Jack et al.- High-throughput plant defense analysis

**A high-throughput method of analyzing multiple plant defensive compounds in minimized sample mass1**

Chandra N. Jack2,6,§, Shawna L. Rowe3,§, Stephanie S. Porter4, Maren L. Friesen2,5

2 Washington State University, Department of Plant Pathology, Pullman, WA, 99164

3 Michigan State University, Department of Plant Biology, East Lansing, MI 48824

4 Washington State University, School of Biological Sciences, Vancouver, WA

5 Washington State University, Department of Crop and Soil Sciences, Pullman, WA

§Co-first authors

Email Addresses: CNJ: [chandra.jack@gmail.com](mailto:chandra.jack@gmail.com)

SLR: [roweshaw@gmail.com](mailto:roweshaw@gmail.com)

SSP: [stephanie.porter@wsu.edu](mailto:stephanie.porter@wsu.edu)

MLF: [m.friesen@wsu.edu](mailto:m.friesen@wsu.edu)

Number of words:

1 Manuscript received \_\_\_\_\_\_\_; revision accepted \_\_\_\_\_\_\_.

6 Author for correspondence: [chandra.jack@gmail.com](mailto:chandra.jack@gmail.com)

**ABSTRACT [need to get to 150 words]**

*Premise of the study:* Current methods for quantifying alterations in plant biochemistry due to herbivory are expensive, time consuming, and typically require large amounts of tissue, thus limiting throughput. We present a cost-effective, high-throughput protocol to quantify multiple biochemical responses from small plant tissue samples.

*Methods and Results:* Using leaves from *Solanum lycopersicum* and *Medicago polymorpha* pre- and post-herbivory, we show that our protocol can quantify differential plant responses in common plant defense assays: peroxidase production, polyphenol oxidase production, ROS production, total protein production, and proteinase inhibition activity.

*Conclusions:* Current protocols can require 500mg of tissue, but our assays detect activity in under 10mg. Using our protocol, in approximately 6 hours, two people can run any of the assays on 300 samples in triplicate, or all of the assays on under 20 samples. Implementation of our protocol enables researchers to plan more complex experiments that compare local versus systemic plant responses, quantify environmental and genetic variation, and measure population level variation.

**Key words:** Microplate; Plant Defense Response; Peroxidase; Polyphenol oxidase; Protease Inhibitors; Protein Quantification

**INTRODUCTION**

Current methods for quantifying molecular responses by host plants to herbivory are expensive, time consuming, and typically require large tissue masses. The ability to quantify these responses over time and compare variation within populations is useful in many research areas from ecology and evolutionary biology to applied agricultural research. However, like many attempts to assay molecular responses, research is limited by the rigorous nature of quantifying subtle physiological changes. Previously developed assays are thus effective but unusable by many researchers due to financial constraints or the need to conduct a given assay on a large set of samples. These constraints have been recognized and addressed in some instances, such as protein quantification [17]. With the development and widespread use of microplate readers, assays, such as protease inhibition and protein quantification (among others), have been scaled and optimized for smaller sample volumes and larger sample numbers resulting in better replication [17, 18].

Assaying multiple responses on individual samples is critical for understanding host responses since many defense responses are interrelated. A prime example of this is the direct relationship between reactive oxygen species production and the induction of peroxidase. Peroxidases such as glutathione peroxidase reduce H2O2 and induced when higher levels of H2O2 begin wreaking havoc on the intercellular space [19]. These enzymes are used to catalyze the oxidation of other molecules using hydrogens as a substrate [20]. Reducing the total amount of tissue required for an expanded array of assays would enable a researcher to perform both small molecule and enzymatic assays during a given investigation and thus allow a researcher to analyze complex responses across large numbers of individuals within single experiments. Early methods of protein quantification such as the Bradford method and the Lowry method were dependent on the use of a spectrophotometer and thus large sample volumes [21]. Currently, assaying for the production of small molecules requires severe buffering conditions that both inhibit and degrade proteins present in suspended tissue samples due to the presence of compounds such as trichloroacetic acid, an acid that causes protein precipitation [22]. Additionally, the large sample masses required for current spectrophotometric techniques limits the total number of technical replications and differing assays that can be performed on a given sample. These problems ultimately result in researchers assaying single physiological changes induced by herbivory as a metric for general herbivory responses.

