THEORY AND PHYSIOLOGY OF PLANT SYMBIOTIC RELATIONSHIPS

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ABSTRACT

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Your acknowledgements here.

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CHAPTER 1

COERCION IN THE EVOLUTION OF PLANT-MICROBE COMMUNICATION

1.1 INTRODUCTION

Communication systems in nature provide a foundation for both intra- and interspecific interactions (Scott-Phillips, 2008). Developing an understanding of how communication systems evolved is therefore key to understanding the evolution of complex relationships between species including mutualism, parasitism, competition, and commensalism. Plants, a sessile group of organisms, are dependent on molecular communication events to interact with the diverse microbial communities present in their environments (Baker et al., 1997). Recent efforts to uncover the functional significance of the plant microbiome have resulted in a new wave of questions about the evolution of the complex associations between plants and microbes. Future advances in this field will depend on a mechanistic understanding of plant-microbe communication and the evolutionary processes that underlie it. Here we discuss coercion, an evolutionary precursor to molecular signaling, in the context of plant-microbe interactions. While human communication relies mainly on the production and detection of visual and auditory stimuli as means of communication, plants and microbes primarily depend on secretion and detection of "infochemicals" (a term we borrow from Barto et al. (2012)) to send and receive information. Infochemicals, biologically-derived molecules that contain information (i.e. those that reduce uncertainty), are secreted by one organism (i.e. the sender) and detected by another (i.e. the receiver); both plants and microbes can play the role of sender or receiver. A formal definition of the terms infochemical, sender, receiver, and others introduced here can be found in Table 1; note that the general communication theory we modify for specificity to plant-microbe interactions was originally presented by Scott-Phillips (2008) and Scott-Phillips et al. (2012). The emergence of novel plant-microbe interactions depends upon two aspects of organismal physiology: 1) both plants and microbes release a variety of chemicals into the environment; 2) both plants and microbes have a variety of chemical receptors that allow

them to detect myriad chemicals. Random mutation of genes encoding metabolic pathways and of cell-surface proteins therefore allow for changes in the respective complements of chemicals both released and detected by plants and microbes. To the extent that the release and perception of these chemicals is associated with survival and reproduction, natural selection can act upon the release of novel chemicals and novel detection mechanisms favoring either sender or receiver accordingly. If detection of an infochemical benefits the receiver, without benefit to the sender, the infochemical is classified as a cue. Note that we only use the term "cue" to describe a class of infochemicals throughout this article; colloquial definitions of the term and those that do not deal with infochemicals (e.g. "environmental cues" such as sunlight) should not be inferred in the text (Table 1). A recent review by van't Padje et al. (2016) focused on the prevalence of cues on both sides of plant-microbe interactions. For example, plants are thought to use quorum sensing autoinducers released by rhizosphere bacteria as cues that convey information about bacterial abundance. Conversely, myco-heterotrophs, parasitic non-photosynthetic plants that extract carbon and nitrogen from arbuscular mycorrhizal (AM) fungi, are thought to use as-of-yet unidentified cues to locate AM fungal hyphae within the soil matrix (Rasmussen et al., 2015). The prevalence of coercion, a formal definition of which is provided in Table 1, in plant microbe interactions has yet to be investigated to the same degree. Coercive infochemicals are those that benefit the sender, without benefit to the receiver. The release of coercive infochemicals often allows the sender to take advantage of an existing evolutionary response in the receiver, which the sender can use to modify the receiver's behavior (Figure 1). The prevalence of coercion is an oft-overlooked interaction despite its apparent presence in commonly studied systems. Here we discuss the mechanism of coercion as it applies to plant microbe interactions, and we provide examples of coercion in natural systems. Furthermore, we discuss the evolutionary outcomes of coercive interactions between plants and microbes focusing on the mechanism by which coercion, which benefits the sender, turns into signaling, which benefits both the sender and receiver, known as "sensory manipulation." (Scott-Phillips, 2008; Scott-Phillips et al., 2012).

Table 1.1: List of definitions specific to plant-microbe interactions dependent on chemical information.*

Term	Definition
Information	a reduction in uncertainty
Infochemical	a biologically-derived molecule that reduces uncertainty
Sender	organism that releases an infochemical
Receiver	organism that detects an infochemical
Cue	any infochemical that (i) affects the behavior of other organisms; (ii) which is effective because the effect (the response) has evolved to be affected by the infochemical; but which (iii) did not evolve because of those effects
Coercive Infochemical	any infochemical that (i) affects the behavior of other organisms; (ii) evolved because of those effects; but (iii) which is effective for some reason other than that the effect has evolved to be affected by the infochemical
Signal	any infochemical that (i) affects the behavior of other organisms; (ii) evolved because of those effects; and (iii) which is effective because the effect (the response) has evolved to be affected by the infochemical
Ritualization	the evolution of signals from preceding cues
Sensory manipulation	the evolution of signals from preceding coercers

*Definitions are largely adapted from Scott-Phillips (2008) and Scott-Phillips et al. (2012). The term "infochemical" was borrowed from Barto et al. (2011).

