Applications in Plant Sciences

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

--Manuscript Draft--

Manuscript Number:	APPS-D-18-00103R1
Full Title:	A high-throughput method of analyzing multiple plant defensive compounds in minimized sample mass
Article Type:	Protocol Note
Keywords:	Microplate; Plant Defense Response; Peroxidase; Polyphenol oxidase; Protease Inhibitors; Protein Quantification
Corresponding Author:	Chandra Jack Washington State University Pullman, WA UNITED STATES
Corresponding Author Secondary Information:	
Corresponding Author's Institution:	Washington State University
Corresponding Author's Secondary Institution:	
First Author:	Chandra Jack
First Author Secondary Information:	
Order of Authors:	Chandra Jack
	Shawna Rowe
	Stephanie Porter
	Maren Friesen
Order of Authors Secondary Information:	
Abstract:	Premise of the study: Current methods for quantifying herbivore-caused alterations in plant biochemistry are expensive, time-consuming, and require large tissue amounts. We present a cost-effective, high-throughput protocol to quantify multiple biochemical responses from small plant tissue samples using spectrophotometric techniques. Methods and Results: Using Solanum lycopersicum and Medicago polymorpha leaves pre- and post-herbivory, we demonstrate that our protocol quantifies common plant defense responses: peroxidase production, polyphenol oxidase production, reactive oxygen species production, total protein production, and Trypsin-like protease inhibition activity. Conclusions: Current protocols can require 500 mg of tissue, but our assays detect activity in under 10 mg. Our protocol takes two people approximately 6 hours to run any of the assays on 300 samples in triplicate, or all of the assays on 20 samples. Our protocol enables researchers to plan complex experiments that compare local versus systemic plant responses, quantify environmental and genetic variation, and measure population level variation.
Funding Information:	Directorate for Biological Sciences (1354878) — Dr. Maren Friesen
	Directorate for Biological Sciences (1355216) — Dr. Stephanie Porter
	MSU Training Program in Plant Biotechnology for Health and Sustainability (T32-GM110523) — Ms. Shawna Rowe
	National Science Foundation (DBI-0939454) — Dr. Chandra Jack

Applications in Plant Sciences Author Agreement Form

Click on the first gray box and start typing. You may use the Tab to get to the next field.

Corresponding Author's Name: Chandra Jack
Date: 9 June 2018

Respond to ALL the statements below either by typing your initials or checking the appropriate box. After you have completed this form, save it to your desktop as a Word document, and upload it with your manuscript submission on the "Attach Files" page in Editorial Manager (http://www.edmgr.com/apps).

1. All Contributing Authors know of and concur with the submission of this manuscript. Single authors please also initial.
Initials: CNJ

2. All authors of this research paper have directly participated in the planning, execution, or analysis of the study;
AND
All authors of this paper have read and approved the final version submitted.
Initials: CNJ

3. The contents of this manuscript have not been copyrighted or published previously and are not now under consideration for publication elsewhere. The contents of this manuscript will not be copyrighted, submitted, or published elsewhere while acceptance by the Journal is under consideration.
Initials: CNJ

4. *APPS* operates under a Creative Commons Attribution 3.0 license. Please specify which license should be applied to publication of this article:

CC-BY-NC-SA: This is the default license under which articles are published in *APPS*. Under the terms of this license, authors retain ownership of the copyright of their articles. Users are allowed to download, print out, extract, reuse, archive, and distribute the article for noncommercial use; this use is contingent upon appropriate credit being given to the authors and the source of the work and upon the new creation being licensed under the same terms.

CC-BY: This license should be selected if mandated by article funding sources. CC-BY permits use, distribution, and reproduction in any medium, provided the original work is properly cited. There is no distinction between reuse for commercial or noncommercial purposes.

CC0: This is the Creative Commons public domain license. Papers authored by one or more US government employees should use this license, which allows unlimited distribution and reuse of the article for any lawful purpose.

Each of these licenses ensure wide availability of the article and that the article can be included in any scientific archive. No permission is required from the author or the Botanical Society of

America. If a CC-BY-NC-SA license is used, those wishing to use material for commercial purposes should contact the author.

Please select the license that should be applied to this article:

☒ **CC-BY-NC-SA**

☐ **CC-BY**

☐ **CC0**

4a. If you select the CC0 license, please confirm the following: One or more authors of this manuscript are US government employees, and this work was prepared as part of the author's official duties as a US government employee. Note: If this work was not prepared as part of the author's official duties as a US government employee, it should be published under a CC-BY-NC-SA or CC-BY Creative Commons license.
Initials:

5. Authors are responsible for recognizing and disclosing any duality of interest that could be perceived to bias their work, acknowledging all financial support and any other personal connections.

☒**No**, there is no duality of interest that I should disclose, having read the above statement.

☐**Yes**, having read the above statement, there is potential duality of interest. This has been fully detailed in my cover letter.

6. Have the results/data/figures in this manuscript been published or are they under consideration for publication elsewhere?

☒**No**, the results/data/figures in this manuscript have not been published elsewhere, nor are they under consideration (from you or one of your Contributing Authors) by another publisher.

☐**Yes**, some portion of the results/data/figures in this manuscript has been published or is under consideration for publication elsewhere.

6a. If you select Yes, please identify results/data/figures taken from other published/pending manuscripts in the textbox below and explain why this does not constitute dual publication. (Note: The existence of pending or previously published articles that use or have used any of the same results presented in the submitted manuscript does not generally prejudice review and acceptance.)

7. All *APPS* papers are published as Open Access articles. Article Processing Charges (APCs) are as follows:

Non-BSA members: $1400 per article
BSA members: $800 per article
BSA members with an active membership for longer than two years: $450 per article

This policy applies to all articles accepted, except for invited articles for which the Editorial Board has agreed to waive fees at the time of article submission. **Authors will be invoiced and payment must be received before online publication.**

☐ **No,** No authors of this manuscript are members of the BSA.

☒ **Yes,** I confirm that at least one author is a BSA member

Please note here the name(s) of author(s) who are BSA members. Membership status will be verified by the editorial office.
Maren Friesen

8. Authors are responsible for covering charges for author-initiated changes made in proof. Authors will be charged when more than 10 changes are requested per article, as well as for figure replacements in proof ($25.00 per figure). This policy applies to BSA members and nonmembers. I have read and understand this policy.
CNJ

9. *APPS* requires that genetic information, such as DNA, RNA, or protein sequences, be submitted to an appropriate data bank (i.e., GenBank or EMBL). Alignments used to produce phylogenies must be submitted to TreeBase or to *APPS* to be published with the paper as supplementary material. Authors are encouraged to archive all sequences generated from next-generation sequencing techniques in a suitable public depository (e.g., the Sequence Read Archive of NCBI, the Sequence Read Archive of ENA, or Dryad). I have read and understand this policy.
CNJ

[last revised 21 June 2017]

August 13, 2018

Re: Resubmission of manuscript *A high-throughput method of analyzing multiple plant defensive compounds in minimized sample mass*, APPS-D-18-00103

Dear Dr. Culley and Dr. Parada:

Thank you for the opportunity to revise and resubmit our manuscript, *A high-throughput method of analyzing multiple plant defensive compounds in minimized sample mass.* We appreciate the review and constructive suggestions. It is our belief that the manuscript is substantially improved after making the suggested edits and we look forward to an additional review from a previous reviewer.

Below we address the referee's comments. The comments are numbered and our responses are in blue. We include section and paragraph number references in our responses to reviewer comments to aid in the review process.

Thank you for your consideration.

Sincerely,

Chandra Jack and Shawna Rowe

Editor comments

1. Greater detail for the methods with clearer organization is needed to better enable someone to use your procedure.
   We have divided the main paragraph of the methods section by assay to give a better idea of how we performed our assays. We have also taken a suggestion by Reviewer 3 and transformed Appendix 1 into a printable protocol that clearly details the steps needed to perform each assay. We hope that our offers clearer organization and a protocol that is able to be accurately followed.
2. The relative importance of this general method (why would one want to obtain this information). Why is your method better than alternative approaches. You say this is faster but how does the quality of the results and speed compare to other methods. Being more specific would be useful.
   Thank you for the helpful feedback. We hope that changes made in text referencing technical issues, specifically changes made in the introduction and validation sections, help us make our point more clearly.
3. Avoid repeating parts of the text in different sections. Please phrase content differently in different sections of the manuscript.
   Repeated sections of text have been removed and reworded throughout, this is especially evident in our revamped Appendix 1.

Reviewer 1

Major Points

The authors write several times that their results confirm other, already published results. Please provide examples for that statement. I guess a table where the new and published results are compared will support the meaning of this new method.
We thank the reviewer for the suggestion and agree that it is important to more clearly explain how our results compare to published literature/ methods. To achieve this, we have included the following explanation (in the Validation section) to say, "Implementation of published protocols (Orians et al. 2000; War et al. 2011) on *S. lycopersicum* provided us with a point of reference for comparison of our modified methods. By first establishing an expected response to a given treatment we are able to determine if the measured microplate response is sufficiently similar and reproducible." We also feel that comparisons to literature values that were generated from experiments will not be useful because those experiments will have used things such as different plant species, different herbivores, no herbivores, different machines, different standards, and different data reporting methods. For instance, the Orians et al paper (2000) uses enzymatic treatments and compares PI induction at different leaf positions which would not be directly comparable to our results. The War et al 2011 paper uses groundnut, a different herbivore, and reports data as IU g/ FW which prevents us from making useful comparisons. These same issues and others, are apparent throughout the literature and we feel that any attempt to consolidate this data into a single table would fail to communicate how our results pair with the literature. Due to these constraints, we compare spectrophotometry data generated by us using published protocols on model species with

data generated by us using our modified method. This builds off the assumptions that previous protocols are able to accurately and effectively measure a given response. This change can be found in paragraph 10 of the "Methods and Results" section.

