PARTNERSHIP AND PREDATION IN PLANT-BIOTIC INTERACTIONS: THEORY, METHODS, AND EVIDENCE

By

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ABSTRACT

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As sessile organisms, plants face an endless series of encounters with other living organisms in their environment. Ranging from the beneficial to the harmful, the pressures of these biotic interactions force plants to rapidly adapt to survive and thrive. This dissertation is aimed at addressing questions about the metabolism of plant interactions with herbivores and nutritional symbionts in legumes. Despite spanning different systems and methods, these questions reflect my interest in understanding the biochemistry underlying ecological and evolutionary function and my desire to develop tools that enable the investigation of such questions. To this end, the work in this dissertation is aimed at building conceptual and methodological tools to enable more thorough investigations of plant symbioses and, more broadly, plant-biotic interactions across levels of biological organization.

First, I present a framework for making predictions on evolutionary trajectories and origins of plant–microbe communication systems. By highlighting the prevalence of coercive interactions in plant–microbe interactions, I demonstrate the plausibility of such interactions types to be an evolutionary precursor to seemingly stable signaling mechanisms. This work aims to provide useful evolutionary context for investigations concerning the evolutionary stability and exploitation of signaling mechanisms in established biological relationships.

Second, I present collaborative work in which we developed and applied a cost-effective, high-throughput protocol for quantifying multiple biochemical defense responses from small quantities of plant tissue using spectrophotometric techniques. This protocol was then applied on two distinct populations of the legume *Medicago polymorpha* to investigate how changes defensive traits in responses to altered selective pressures have manifested over the course of novel range expansion. Our work demonstrated the feasibility and potential of assessing defense responses across plant

populations. Further, the work documents a shift in herbivore preference of plants from familiar and unfamiliar ranges by demonstrating a herbivore preference for plant tissues with pre-induced defenses over uninduced tissues from an unfamiliar geographic range.

Finally, I demonstrate the creation, construction, and validity of a novel microcosm system for assessing nutrient exchange in the symbiotic mutualism between plants and arbuscular mycorrhizal fungi (AMF). The novel system is reliable, biologically-relevant, durable, and sufficiently simple and cost-effective to deploy. I demonstrate the validity of the microcosm system and discuss in-progress work which demonstrates its potential to rigorously investigate unknown aspects of the plant-AMF mutualism. Taken together, these developments and suggestions contribute to the growing set of methods and frameworks developed for improving our understanding a various plant-biotic interactions.

For those who fall down rabbit holes.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1

INTRODUCTION

The research presented in this dissertation concerns plant-biotic interactions and their underlying metabolic processes. Since both the interactions considered and research approaches taken vary between chapters, each chapter contains its own introduction to orient the reader. This chapter aims to provide broader context with the goal of presenting unifying themes across the various types of plant-biotic interactions by drawing extensively on selected examples of environmental information perception (i.e. sensing and signaling, the key mechanisms explored in chapter 2) and applicable theory. The goals of each chapter are unified by interest in the following key themes: 1) the metabolic features related to perception of and response to environmental challenges faced by plants; 2) the numerous parallels and differences between types of biotic interactions; 3) evolutionary and ecological theory used to understand the natural histories of such interactions (explored in chapters 2 and 4); and 4) the practical challenges and urgent need to better understand the mechanisms that underlie phenomena discussed at ecological and evolutionary scales (considered and addressed in chapters 3 and 5).

1.1 Plant metabolism and environmental stress

The diversity of metabolic compounds produced by the members of the plant kingdom is magnificent in scale; as organisms that largely remain fixed to a single point over the course of their lifetimes, plants face a distinct set of challenges when interacting with and responding to the world. These interactions are mediated by unique mechanisms of exchanging information and resources, which continuously develop over evolutionary time (Austin and Ballaré 2014; Baker et al. 1997) and are predominantly mediated through regulation and specialization of metabolism (Bednarek and Osbourn 2009).