1.2 INTERSPECIFIC COERCION

Although not always recognized as such, many well-characterized molecular interactions between plants and their associated microbes fall into the category of coercion, where the sender benefits at the potential expense of the receiver. Two pervasive types of coercion are plant coercion of microbes via manipulation of quorum sensing and microbial coercion of plants via modulation of phytohormones. In this section we provide specific examples of each behavior within the conceptual framework of communication theory.

1.2.1 Plant coercion of microbes— Manipulation of quorum sensing

Rhizosphere microbes use quorum sensing, a form of cell-to-cell communication, to regulate a variety of behaviors including exoenzyme release (e.g. Chernin et al. (1998)), biofilm formation (e.g. Danhorn and Fuqua (2007)), and motility (e.g. Köhler et al. (2000)). Quorum sensing relies on infochemicals known as autoinducers to provide information about the density of microbes that release these chemicals in a given environment or the diffusion environment of the microbes; this ensures that the behaviors of the aforementioned group only occur under optimal conditions. Once enough bacteria are present (or there is a low rate of diffusion, such as when the microbes are adjacent to a root surface) the total amount of autoinducers present will exceed the necessary threshold for a bacterial response to occur ((Bassler, 1999; Redfield, 2002). Autoinducers often times directly bind to transcription factors or act upstream of transcription factors and regulate gene expression (Engebrecht and Silverman, 1987). Plants can manipulate quorum sensing by releasing molecules that either mimic bacterial autoinducers or interfere with the ability of microbes to produce or detect autoinducers; we provide further examples of these phenomena below. Plants can decouple quorum sensing-controlled behaviors from microbial density thereby ensuring that those behaviors that positively affect plant fitness are favored, while those that negatively affect plant fitness are not. We suggest that the ubiquitous phenomenon of plant manipulation of quorum-sensing is a coercive act.

Extracts and exudates from plants have been shown to elicit quorum sensing-dependent responses in bacterial indicator strains (e.g. *Chromobacterium violaceum*, which uses quorum sensing to regulate production of the pigment violacin; (McClean et al., 1997)) suggesting that plants produce molecules that mimic bacterial autoinducers; this behavior could prevent biofilm formation which is a key component of pathogenesis for many pathogens (Pérez-Montaño et al., 2013). This type of coercive behavior in higher plants was first demonstrated in pea (*Pisum sativum*) (Teplitski et al., 2000) and has since been shown in rice (*Oryza sativa*) (Degrassi et al., 2007; Pérez-Montaño et al., 2013).), common bean (*Phaseolus vulgaris*) (Pérez-Montaño et al., 2013), barrel medic (*Medicago truncatula*) (Gao et al., 2003), and a variety of medicinal plants (Tolmacheva

