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Title: Evaluating Leaf Responses to Microbial Secondary Metabolites Using a High-Throughput Format

Authors and Affiliations:
Whynn Bosnich[1], Natalie Hoffmann[1,2], Siddharthan Lakshmanan[1,3], Elizabeth K. Brauer[1,3]

[1]Ottawa Research and Development Centre, Agriculture and Agri-Food Canada
[2]The Boyce Thompson Institute for Plant Research
[3]Department of Biology, University of Ottawa

Corresponding Authors:
Elizabeth K. Brauer elizabeth.brauer@agr.gc.ca

Email Addresses for All Authors:
Whynn Bosnich whynn.bosnich@agr.gc.ca
Natalie Hoffmann njh98@cornell.edu
Siddharthan Lakshmanan siddharthan.chinnaiyanlakshmanan@agr.gc.ca
Elizabeth K. Brauer elizabeth.brauer@agr.gc.ca

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Author Questionnaire

1. **Microscopy**: Does your protocol require the use of a dissecting or stereomicroscope for performing a complex dissection, microinjection technique, or something similar? **No**

2. **Software:** Does the part of your protocol being filmed include step-by-step descriptions of software usage? **No**

3. **Filming location:** Will the filming need to take place in multiple locations? **No**

4. **Testimonials (optional):** Would you be open to filming two short testimonial statements **live during your JoVE shoot**? These will **not appear in your JoVE video** but may be used in JoVE's promotional materials. **No**

Current Protocol Length
Number of Steps: 14
Number of Shots: 30

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Introduction

Videographer: Obtain headshots for all authors available at the filming location.

INTRODUCTION:

1.1. **Elizabeth Brauer:** My lab is trying to understand how plants respond to microbes with the ultimate goal of improving cereal crops.

1.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

1.2. **Elizabeth Brauer:** Recent work shows that microbial secondary metabolites have a key role in promoting either plant host resistance or susceptibility.

1.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

CONCLUSION:

1.3. **Elizabeth Brauer:** Our high-throughput assay enables researchers to measure three plant stress responses from the same leaf to make the most of limited amounts of microbial secondary metabolites.

1.3.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

1.4. **Elizabeth Brauer:** The ability to compare up to 96 samples within the same plate enables genetic mapping in plants and systems-level approaches for understanding microbial secondary metabolites and their effects on plants.

1.4.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

1.5. **Elizabeth Brauer:** We will continue to find the plant genes that underlie QTL regions associated with secondary metabolite responses in barley and to characterize the molecular mechanisms involved in these responses.

1.5.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

Videographer: Obtain headshots for all authors available at the filming location.

Protocol

2. Sample Preparation and Treatment

Demonstrator: Whynn Bosnich

2.1. To begin, use a cork borer with a 4-millimeter diameter to collect three leaf disks per plant while avoiding the midvein **[1-TXT]**. With the sharp end, apply a twisting motion to prevent macerating the tissue **[2]**.

2.1.1. WIDE: Talent positioning a 4-millimeter cork borer on a leaf disc. **TXT: Arabidopsis and Barley were used here**

2.1.2. Close-up of the twisting motion as the sharp cork borer avoids damaging the tissue.

2.2. Add secondary metabolites or solvent treatments at the desired concentration into individual wells of a 96-well plate to reach a final volume of 200 microliters **[1].** Gently place the leaf disks into the wells **[2-TXT]**.

2.2.1. Talent pipetting treatment solution into wells of the 96-well plate, adjusting to a final volume of 200 microliters.

2.2.2. Talent gently placing freshly collected leaf disks into one well of a 96-well plate filled with solution. **TXT: Keep adding disks until the required samples are collected**

2.3. Place the 96-well plate, with the lid removed, into a bell jar equipped with a vacuum nozzle **[1]**.

2.3.1. Talent placing the open 96-well plate inside the bell jar.

2.4. Then, attach the vacuum hose to the bell jar nozzle **[1]** and turn on the vacuum pressure at 9.8 pounds per square inch for 10 seconds **[2]**. Turn off the vacuum for 10 seconds, then repeat the vacuum cycle once more **[3]**.

2.4.1. Talent connecting the vacuum hose to the nozzle of the bell jar.

2.4.2. Talent switching on the vacuum and monitoring pressure.

2.4.3. Talent turning off the vacuum after 10 seconds.

2.5. Now, place the lid back on the 96-well plate **[1]** and incubate it on an orbital shaker

under a light source, positioned 23 centimeters above the shaker for 4 hours **[2]**.