Like all living things, plants possess a variety of sensory mechanisms that for the purpose of decoding information available in their environment (Trewavas 2014; Karban 2008). This informa-

tion can come in the forms of chemical concentration gradients (Karban 2008), nutrient availability (Kumar, Memo, and Mastinu 2020), secreted compounds by neighboring organisms (Pierik, Mommer, and Voesenek 2013; Pierik, Ballaré, and Dicke 2014; Kong et al. 2018), electromagnetic radiation (Tafforeau et al. 2004; Soran et al. 2014), wavelengths of light (Franklin, Larner, and Whitelam 2004; Legris et al. 2017), and even vibrations generated by physical movement (de Wit et al. 2012; Appel and Cocroft 2014). The unique biochemistry of a given receptor is in part a result of the co-evolutionary pressure exerted by the unique architecture (biochemical, energetic, or physical) of the information requiring detection (Austin and Ballaré 2014; Karban 2008); the extraordinary diversity of plant metabolites reflects the diversity of adaptations to various ecological niches (Li et al. 2020). The collection of this information enables plants to reduce uncertainty in their environmental context and, ultimately, better enable their survival (Adami 2012). Collectively, the study of these metabolic processes underlie critical works in topics of plant biology as diverse as agriculture, ecophysiology, defense, mutualism studies, and evolution (Nelson and Sadowsky 2015; Cheynier et al. 2013; Dudareva et al. 2013).

1.1.1 Metabolic regulation of abiotic and biotic responses

Broadly, the types of challenges plants face can be broken down into the abiotic (non-living) and the biotic (living). Abiotic stress is caused by non-optimal environmental conditions related to environmental features like light, water stress, nutrient availability, soil salinity, temperature, and heavy metal pollution. In contrast, biotic challenges present in the form of competition, predation, parasitism, pathogen attack, and detection of mutualists that coexist in a given environment (Oldroyd 2013; Rajendran et al. 2014). Despite originating from different stimuli, effective coping is dependent on quick perception of and response to both broad classes of environmental challenges (Fujita et al. 2006). The first step of the process is perception of unique chemical patterns to acquire information about the nature of the threat (Plett and Martin 2018). Though typically associated with detrimental interactions, the process of pattern recognition by cells is a mechanism that has been repeatedly selected to help plants cope with a wide variety of signals and cues they encounter over

the course of their lives. The parallels between perception and response to various environmental factors is likely a consequence of shared evolutionary history (Oldroyd 2013).

1.1.2 Signaling

In plants, this pattern recognition process occurs largely through a variety of membrane-bound protein receptors that are constitutively present on cell surfaces for the detection of stress-associated chemical or physical cues (Shiu and Bleecker 2001; Day and T. Graham 2007; Osakabe et al. 2013). The most widely implicated family of membrane-bound receptor proteins are the receptor-like kinasas (RLKs) found throughout the plant kingdom (Shiu and Bleecker 2001). Over the last 3 decades, RLKs have been found to be a prominent point of recognition for both abiotic and biotic stressors; for instance, RLKs have been found to control water stress signaling (Osakabe et al. 2013) and the initial detection of plant pathogens via recognition of conserved bacterial peptide sequences known as microbe/pathogen-associated molecular patterns (MAMPS/PAMPs) (Boller and Felix 2009). Further, RLKs have also been implicated in the perception of early signaling molecules released by beneficial soil bacteria (Indrasumunar et al. 2010). The similarities continue as the recognition of these patterns then begins a downstream signal cascade such as signal propagation through the highly-conserved mitogen-activated protein kinase (MAPK) pathway leading to changes in transcriptional regulation (Fujita et al. 2006; Zipfel and Robatzek 2010). Additional key signaling and regulatory mechanisms occur via phytohormones and small molecules such as small signal peptides, calcium ions, and reactive oxygen species (Apel and Hirt 2004; A. R. War, Paulraj, M. Y. War, et al. 2011).