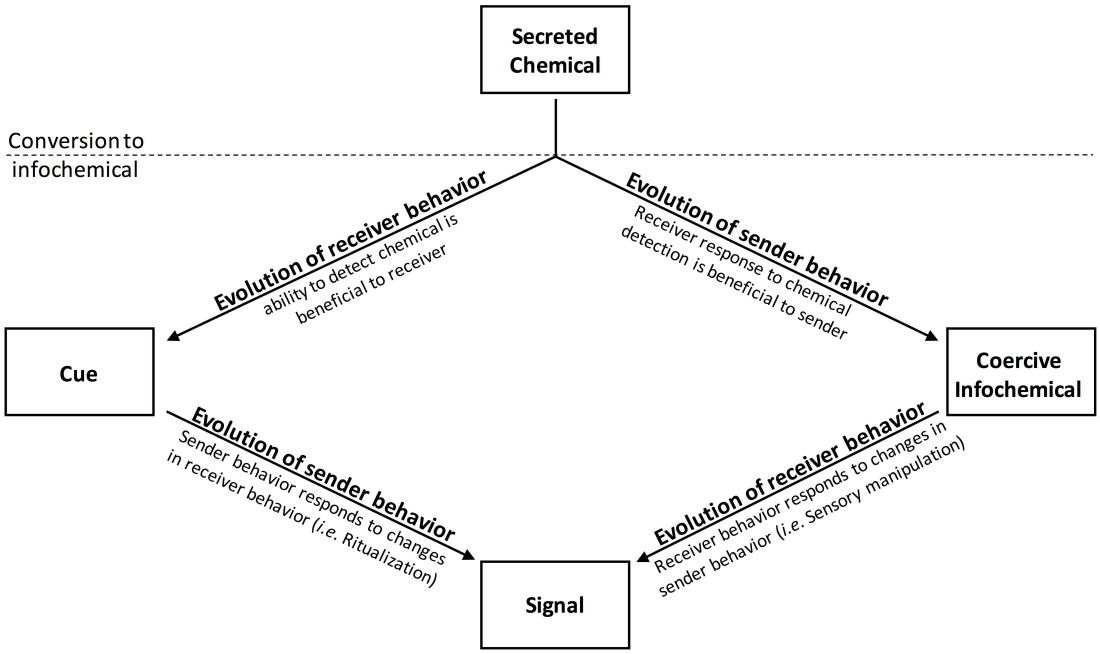

Figure 1.1: Evolution of signaling in plant-microbe interactions. The left side of the figure shows the process through which signals evolve from cues, known as ritualization. This process was discussed by van't Padje et al. (2016). The right side of the figure shows the process through which signals evolve from coercion, known as sensory manipulation. Coercion and sensory manipulation are the focus of this paper.

et al., 2014). (Annapoorani et al., 2012) predicted that a variety of compounds produced by plants would bind to quorum-sensing regulator genes suggesting that these compounds were autoinducer mimics. Recently, (Corral-Lugo et al., 2016) tested these predicted effects of one such compounds, rosmarinic acid, and showed that this compound activates quorum sensing-controlled behavior by a variety of bacterial indicator strains. Plants also coerce microbes by interrupting quorum sensing through a variety of processes collectively known as quorum quenching. Mechanisms of quorum quenching found in nature include the release of enzymes such as lactonases and acylaces that degrade autoinducers, and the release of inhibitory chemicals that affect the ability of microbes to synthesize, exchange, perceive, and respond to, autoinducers (Grandclément et al., 2016). In higher plants, quorum quenching effects were first demonstrated in pea plants (*Pisum sativum*) (Teplitski et al., 2000), and have since been shown in garlic (*Allium sativum*) (Rasmussen and Givskov, 2006), various medicinal plants (Adonizio et al., 2006) and more-recently in tarragon (*Artemisia*

dracunulus), radish (*Raphanus raphanistrum*), and hollyhock (*Althea officinalis*.) (Mahmoudi et al., 2014) among others. Enzymatic degradation of autoinducers in the extracellular environment, a trait common to many bacterial taxa ((Rasmussen et al., 2015) and references therein), has yet to be shown in plants, though some are capable of intracellular enzymatic degradation of autoinducers (Palmer et al., 2014). However, plants from 21 families produce various phenolics, terpenoids, organosulfur phytochemicals, coumarins, quinones, and alkaloids that inhibit quorum sensing by a variety of mechanisms (Ta and Arnason (2016) and references therein), suggesting that plant coercion of microbes by quorum quenching may be widespread. While the ability of plants to manipulate quorum sensing is well documented, mostly through the use of indicator strains like *Chromobacterium violaceum*, we are unaware of any study that documents the *in situ* benefits of quorum sensing manipulation to naturally-occurring plants. However, studies involving transgenic crops that have been modified to manipulate quorum sensing give insight into the potential benefits of this form of coercion. Tobacco plants modified to produce 3-oxo-C6-Acyl-homoserine-lactone (an autoinducer used to coordinate pathogenesis by *Pectobacterium cartovora*, which causes bacterial soft rot in a variety of plants) were more resistant to infection by *P. cartovora* than unmodified plants when inoculated with the pathogen (Mäe et al., 2001). The authors speculated that the high concentration of autoinducer produced by the tobacco plants caused *P. cartovora* to release virulence factors at lower-than-normal cell densities, thereby triggering the plant's immune response early enough to fight off the infection. Interestingly, potato plants modified to produce a bacterial lactonase, which degrades autoinducers including 3-oxo-C6-Acyl-homoserine-lactone, were also more resistant to infection by *P. cartovora* than unmodified plants, presumably due to interrupting virulence responses at high cell density (Dong et al., 2001). While quorum sensing manipulation may provide unmodified plants with pathogen resistance, there are numerous other potential benefits of this form of coercion. For example, since many bacterial strains use quorum sensing to coordinate biofilm formation (Bassler, 1999), plants could release specific autoinducer mimics to encourage biofilm formation, and thus colonization, by beneficial microbes along the root surface (e.g. diazotrophs, which have been shown to increase plant growth; (Norman et al., 2017)). Future

work should seek explore the benefits of this form of coercion in natural plants in a field setting.