For the Solanum experiments (p. 20), I understand that you combined leaves that were infested with the insect and leaves that were wounded/regurgitate-treated. Why putting both treatments together? For me that makes no sense.
Due to variable feeding patterns, use of regurgitate was required to simulate herbivory conditions for parts of the experiment. Mechanical wounding using regurgitant and/or saliva has been shown to induce herbivory responses in a similar fashion (e.g. Santiago et al 2017; Kant et al 2015; Lawrence et al 2008; Engelberth et al 2004; Erb et al 2000; Korth & Dixon, 1997; Mattiacci et al 1994). We have modified that sentence to include the explanation. This change can be found in paragraph 2 of the "Methods and Results" section.

The Medicago experiments have nothing to do with any type of herbivory or herbivory-like treatments. Using whole larvae homogenate-extract in combination with wounding cannot mimic insect feeding. Please rephrase this approach and write "wounding".
We have revised our manuscript to make this change. We have replaced references in these instances to say "mechanical wounding with regurgitant and/or homogenate to simulate herbivory treatment" and variations of this when appropriate. References throughout have been replaced.

On page 10 line 18ff the authors argue for individually assaying plants but refer to table 2, which compares spectrophotometer and microplates. I do not see any connection here. The statement on page 10 line 31ff ("The dramatic difference in absorbance means between the microplate method….") contradicts what is shown in Figure 1. Please explain.
Figure 1 is a representation of the standard curves of POD and PPO and comparing the absorbance values and r^2 values using both the microplate method and the published spectrophotometer using known concentrations of enzymes. We have modified our figure legend to explicitly state that the figure is of our standard curves.

Minor points:

1. Write "trypsin-like protease inhibitors" not "protease inhibitors", which is too general.
   This suggestion was implemented. Changes were made throughout, when appropriate.
2. Page 6 line 40 ff: During the 15 min of homogenizing of the plant tissue, is here no cooling at all?
   We homogenized tissue using holders that are made of teflon and stored at -80C. All samples and holders were also dipped in liquid N before homogenizing. We have made in text changes (Paragraph 4 of the "Methods and Results" section)
3. Page 6 line 38: don't you mean table 3?
   We have changed the reference to Table 2 in paragraph 3 of the "Methods and Results" section.

4. Page 10 line 22: write Appendix 3 not 2.
   Line was fixed to say Appendix 3 instead of Appendix 2
5. Table 1: Mention the plant that was used and explain better what is shown here (in the Text and more info in the legend).
   We now say (paragraph 13 of "Methods and Results") that "Current practice for many of these assays is to pool tissue samples from multiple plants. Table 1 shows the results of an ANOVA where we compare Absorbance values based on plant sample pre- and post- herbivory and show that there is significant variation between plants of the same ecotype that were grown in the same environment. This variation highlights the strength of our assay, which does not require plant tissue to be pooled.
6. Table 2: Which plant?
   Included "Values taken from *Solanum lycopersicum* samples." in the legend.

7. Appendix 3: You wrote: "E is calculated by subtracting post-herbivory values from pre-herbivory values". Isn't it the other way around? How can you get positive results? Compare with Appendix 4E.
   Inhibition is often shown in terms of proteolytic enzyme activity. We get positive values here because the activity of the enzymes we are assaying would be higher pre-herbivory due to the lower concentrations of PI (inhibitor) present in our samples. Post-herbivory, we expect there to be an increase in PI concentration, resulting in reduced enzyme activity. Showing the difference as pre-herbivory(high activity) - post-herbivory (low activity) results in a net positive amount of activity. We have modified the figure to read as "PI trypsin activity in IU" on the Y-axis to prevent further confusion. While in Tomato, wounding always resulted in increased activity (due to a, in Medicago (Appendix Fig 4E) the response to wounding was genotype dependent.

Reviewer 2

Major points

I was expecting to see a comparison between the developed method and the previous methods. We see that the develop is effective using small sample sizes, but when compared to the older methods that require more sample, are they still just as accurate?
We thank the reviewer for the suggestion and agree that it is important to more clearly explain how our results compare to published literature/ methods. To achieve this, we have included the following explanation (in the Validation section) to say, "Implementation of published protocols (Orians et al. 2000; War et al. 2011) on *S. lycopersicum* provided us with a point of reference for comparison of our modified methods. By first establishing an expected response to a given treatment we are able to determine if the measured microplate response is sufficiently similar and reproducible." We also feel that comparisons to literature values that were generated from experiments will not be useful because those experiments will have used things such as different plant species, different herbivores, no herbivores, different machines, different standards, and different data reporting methods. For instance, the Orians et al paper (2000) uses enzymatic treatments and compares PI induction at different leaf positions which would not be directly comparable to our results. The War et al 2011 paper uses groundnut, a

different herbivore, and reports data as IU g/ FW which prevents us from making useful comparisons. These same issues and others, are apparent throughout the literature and we feel that any attempt to consolidate this data into a single table would fail to communicate how our results pair with the literature. Due to these constraints, we compare spectrophotometry data generated by us using published protocols on model species with data generated by us using our modified method. This builds off the assumptions that previous protocols are able to accurately and effectively measure a given response. This change can be found in paragraph 10 of the "Methods and Results" section.

I also would have liked to see some comments about the trend that more studies use genomic data to examine the genetic variation underlying the differences in herbivory-response, so the readers are convinced that chemical assays are still an important aspect of this field.
We have now included the following text in the introduction (paragraph 6):
"Since the onset of the next generation sequencing revolution, many studies use genomic data as evidence of variation. Although this has proven to be a highly useful tool in many studies, it is important to assess functional variation as well. Studies have indicated that transcript abundance doesn't necessarily match functional activity in a tissue samples (Greenbaum et al. 2013). This finding and others like it are important to consider when assessing functional diversity in physical populations of organisms."

Minor point
Introduction

Paragraph 1, line 1: The first sentence is almost identical to one in the abstract. Please rephrase it to avoid repetitions.
The first two lines of the introduction now read, "The ability to quantify plant molecular responses to herbivory over time and compare variation within populations is useful in many research areas from ecology and evolutionary biology to applied agricultural research. Unfortunately, current methods are expensive, time consuming, and typically require large tissue masses."

Paragraph 1, line 6: "Previously developed assays" Could you provide some examples and citations?
We feel that this is addressed sufficiently throughout the remaining text of paragraph 1. We thank the reviewer for their suggestion.

Paragraph 1, line 7: "Unusable due to financial constraints" Is this due to the cost of assay reagents/kits or due to the large amounts of samples required for the assays?
The sentence has been changed to read (paragraph 1 of "Introduction"), " Previously developed assays are thus effective but unusable by many researchers due to financial constraints (i.e. lack of access to spectrophotometers or large quantities of reagents) or the need to conduct a given assay on a large set of samples."

Paragraph 1, line 10: Are there more recent reviews on this? Methods improve in such short time periods that a decade-old reviews may not be relevant for an up-to-date comparison of available methods.
Many of the current methods still rely on high volume spectrophotometer methods, which were not feasible for the number of experimental tissue samples we needed to process for our research, so we set out to modify those protocols and developed a system that we thought would be beneficial to the greater scientific community.

Paragraph 2, line 5: H2O or H2O2?
This line has been changed to H2O2 instead of H2O. We thank the reviewer for noticing this.

Paragraph 2, line 17: What is meant by "large sample masses"? Most of the methods are capable of detecting small amounts using microplate readers.
Large sample masses are required due to the 3ml reaction volume required for common spectrophotometric assays. To express this, the sentence has been changed to read, "Additionally, as with protein-based assays, the large sample masses required as a result of large reaction volumes for current spectrophotometric techniques limits the total number of technical replications and assays that can be performed on a given sample." This is found in paragraph 5 of "introduction."