Ultimately, the purpose of these communication events is to enable a plant to detect information and react accordingly. Such final responses can include programmed cell death (Coll, Epple, and J. L. Dangl 2011), growth inhibition (Jones 2004), root system expansion (Araya et al. 2014), accommodation of endosymbionts (Parniske 2008), or protein interlinking for anti-nutritional purposes (A. R. War, Paulraj, Ahmad, et al. 2012). For all of these end responses and more, we can follow the sequence of 1) perception of environmental pattern/ information, 2) intermediate

signaling response, and 3) physiological effect. In the instance of nitrogen foraging by plant roots, the environmental information (1) may come in the form of pH changes as a result of changes in ion flux along an ammonium gradient. Taking in this information, it has been shown that plants can locally accumulate auxin (2) for the purpose of (3) triggering lateral root branching for improved nitrogen uptake (Meier et al. 2020). When faced with pathogenic bacteria, plants can detect MAMPs (1) via membrane receptors. This detection leads to (2) a rapid influx of calcium, accumulation of ROS, and a MAPK phosphorylation cascade. These metabolic steps can lead to (3) changes such as cell wall alterations, defense gene expression, and the hypersensitive response (M. D. Jones and Sally E Smith 2004; Tóth and Stacey 2015; Nishad et al. 2020). As a final example, the endosymbiotic association formed between plants and the microbial arbuscular mycorrhizal fungi (AMF) requires host plants to accommodate the fungi within the root cells while still protecting against exploitation. This process happens via (1) an exchange of signals through the release of strigolactones, a plant hormone, and fungal release of lipochitooligosaccharides. This exchange results in (2) plant transcriptional upregulation of symbiosis genes, slight increases in lateral root elongation, and calcium oscillations as well as the fungal formation of a modified hyphal organ (appressorium) to facilitate cell penetration. Finally, (3) the plant cell nucleus moves ahead of the fungal pre-penetration apparatus before the fungi breach the plant cell wall and create a highly branched structure that does not breach the plant cell plasma membrane (Parniske 2000).

These examples, though far from exhaustive, exemplify the magnificent biochemical diversity that arose despite the similarities between the example processes. Examples using plant responses like those of herbivory, interspecific competition, or water stress would have similarly followed the perception–signal– response sequence. Despite the monumental amount of information uncovered by research efforts over recent decades, much remains unknown both about the specific details of the individual mechanisms and the broader processes of which they are apart. Future work will hopefully reveal additional information on the extent of molecular crosstalk, the evolutionary origins, the context-dependency, and the biogeographical variation of these pathways. Such work will inevitably involve tight collaboration between researchers of mechanisms and researchers of

ecosystem dynamics.

1.1.3 Symbioses on a spectrum

A particularly interesting driver of this metabolic development is the interactions between plants and other organisms (Austin and Ballaré 2014). The intentional exchange (signals) and the opportunistic receiving (cues) of information between organisms is an evolutionary dance that began billions of years ago (Otte 1974; Scott-Phillips et al. 2012; Han 2019; Harris, Katharina Pawlowski, and Mathesius 2020). Interspecific plant-biotic interactions exist on a spectrum from mutually beneficial (i.e. mutualism) to mutually detrimental (i.e. competition) (Haskell 1949) and demonstrate the ability to shift along this spectrum within the lifetimes of individuals (A. E. Douglas 2021). A given plant's success (i.e. fitness) is thus highly dependent on its ability to discern a difference between commensalists, mutualists, and antagonists. The collection of processes largely responsible for receiving and translating information needed to distinguish friend from foe is the plant immune system (M. D. Jones and Sally E Smith 2004; Chisholm et al. 2006; Plett and Martin 2018; Han 2019; Nishad et al. 2020).

"Symbiosis" is an inconsistently defined term that is often misconstrued to mean "mutualist" (J. Bronstein 2015). Symbioses are any type of physically close, long-term relationship between two or more living organisms (de de Bary 1879) and can function as both mutualisms and pathogenic relations (J. Bronstein 2015). Though there still remains much debate about whether pathways for accommodating mutualists or pathways for protecting against antagonists came first (Parniske 2008; Han 2019) it's clear from their functional, molecular, and genetic similarities that they share common evolutionary origins (Tóth and Stacey 2015; Plett and Martin 2018; Han 2019). Symbioses have made dramatic contributions towards the evolutionary success of modern plants; notable examples include the endosymbiotic event that gave rise to the chloroplast an estimated 1.6 billion years ago (Hedges et al. 2004; Mereschkowsky 1905; S. E. Douglas 1994; McFadden 2001) and the symbioses between early diverging fungi belonging to the Glomeromycotina and potentially the Mucoromycotina that facilitated the terrestrialization of plants an estimated 475 million years