Paired together, assaying both small molecule production and enzyme production would allow for a thorough and more holistic assessment of herbivory-associated plant immunity responses. Here, we present a cost-effective method to assay multiple molecular responses in limited sample masses. The selected assays include total protein content, peroxidase, polyphenol oxidase, H2O2, and protease inhibitors. Many defense responses can be assayed individually with few logistical issues in plants. However, these assays often require diverse tissue extraction methods that are mutually exclusive. For our purposes, we selected induced responses that were both diverse and able to be assayed from a common sample extract. We tested our method on leaves taken from *Solanum lycopersicum* pre- and post- herbivory to show that our assays can quantify differential plant responses. *S. lycopersicum* is a popular species that is often used to test biochemical defense responses, which allows us to compare our results to published studies. After validating our assays using *S. lycopersicum,* we tested our protocol using *Medicago polymorpha*. *M. polymorpha* is a leguminous plant whose biochemical responses to herbivory have not been quantified. As a non-model organism with trifoliate leaves that may weigh less than 50mg, it is representative of a “non-model” plant that researchers may wish to characterize. Implementation of this protocol paves the way toward more comprehensively assaying plant biochemical responses to herbivory in non-model plants and in plants that develop few or small leaves. Furthermore, this protocol allows for greater sample capacity, which would allow for improved statistics, time course experiments, expanded treatment options, and other more complex experiments.

**METHODS AND RESULTS**

**Pre-experiment tissue preparation-** To test our protocol and compare it to current spectrophotometer protocols, we used Tomato (*Solanum lycopersicum,* ecotype M82*)*, a model plant often used for testing defense responses. We then quantified defense responses in a non model species. Tomatoseeds were scarified with 600 grit sandpaper. The seeds were imbibed in dH2O for three days at 4°C in the dark to stratify. We removed the seeds from the cold and placed them in a dark cabinet overnight. The germinated seedlings were grown for three weeks in a grow room before inducing defensive responses. Burr medic (*Medicago polymorpha*) seeds (Appendix 2) were scarified as above and planted into 158-ml pots filled with Sungro Sunshine Mix #1. Plants were inoculated a week after planting with a rhizobium strain mixture of 107 cells of equal parts *Ensifer medicae* strain WSM419 and *E. meliloti* strain 1021, necessary to ensure healthy plants.

To compare changes in plant responses and ensure that our protocol could adequately detect expression both pre- and post herbivory, we mimicked herbivore attack by manually inducing plant responses using caterpillar regurgitant. Regurgitant was generated by compressing soybean looper (*Chrysodeixis includens*) stomachs with forceps post feeding on corresponding host plants. Leaves were manually wounded with scissors that were dipped in soybean looper regurgitant. Leaf samples were taken from each plant at 0 and 24 hours and flash frozen in liquid nitrogen for storage at -80°C until processing. Leaf tissue from additional tomato plants was harvested for validation.

**Extraction and homogenization-** One of the challenges of attempting to assay multiple enzymes and small molecules from a single source is finding an appropriate extraction buffer that will preserve the integrity of the metabolites while not creating conditions inhibitory for other assays.Initially, we aimed to perform all assays on a single plant extract/ enzyme source but were unable to due to the array of assays we wished to run. Ultimately, we were able to reduce down to two buffers: a trichloroacetic acid (TCA) buffer and a protein extraction (PE) buffer. The TCA buffer provides the appropriate conditions for assaying the production of hydrogen peroxide [29]. The PE buffer was designed to provide the best crude extraction without the presence of interfering compounds. Phenylmethane sulfonyl fluoride (PMSF), the serine protease inhibitor commonly present in protein extraction buffers [41], was removed due to the need to assay the production of protease inhibitors. Β-mercaptoethanol, also a common protein buffer ingredient [41] used as a reducing agent to ensure analysis of strictly monomeric proteins, was removed due to interference with the Thermo ScientificPierceTM BCA Protein Assay Kit. Previous studies, specifically ones from which we modified original assays [27, 28] used extraction buffers lacking protease inhibitors and/or reducing agents with no significant change to final results. Our PE buffer thus results in a crude extract that provides predictable results when published assays were replicated for validation purposes (Table 1). All protein extractions were diluted 10-fold to enable additional assays to be run on individual samples. Leaf tissue from each plant was placed into two eppendorf tubes and weighed. The tubes were homogenized for 15 minutes at 300 rpm in a tissuelyser (QIAGEN TissueLyser II)**.** One tube received 1ml of the 0.1% TCA buffer, while the other received 1 ml of the PE buffer (1mM EDTA, 88mM Trizma Base, 10% glycerol). Appendix 1 has a table that lists what buffer samples were used in each assay. The tubes were centrifuged at 4°C for 10 minutes at 15000 rpm in an accuSpin Micro 17 centrifuge (Fisher Scientific) and the supernatant pipetted into clean tubes. The PE buffer tubes were then diluted to 1/10X. All absorbance values were run on a SpectraMax M2 spectrophotometer and microplate reader.