Paragraph 3, line 5: Please cite some examples for these defense response assays.
One of the earliest biochemical responses to herbivory is the production of reactive oxygen species (ROS) such as superoxide ($O^{\cdot}$), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($HO^{\cdot}$) after depolarization of the plasma membrane due to leaf damage (Maffei et al 2012; War et al 2012; Zebelo & Maffei 2015). Both chemical treatments and mechanical wounding can elicit ROS production (Maffei et al 2007). H_2O_2 production is both used as a local signal to induce the hypersensitive response when plants are subjected to mechanical damage as well as a systemic signal for the induction of additional defense responses (Orozco-Cárdenas & Ryan 1999). The presence and activity of ROS additionally results in the production of a group of enzymes, peroxidases (POD), that are upregulated to perform a diverse set of physiological processes such as metabolism of ROS, restructuring of cellular walls, cross-linking of complex polymers, and other critical functions (War et al 2012). Increases in POD activity also decrease the nutritional quality of leaf tissue, which significantly reduces the growth and development of insect larvae. Finally, the presence of plant PODs in insect guts may also be toxic to insects.
Two additional compounds that are produced in response to herbivory are polyphenol oxidase (PPO) and proteinase inhibitors (PI) (Mithöfer & Boland 2012; War et al 2012). PPO is upregulated directly by the presence of herbivore-associated signaling compounds such as methyl-jasmonate (Koussevitzky et al 2004). PPO breaks down diphenolic compounds to produce more reactive phenolic compounds that have anti-insect activity once consumed (War et al 2012). Protease inhibitors (PI) are small molecules that prevent proteolytic activity. In response to herbivores, plants will produce PIs to inhibit protein catabolism in insect guts which can halt the degradation of proteins that may serve as precursors used for various physiological processes (Mithöfer & Boland 2012).

Paragraph 3, line 6: Please clarify whether these selected responses are the ones listed in line 3 (protein content, peroxidase, polyphenol oxidase, H2O2, and protease inhibitors). It would also be beneficial to briefly explain how quantifying these responses are relevant to herbivory detection.
The paragraph above was added and we believe that it addresses both this comment and the previous comment.

Methods and Results

Paragraph 1, line 3: Does *Medicago polymorpha* have defense response mechanisms similar to *Solanum lycopersicum*? The reason for choosing *Solanum lycopersicum* is clearly stated, but the reason for selecting *Medicago polymorpha* is not justified.
We use MP as an example of a plant that has not been well characterized and tends to have small leaves, making it more difficult to assay using spectrophotometric methods. Many species would have worked here but because of the specific expertise of our lab we selected *M. polymorpha.*

Paragraph 2, line 6: Are 0 and 24 hrs sufficient time points for detecting herbivory defense response?
Yes. The literature we reference indicates that this is common practice in the plant-herbivore interactions field.

Paragraph 3, line 18: How does Table 1 validate that the replicated assays provide predictable results?
Please see our response to the first major comment.

Paragraph 4: There are several sentences identical to the ones in the Appendix 1. Please revise this section.
Appendix 1 has been modified to read more as a protocol as per reviewer suggestions.
Paragraph 5, line 12: 2/5 should be 2.5.
This has been changed.
Paragraph 6, line 6: Appendix 2 should be Appendix 3.
This has been changed.
Paragraph 6, line 7: I am not sure I follow the reason for referring to Table 2 here. The text says assaying individual samples reduce experimental error, but the table shows the difference between spectrophotometer and microplate assays. Does this mean spectrophotometer assays use pooled samples whereas microplate assays use individual samples? If so, please clarify. Can you also perform a statistical test to state whether these differences are significant?
We have modified this area to read as follows: “Pooling tissue samples, such as what is required to get adequate tissue masses for spectrophotometric assays, increases variability due to the sample pool containing multiple individual plant responses and makes a strong argument for assaying individual plants.” This is to indicate that the spectrophotometer assays are pooled. This change was made in paragraph 13 of “Methods and Results.”

Paragraph 6, line 10-12: Please explain the observed "dramatic differences" between the spectrophotometric and microplate methods. It is previously explained in the manuscript that both readings were performed on the same machine (SpectraMax M2), so the differences cannot be explained as "a consequence of using different machines".
This sentence has now been changed to "different detection methods." Although the machine is the same, the functions for reading cuvette samples and microplate samples are different and also output the data separately. This change was made in paragraph 13 of "Methods and Results."

Paragraph 6, line 16: "we were able to detect expression in as little as 3.8 mg of issue" Figure 2 and Appendix 3 is cited here, but the detection threshold of 3.8 mg is not clearly shown anywhere.
We have modified our reference to Figure 2 to include more explanation. Specifically, "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. This was determined by doing a series of dilutions on a tissue sample to determine the linear range of the microplate assay (Fig. 2)."

Appendix 1

· Table 1 in the Appendix should be given a different name. Table 1 already exists in the manuscript.
Table 1 is now named Table A1.

Appendix 3

· The legend for time (pre vs post) should be present for A, B, C, and D. Alternatively, place the legend next to the 4-plot panel.
The figure has been changed to reflect this.
· Please indicate that five samples of the same ecotype were used for the plots.
The figure has been changed to reflect this.
· A statistical test should be performed to state that the variation was "significant".
We have added a line that now says, "(see Main Document Table 1)" to reference the results of statistical tests in main document table 1.
· The plots are too small. Can they be enlarged to match the ones in Appendix 4?
The plots have been made larger

Appendix 4

· The first two comments for Appendix 3 also apply to Appendix 4.
The figure has been changed to reflect this.
· The Y axis for plots C and D are written in different format between Appendix 3 and Appendix 4 (g/FW vs g FW-1). Please keep the format consistent to avoid confusion.
The figure has been changed to reflect this.
· There are vast differences between the tomato and *Medicago* plots for each assay (Appendix 3 versus Appendix 4). Any comments?

These are different species and are expected to have different responses to wounding. Additionally, the tomato plants are the same ecotype, while we tested 5 genetically distinct *Medicago polymorpha* ecotypes.

Figure 1:

- State the wavelength at which the absorbance was measured for each assay. This information should be given in both the figure legend and the Y-axis on the plots.

We have changed the figure legend to include the wavelengths for each assay and they have been added to the figure y axis.

- Please change "dot" to "data point" or something similar. Data points are represented by "dots" and "triangles" on the plots.

We have switched from dot to data point.

- Please rephrase this sentence: "The line All concentrations were done in triplicate."

"The line" has been removed.

Figure 2:

- The first comment for Figure 1 also applies to Figure 2.

We have changed the figure legend to include the wavelengths for each assay and they have been added to the figure y axis.

- What does FW stand for? Fresh weight? It is not explained anywhere in the text.

FW stands for fresh weight and we have now defined it in the last line of the assays section in the methods.

- Again, the lower threshold for accurate detection is not clear here. The X axis does not seem to match with the mentioned starting point of 0.38 g/ml.

We have clarified this in the text.

Reviewer 3

Major points

1) Compare the techniques to other techniques in the literature or tested against samples with known concentrations for all studies. The authors do this with POD and PPO, but not with H2O2, PI, or Protein Quantification. Known concentrations of H2O2 were used to compute a calibration curve, but nothing was confirmed on other samples. If these protocols have been well tested in the literature already, then please incorporate that into a discussion. This is especially important because of the variation that was observed across ecotypes (which is really interesting and a great addition that sells the importance of not combining samples from multiple individuals).

On page 4, line 49, tomato was used to be able to compare to other published studies, but this comparison was not done.

We thank the reviewer for the suggestion and agree that it is important to more clearly explain how our results compare to published literature/ methods. To achieve this, we have included the following explanation (in the Validation section) to say, "Implementation of published protocols (Orians et al. 2000; War et al. 2011) on *S. lycopersicum* provided us with a point of reference for comparison of our modified methods. By first establishing an expected response to a given treatment we are able to

determine if the measured microplate response is sufficiently similar and reproducible." We also feel that comparisons to literature values that were generated from experiments will not be useful because those experiments will have used things such as different plant species, different herbivores, no herbivores, different machines, different standards, and different data reporting methods. For instance, the Orians et al paper (2000) uses enzymatic treatments and compares PI induction at different leaf positions which would not be directly comparable to our results. The War et al 2011 paper uses groundnut, a different herbivore, and reports data as IU g/ FW which prevents us from making useful comparisons. These same issues and others, are apparent throughout the literature and we feel that any attempt to consolidate this data into a single table would fail to communicate how our results pair with the literature. Due to these constraints, we compare spectrophotometry data generated by us using published protocols on model species with data generated by us using our modified method. This builds off the assumptions that previous protocols are able to accurately and effectively measure a given response. This change can be found in paragraph 10 of the "Methods and Results" section.

2) Consolidate and Clean up the Methods and Appendix 1. I view Appendix 1 as recipe that I need to print out and follow, so the Reagents should be closer to the respective protocol (as opposed to separated by the plant growth and plant damage methods). I think the plant growth and damage protocol can be moved to their own Appendix or removed completely. The plant growth and damage protocol (page 19 line 20 to page 20 line 58) occur in the Methods (page 5 lines 13-52). Moreover, some of these descriptions in the Appendix do not line up with the Methods. For example, the Methods on page 5, line 47 mentions regurgitant, yet page 20 line 34 mentions homogenate and page 20 lines 11-16 mention that both live organisms and scissors were used.

In the Methods, please split up the two paragraphs under Assays (page 5 line 56 to page 9 line 23). I recommend deleting page 6 lines 38 and 40 ("All protein extractions were diluted...") because it appears at at the bottom of page 6 and top of page 7. The new paragraph would then start with "Frozen leaf tissue...". Page 7 and page 8 can be broken up into the various tests: page 7 line 29 "POD activity..."; page 7 line 45 "PPO activity"; page 7 line 58 "Proteinase inhibition activity..."; and page 8 line 34 "The hydrogen peroxide...". At the end of each paragraph, I would like to see what was measured (in many cases absorbance was measured, but amount could be computed as g / g FW). PI and H2O2 does this.
We have taken your suggestion and reorganized this section.