ago (Parniske 2008; Hoysted et al. 2019). Regardless of the net effect on the hosts, microbial symbiotic associations seem to involve a common set of ordered features: 1) the pre-symbiotic exchange of signals and cues (described in previous section); 2) initial microbial attachment to the host plant cells; 3) growth on, in, or between the plant cells; and 4) the exchange or extraction of nutrients (Plett and Martin 2018).

When comparing the generally mutualistic arbuscular mycorrhizal fungi (AMF) described above with some soil-dwelling fungal pathogens, it's interesting to note that symbiotic establishment steps are more similar than the initial step of pre-symbiotic signal exchange and the final step involving the exchange or extraction of nutrients. Like AMF, a subset of pathogenic fungi fully attach to the host cell post initial adhesion using the modified hyphal structure known as the appresorium (Deising, S. Werner, and Wernitz 2000). The appressoria penetrates the plant cuticle and expands into the host cell (Howard et al. 1991). The similarities diverge slightly with the pre-symbiotic information exchange. Though fungal mutualists and pathogens use hyphal chemotropism to sense plant hosts, AMF are known to sense a phytohormone that likely evolved because of its effect on AMF (Parniske 2008). This is in contrast to opportunistic cue detection by some fungal pathogens, such as *Fusarium*, which may detect peroxidase and other root exudates that are secreted for non-signaling purposes (Tsai, Oota, and Sawa 2020). The similarities diverge further still when considering the difference between nutrient exchange of mutualistic symbioses and nutrient extraction/ necrotrophy of plant pathogens.

Finally, to demonstrate this dynamic at the molecular level, consider the exchange/ extraction of host sugars by various symbionts. At the community level, plants exchange carbon with their mutualistic symbionts in exchange for other resources; in contrast, plant are the nutritional resource when engaging with their pathogenic symbionts. Plants possess a family of sugar transporters known as SWEET transporters; during colonization by both mutualists and pathogens these transporters are upregulated. In what proves to be a common theme when comparing the metabolic differences in plants responding to mutualists and pathogens, slight differences exist between the profile of sugars transported out of the cell during the two interaction types. Further exemplifying this,

plant cell vacuoles appear to hoard less sugar during colonization by mutualists than they do when attacked by pathogens; these differences reflect differences in the apparent willingness of the host plant to part with its resources (Plett and Martin 2018). In any given environment, plant defenses are critical for survival. These responses are known to vary between species, ecotypes, and pathogen type but little is known about what underpins this variation (Chisholm et al. 2006) and the full extent of the molecular crosstalk deserves more investigation.

1.1.4 Pathogens and pests

As introduced previously, plants can weaponize metabolites and target attacking organisms to reduce the severity of damage (Dodds and Rathjen 2010). The mechanisms involved in responding to dangerous biotic interactions are primarily modulated by phytohormones (Jeffery L. Dangl and J. D. G. Jones 2001) and, again, there is extensive overlap between plant responses to microbial pathogens and insect herbivores (A. R. War, Paulraj, Ahmad, et al. 2012). The early perception of cues associated with pathogens is the first important step in responding to biotic foes (Jeffery L. Dangl and J. D. G. Jones 2001). Plants can detect environmental cues in the forms of highly-conserved chemical motifs, such as peptide sequences of flagella, known as microbe-associated molecular patterns (MAMPs). When interacting with herbivores, plants have the ability to perceive various cues such as oral secretions dubbed herbivore-associated molecular patterns (HAMPs) or the self-recognition of damaged tissues dubbed damage-associated molecular patterns (DAMPs) (Acevedo et al. 2015; Heil and Land 2014). These variously associated patterns are detected by surface membrane-localized receptors that trigger defense responses via signal cascades (He, Shan, and Sheen 2007; J. Zhang and Zhou 2010).