**Assays-**

A detailed description of the steps for our protocol can be found in Appendix 1. Total protein content was measured using the Thermo ScientificPierceTM BCA Protein Assay Kit (Product number: 23337) according to manufacturer instructions for microplate samples and was included to test the efficacy of our protein extraction buffer and to quantify the total time necessary to allocate to data collection because this assay is usually included when measuring plant defense responses. Polyphenol oxidase (PPO) and peroxidase (POD), the two enzymes assayed, underwent similar modifications during the scaling process. Previous protocols required between 0.025 ml to 0.100 ml of 1X crude extract to be assayed in a final volume of between 2.5 ml and 3.1 ml of solution [27, 28]. When scaling our total assay volumes down to fit the requirements of a standard 96-well microplate the volumes were reduced ~100 fold. *POD* *activity* was measured in triplicate for each sample and also included a tissue specific control. Wells of the microplate designated as treatment wells received 143 uL of peroxidase reaction buffer (100 mM sodium phosphate buffer (pH 6.5) containing 5 mM guaiacol). Wells designated for controls received 143 uL of 100mM sodium phosphate buffer (ph 6.5). 25 uL of supernatant (enzyme source) was added to each well.  We then added 32 uL of 5 mM H2O2(final concentration 0.8 mM)  to start the reaction. The plates were incubated in the dark for 15 minutes at room temperature before reading absorbance values at 470 nm. *PPO activity* was also measure in triplicate per sample with a tissue specific control. Wells of the microplate designated as treatment wells received 115 uL of 100 mM sodium phosphate buffer (pH 6.8) and 60 uL of 50 mM pyrocatechol. Control wells received 175 uL of 100 mM sodium phosphate buffer (pH 6.8). 25 uL of supernatant (enzyme source) was added to all wells. Samples were incubated for 5 minutes before reading absorbance values at 420 nm. *Proteinase Inhibition activity* was quantified using an adapted method from [30] where activity is represented by the inhibition of trypsin. This assay requires the preparation of two reaction buffers per sample. Reaction buffer 1 was prepared in tubes with 133.3 uL of Trizma Base buffer, 83.3 uL of 2% azocasein dissolved in Trizma Base buffer, and 33.3 uL of 0.001 M HCl solution containing 200 ng of trypsin. Reaction buffer 2 was the same as reaction buffer 1, but additional Trizma base was substituted for the trypsin solution. 100ul of the sample extract was added to each tube. These serve as the sample measurement tube and the sample control tube. Reaction buffers 1 and 2 were also used for positive and negative assay controls respectively. The assay controls received 100ul of Trizma base instead of enzyme source. All tubes were incubated at 30°C for 25 minutes. Post-incubation, 133 uL of 100% w/v TCA was added and tubes were centrifuged at 8000 rpm for 10 minutes. After centrifugation, 100 uL of the supernatant were added to wells of a microplate that contained 100 uL of 1M NaOH and absorbance was measured at 450 nm. As with the other assays, samples were run in triplicate. *Hydrogen Peroxide (H2O2)Quantification-* The hydrogen peroxide assay (H2O2) was implemented with few modifications. Our primary change was to the measurement wavelength. As measured in [29], the triiodide produced as a result of the reaction mechanism has optimal absorbance at 285 nm with significant differences able to be determined at wavelengths up to 410 nm. We selected 390 nm due to previously published H2O2 assays [29, 31], the results of a spectral scan that indicated no significant differences in absorbance values at 390 nm relative to surrounding wavelengths (t(5) = -1.608, p = 0.169) and the fact that 285 nm is cleanly in the ultraviolet range which requires special plates for to avoid issues of interference from the standard polymers used in 96-well plates. Our measurements at 390 nm in standard plates yielded clean data consistent with previous results showing H2O2 induction after induction by herbivores. Sample aliquots were taken from the 0.1% TCA buffer extraction. All sample wells received 100 uL of 1M potassium iodide (KI), 50 uL of 10mM Potassium Phosphate buffer (pH 6.5), and 50 uL of sample aliquot. Control wells received 100 uL of dH2O, 50 uL of 10mM Potassium Phosphate buffer (pH 6.5), and 50 uL of sample aliquot to account for tissue coloration. Samples were incubated in the dark for 20 minutes at room temperature. A standard curve was prepared by preparing wells with 100 uL of 1M potassium iodide (KI), 50 uL of 10mM Potassium Phosphate buffer (pH 6.5), and 50 uL of 0.1% TCA then seeding with known amounts (5-20 nmoles) of H2O2. Absorbance was measured at 390 nm and values were compared to the standard curve for quantification in nmoles.