1.2.2 Microbial coercion of plants—Manipulation of phytohormones

Phytohormones, plant hormones involved in a variety of developmental and regulatory processes, are well documented to be commonly modulated by microorganisms (Chanclud and Morel, 2016). While phytohormones can have microbial functions (Glick, 2020), most studies have discussed the production of phytohormones by microbes in a mutualistic fashion where microbes are interacting with plants in a beneficial manner that results in equal fitness trade-offs (Hardoim et al., 2008). Evolutionary theory demonstrates that, to understand these types of traits, we need to take the perspective of the microbial genes and ask how natural selection will act on variation that arises. If a mutation arises that alters a plant's physiology in a way that enhances microbial fitness, then this mutation will spread through the population. Studies showing microbial modulation of plant growth typically focus on plant growth promotion and rarely consider the complex relationship that resulted in a given phenotype. Microbial production of phytohormones is nearly ubiquitous in soil-dwelling organisms and occurs in both prokaryotic and eukaryotic microorganisms including heterotrophs, phototrophs, algae, and fungi (Tsavkelova et al., 2005). However, it can be difficult to tease apart which interacting partner is acting coercively. Below, we offer examples of two phytohormones used by microbes for the manipulation of plants. Gibberellins (GA) are a group of phytohormones that regulate growth and developmental processes in plants such as germination, senescence, and flowering (Sun, 2010). GA were first discovered by Kurosawa (1926) in the fungal pathogen *Gibberella fujikuroi*. GA production was shown to be a critical factor in the excessive stem elongation in rice plants, known as "foolish seedling syndrome," infected with *G. fujikuroi* and GA levels also positively correlate with virulence of the pathogen (Desjardins et al., 2000). Since GA production might be occurring to increase the total biomass available for exploitation, GA production is likely a coercive method used by microbes to facilitate plant growth. Ethylene, the well-characterized phytohormone responsible for fruit ripening and numerous other plant processes ((Bleecker and Kende, 2000) and references therein), is another molecule used in coercive plant-

microbe interactions. Some pathogenic bacteria produce a molecule called rhizobitoxine that inhibits the rate limiting enzyme in the metabolic pathway responsible for ethylene production in plants (Yasuta et al., 1999) and induces chlorosis in leaf tissue. Surprisingly, some members of the beneficial group of bacteria known as rhizobia produce rhizobitoxine (Yuhashi et al., 2000). During symbiotic associations between leguminous plants and their rhizobial counterparts, the inhibition of ethylene biosynthesis results in a hypernodulation response (i.e. more nodules are formed on root systems when ethylene is inhibited) (Penmetsa and Cook, 1997). Thus, it appears that the production of rhizobitoxine may be a coercive act on the part of rhizobial partners; decreased ethylene levels increases the ability of a particular rhizobial strain to colonize the root system of host likely resulting in a net fitness gain for that particular strain. Furthermore, rhizobitoxine producers are able to hoard carbon resources in the form of poly-3-hydroxybutyrate while simultaneously reducing plant growth (Ratcliff and Denison, 2009). The fitness gain at the expense of the host strongly indicates that this interaction is coercive. Developing an improved framework to understand these complex associations will allow a greater understanding of the directionality of various communication mechanisms.

1.3 EVOLUTIONARY OUTCOMES OF COERCION

Coercive interactions currently observable in plant-microbe systems are merely a snapshot along an evolutionary trajectory with a variety of possible outcomes. The very existence of these interactions indicates a past evolutionary response by the sender. For example, while a random mutation may have initially caused a particular plant to excrete a quorum sensing mimic, fitness benefits to that plant favored the proliferation of this mutation, therefore increasing the prevalence of this coercive behavior in plant populations over time. Just as evolution has played a role in the emergence of these interactions, evolution of the interacting organisms also determines the eventual fate of coercive plant-microbe interactions. Scott-Phillips et al. (2012) introduced a framework by which coercive interactions either remain coercive, disappear, or transform into signaling through the process of sensory manipulation, which depends on evolution of sender and/or receiver. While the

evolution of signals from cues, a process known as ritualization, has recently been explored in the context of plant microbe interactions (van't Padje et al., 2016), sensory manipulation, an alternate pathway for the evolution of signaling that we explore here, has not. Both ritualization and sensory manipulation are shown in Figure 1.

1.3.1 Stabilized coercion

Scott-Phillips et al. (2012) posited that coercive interactions can continue indefinitely under a given set of environmental conditions if there is no net effect of coercion on the fitness of the receiver and therefore no evolutionary pressure to change the receiver's behavior. Though, on the surface, coercion seems to benefit the sender at the expense of the receiver, one can imagine plant-microbe interactions where the net fitness of the receiver is unaffected. Building on real examples from the literature, we propose hypothetical scenarios to examine possible evolutionary outcomes. For example, soil microbes may release a phytohormone that increases a plant's growth rate thereby increasing the exudation of carbon subsidies to the soil environment. This increased growth rate may be advantageous to the plant, allowing it to outcompete neighboring plants for light. By increasing phytohormone levels through coercion, microbes have caused both decreases in plant fitness associated with increased carbon costs belowground and increases in fitness due to the competitive advantages associated with increased height aboveground; if these fitness effects balance out over time, and environmental conditions remain relatively stable, coercion will continue.