Specifically for herbivorous insects, immunity responses can range from thickened cuticles and other structural changes to the production of reactive phenolic compounds and reactive oxygen species (ROS) (M. D. Jones and Sally E Smith 2004). 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, Arimura, and Mithöfer 2012; A. R. War, Paulraj, Ahmad, et al. 2012; Zebelo and Maffei 2015). Both chemical treatments and mechanical wounding can elicit ROS production (Maffei, Mithöfer, and Boland 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 C. A. Ryan 1999). Many of these processes are highly-conserved across species and numerous similarities exist between herbivore defense strategies and defenses against microbial pathogens (Taylor, Hatcher, and Paul 2004).

1.1.5 Mutualism

The establishment of mutualism, or mutually beneficial relationships between species, is also dependent on plant-derived metabolic processes (Nelsosn and Cox 2012). Plants are known to excrete metabolites and hormones from their roots with the aim of attracting nutritional mutualists from the environment. Mutualisms can be symbiotic or non-symbiotic, obligate or facultative, and symbionts range from microbes to the "macrobes" (J. Bronstein 2015). Plant mutualisms have key roles in global nutrient cycles, ecosystem functions, species diversity, and plant evolution (Bond 1994; Wall and Moore 1999). Plant mutualisms come in a variety of forms: plant-pollinator interactions involved plants as a nutrient or shelter source for animals like insects, birds, and bats in exchange for reproductive assistance via pollination (Bond 1994) while many plant-microbe mutualisms are founded in nutrient exchange.

For these so-called nutritional mutualisms, the prime benefit is the exchange of essential metabolic resources containing carbon (C), nitrogen (N), phosphorus (P), and other essential nutrients (Nelson and Sadowsky 2015; Bonfante and Genre 2010). These can be distinguished from plant growth-promoting bacteria by the apparent evolutionary emphasis on nutrient transfer rather than other plant growth-promoting mechanisms (e.g. release of phytohormones to stimulate plant growth (Glick 2012)). It is important to note that the exact mechanisms of many plant growth-promoting microbes have yet to be elucidated; as such, there are likely to be many more

nutritional mutualists that have yet to be identified (Glick 2012). Further complicating matters is the difficulty of categorizing some symbioses as mutualists. Changes in the effects of biotic associations between partners under environmental perturbations have been observed in associations of all types; unfortunately, there remain large knowledge gaps regarding the causes, mechanisms, and evolutionary stability of such shifts (M. D. Jones and Sally E Smith 2004). This general lack of knowledge is true for the vast majority of observed plant-biotic interactions of the natural world (Vandvik et al. 2020); despite the clear evidence that biotic interactions affect species responses to nearly every type of environmental perturbation, unraveling the mechanisms of these interactions and generating accurate predictions regarding species coexistence remains a major challenge (Wisz et al. 2013).

General attention to nutritional mutualisms has increased in recent decades; despite this, many often don't receive significant research attention (Bidartondo et al. 2011). Recent research on the fungal phylum Mucoromycotina has yielded evidence of nutrient transfer but the mechanisms are far from elucidated (K. J. Field et al. 2019; S. Zhang et al. 2019). Additional examples of known plant-microbe nutritional mutualists include the two groups of actinorhizal nitrogen-fixing bacteria, Frankia and Rhizobia (Peter, Young, and Haukka 1996) and the various types of mutualistic mycorrhizal fungi such as many of the arbuscular, orchid, ericoid, ecto-mycorrhizal species (Sally E. Smith and Read 2010). Two nutritional symbiotic mutualists of growing agricultural interest are the group of nitrogen-fixing bacteria collectively known as rhizobia and the arbuscular mycorrhizal fungi (AMF) (Udvardi and Poole 2013; Parniske 2008). These mutualists are root endosymbionts and are predominantly known for trading biologically available N and P for photosynthetically fixed C (Udvardi and Poole 2013; Parniske 2008). N and P represent the most limiting elements for plant productivity in natural systems and are two of the three primary components of synthetic fertilizers applied to agricultural fields (Vitousek et al. 2010).