The success of our protocol hinges on three points that we address through different validation methods. First, is our assay able to accurately quantify the same amount of enzyme activity compared to assays run using a spectrophotometer. This validation was focused on our POD and PPO assays given these were the most modified protocols. For each assay we generated standard curves from enzymes obtained from Worthington Biochemical Corporation (USA). Horseradish peroxidase with an activity of 220 U/mg dry weight was diluted to a stock concentration of of 100 mU/ml in PE buffer. Serial dilutions were performed to get the concentration values as follows: 100 mU/ml, 50 mU/ml, 25 mU/ml, 10 mU/ml, 5 mU/ml, 2/5 mU/ml, 1.25 mU/ml, 0.625 mU/ml, and 0 mU/ml. Mushroom polyphenol oxidase with an activity of 630 U/mg dry weight was diluted to a stock concentration of 100 U/ml in PE buffer. Serial dilutions were performed to get the concentration values as follows: 1000 U/ml, 500 U/ml, 250 U/ml, 125 U/ml, 62.5 U/ml, 31.625 U/ml, 15.625 U/ml, 7.81 U/ml, 3.91 U/ml, 1.95 U/ml, 0.977 U/ml. Absorbance values were measured using the SpectraMax M2 and the r2 values are similar for both standard curves (Fig 1).

Given the large quantities of tissue that are required for spectrophotometric based assays, researchers are often forced to pool tissue samples from different plants. Our microplate protocols require much smaller quantities of plant tissue, allowing us to measure each plant individually. We show that there is significant variation in expression both pre and post herbivory between the five genetically identical tomato plants used in all five assays (Appendix 2 and Table 1) and that assaying plants individually for both POD and PPO, we are able to reduce experimental error (Table 2).

We show in the previous paragraph that pooling tissue samples increase variability and makes a strong argument for assaying individual plants. However, researchers are limited in what they can measure using spectrophotometers if their study system does not develop large or many leaves. We serially diluted tomato tissue to measure the lower limits of detection for our POD and PPO microplate assays and found that we were able to detect expression in as little as 3.8mg of tissue (Appendix 3). Then, we ran all of our assays on trifoliate leaves collected from *M. polymorpha* as proof of concept that we could detect expression in actual small leaf tissue samples (Appendix 4).

**Conclusion**

The measurement of plant biochemical variation in response to insect herbivory has previously faced several substantial limitations that have hindered the progress of the field. In particular, current practice in many molecular biology labs is to use a single lab ecotype to measure differences between experimental treatments and to pool tissue from multiple leaves and individuals to obtain sufficient sample mass [32-34]. However, this approach has precluded the study of variation within and between individuals, which is what is relevant for real-world interactions [35-37]. In contrast to previous techniques [29, 30, 32], our protocol offers the ability to implement multiple assays on a large sample set by consolidating sample preparation buffers and running all assays on a microplate reader. This not only dramatically reduces the amount of tissue needed for a given assay, but also reduces the total time required to perform a given assay set.

Using our high-throughput protocols, researchers can now compare variation both within and between individuals, genotypes, and populations. Subsampling can be performed on populations and intraspecific variation can be more thoroughly explored. Genetic and evolutionary biology studies often focus on variation between genotypes, which requires the higher levels of experimental replication afforded by our method. Understanding the genetic variation associated with plant biochemical responses is critical both for understanding how coevolution has shaped these interactions as well as for the success of molecular plant breeding for enhancing these interactions in agronomic settings. Importantly, our protocol also enables paired measurements of multiple defense responses on the same tissue. This has multiple advantages over testing pools of tissue or defense responses on separate tissues–notably, we find high inter-individual variation in biochemical responses within a single genotype of *Solanum lycopersicum*, underscoring how critical it is to perform paired assays. For example, measuring the production of multiple metabolites within a given sample enables researchers to quantify potential tradeoffs in phytochemical production at the level of individual leaves, which is the scale at which insects interact with their plant hosts. Our protocol also enables researchers to compare systemic versus localized defense responses within the same plant, since individual leaves can be assayed in parallel. Moreover, increased biological replication provides researchers with the opportunity to seriously consider statistical power when designing experiments--an option often overlooked due to pragmatic considerations.