1.3.2 Elimination of coercion

If the fitness of the receiver is negatively impacted by coercion, then Scott-Phillips et al. (2012) predict that coercion will not continue indefinitely. Rather, evolutionary pressure will favor mutations that change receiver behavior such that the receiver will stop responding to coercive infochemicals. In the aforementioned example of a microbe releasing plant growth promoting phytohormones, we suggested that plant carbon losses associated with increased growth were ameliorated by the competitive advantages associated with increased stem height; were this not the case, one would

not expect coercion to last indefinitely. If the coercive infochemical is not identical to the phytohormone that it mimics in the previous example, then slight mutations in the active sites of proteins to which the phytohormone binds that allow the plant to distinguish between the coercive infochemical and the phytohormone may be favored. If the coercive infochemical is not chemically-distinct from the phytohormone it mimics, then the mutations favored would be those that either change downstream responses to the production of said phytohormone, or those that change the hormone itself. Since drastic changes in hormonal pathways may not be possible in every case, coercion may be eliminated in some cases by the extinction of the receiver.

1.3.3 Sensory manipulation

Sensory manipulation is an evolutionary process by which coercion becomes communication (Figure 1). Scott-Phillips et al. (2012) constructed an evolutionary model to show that a coercive interaction turns communicative (i.e. a coercive infochemical becomes a signal) only if the receiver's response to coercion has a fitness benefit for the receiver; if the receiver's response has neutral or negative fitness effects, the coercive interaction will either stabilize or disappear over evolutionary time. A possible example of this can be found in microbially-secreted phytohormones. The auxin, indole-3-acetic acid (IAA) is one of the most-studied microbially-derived phytohormones. Auxins are the primary regulators of plant growth and production of IAA has been shown to significantly increase root biomass in associated plants. Studies conducted on *Klebsiella planticola*, a representative plant growth promoting rhizobacterium, show that secretion of IAA in the presence of plants improves germination, root growth, and robustness to environmental factors (Blinkov et al., 2014 Sep-Oct). The improvement of these traits specifically benefits the associated rhizobacteria. Additionally, the gall-forming plant pathogen, *Agrobacterium tumefacians*, releases IAA during the infection process; this activity has been strongly correlated with increased virulence (Morris, 1986). Research shows that IAA production by microbes is generally used to modulate plant growth. Pathogenic interactions should serve as reminders that this act is not altruistic on the side of the microbe and thus represents a coercive interaction. However, when additional

information is considered, it is apparent that IAA biosynthesis by microbes represents only half of a communication system used by plants and microbes (Lambrecht et al., 2000). It has been shown that the root secretion of L-tryptophan results in greater production of IAA (Karnwal, 2009). Extending this further, it is possible that the two organisms have developed a communication system as a byproduct of previously coercive acts. Thus, what began as coercion may have evolved into a signal and can be categorized as sensory manipulation. Developing an improved framework to understand these complex associations will allow a greater understanding of the directionality of various communication mechanisms. While Scott-Phillips et al. (2012) posit that ritualization, the evolution of signals from cues (Figure 1), is a more common pathway for the evolution of signaling than sensory manipulation, this theory is rooted in animal communication rather than plants and microbes. Since microbes have much faster evolutionary rates than plants, and can act as either sender or receiver of cues, we argue that sensory manipulation could play a major role in the evolution of plant-microbe communication. As our previous examples demonstrate, interspecific interactions are complex and many of their evolutionary histories and trajectories are unknown. We presented the release of autoinducers by plants and the modulation of phytohormones by microbes as coercive acts in interspecific interactions between plants and microbes. Given the right evolutionary pressure, both of these interactions could evolve into signals via sensory manipulation. Though the aforementioned examples could be stable over evolutionary time, other forms of signaling may have evolved by similar means. The sensory manipulation process could provide a functional framework to understand the evolutionary fates of coercive interactions as well as the evolution of certain signaling mechanisms and defection within seemingly established relationships.