AMF are an early diverging clade of fungi that are believed to have played a pivotal role in the evolution of plants from aquatic ecosystems to dry land (Tisserant et al. 2013). These obligate, root-associated symbionts possess extensive hyphal networks with more surface area and physical reach

of plant root systems, resulting in improved nutrient uptake, communication between plants, and resiliency to a wide range of biotic and abiotic stresses (Parniske 2008). AMF are considered the most ecologically relevant plant mutualists due to their ubiquity and net benefit to hosts (Tisserant et al. 2013). It is estimated that approximately 75% of all vascular plant species form associations with AMF with interactions occurring in nearly all terrestrial ecosystems (Sally E. Smith and F. A. Smith 2011). Recent studies have focused on defining pathways dedicated to nutrient movement and elucidating additional resources transported via AMF; the uptake and transfer of N to host plants is of special intrigue due to its overlapping niche with rhizobia. Rhizobia perform the energetically expensive process of fixing atmospheric dinitrogen (N_2) in the bioavailable form of ammonium (NH_4^+) for leguminous hosts (Udvardi and Poole 2013). In contrast to rhizobia, AMF are able to take in and translocate N in the forms of nitrate (NHO_3^-) and ammonium (NH_4^+) directly from the soil environment (Tian et al. 2010). Unfortunately, the specifics of how host plants regulate the distribution of C to multiple symbiotic partners, mechanisms underlying the N transfer process, host regulation of the symbiosis under variable nutritional conditions, and more remain unclear (Tisserant et al. 2013; Tian et al. 2010). Thorough characterization of N nutrient pathways and their physiological regulation, host regulatory mechanisms of symbionts, and exchange of resources between plants via AMF is still needed. Challenges associated with such studies are further described in chapter 5.

1.1.6 Modulating multiples: The interesting case of legumes

Although there is significant overlap in genetic immune responses, defenses raised against bacterial pathogens, bacterial mutualists, insect herbivores, and fungal pathogens all vary (Newton et al. 2010). Furthermore, the specific nature of an individual plant's immune responses can be influenced by co-evolutionary histories and the nature of the interaction (Dodds and Rathjen 2010).

The symbiosis between legumes, AMF, and rhizobia serves as a fascinating system to study tripartite associations due to its exchange of common resources (Tian et al. 2010; Ossler, Zielinski, and Heath 2015; Afkhami and Stinchcombe 2016). Legumes are members of the third-largest land

plant family, Fabaceae (Hasanuzzaman, Araújo, and Gill 2020). Legumes are notable for their mutualistic symbiosis with the nitrogen-fixing bacteria collectively known as rhizobia (Suzaki, Yoro, and Kawaguchi 2015). Biological nitrogen fixation is of high agricultural importance because nitrogen is one of the most limiting nutrients for plant growth in natural environments and because of the high energy input (16 ATP per unit of N_2) required to break the triple covalent bond of diatomic nitrogen (Raymond et al. 2004; Mylona, Pawlowski, and Bisseling 1995). Since the legume-rhizobia symbiosis is almost always found in the presence of AMF, investigations of tripartite associations and regulation of exchange of common nutrition resources is warranted (E. Wang et al. 2012). The interesting positionality of legumes makes it an interesting, if not complicated, study system and thus the plant family that is used throughout the studies presented in this dissertation. Past work on this tripartite association has focused on assessing carbon sink strength and effects of altered P supply to the system (Antunes and Goss 2005; Ossler, Zielinski, and Heath 2015; G. Wang et al. 2016; Primieri et al. 2022). Other work has employed numerous –omics techniques to study regulatory changes in response to one (Afkhami and Stinchcombe 2016) or both symbionts, or focused on assessing functional diversity in ecological experiments (Ossler, Zielinski, and Heath 2015). These experiments share the common goal of identifying overlap between mutualism pathways and host responses to microbial nutritional mutualists. However, very little is known about the nutrient exchange fluxes and their environmental regulations due to the various technical challenges involved with application of quantitative assessments required for elucidating metabolic flux under multiple environmental factors. Difficulties like these are addressed through methodological developments presented in chapters 3 and 5.