**Acknowledgements**

This research was supported the National Science Foundation awards DEB-1354878 (MLF) and DEB-1355216 (SSP); a fellowship from Michigan State University under the Training Program in Plant Biotechnology for Health and Sustainability (T32-GM110523; SLR). This material is based in part upon work supported by the National Science Foundation under Cooperative Agreement No. DBI-0939454 (CNJ). Any opinions, findings, and conclusions or recommendations expressed in this material are those of the author(s) and do not necessarily reflect the views of the National Science Foundation.

**Author Contributions**

CNJ and SLR conceived of and designed the study in discussion with MLF. CNJ and SLR performed the experiments. CNJ analyzed the data. SSP contributed with sample preparation. CNJ and SLR drafted the manuscript. CNJ, SLR, SSP, and MLF provided critical feedback and revisions to the manuscript. CNJ, SLR, SSP, and MLF gave final approval of the version to be published

**Literature Cited (No more than 25)**

**Tables (no more than 2)**

**Table 1.** Amount of variation explained by between plant tissue samples pre- and post-herbivory.

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| **Assay** | **Pre-herbivory** | | |  | **Post-herbivory** | |
| % Variation explained by plant | F stat | *p* | % Variation explained by plant | F stat | *p* |
| Protein Quantification | 95.8% | 80.42 | <0.001 | 99.9% | 3753 | <0.001 |
| H2O2 | ~ 0%a | 0.911 | 0.404 | 99.98% | 1.476e4 | <0.001 |
| POD | 51.7% | 6.076 | <0.01 | 37.8% | 3.887 | <0.05 |
| PPO | 70.1% | 9.218 | <0.01 | 81.3% | 16.25 | <0.001 |
| PI | 60.8% | 3.167 | 0.0875 | 77.7% | 10.59 | <0.01 |

aH2O2 production prior to herbivory was negligible.

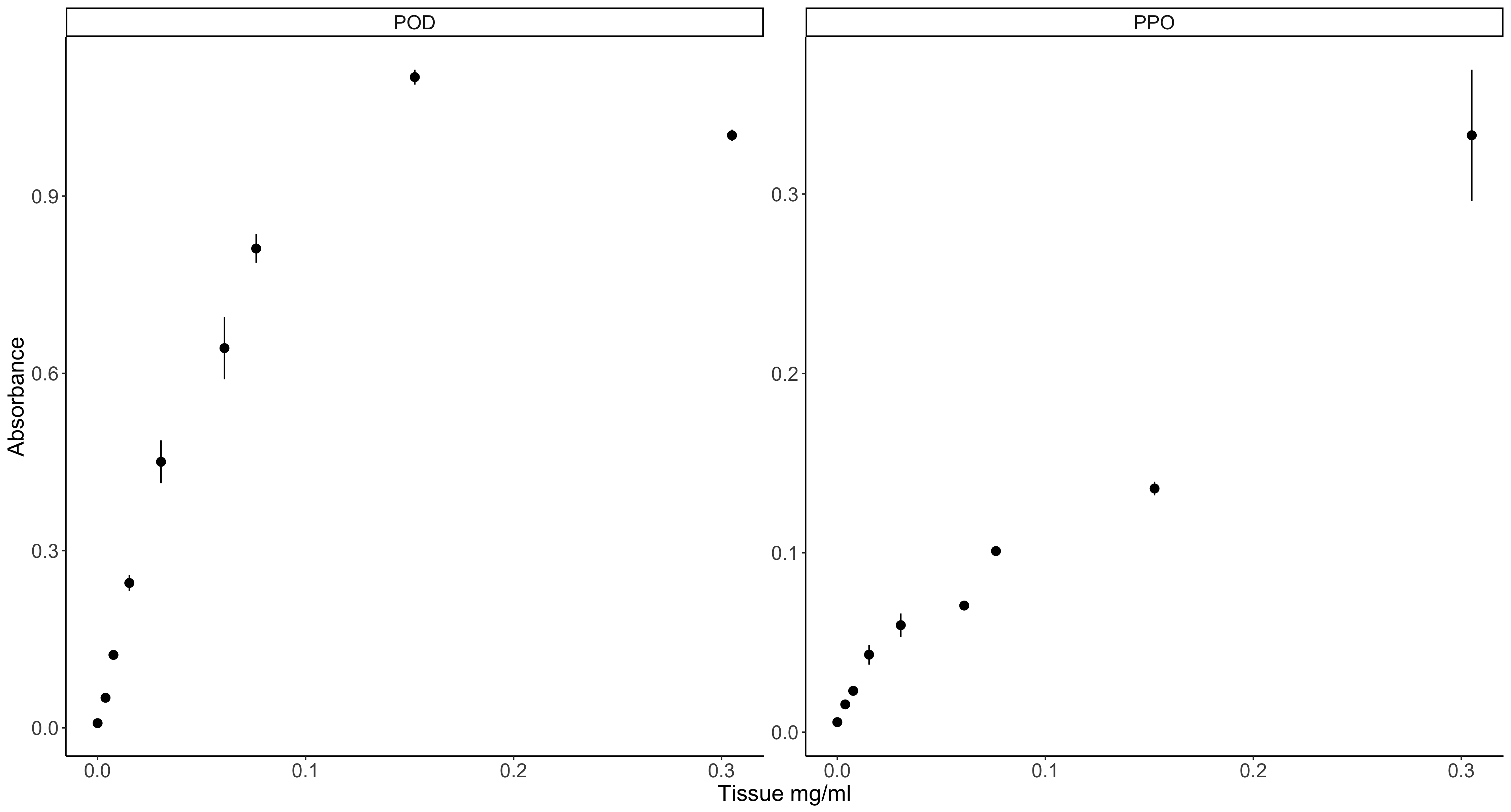
**Table 2.** Comparison of data between spectrophotometer and microplate assays for POD and PPO.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Assay** | **Experimental mean** | | **Experimental SE as % of mean** | |
| Pre-herbivory | Post-herbivory | Pre-herbivory | Post-herbivory |
| POD  Spec | 1.17 | 46.8 | 68.14% | 34.85% |
| POD  Micro | 6.31 | 256.47 | 3.34% | 1.97% |
| PPO  Spec | 0.79 | 34.64 | 26.88% | 26.74% |
| PPO  Micro | 1.98 | 122.26 | 14.6% | 10.15% |