1.4 CONCLUSIONS

Developing an understanding of coercion is critical to forming a complete framework for the study of plant-microbe interactions, including the evolution of signaling. Here, we show that two commonly studied plant-microbe interactions, plant manipulation of microbial quorum sensing and microbial manipulation of plant hormones, are properly categorized as coercive interactions. Fur-

thermore, currently-observable coercive interactions such as these may meet multiple evolutionary fates: stabilize and remain coercive, destabilize and disappear, or turn into signaling through the process of sensory manipulation. A complete understanding of coercion is required for a comprehensive view of the drivers of plant and microbe behaviors in natural systems, understanding future evolutionary trajectories of currently observable plant-microbe interactions, and past evolutionary histories of plant-microbe signaling. Plants are constantly in association with a vast number of organisms representing multiple kingdoms; although we present this framework in the context of two agent interactions, we acknowledge that there may be emergent properties resulting from the inherent biodiversity of microbial communities in association with plants beyond what are discussed here. Finally, future investigations should consider sensory manipulation alongside ritualization, the evolution of signals from cues, when delving into the evolutionary history of plant-microbe signaling.

CHAPTER 2

METHOD DEVELOPMENT AND APPLICATION FOR INVESTIGATING BIOCHEMICAL PLANT DEFENSES TO HERBIVORY AT THE POPULATION LEVEL

INTRODUCTION

SHAWNA TO FILL IN SOME PREAMBLE

2.1 A High-Throughput Method of Analyzing Multiple Plant Defensive Compounds in Minimized Sample Mass

2.1.1 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 (H^-) after depolarization of the plasma membrane due to leaf damage (Maffei et al., 2012; War et al., 2012; Zebelo and 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-Cardenas and Ryan, 1999). The presence and activity of ROS additionally results in the production of a group of enzymes, peroxidases (POD), that are up-regulated 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 and Boland, 2012; War et al., 2012). PPO is up-regulated 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 and 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.

2.1.2 METHODS AND RESULTS

2.1.2.1 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 107 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.

2.1.2.2 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. β-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 PierceTM 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

Table 2.1: 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 (plant)	F stat	*p*	%Variation (plant)	F stat	*p*
Protein Quantification	95.8%	80.42	<0.001	99.9%	3753	<0.001
H_2O_2	0%	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

Note: H2O2 production prior to herbivory was negligible.

Table 2.2: 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%

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.

2.1.2.3 Protein Quantification

Total protein content was measured using the Thermo Scientific Pierce^{TM} 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.

2.1.2.4 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.

2.1.2.5 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.

2.1.2.6 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.

2.1.2.7 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.

2.1.2.8 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.

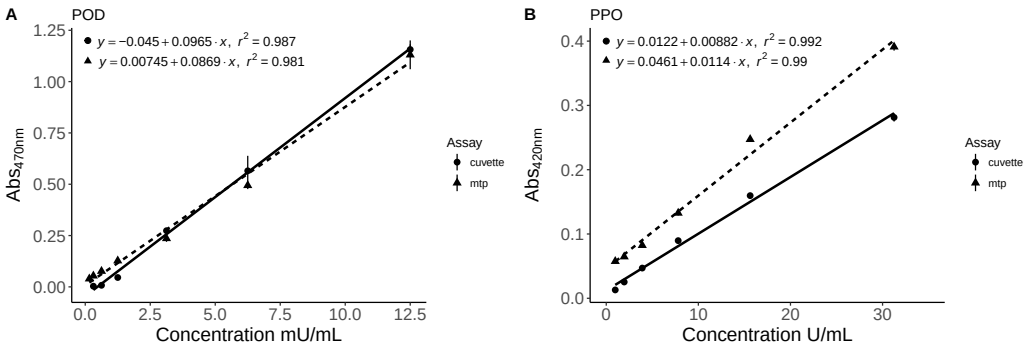

Figure 2.1: Comparison of absorbance values for A) POD and B) PPO when measured using either a spectrophotometer (cuvette) or microplate reader (mtp) to generate a standard curve using horseradish peroxidase for POD (measured at 470 nm) and mushroom polyphenol oxidase for PPO (measured at 420 nm). Line equations and r2 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.

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

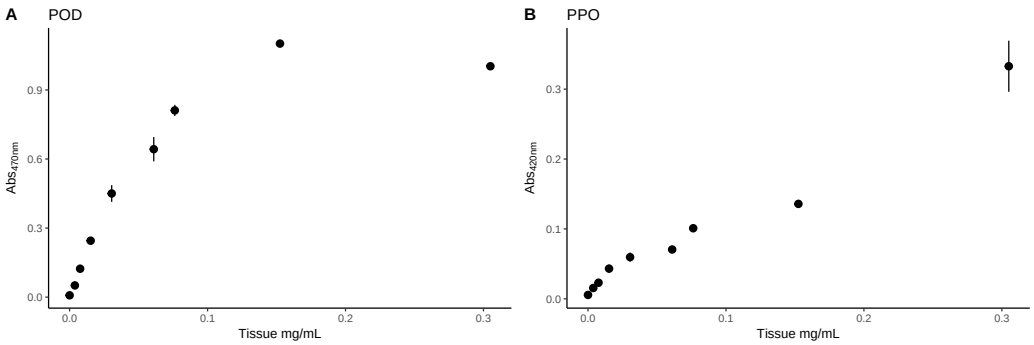

Figure 2.2: Serial dilutions of uninduced tomato tissue. We serially diluted a homogenized tissue sample initially at a concentration of 0.38 g FW/mL to determine the lower limit of detection for the A) POD and B) PPO assays. We used uninduced tissue with low expression of defense compounds and measured 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.