Minor suggestions:
page 2 line 22: define ROS instead of acronym
This line now reads "reactive oxygen species"

page 3 line 45: should H2O be H2O2?
Yes, this has been corrected to reflect this suggestion. We thank the reviewer for finding this typo.

page 3 line 59: transition between sentences. Disconnect between complex responses and requiring a large sample.
This has been changed to read as follows: “Additionally, reducing the total amount of tissue required for an expanded array of assays enables researchers to perform both small molecule and enzymatic assays during a given investigation by allowing two separate extraction buffers to be used resulting in smaller amounts of tissue being assayed in more ways. This allows complex responses across large numbers of individuals to be analyzed within a single experiment.” This change can be found in paragraph 5 of “Introduction.”

page 4 line 7: transition between sentences. Disconnect between protein sampling and buffers for small molecules
This portion has been changed to read, “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, which causes protein precipitation [5] preventing the use of a single buffer. Additionally, as with protein-based assays, the large sample masses required as a result of large reaction volumes for current spectrophotometric techniques limits the total number of technical replications and assays that can be performed on a given sample.”

page 4 line 36: Introduce acronyms here or remove acronyms from paper.
H2O2 has now been introduced as hydrogen peroxide in the second paragraph of the introduction.

page 4 line 37: Include genus in scientific name of tomato
We first introduce *Solanum lycopersicum* in the line preceding this and feel that *S. lycopersicum* is sufficient throughout the remaining discussions in the paper. This is common practice in studies using plants.

page 5 lines 31-36: Why were plants inoculated?
Since *M. polymorpha* is a legume, inoculum was used to mimic natural conditions. Fertilizer could have also been used here to prevent nitrogen deficiency. We have modified the line to read, “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 to prevent nitrogen starvation and to mimic natural conditions.” This change can be found in paragraph 1 of “Methods and Results.”

page 6 line 41: how much tissue was used? Relates to merging appendix and methods
We hope that the reorganization of the manuscript addresses this concern.

pages 7-9 and Appendix 1: missing a prefix (I assume micro), everything in Liters
We have corrected the prefixes.

page 7 line 59: change (Orians et al. 2000) to Orians et al. (2000)
This citation has been fixed.

page 8 line 38: what is [6]?

This was citation that was left in from a previous draft. The citation has been added in the appropriate form.

page 8 line 49: More information about what wavelengths were tested. t suggests two wavelengths.
We have added that we compared absorbance at 350 nm and 390 nm. Paragraph 9 of "Methods and Results."

page 8 line 52: "special plates for to avoid issues" remove for.
We have made this change.

page 8 line 56: Is there an article to be compared to? Do these results appear in a table?
The spectrophotometer method we use serves as our data for comparison. There is no table to compare to literature values due the variability in sample preparation, analysis, and reporting.

page 9 line 52: 2/5 is 2.5?
This has been fixed

page 10 line 23: reference to appendix 2, but that shows M. polymorpha information when the sentence discusses tomato. Should this be appendix 3?
This has been changed to appendix 3.

page 10 line 23: Table 1 and the figure in appendix 3 show the same idea. I prefer the figure in appendix 3.
We are recommended to have only 2 figures for this journal and had a hard time picking the appropriate ones. Because of this, we opted for figures that had information that was difficult to also communicate in table form. Since we are capable of communicating this as a table we have decided to leave it as such. We thank the reviewer for their input.

page 10 lines 31-36: Mentions difference in values between microplate method and spectrophotometric method, but measures absorbance. Compute concentration and compare.
This sentence has now been changed to "different detection methods." Although the machine is the same, the functions for reading cuvette samples and microplate samples are different and also output the data separately. We have modified this paragraph to say "that assaying plants individually for both POD and PPO, we are able to reduce experimental error. This is important because it indicates that our readings are more reproducible than alternative methods (Table 2)" and we have modified the Table 1 heading to reflect the important comparison is between standard errors.

Table 1: Define acronyms (or use acronyms more in paper). Think about replacing with Figure from appendix 3.
We hope we have made this more clear with our reorganization of the paper.

page 20 line 20: Where the 4 replicate leaves combined into one sample?

We included the word “separately” to indicate that the replicate leaves were not combined.

page 20 line 38: Where were the leaves cut? Along the blade?
We added “blade” for clarity.

Appendix 1: in two places N2 needs subscripted 2
This change has been made in the manuscript.

page 23 line 14: H2O2 needs subscripts
This change has been made in the manuscript.

Appendix 2: Is this referenced in the article? I was confused as to why this was included.
This is referenced in the Methods & Results section. The journal requires location information for all collected germplasm.

Appendix 3: Figure: POD and PPO units should be Abs / g Fw (from table 2 in main article)
We have changed this.

Appendix 3: Please define what 1-5 on the x axis mean. Maybe as simple as "Results of our microplate-based protocols for five tomato plants of ecotype M82..."
We have modified our figure legend to say, “Results of our microplate-based protocols using *S. lycopersicum,* ecotype M82 for A) H_2O_2, B) Protein Quantification, C) POD, D) PPO, and E) PI. The numbers 1-5 on the x-axis reflect individual plants used for sampling.”

page 24 line 54: Are error bars standard error bars?
We have now included “standard error” in the legend for clarity.

page 25 line 56: Are error bars standard error bars?
We have now included “standard error” in the legend for clarity.

page 26 line 14: what is the line "All concentrations"
We have removed the words “the line.”

Jack et al.- High-throughput plant defense analysis

A high-throughput method of analyzing multiple plant defensive compounds in minimized sample mass[1]

Chandra N. Jack[2,6,§], Shawna L. Rowe[3,§], Stephanie S. Porter[4], Maren L. Friesen[2,5]

[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

SLR: roweshaw@gmail.com

SSP: stephanie.porter@wsu.edu

MLF: m.friesen@wsu.edu

Number of words: 3493

[1] Manuscript received _____; revision accepted _____.

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

ABSTRACT

Premise of the study: Current methods for quantifying herbivore-caused alterations in plant biochemistry are expensive, time-consuming, and require large tissue amounts. We present a cost-effective, high-throughput protocol to quantify multiple biochemical responses from small plant tissue samples using spectrophotometric techniques.

Methods and Results: Using *Solanum lycopersicum* and *Medicago polymorpha* leaves pre- and post-herbivory, we demonstrate that our protocol quantifies common plant defense responses: peroxidase production, polyphenol oxidase production, reactive oxygen species production, total protein production, and Trypsin-like protease inhibition activity.

Conclusions: Current protocols can require 500 mg of tissue, but our assays detect activity in under 10 mg. Our protocol takes two people approximately 6 hours to run any of the assays on 300 samples in triplicate, or all of the assays on 20 samples. Our protocol enables researchers to plan 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

The ability to quantify plant molecular responses to herbivory over time and compare variation within populations is useful in many research areas from ecology and evolutionary biology to applied agricultural research. Unfortunately, current methods are expensive, time consuming, and typically require large tissue masses. 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 - (i.e. lack of access to spectrophotometers or large quantities of reagents) 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 (Olson and Markwell 2007). With the development and widespread use of microplate readers, some assays such as protease inhibition and protein quantification, have been scaled and optimized for smaller reaction volumes and larger sample numbers resulting in better replication (Pande and Murthy 1994; Olson and Markwell 2007).

One of the earliest biochemical responses to herbivory is the production of reactive oxygen species (ROS) such as superoxide (O^-), hydrogen peroxide (H_2O_2), and hydroxyl radicals (HO^-) after depolarization of the plasma membrane due to leaf damage (Maffei et al 2012; War et al 2012; Zebelo & Maffei 2015). Both chemical treatments and mechanical wounding can elicit ROS production (Maffei et al 2007). H_2O_2 production is both used as a local signal to induce the hypersensitive response when plants are subjected to mechanical damage as well as a systemic signal for the induction of additional defense responses (Orozco-Cárdenas & Ryan 1999). The presence and activity of ROS additionally results in the production of a group of enzymes, peroxidases

(POD), that are upregulated to perform a diverse set of physiological processes such as metabolism of ROS, restructuring of cellular walls, cross-linking of complex polymers, and other critical functions (War et al 2012). Increases in POD activity also decrease the nutritional quality of leaf tissue, which significantly reduces the growth and development of insect larvae. Finally, the presence of plant PODs in insect guts may also be toxic to insects.

Two additional compounds that are produced in response to herbivory are polyphenol oxidase (PPO) and protease inhibitors (PI) (Mithöfer & Boland 2012; War et al 2012). PPO is upregulated directly by the presence of herbivore-associated signaling compounds such as methyl-jasmonate (Koussevitzky et al 2004). PPO breaks down diphenolic compounds to produce more reactive phenolic compounds that have anti-insect activity once consumed (War et al 2012). Protease inhibitors (PI) are small molecules that prevent proteolytic activity. In response to herbivores, plants will produce PIs to inhibit protein catabolism in insect guts which can halt the degradation of proteins that may serve as precursors used for various physiological processes (Mithöfer & Boland 2012).