**Figures and legends (no more than 2)**

****

**Fig 1.** Comparison of absorbance values for A)POD and B)PPO when measured using either a spectrophotometer (cuvette) or microplate reader (mtp). Each dot represents mean plus standard error. All concentrations were done in triplicate.



**Fig 2. Serial dilutions of uninduced tomato tissue.** We serially diluted a homogenized tissue sample that was initially at a concentration of 0.38 gFW/ml of buffer to determine the lower limits of tissue mass that could be detected. We used uninduced tissue because that has the lowest expression of the defense compounds and will determine sample mass that researchers will use in comparative assays. Each dilution was measured in triplicate, and values shown are means plus standard errors.

**=== End of Word Count ===**

**Appendix**

**APPENDIX 1**

**Methods**

*Plant Growth*

Tomato (*Solanum lycopersicum,* ecotype M82*)* seeds were scarified with 600 grit sandpaper. The seeds were imbibed in dH2O for three days at 4°C in the dark to stratify. We removed the seeds from the cold and placed them in a dark cabinet overnight. The germinated seedlings were grown for three weeks in a grow room (Conditions: 16 hour days at 22°C with light levels at ~ 300µEinsteins in autoclaved soil substrate (2 part SuremixTM: ½ part sand) in 5” pots. The plants were watered weekly with dH2O.

Burr medic (*Medicago polymorpha*) seeds were scarified as above and planted into 158-ml pots filled with Sungro Sunshine Mix #1. Three seeds were planted into each cone 5 mm below the soil surface in the Washington State University, Vancouver greenhouse. Plants were fertilized with slow release fertilizer (Osmocote Plus Outdoor & Indoor fertilizer pellets) and inoculated a week after planting with a rhizobium strain mixture of 107 cells of equal parts *Ensifer medicae* strain WSM419 and *E. meliloti* strain 1021. During germination, seeds were mist irrigated twice a day for 20 minutes, and then mist-irrigated daily as needed.

*Herbivore Treatment*

*S. lycopersicum*

Three weeks post-germination, five plants were treated with a combination of regurgitant and herbivory from soybean loopers (*Pseudoplusia includens)* for 24 hours. A single soybean looper was placed in a clip cage on a mature leaf. A second leaf near the leaf subjected to herbivory was manually wounded with scissors that were dipped in soybean looper regurgitant. Four replicate leaf samples were taken from each host at 0 and 24 hours and flash frozen in liquid nitrogen for storage at -80°C until processing. Leaf samples were taken from the top half of young leaves.

