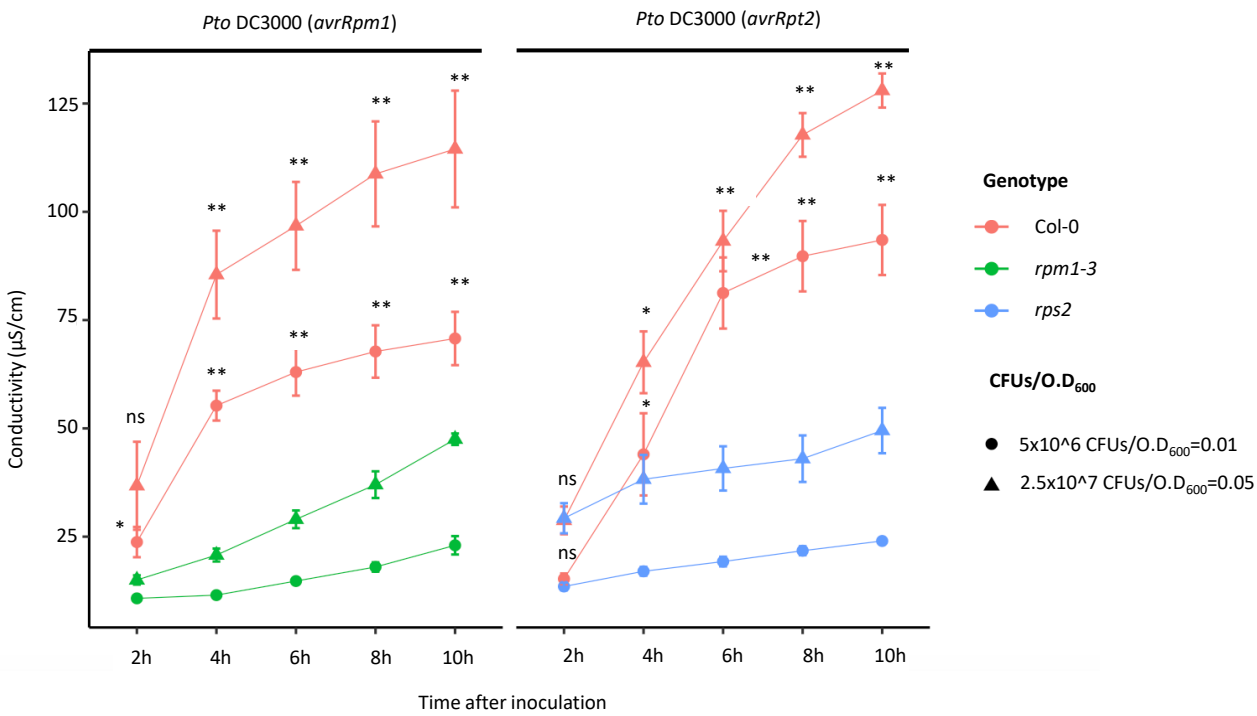
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Pto DC3000 (*avrRpt2*)
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Genotype
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rpm1-3
rps2
CFUs/O.D$_{600}$
5x10^6 CFUs/O.D$_{600}$=0.01
2.5x10^7 CFUs/O.D$_{600}$=0.05