1.2 Evolutionary theory of plant-biotic interactions

Relationships between dissimilar species have been key players in the evolution of countless biological phenomena; from the rise of eukaryotes and multicellularity (López-García, Eme, and Moreira 2017) to the cornucopia of bioactive metabolites produced by plants, interspecific interactions have shaped evolutionary histories across the tree of life (Gilbert, Bosch, and Ledón-Rettig

2015; Becerra, Noge, and Venable 2009; Raina et al. 2018). In attempts to explain the origins of such diverse and highly specialized features apparent in extant species, many evolutionary explanations of plant immune responses and corresponding relationships make use of fields such as game theory (Hammerstein and Selten 1994; Weibull 1997) and information theory (Adami 2012; Li et al. 2020).

1.2.1 Co-evolution and game theory

"All models are wrong, but some are useful" - George Box

Natural systems are highly-complex and vary on axes both known and unknown (Yates et al. 2018). Consequently, many researchers are faced with the challenge of investigating data-poor phenomena, environments, interactions, and other biological dynamics. One such way of attempting to navigate this issue is through the creation and analysis of mathematical and statistical models. Though an exciting prospect, modeling efforts are also challenged by issues of complexity (Lopatkin and Collins 2020). Models are nearly always intentional simplifications of some or many components of the natural world. The hallmark of a good model is its ability to provide insights on a particular, distilled phenomenon about which it makes key assumptions (McNamara 2013).

The borrowing and application of theory from different disciplines, such as game theory from microeconomics, has proven to be useful in evolutionary modeling efforts (J. M. Smith 1982; Archetti et al. 2011). Models of game theory generally possess two common features: 1) they assume the existence of an optimal strategy or selectable feature that maximizes plant fitness and 2) they capture the selective pressure exerted by an external factor (McNickle and Dybzinski 2013). Biologists have found easy application of game theory because of the quick economic connections to determining optimal strategies during interactions such as competition over resources (Tilman 1982), investment in a diverse set of long-term cooperative relationships (Hoeksema and Bruna 2000), and enemy attack (Bixenmann et al. 2016). A widely-used application of game theoretic approaches is the use of the Red Queen Hypothesis. The Red Queen Hypothesis aims to describe the constant, co-occurring adaptations that individual species undergo as they compete through

evolutionary time (Van Valen 1973; Langerhans 2008). Van Valen's theory derives its name from Lewis Carroll's Through the Looking-Glass. The character of the Red Queen describes the world as, "... here, you see, it takes all the running you can do, to keep in the same place." In the world of biological adaptations, co-evolving organisms engage in a so-called "arms race" over time which manifests as organisms sharing a long evolutionary history with specialized responses to one another (Edger et al. 2015). This Theory relies on the assumption that development of a novel immune response will inevitably select for predators that can overcome a given defense (Clay and Kover 1996). Without such simultaneous escalations, a given organism may be threatened with eventual extinction upon losing the evolutionary game (Van Valen 1973). Like all models, the Red Queen Hypothesis has been proven to be overly simplistic in model form; however, use of it as a conceptual framework helped encourage investigations like parasites as an evolutionary selection pressure (Clay and Kover 1996). The importance of considering numerous possible selection pressures for a given relationship, in part, brought about the work presented in chapter 2.

Unfortunately, researchers are realistically only able to assess dynamic environmental responses in extant species and assessing the formation and breakdown of various plant-biotic associations has proven challenging (J. Bronstein 2015). Consequently, it is also extremely difficult to predict the future effects of the modern selection pressures on established biotic relationships. The co-evolution theory that predicts continual escalation of defenses and mechanisms of exploitation is contingent on the assumption that the strongest selection pressures in a given relationship are dependent on the primary interacting species (Mello and Silva-Filho 2002).