*M. polymorpha*

Twenty-four hours prior to tissue collection, soybean looper caterpillars were fed *M. polymorpha* leaf tissue. Immediately prior to leaf tissue collection, herbivore homogenate was prepared by grinding two caterpillars in 500 uL of deionized water with a mortar and pestle. Full trifoliate leaves were cut with sterilized scissors that had been dipped in the herbivore homogenate, and 50-75 mg of tissue was collected per plant and placed into a 96-well plate on ice. Leaf samples were taken from host plants at 0 and 24 hours and were flash frozen by setting filled plates into liquid nitrogen. Plates were stored at -80°C and shipped on dry ice to Michigan State University.

*Regurgitant collection*

Regurgitant was generated by compressing soybean looper stomachs with forceps post feeding on corresponding host plants. Regurgitant was deposited directly into sterile eppendorf tubes and frozen at -20°C.

*Biochemical Assays*

*Extraction and homogenization*

Leaf tissue from each plant was placed into two eppendorf tubes and weighed. The tubes were homogenized for 15 minutes at 300 rpm in a tissuelyser (QIAGEN TissueLyser II)**.** One tube received 1ml of the 0.1% TCA buffer, while the other received 1 ml of the PE buffer (1mM EDTA, 88mM Trizma Base, 10% glycerol) (Figure 1). Table 1 lists what buffer samples were used in each assay. The tubes were centrifuged at 4°C for 10 minutes at 15000 rpm in an accuSpin Micro 17 centrifuge (Fisher Scientific) and the supernatant pipetted into clean tubes. The PE buffer tubes were then diluted to 1/10X. All absorbance values were run on a SpectraMax M2 spectrophotometer and microplate reader.

**Table 1. List of buffer conditions for each assay**

|  |  |  |
| --- | --- | --- |
| **Assay** | **Buffer** | **Dilution** |
| Protein Quantification | PE | 0.1x |
| Polyphenol Oxidase | PE | 0.1x |
| Peroxidase | PE | 0.1x |
| Proteinase Inhibitor | PE | 0.1x |
| Hydrogen Peroxide | TCA | 1x |

*Protein Quantification*

Protein quantification was performed using the Thermo ScientificPierceTM BCA Protein Assay Kit (Product number: 23337) according to manufacturer instructions for microplate samples.

*Peroxidase (POD) Activity*

POD activity was measured using a modified quantification assay previously described by [27]. Sample aliquots were taken from the 1/10x PE buffer extraction. Each sample was processed in triplicate with a tissue specific control containing no guaiacol. Wells of the microplate designated as treatment wells received 143 uL of peroxidase buffer (100 mM sodium phosphate buffer (pH 6.5) containing 5 mM guaiacol). Wells designated for controls received 143 uL of 100mM sodium phosphate buffer (ph 6.5). 25 uL of supernatant (enzyme source) was added to each well.  We then added 32 uL of 5 mM H2O2(final concentration 0.8 mM) and incubated the plates in the dark for 15 minutes at room temperature. Absorbance was read at 470 nm and enzyme content was expressed as [(AbsTrt - AbsCtrl) FW-1] (Abs g-1).

*Polyphenol Oxidase (PPO) Activity*

PPO activity was measured using a modified quantification assay previously described by [28]. Sample aliquots were taken from the 1/10x PE buffer extraction. Each sample was processed in triplicate with a tissue specific control. Wells of the microplate designated as treatment wells received 115 uL of 100 mM sodium phosphate buffer (pH 6.8) and 60 uL of 50 mM pyrocatechol. Control wells received 175 uL of 100 mM sodium phosphate buffer (pH 6.8). 25 uL of supernatant (enzyme source) was added to all wells and samples were incubated for 5 minutes. Absorbance was read on the microplate reader at 420 nm and enzyme content was expressed as [(AbsTrt - AbsCtrl) FW-1] (Abs g-1).

*Hydrogen Peroxide (H2O2)Quantification*

Hydrogen peroxide was quantified using a modified assay previously described by [29]. Sample aliquots were taken from the 0.1% TCA buffer extraction. All sample wells received 100 uL of 1M potassium iodide (KI), 50 uL of 10mM Potassium Phosphate buffer (pH 6.5), and 50 uL of sample aliquot. Control wells received 100 uL of dH2O, 50 uL of 10mM Potassium Phosphate buffer (pH 6.5), and 50 uL of sample aliquot to account for tissue coloration. Samples were incubated in the dark for 20 minutes at room temperature. A standard curve was prepared by preparing wells with 100 uL of 1M potassium iodide (KI), 50 uL of 10mM Potassium Phosphate buffer (pH 6.5), and 50 uL of 0.1% TCA then seeding with known amounts (5-20 nmoles) of H2O2. Absorbance was measured at 390 nm and values were compared to the standard curve for quantification in nmoles.