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 peroxidases. Peroxidases such as glutathione peroxidase reduce H_2O_2 and are induced in response to high levels of H_2O_2 and catalyze the oxidation of other molecules (Quan et al. 2008); measuring both peroxide and peroxidase in the same sample thus gives additional insight into this cellular process.

Additionally, reducing the total amount of tissue required for an expanded array of assays enables researchers to perform both small molecule and enzymatic assays

during a given investigation by allowing two separate extraction buffers to be used resulting in smaller amounts of tissues being assayed in more ways. This allows complex responses across large numbers of individuals to be analyzed within a single experiment. 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 (Bradford 1976). 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, which causes protein precipitation (Rajalingam et al. 2009) preventing the use of a single buffer. Additionally, as with protein-based assays, the large sample masses required as a result of large reaction volumes for current spectrophotometric techniques limits the total number of technical replications and 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 more holistic assessment of herbivory-associated plant immunity responses.

Since the onset of the next generation sequencing revolution, many studies use genomic data as evidence of variation. Although this has proven to be a highly useful tool in many studies, it is important to assess functional variation as well. Studies have indicated that transcript abundance doesn't necessarily match functional activity in a tissue sample (Greenbaum et al. 2003). This finding and others like it are important to consider when assessing functional diversity in physical populations of organisms.

Here, we present a cost-effective method to assay multiple molecular responses in small sample masses (Appendix 1). The assays include total protein content,

peroxidase, polyphenol oxidase, H_2O_2, and trypsin-like protease inhibitors. Many defense responses can be assayed individually but 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 often used to test biochemical defense responses, which we used to compare data we generated using our method and published protocols. After validating our assays using *S. lycopersicum,* we tested our protocol using *Medicago polymorpha*, a leguminous plant whose biochemical responses to herbivory have not been quantified. With trifoliate leaves that may weigh less than 50 mg, *M. polymorpha* is representative of a "non-model" plant. This protocol paves the way toward more comprehensively assaying plant biochemical responses to herbivory in non-model plants and allows for greater sample capacity, which would allow for improved statistics, time course experiments, and more complex experimental designs.

METHODS AND RESULTS

Tissue preparation— To compare our protocol to current spectrophotometer protocols, we used tomato (*Solanum lycopersicum,* ecotype M82*)*, a model plant often used for testing defense responses, and the non-model plant *Medicago polymorpha*. Tomato seeds were scarified with 600 grit sandpaper, imbibed in dH_2O for three days at 4°C in the dark to stratify, then placed in a dark cabinet overnight. 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 described above and planted into 158-mL pots filled with Sungro Sunshine Mix #1 (SunGro® Horticulture, Michigan, USA). Plants were inoculated a week after planting with a rhizobium strain

mixture of 10^7 cells of equal parts *Ensifer medicae* strain WSM419 and *E*. *meliloti* strain 1021 to prevent nitrogen starvation and to mimic natural conditions.

To ensure that our protocol could adequately detect plant defense responses both pre- and post-herbivory, we allowed soybean loopers (*Chrysodeixis includens*) to feed on leaves and also manually induced plant responses using caterpillar regurgitant to account for variable insect feeding patterns. Regurgitant was generated by compressing stomachs with forceps post feeding on corresponding host plants. Leaves were manually wounded with scissors dipped in 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.

Assays— One challenge of attempting to assay multiple enzymes and small molecules from a single sample is finding an appropriate extraction buffer that will preserve the integrity of the metabolites while not creating conditions inhibitory for other assays. We were able to utilize two extraction 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 (Junglee et al. 2014). 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 (Grimplet et al. 2009), was removed due to the need to assay the production of trypsin-like protease inhibitors. B-mercaptoethanol, also a common protein buffer ingredient (Grimplet et al. 2009) used as a reducing agent to ensure analysis of strictly monomeric proteins, was removed due to interference with the Thermo Scientific Pierce™ BCA Protein Assay Kit. Previous studies, specifically ones from which we modified original assays (Cavalcanti et al. 2004;

Goud and Kachole 2012) 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 2).

Frozen leaf tissue from each plant was placed into two microcentrifuge tubes and weighed. The tubes were homogenized for 15 minutes at 300 rpm in a tissuelyser (QIAGEN TissueLyser II, QIAGEN, Maryland, USA)**.** The tube holders were made of Teflon and stored at -80°C. All samples and holders were also dipped in liquid N_2 before homogenizing. 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). Tubes were centrifuged at 4°C for 10 minutes at 15000 rpm in an accuSpin Micro 17 centrifuge (Thermo Fisher Scientific, Waltham, USA) and the supernatant pipetted into clean tubes. The PE extract samples were then diluted to 1/10X. All absorbance values were run on a SpectraMax M2 combination spectrophotometer and microplate reader and standardized for fresh weight (FW). A detailed description of our protocols can be found in Appendix 1.

Protein Quantification

Total protein content was measured using the Thermo Scientific Pierce™ BCA Protein Assay Kit (Product number: 23337, Thermo Fisher Scientific) according to manufacturer instructions for microplate samples and was included to test the efficacy of our protein extraction buffer.

Peroxidase (POD) Activity

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 µL of peroxidase reaction buffer (100 mM sodium phosphate buffer (pH 6.5) containing 5 mM guaiacol). Control wells received 143 µL of 100 mM sodium phosphate buffer (ph 6.5). 25 µL of supernatant (enzyme source) was added to each well. We then added 32 µL of 5 mM H_2O_2 (final concentration 0.8 mM) to start the reaction. Plates were incubated in the dark for 15 minutes at room temperature before reading absorbance values at 470 nm.

Polyphenol oxidase (PPO) Activity

PPO activity was also measured in triplicate per sample (biological replicate) with a tissue specific control. Sample wells received 115 µL of 100 mM sodium phosphate buffer (pH 6.8) and 60 µL of 50 mM pyrocatechol. Control wells received 175 µL of 100 mM sodium phosphate buffer (pH 6.8). 25 µL of supernatant (enzyme source) was added to all wells. Samples were incubated for 5 minutes before reading absorbance values at 420 nm.

Protease inhibition (PI) Activity

Protease inhibition activity was quantified using an adapted method from (Orians et al. 2000) 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 µL of Trizma Base buffer, 83.3 µL of 2% azocasein dissolved in Trizma Base buffer, and 33.3 µL 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. 100 µL 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 used for positive and negative assay controls, respectively. The assay controls received 100 µL of Trizma base instead of enzyme source. All tubes were incubated at 30°C for 25 minutes. Post-incubation, 133 µL of 100% w/v TCA was added and tubes were centrifuged at 8000 rpm for 10 minutes. After centrifugation, 100 µL of the supernatant were added to wells of a microplate that contained 100 µL of 1M NaOH and absorbance was measured at 450 nm. As with the other assays, samples were run in triplicate.

Hydrogen peroxide (H_2O_2) Quantification

The hydrogen peroxide quantification assay (H_2O_2) was implemented with few modifications. The primary change was to the measurement wavelength. As measured in Junglee et al. (2014), 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 H_2O_2 assays (Velikova et al. 2000; Junglee et al. 2014), the results of our spectral scan that indicated no significant differences in absorbance values at 390 nm relative to absorbance at 350 nm ($t_{(5)} = -1.608$, $p = 0.169$) and the fact that 285 nm is cleanly in the ultraviolet range which requires special plates to avoid issues of interference from the standard polymers used in 96-well plates. Sample aliquots were taken from the 0.1% TCA buffer extraction. Sample wells received 100 µL of 1M potassium iodide (KI), 50 µL of 10mM potassium phosphate buffer (pH 6.5), and 50 µL of sample aliquot. Control wells received 100 µL of dH_2O, 50 µL of 10mM potassium phosphate buffer (pH 6.5), and 50 µL 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 µL of 1M potassium iodide (KI), 50 µL of 10mM potassium phosphate buffer (pH 6.5), and 50 µL of 0.1% TCA then seeding with known amounts (5-20 nmoles) of H_2O_2. Absorbance was measured at 390 nm and values were compared to the standard curve for quantification in nanomoles.

Protocol Validation— The success of our protocol hinges on three points that we address through different validation methods. First, we validated that our assay is able to accurately quantify the same amount of enzyme activity compared to assays run using a spectrophotometer. We focused on POD and PPO, the two enzymes assayed given these were the most modified protocols. Implementation of published protocols (Orians et al. 2000; War et al. 2011) on *S. lycopersicum* provided us with a point of reference for comparison of our modified methods. By first establishing an expected response to a given treatment we are able to determine if the measured microplate response is sufficiently similar and reproducible. Both assays 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 (Cavalcanti et al. 2004; Goud and Kachole 2012). When scaling our total assay volumes down to fit the requirements of a standard 96-well microplate the volumes were reduced ~100 fold.

For each assay we generated standard curves from enzymes obtained from Worthington Biochemical Corporation (New Jersey, USA). Horseradish peroxidase with an activity of 220 U/mg dry mass was diluted to a stock concentration of 100 mU/mL in PE buffer. Standard curves were used to verify that the protocol was detecting analyte quantities within the detection limits of the machines used for absorbance measurements (Fig. 1). 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 mass 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 r^2 values are similar for both standard curves (Fig 1). This suggests that both machines are able to accurately predict concentrations given an absorbance due to the high r^2 values.