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).

2.1.3 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; Rajalingam et al., 2009). 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 trade-offs 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.

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2.2 Rapid Evolution of Herbivore Interactions Despite Conserved Trade-offs and Biomarkers in Burr Clover

APPENDICES

APPENDIX 2A

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.

Table 2A.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
Protease Inhibitor	PE	0.1x
Hydrogen Peroxide	TCA	1x

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.

Assays

Protein Quantification

- Protein quantification was performed using the Thermo Scientific PierceTM 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: are as follows: 143 μL of 100 mM 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 2 O 2 (final concentration 0.8 mM).

6. Incubate the plates in the dark for 15 min at room temperature.

7. Read absorbance at 470 nm on the microplate reader and ex- press enzyme content as ([AbsSpl – AbsCtrl]/FW) (Abs/g). (FW denotes fresh weight.)

Polyphenol oxidase (PPO) activity

1. Sample aliquots are taken from the 1/10× PE buffer extraction. All reactions are run in triplicate.

2. Create sample master mix by multiplying reaction components by the 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 the total number of control reactions + 1. Reaction compo- nents 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 incu- bate for 5 min.

6. Read absorbance on the microplate reader at 420 nm and ex- press enzyme content as ([AbsSpl – AbsCtrl]/FW) (Abs/g).

Hydrogen peroxide (H_2O_2) quantification

1. Generate a standard curve using a mix containing 100 μL of 1 M potassium iodide, 50 μL of 10 mM 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 the total number of reactions + 1. Reaction components are as follows: 100 μL of 1 M potassium iodide, 50 μL of 10 mM po- tassium phosphate buffer (pH 6.5), and 50 μL of enzyme source.

4. Create control master mix by multiplying reaction components by the total number of reactions + 1. Reaction components are as follows: 100 μL of dH 2 O, 50 μL of 10 mM 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 min at room temperature.

7. Read absorbance at 390 nm and compare values to the standard curve for quantification in nanomoles.

Trypsin-like protease inhibition activity

1. Activity is represented by the inhibition of trypsin in sample aliquots taken from the 1/10× PE buffer. All reactions are run in triplicate.

2. Create sample master mix by multiplying reaction components by the total number of reactions + 1. Reaction components are as follows: 100 μL of enzyme source, 133.3 μL of Trizma base buffer (Sigma-Aldrich), 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 com- ponents by the total number of reactions + 1. Reaction com- ponents are as follows: 100 μL of enzyme source, 166.6 μL of Trizma base buffer (Sigma-Aldrich), and 83.3 μL of 2% azoca- sein dissolved in Trizma base buffer.

4. Create assay control master mix by multiplying reaction components by the total number of reactions + 1. Reaction components are as follows: 233.3 μL of Trizma base buffer (Sigma-Aldrich), 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 the total number of reactions + 1. Reaction components are as follows: 266.6 μL of Trizma base buffer (Sigma- Aldrich) and 83.3 μL of 2% azocasein dissolved in Trizma base buffer.

6. Incubate samples at 30°C for 25 min.

7. Post-incubation, add 133 μL of 100% w/v TCA to all samples and centrifuge at 6146 × g for 10 min.

8. Aliquot 100 μL of 1 M 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. Protease inhibition 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 the values are reported as post-herbivory minus pre-herbivory.

APPENDIX 2B

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 2C

THE STANDARD CURVE GENERATED TO QUANTIFY HYDROGEN PEROXIDE PRODUCTION.

Absorbance values were measured at 390 nm.