2.5.1. Talent covering the 96-well plate with its lid.

2.5.2. Talent placing the plate on the orbital shaker beneath the light source.

3. Ion Leakage Measurements

Demonstrator: Natalie Hoffman

3.1. Wash the probe in Ultrapure water **[1]** and dab it lightly on a lint free wipe to dry **[2]**. Note the conductivity reading of the water for future reference **[3]**.

3.1.1. Talent rinsing the probe in a container of Ultrapure water.

3.1.2. Talent gently dabbing the clean probe on a lint free wipe to dry.

3.1.3. Show the conductivity reading of the Ultrapure water displayed on the device screen.

3.2. Between 4 to 6 hours after treatment, measure and record conductivity in individual wells by immersing the probe into the liquid surrounding the leaf disk **[1]**. Hold the plate at a 45-degree angle to ensure the probe is fully immersed **[2]**.

3.2.1. Talent inserting the probe into a well to measure conductivity.

3.2.2. Talent tilting the 96-well plate to a 45-degree angle during measurement.

3.3. After rinsing the probe as demonstrated earlier, occasionally test the conductivity of the water to confirm probe stability **[1]**.

3.3.1. Show the conductivity reading of the Ultrapure water to confirm consistent values.

3.4. Then, place a plastic sealing film over the 96-well plate **[1]** and return it to the orbital shaker for overnight incubation **[2]**.

3.4.1. Talent sealing the plate with a plastic film.

3.4.2. Talent placing the sealed plate back on the orbital shaker.

4. Peroxidase Activity Measurements

Demonstrator: Siddharthan Lakshmanan

4.1. To prepare the peroxidase substrate, heat 40 milliliters of distilled sterile water to 70 degrees Celsius in a beaker with a stir bar **[1]**. Dissolve 50 milligrams of 5-aminosalicylic acid in heated water **[2].** Then, add additional water to bring the total volume to 50 milliliters **[3]** and adjust the pH to 6 using sodium hydroxide **[4]**. Protect the light-sensitive solution by covering the container with aluminum foil **[5]**.

4.1.1. WIDE: Talent heating 40 milliliters of distilled water to 70 degrees Celsius in a beaker with a stir bar.

4.1.2. Talent adding 50 milligrams of 5-aminosalicylic acid to the heated water and stirring.

4.1.3. Talent topping up the beaker to 50 milliliters with additional distilled water.

4.1.4. Talent adjusting the pH to 6 using sodium hydroxide.

4.1.5. Talent covering the beaker with aluminum foil.

4.2. In a 50-milliliter amber conical tube, prepare a 1 percent hydrogen peroxide solution in Ultrapure water **[1]**.

4.2.1. Talent pipetting 1.7 milliliters of 30 percent hydrogen peroxide into a 50-milliliter amber conical tube containing 48.3 milliliters of Ultrapure water.

4.3. At the desired time point, take 50 microliters from each well **[1]** and transfer to a clear, flat-bottomed 96-well plate **[2]**. Add 50 microliters of the assay medium, mix by pipetting five times, and incubate at room temperature for 3 minutes **[3]**.

4.3.1. Talent pipetting 50 microliters of liquid from each well of the treatment plate.

4.3.2. Talent transferring the samples to a clear 96-well plate.

4.3.3. Talent adding 50 microliters of assay medium to each well and pipetting up and down five times to mix.

4.4. Then, add 10 microliters of the 1 percent hydrogen peroxide solution per milliliter of 5-aminosalicylic acid solution required to generate the assay medium **[1]**.

4.4.1. Talent pipetting 10 microliters of the 1 percent hydrogen peroxide solution and mixing to prepare the assay medium.

4.5. To stop the reaction, add 20 microliters of 2 normal sodium hydroxide to each well **[1]** and use a plate reader to measure the absorbance of each well at 595 nanometers **[2]**.

4.5.1. Talent adding 20 microliters of 2 normal sodium hydroxide to each well to stop the reaction.

4.5.2. Talent placing the plate into a plate reader and initiating the absorbance measurement at 595 nanometers.

Results

5. Results

5.1. Water and 0.1 percent dimethyl sulfoxide produced similar ion leakage and peroxidase activity levels, validating their use as negative controls **[1].**

5.1.1. LAB MEDIA: Figure 2. *Video editor: Highlight the 2nd and 3rd bars from the left for "flg22 + / DMSO - / MeOH -" and "flg22 + / DMSO + / MeOH -" in the left and right graphs*.