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. Current practice for many of these assays is to pool tissue samples from multiple plants. We show that there is significant variation in expression both pre- and post-herbivory between the five tomato plants of the same ecotype used in all five assays (Appendix 3 and Table 1). Table 1 shows the results of an ANOVA where we compare absorbance values based on plant sample pre- and post-herbivory and show that there is significant variation between plants of the same ecotype that were grown in the same environment. This variation highlights the strength of our assay, which does not require plant tissue to be pooled This is important because it indicates that our readings are more reproducible than alternative methods (Table 2). Pooling tissue samples, such as what is required to get adequate tissue masses for spectrophotometric assays, increases variability due to the sample pool containing multiple individual plant responses and makes a strong argument for assaying individual plants (Zhang and Gant 2005). The difference in absorbance means between the

microplate method and the spectrophotometric method are not of concern since differences can be explained as a consequence of using different detection methods. 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 3.8 mg of tissue. This was determined by doing a series of dilutions on a tissue sample to determine the linear range of the microplate assay (Fig. 2). Then, we ran all of our assays on trifoliate leaves (ranging in mass from 12 mg to 56 mg) collected from *M. polymorpha* as proof of concept that we could detect expression in actual small leaf tissue samples (Appendix 4).

CONCLUSIONS

The measurement of plant biochemical variation in response to insect herbivory previously faced substantial limitations that have hindered the progress of the field. In particular, current practice in many labs is to use a single ecotype to measure differences between experimental treatments and to pool tissue from multiple leaves and individuals to obtain sufficient sample mass (War et al. 2011; Rajendran et al. 2014; Ferrieri et al. 2015). However, this approach has precluded the study of variation within and between individuals, which is what is relevant for real-world interactions (Whitham 1983; Winn 1996; Bolnick et al. 2011). In contrast to previous techniques (Orians et al. 2000; War et al. 2011; Junglee et al. 2014), 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. It took 1.5 hours to run the spectrophotometer protocols for the standard curves for just eleven samples. In contrast, between two researchers, we were able to perform each assay on three hundred samples in triplicate in under six hours.

Using our high-throughput protocols, researchers can now compare variation both within and between individuals, genotypes, and populations. Genetic and evolutionary biology studies often focus on variation between genotypes [e.g. (Fitzpatrick et al. 2015; Kerwin et al. 2015)], which requires 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 several 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. Furthermore, measuring the production of multiple metabolites/enzymes within a single sample will enable researchers to quantify tradeoffs in phytochemical production at the level of individual leaves, 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 multiple leaves can be assayed in parallel. Moreover, increased biological replication provides researchers the opportunity to test hypotheses with enhanced statistical power.

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

Bolnick DI, Amarasekare P, Araújo MS, Bürger R, Levine JM, Novak M, Rudolf VH, Schreiber SJ, Urban MC, Vasseur DA (2011) Why intraspecific trait variation matters in community ecology. **Trends in ecology & evolution** 26: 183-192

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. **Analytical biochemistry** 72: 248-254

Cavalcanti FR, Oliveira JTA, Martins-Miranda AS, Viégas RA, Silveira JAG (2004) Superoxide dismutase, catalase and peroxidase activities do not confer protection against oxidative damage in salt-stressed cowpea leaves. **New Phytol** 163: 563-571

Ferrieri AP, Appel HM, Schultz JC (2015) Plant vascular architecture determines the pattern of herbivore-induced systemic responses in Arabidopsis thaliana. **Plos One** 10: e0123899

Fitzpatrick CR, Agrawal AA, Basiliko N, Hastings AP, Isaac ME, Preston M, Johnson MT (2015) The importance of plant genotype and contemporary evolution for terrestrial ecosystem processes. **Ecology** 96: 2632-2642

Goud PB, Kachole MS (2012) Antioxidant enzyme changes in neem, pigeonpea and mulberry leaves in two stages of maturity. **Plant signaling & behavior** 7: 1258-1262

Greenbaum D, Colangelo C, Williams K, Gerstein M. 2003. Comparing protein abundance and mRNA expression levels on a genomic scale. **Genome biology** 4:117.

Grimplet J, Wheatley MD, Jouira HB, Deluc LG, Cramer GR, Cushman JC (2009) Proteomic and selected metabolite analysis of grape berry tissues under well-watered and water-deficit stress conditions. **Proteomics** 9: 2503-2528

Junglee S, Urban L, Sallanon H, Lopez-Lauri F (2014) Optimized assay for hydrogen peroxide determination in plant tissue using potassium iodide. **American Journal of Analytical Chemistry** 5: 730

Kerwin R, Feusier J, Corwin J, Rubin M, Lin C, Muok A, Larson B, Li B, Joseph B, Francisco M (2015) Natural genetic variation in Arabidopsis thaliana defense metabolism genes modulates field fitness. **Elife** 4: e05604

Maffei ME, Mithöfer A, Boland W. 2007. Before gene expression: early events in plant–insect interaction. **Trends in plant science** 12:310-316.

Maffei ME, Arimura G-I, Mithöfer A. 2012. Natural elicitors, effectors and modulators of plant responses. **Natural product reports** 29:1288-1303.

Mithöfer A, Boland W. 2012. Plant defense against herbivores: chemical aspects. **Annual review of plant biology** 63:431-450.

Olson BJ, Markwell J (2007) Assays for determination of protein concentration. **Current protocols in protein science**: 3.4. 1-3.4. 29

Orians CM, Pomerleau J, Ricco R (2000) Vascular architecture generates fine scale variation in systemic induction of proteinase inhibitors in tomato. **Journal of Chemical Ecology** 26: 471-485

Orozco-Cardenas M, Ryan CA. 1999. Hydrogen peroxide is generated systemically in plant leaves by wounding and systemin via the octadecanoid pathway. **Proceedings of the National Academy of Sciences** 96:6553-6557.

Pande SV, Murthy MS (1994) A modified micro-Bradford procedure for elimination of interference from sodium dodecyl sulfate, other detergents, and lipids. **Analytical biochemistry** 220: 424-426

Peterson GL. 1977. A simplification of the protein assay method of Lowry et al. which is more generally applicable. **Analytical biochemistry** 83:346-356.

Quan LJ, Zhang B, Shi WW, Li HY (2008) Hydrogen peroxide in plants: a versatile molecule of the reactive oxygen species network. **Journal of Integrative Plant Biology** 50: 2-18

Rajalingam D, Loftis C, Xu JJ, Kumar TKS (2009) Trichloroacetic acid-induced protein precipitation involves the reversible association of a stable partially structured intermediate. **Protein science** 18: 980-993

Rajendran S, Lin I-W, Chen M-J, Chen C-Y, Yeh K-W (2014) Differential activation of sporamin expression in response to abiotic mechanical wounding and biotic herbivore attack in the sweet potato. **BMC plant biology** 14: 112

Velikova V, Yordanov I, Edreva A (2000) Oxidative stress and some antioxidant systems in acid rain-treated bean plants: protective role of exogenous polyamines. **Plant science** 151: 59-66

War AR, Paulraj MG, War MY, Ignacimuthu S (2011) Herbivore-and elicitor-induced resistance in groundnut to Asian armyworm, *Spodoptera litura* (Fab.) (*Lepidoptera: Noctuidae*). **Plant signaling & behavior** 6: 1769-1777

War AR, Paulraj MG, Ahmad T, Buhroo AA, Hussain B, Ignacimuthu S, Sharma HC. 2012. Mechanisms of plant defense against insect herbivores. **Plant signaling & behavior** 7:1306-1320.

Whitham TG (1983) Host manipulation of parasites: within-plant variation as a defense against rapidly evolving pests. **Variable plants and herbivores in natural and managed systems**: 15-41

Winn AA (1996) Adaptation to fine-grained environmental variation: an analysis of within-individual leaf variation in an annual plant. **Evolution** 50: 1111-1118

Zebelo SA, Maffei ME. 2015. Role of early signalling events in plant–insect interactions. **Journal of Experimental Botany** 66:435-448.

Zhang S-D, Gant TW (2005) Effect of pooling samples on the efficiency of comparative studies using microarrays. **Bioinformatics** 21: 4378-4383

Tables

Table 1. ANOVA table comparing absorbance values of *Solanum lycopersicum* tissue samples pre- and post- herbivory and show that there is significant variation between plants of the same ecotype that were grown in the same environment.

Assay	**Pre-herbivory**				**Post-herbivory**	
	% Variation explained by plant	F stat	*p*	% Variation explained by plant	F stat	*p*
Protein	95.8%	80.42	<0.001	99.9%	3753	<0.001

Quantification						
H_2O_2	~ 0%	0.911	0.404	99.98%	$1.476e^4$	<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

Note: H_2O_2 production prior to herbivory was negligible.

Table 2. Comparison of technical replicate standard errors between spectrophotometer and microplate assays for POD and PPO of *Solanum lycopersicum* plants. By not pooling tissue samples, we are able to decrease replicate experimental error.