Proteinase Inhibition (PI) Activity

Proteinase Inhibition activity was quantified using an adapted method from [30]. Activity is represented by the inhibition of trypsin. Sample aliquots were taken from the 1/10x PE buffer. Reactions were prepared using 100 uL of enzyme source, 133.3 uL of Trizma Base buffer, 83.3 uL of 2% azocasein dissolved in Trizma Base buffer, and 33.3 uL of 0.001 M HCl solution containing 200 ng of trypsin. Sample controls were prepared the same way, except that the additional Trizma Base was substituted for the trypsin. Assay controls were prepared with 100 uL of Trizma Base instead of enzyme source. The negative control also substituted Trizma Base for the trypsin solution. Samples were incubated at 30°C for 25 minutes. Post-incubation, 133 uL of 100% w/v TCA was added and samples were centrifuged at 8000 rpm for 10 minutes. 100 uL of supernatant were added to wells of a microplate that contained 100 uL of 1M NaOH and absorbance was measured at 450 nm. PI activity was calculated for pre- and post- herbivory as 1-((AbsSample/AbsSampControl)/(AbsPosContrlol/AbsNegControl)), standardized by tissue weight and then the values for post herbivory minus pre- herbivory were reported.

**Appendix 2**

**Medicago polymorpha genotype with country and GPS coordinates**

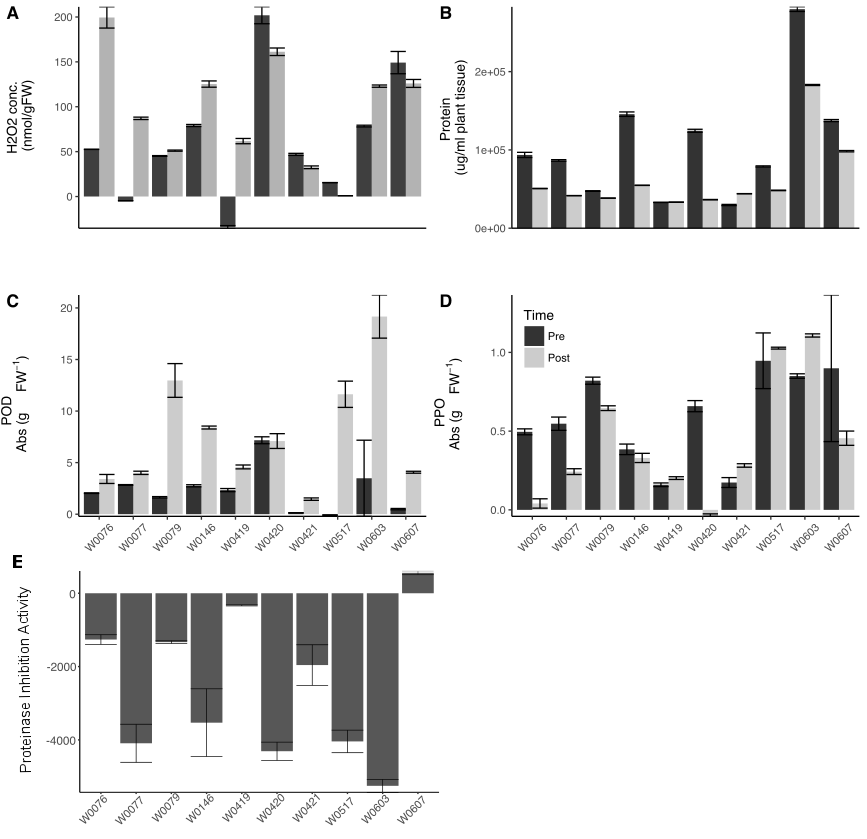
W0419 (), W0420 (), W0077 (), W0607 (), W0079 (), W0076 (), W0517 (), W0603 (), W0146 (), W0421 ()

**Appendix 3**



Results of our microplate-based protocols using tomato for A) H2O2, B) Protein Quantification, C) POD, D) PPO, and E) PI. The significant variation between biological replicates makes a strong argument against pooling tissue samples from different plants and highlights the benefit to using a protocol that requires a much smaller quantity of tissue.

**Appendix 4**



**Assays tested on *Medicago polymorpha*.** As proof of concept, we ran all of the assays on a non-model plant, *Medicago polymorpha*. A) H202 B) Protein Quantification C) POD D) PPO E) PI. Unlike tomato, *M. polymorpha* does not always show increase in production of defensive phytochemicals.