APPENDIX 2D

RESULTS OF OUR MICROPLATEBASED PROTOCOLS USING *SOLANUM LYCOPERSICUM* (ECOTYPE M82)

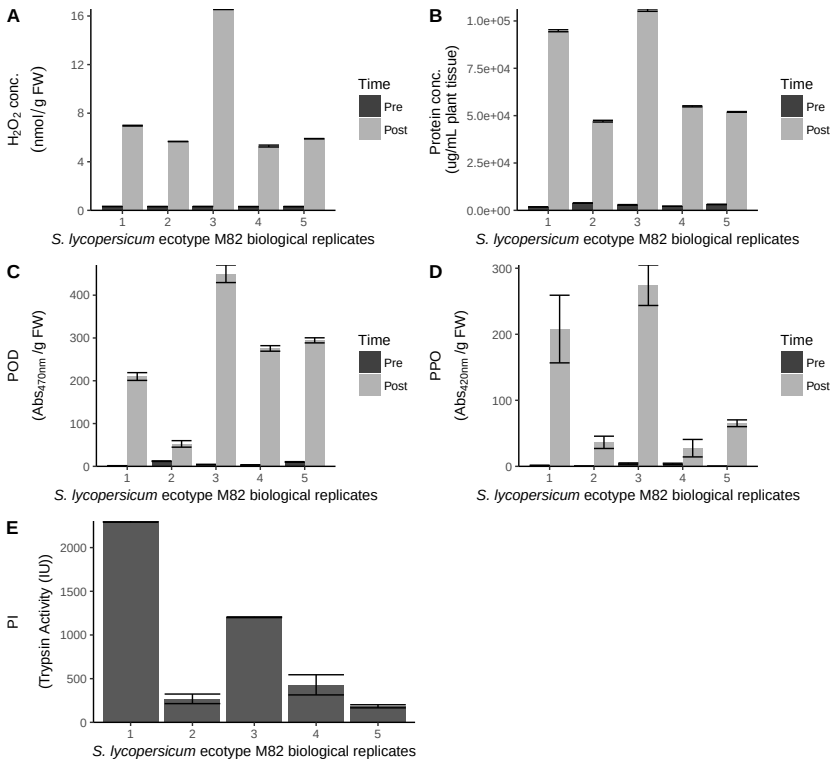

Figure 2D.1: for H_2O_2 (A), protein quantification (B), peroxidase (C), polyphenol oxidase (D), and protease inhibition (E). The numbers 1–5 on the x-axis reflect individual plants used for sampling. The significant variation between biological replicates makes a strong argument against pooling tissue samples from different plants and highlights the benefit of using a protocol that requires a much smaller quantity of tissue (see Table 1). A–D show values preand postherbivory. Trypsin activity is determined by the difference in inhibition preand postherbivory, and thus E only displays one bar per replicate. Bars in all panels show the mean with standard error bars for three technical replicates. PI = protease inhibitors; POD = peroxidase; PPO = polyphenol oxidase.

APPENDIX 2E

ASSAYS TESTED ON *MEDICAGO POLYMORPHA*

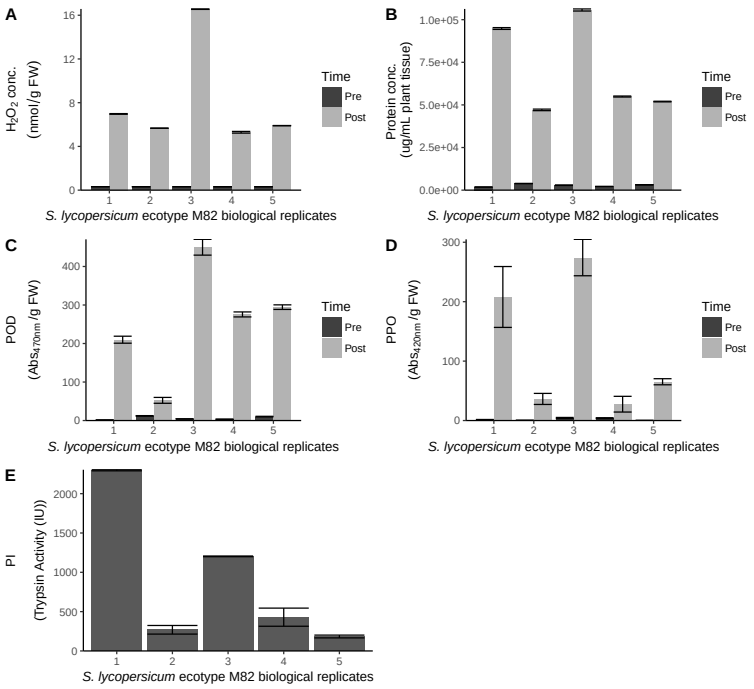

Figure 2E.1: H_2O_2 (A), protein quantification (B), peroxidase (C), polyphenol oxidase (D), protease inhibition (E). Unlike Solanum lycopersicum, M. polymorpha does not always show increase in production of defensive phytochemicals. A–D show values preand postmechanical wounding with regurgitant to simulate herbivory. Trypsin activity is determined by the difference in inhibition preand postmechanical wounding, and thus E only displays one bar per replicate. Bars in all panels show the mean ± standard error bars for three technical replicates. PI = protease inhibitors; POD = peroxidase; PPO = polyphenol oxidase.

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