Assay	**Experimental mean (Abs/ g FW)**		**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%

APPENDIX

Appendix 1- Protocol for analyzing multiple plant defensive compounds using a microplate reader

Buffers (all stored at room temperature)

Protein Extraction (PE) Buffer:

4 mL of 25 mM EDTA (final concentration of 1mM)

88 mL of 100 mM Trizma-Base (final concentration 88mM)

8 mL of 80% Glycerol (final concentration 10%)

Trichloroacetic Acid (TCA) Extraction Buffer:

0.1% w/v Trichloroacetic acid in H_2O

Reagents

Protein Quantification Assay:

Pierce BCA Protein Assay Kit

Peroxidase (POD) Assay:

100 mM sodium phosphate buffer (pH 6.5)

5 mM guaiacol made in 100mM sodium phosphate buffer (pH 6.5)

- May be liquid at room temperature; stock must be stored under inert gas (N_2, Ar)
- Solution is light sensitive

5 mM H_2O_2

- 3% stock solution used; good for 4 weeks
- Light sensitive

Polyphenol Oxidase (PPO) Assay:

100 mM sodium phosphate buffer, pH 6.8

50 mM pyrocatechol

- Stock must be stored under inert gas (N_2, Ar)
- Solution is light sensitive
- Solution only good for ~ 2 days
- Soluble in sodium phosphate buffer

H_2O_2 Quantification Assay:

0.1% w/v TCA

1 M potassium iodide

10 mM potassium phosphate buffer (pH 6.5)

3% w/v H_2O_2 (0.988 M)

- Only good for 30 days
- Light sensitive and must be kept at 4C

Protease Inhibition (PI) Assay:

100 mM Trizma-Base buffer (pH 7.8)

2% azocasein in Trizma-Base buffer (100mM)

1 mM HCl solution (Trizma-Base) containing 200 ng of trypsin (0.1mg/ml)

100% w/v TCA

1 M sodium hydroxide

Extraction and homogenization

1. Snap freeze harvested leaf tissue from each plant in microcentrifuge tubes and weigh.

2. Homogenize tubes for 15 minutes at 300 rpm in a tissuelyser (QIAGEN TissueLyser II, QIAGEN, Maryland, USA) using Teflon coated adaptors that are stored at -80°C to prevent additional accumulation of stress-related compounds**.**
3. Add 1mL of the 0.1% TCA buffer (Table A1) to microcentrifuge tubes with plant samples to be used for the hydrogen peroxide assay.
4. Add 1mL of PE buffer (Table A1) to microcentrifuge tubes with plant samples to be used for all the other assays.
5. Centrifuge tubes at 4°C for 10 minutes at 15000 rpm in an accuSpin Micro 17 centrifuge (Fisher Scientific ®) and pipette the supernatant into clean tubes. The PE buffer tubes were then diluted to 1/10X.

Table A1. List of buffer conditions for each assay

Assay	Buffer	Dilution
Protein Quantification	PE	0.1x
Polyphenol Oxidase	PE	0.1x
Peroxidase	PE	0.1x
Protease Inhibitor	PE	0.1x
Hydrogen Peroxide	TCA	1x

Assays

Protein Quantification

- Protein quantification was performed using the Thermo Scientific Pierce™ BCA Protein Assay Kit (Product number: 23337) according to manufacturer instructions for microplate samples. Due to the general nature of our buffer, other protein quantification methods (e.g. Bradford 1976; Peterson 1977) can be used.

Peroxidase (POD) Activity

1. Sample aliquots are taken from the 1/10x PE buffer extraction. All reactions are run in triplicate.
2. Create sample master mix by multiplying reaction components by total number of reactions + 1. Reaction components are as follows: 143 µL of peroxidase buffer (100 mM sodium phosphate buffer (pH 6.5) containing 5 mM guaiacol.
3. Create control master mix by multiplying reagent components by total number of control reactions + 1. Reaction components are as follows: 143 µL of 100mM sodium phosphate buffer (ph 6.5).
4. Aliquot 143 µL of each master mix (triplicate) to separate wells in a 96 well plate.
5. Add 25 µL of supernatant (enzyme source) to each well and then add 32 µL of 5 mM H_2O_2 (final concentration 0.8 mM).
6. Incubate the plates in the dark for 15 minutes at room temperature.
7. Read absorbance at 470 nm on the microplate reader and express enzyme content as [(Abs_{Spl} - Abs_{Ctrl})/FW] (Abs/g). FW denotes fresh weight.

Polyphenol Oxidase (PPO) Activity

1. Sample aliquots are taken from the 1/10x PE buffer extraction. All reactions are run in triplicate.
2. Create sample master mix by multiplying reaction components by total number of reactions + 1. Reaction components are as follows: 115 µL of 100 mM sodium phosphate buffer (pH 6.8) and 60 µL of 50 mM pyrocatechol.
3. Create control master mix by multiplying reagent components by total number of control reactions + 1. Reaction components are as follows: 175 µL of 100 mM sodium phosphate buffer (pH 6.8).

4. Aliquot 175 µL of each master mix (triplicate) to separate wells in a 96 well plate.
5. Add 25 µL of supernatant (enzyme source) to all wells and incubate for 5 minutes.
6. Read absorbance on the microplate reader at 420 nm and express enzyme content as $[(Abs_{Spl} - Abs_{Ctrl})/FW]$ (Abs/g).

Hydrogen Peroxide (H_2O_2) Quantification

1. Generate a standard curve using a mix containing 100 µL of 1M potassium iodide (KI), 50 µL of 10mM Potassium Phosphate buffer (pH 6.5), and 50 µL of 0.1% TCA per well. Spike each well with a known quantity of hydrogen peroxide from dilutions of 3% stock.
2. Sample aliquots are taken from the 0.1% TCA buffer extraction. All reactions are run in triplicate.
3. Create sample master mix by multiplying reaction components by total number of reactions + 1. Reaction components are as follows: 100 µL of 1M potassium iodide (KI), 50 µL of 10mM Potassium Phosphate buffer (pH 6.5), and 50 µL of enzyme source.
4. Create control master mix by multiplying reaction components by total number of reactions + 1. Reaction components are as follows: 100 µL of dH_2O, 50 µL of 10mM Potassium Phosphate buffer (pH 6.5), and 50 µL of enzyme source.
5. Aliquot 200 µL of each master mix (triplicate) to separate wells in a 96 well plate.
6. Incubate samples plus standard curve in the dark for 20 minutes at room temperature.
7. Read absorbance at 390 nm and compare values to the standard curve for quantification in nmoles.

Trypsin-like protease Inhibition (PI) Activity

1. Activity is represented by the inhibition of trypsin in sample aliquots taken from the 1/10x PE buffer. All reactions are run in triplicate.
2. Create sample master mix by multiplying reaction components by total number of reactions + 1. Reaction components are as follows: 100 µL of enzyme source, 133.3 µL of Trizma Base buffer, 83.3 µL of 2% azocasein dissolved in Trizma Base buffer, and 33.3 µL of 0.001 M HCl solution containing 200 ng of trypsin
3. Create sample control master mix by multiplying reaction components by total number of reactions + 1. Reaction components are as follows: 100 µL of enzyme source, 166.6 µL of Trizma Base buffer and 83.3 µL of 2% azocasein dissolved in Trizma Base buffer.
4. Create assay control master mix by multiplying reaction components by total number of reactions + 1. Reaction components are as follows: 233.3 µL of Trizma Base buffer, 83.3 µL of 2% azocasein dissolved in Trizma Base buffer, and 33.3 µL of 0.001 M HCl solution containing 200 ng of trypsin.
5. Create a negative control by multiplying reaction components by total number of reactions + 1. Reaction components are as follows: 266.6 µL of Trizma Base buffer and 83.3 µL of 2% azocasein dissolved in Trizma Base buffer.
6. Incubate samples at 30°C for 25 minutes.
7. Post-incubation, add 133 µL of 100% w/v TCA to all samples and centrifuge at 8000 rpm for 10 minutes.
8. Aliquot 100 µL of 1M NaOH to all wells of a 96 well plate and then aliquot 100 µL of the supernatant to each well.