5.2. Treatment with 1 micromolar flg22 significantly induced ion leakage **[1],** peroxidase activity **[2],** and callose production, confirming it as a robust positive control **[3].**

5.2.1. LAB MEDIA: Figure 3. *Video editor: Highlight the box labeled "flg22" under the "Ion Leakage" panel.*

5.2.2. LAB MEDIA: Figure 3. *Video editor: Highlight the box labeled "flg22" under the "POX Activity" panel.*

5.2.3. LAB MEDIA: Figure 3. *Video editor: Highlight the box labeled "flg22" under the "Callose" panel.*

5.3. Gramillin treatment induced significant ion leakage **[1]** and callose production **[2]** but had variable effects on peroxidase activity **[3].**

5.3.1. LAB MEDIA: Figure 3. *Video editor: Highlight the box labeled "Gramillin" under the "Ion Leakage" panel.*

5.3.2. LAB MEDIA: Figure 3. *Video editor: Highlight the box labeled "Gramillin" under the "Callose" panel.*

5.3.3. LAB MEDIA: Figure 3. *Video editor: Highlight the box labeled "Gramillin" under the "POX Activity" panel.*

5.4. Surfactin at 10 micromolar induced both peroxidase activity **[1]** and callose production **[2],** while ion leakage remained unchanged **[3].**

5.4.1. LAB MEDIA: Figure 3. *Video editor: Highlight the box labeled "Surfactin" under the "POX Activity" panel.*

5.4.2. LAB MEDIA: Figure 3. *Video editor: Highlight the box labeled "Surfactin" under the "Callose" panel.*

5.4.3. LAB MEDIA: Figure 3. *Video editor: Highlight the box labeled "Surfactin" under the "Ion Leakage" panel.*

5.5. T-2 toxin treatment suppressed peroxidase activity **[1]** while inducing callose production **[2]** and having no impact on ion leakage **[3].**

5.5.1. LAB MEDIA: Figure 3. *Video editor: Highlight the box labeled "T-2 toxin" under the "POX Activity" panel, showing a low value.*

5.5.2. LAB MEDIA: Figure 3. *Video editor: Highlight the box labeled "T-2 toxin" under the "Callose" panel.*

5.5.3. LAB MEDIA: Figure 3. *Video editor: Highlight the box labeled "T-2 toxin" under the "Ion Leakage" panel.*

- Cork borer

Pronunciation link: https://www.howtopronounce.com/cork-borer How To Pronounce+1
IPA: /ˈkɔːrkˌbɔːrɚ/
Phonetic Spelling: KORK-bor-er

- Midvein

Pronunciation link: No confirmed link found
IPA: /ˈmɪdˌveɪn/
Phonetic Spelling: MID-vayn

- Orbital shaker

Pronunciation link: https://youglish.com/pronounce/orbital%2Bshaker/english youglish.com
IPA: /ˈɔːr.bɪ.təl ˈʃeɪ.kɚ/
Phonetic Spelling: OR-bi-tul SHAY-ker

- Vacuum

Pronunciation link: https://dictionary.cambridge.org/us/pronunciation/english/vacuum Cambridge Dictionary (for "borer" but similar vowel pattern)
IPA: /ˈvæk.juːm/
Phonetic Spelling: VAK-yoom

- Microliter

Pronunciation link: No confirmed link found
IPA: /ˈmaɪ.kroʊˌliːtər/
Phonetic Spelling: MY-kroh-LEE-ter

- Ultrapure

Pronunciation link: No confirmed link found
IPA: /ˈʌl.trəˌpjʊr/
Phonetic Spelling: UL-truh-pure

- Peroxidase

Pronunciation link: https://www.merriam-webster.com/dictionary/peroxidase merriam-webster.com
IPA: /ˌpɛr.əˈksɪ.deɪs/
Phonetic Spelling: puh-ROKS-ih-days

- 5-aminosalicylic (acid)

Pronunciation link: No confirmed link found
IPA: /ˌfaɪv-əˌmaɪ.noʊˌsælɪˈsɪlɪk/
Phonetic Spelling: five-uh-MY-noh-sal-ih-SIL-ik

- Dimethyl sulfoxide

Pronunciation link: No confirmed link found
IPA: /ˌdaɪˈmɛθ.əl sʌlˈfɒk.saɪd/
Phonetic Spelling: dye-METH-uhl sul-FOK-syde

- Callose

Pronunciation link: No confirmed link found
IPA: /ˈkæl.oʊs/
Phonetic Spelling: KAL-ohs