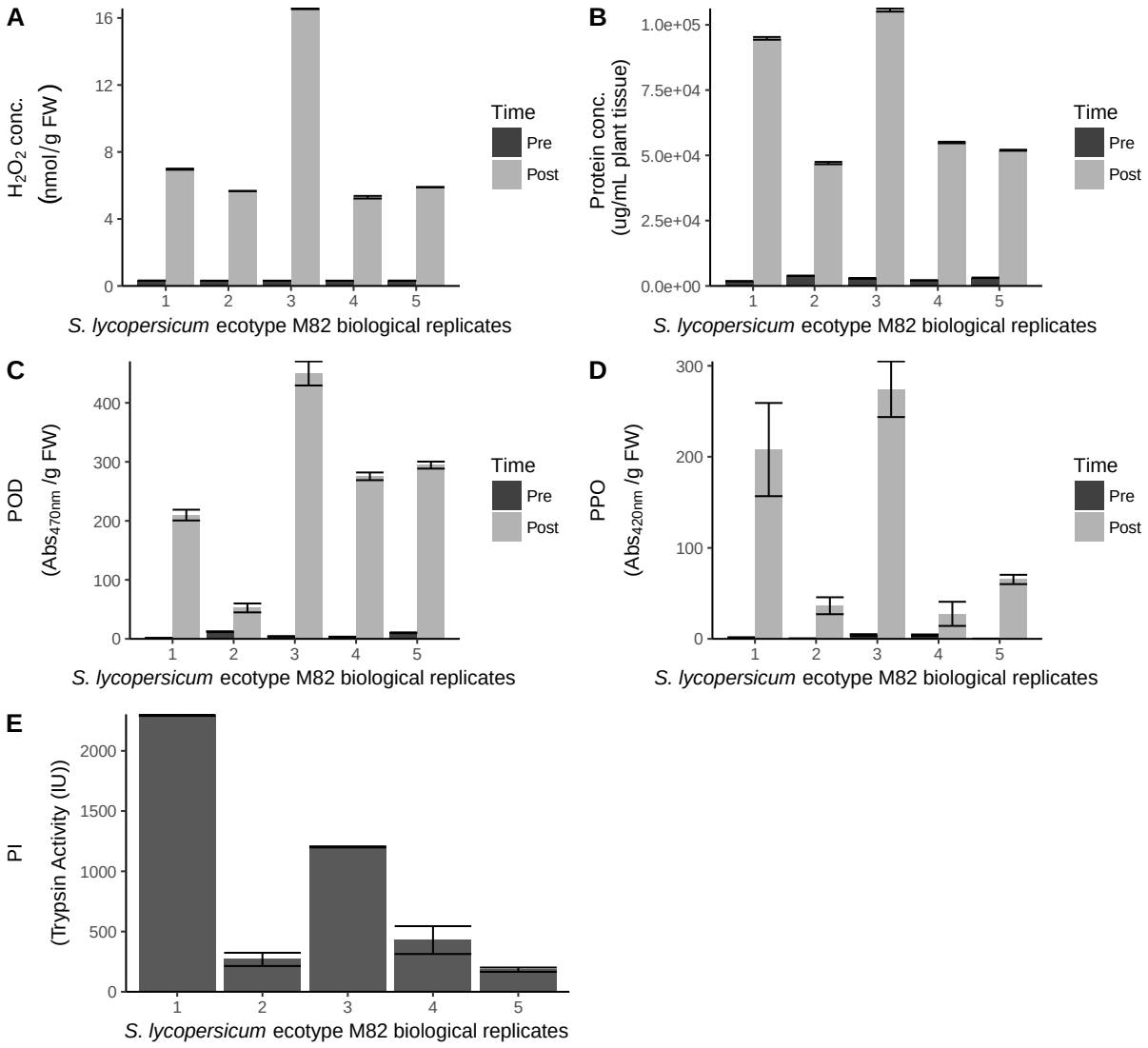
9. Read absorbance at 450 nm. PI activity is calculated for pre- and post-herbivory as 1- ((Sample absorbance/Sample Control Absorbance)/(Assay Control Absorbance/Negative Control Absorbance)), standardized by tissue mass and then report the values as post-herbivory minus pre-herbivory.

Appendix 2

Medicago polymorpha genotype with country and GPS coordinates

W0419 (France; 43.618907, 4.813317), W0420 (Spain; 43.45713, 4.353194), W0077 (Spain; 43.301433, 2.344602), W0607 (USA; 43.221144, -123.406702), W0079 (France; 43.67624, 3.352244), W0076 (USA; 40.87011, -124.11282), W0517 (USA; 40.87011, -124.11282), W0603 (USA; 40.87011, -124.11282), W0146 (USA; 40.87011, -124.11282), W0421 (Turkey; 42.643558, 11.850325)

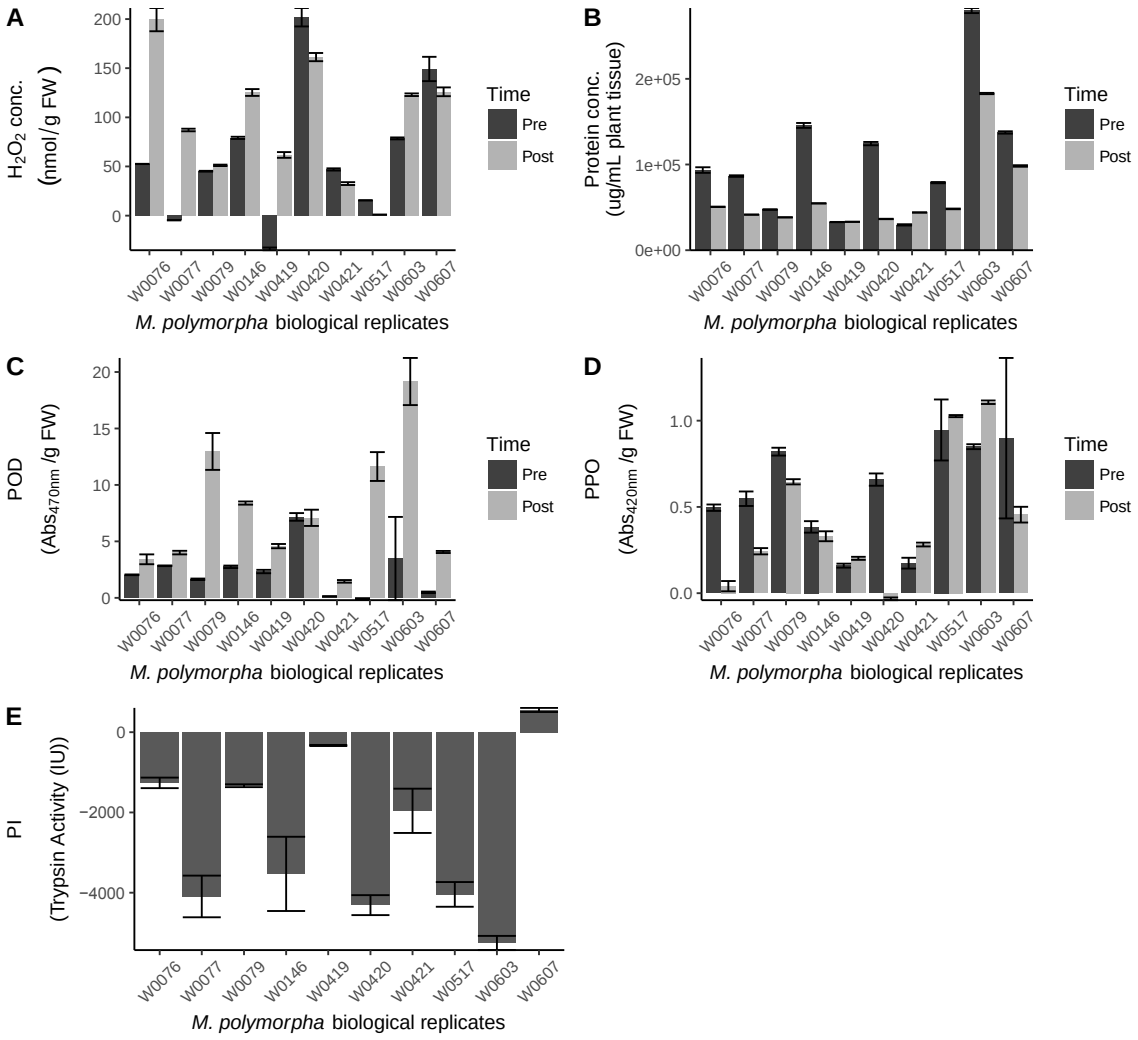
Appendix 3



Results of our microplate-based protocols using *S. lycopersicum,* ecotype M82 for A) H_2O_2, B) Protein Quantification, C) POD, D) PPO, and E) PI. The numbers 1-5 on the x-axis reflect individual plants used for sampling. 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 (see Main Document Table 1). A-D show values pre- and post-herbivory. E is calculated by subtracting post-herbivory values from pre-herbivory

values. Bars in all panels show the mean with standard error bars for three biological replicates.

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) H_2O_2 B) Protein Quantification C) POD D) PPO E) PI. Unlike tomato, *M. polymorpha* does not always show increase in production of defensive phytochemicals. A-D show values pre- and post-mechanical wounding with regurgitant to simulate herbivory. E is calculated by subtracting post-

mechanical wounding values from pre-mechanical wounding values. Bars in all panels show the mean plus/minus standard error bars for three biological replicates.

Figures and legends

Fig 1. Comparison of absorbance values for A) POD and B) PPO when measured using either a spectrophotometer (cuvette) or microplate reader (mtp) to generate a standard curve using horseradish peroxidase for POD (measured at 470 nm) and mushroom polyphenol oxidase for PPO (measured at 420 nm). Line equations and r^2 values were generated by fitting data using a linear model. Each data point represents mean plus/minus standard error. All concentrations were done in triplicate.

Fig 2. Serial dilutions of uninduced tomato tissue. We serially diluted a homogenized tissue sample initially at a concentration of 0.38 g FW/mL to determine the lower limit of detection for the A) POD and B) PPO assays. We used uninduced tissue with low expression of defense compounds and measured absorbance at 470 nm for POD and 420 nm for PPO. Each dilution was measured in triplicate; data points shown are means plus/minus standard errors.