1.2.2 Context-dependency

In investigating extreme pressures on plant mutualisms, new research has demonstrated the prevalence of strong external selection pressures and found evidence for the breakdown of mutualism over the course of plant domestication (Xing et al. 2012; Turrini et al. 2016; S. Zhang et al. 2019); so just as shared evolutionary history can cause escalations, relationships between organisms can also deescalate and destabilize over shared evolutionary time (Kiers, Hutton, and Denison 2007; Martín-

Robles et al. 2018). Outside of domestication, trade-offs have been found in natural environments in which mutualism breakdown can occur as a result of successful asymbiotic nutrient extraction or association with metabolically cheaper partners (G. D. A. Werner et al. 2018). These shifts are likely a result of variation in performance and benefit under variable contexts (Chamberlain, J. L. Bronstein, and Rudgers 2014). Though this context-dependency has been observed in all types of biotic interactions (Chamberlain, J. L. Bronstein, and Rudgers 2014) different interaction types seem to be more sensitive to variations in context than others; notably, mutualisms appear to demonstrate greater sensitivity to context than other interaction types (Chamberlain, J. L. Bronstein, and Rudgers 2014). This reality is exemplified by the arbuscular mycorrhizal fungi (AMF) symbiosis. AMF are generally described as root-associated mutualists that benefit plants by improving nutrient uptake from the soil (Parniske 2008). However, the growing body of work on AMF has clearly demonstrated that these symbioses exist on a continuum between mutualism and parasitism (M. D. Jones and Sally E Smith 2004) with shifts along the continuum likely determined by environmental contexts (Stribley, Tinker, and Rayner 1980). Many researchers have found that under too luxurious of nutrient conditions (high phosphorus and nitrogen) the assumed growth benefit of AMF is reversed and AMF appears to have a negative effect on host biomass (Sally E. Smith and F. A. Smith 2011). Considering the context-dependency in mechanistic mutualism research is critical for evaluating mutualistic effects (terHorst, Lennon, and Lau 2014); this context-dependency is hinted at in the results of the nutrient limitation testing of chapter 5 (Figure 5.3).

The breakdown of plant-biotic associations that provide essential ecosystem services such as nutrient cycling and soil structuring would leave both natural and agricultural ecosystems increasingly vulnerable to rapid changes in their environments (Shennan 2008). With growing interest in applications of beneficial soil microbes for improved agricultural productivity, understanding the potential symbiotic trade-offs that may result from agricultural interventions will be essential for future successes (Porter and Sachs 2020). Continual development of evolutionary hypotheses and subsequent testing will help shed light on how modern selective pressures may or may not be destabilizing various plant-biotic interactions (Thrall et al. 2011).

1.2.3 Invasion biology: evolutionary pressures by herbivores

The evolutionary arms race dynamic described previously is especially apparent in plant-herbivore relationships and multiple hypotheses have been proposed detailing how plant species will interact with novel herbivores (Mello and Silva-Filho 2002; Zu et al. 2020). Like other mechanistic investigations at higher levels of biological organization, researching these various hypotheses is burdened by limitations regarding the feasibility of testing defensive responses at the population level (Poisot et al. 2012; Zu et al. 2020). Making accurate predictions about host plant responses to a given environment requires careful consideration of pest type, environmental context, and co-evolutionary histories. Applications of evolutionary theory to modern obstacles could prove to be a powerful tool for understanding how human interventions and anthropogenic changes are able to alter biotic interactions and biological processes on which we depend (Thrall et al. 2011). More work will need to be done to test these evolutionary theories across distinct biogeographic regions (Zu et al. 2020) and future work similar to that described in chapters 3 and 5 will help these efforts.

An understanding of the environmental context and relevant evolutionary history is especially useful in invasion biology, where research has focused on understanding which biotic and abiotic factors make an invasive species successful in a given environment (Theoharides and Dukes 2007). Invasive species from all kingdoms have widespread effects on local populations when they begin to establish novel niches. The effect of novel insect herbivores has been demonstrated by invasive species such as the emerald ash borer in North America which has decimated local populations of ash trees around the continent (Poland and McCullough 2006). While it is known that these resulting effects dramatically alter communities and ecosystems, current theory and evidence still struggles to make predictions about the success of a species in a novel biogeographical environment (Paini et al. 2016; Srivastava, Lafond, and Griess 2019). Unraveling the factors that contribute to a given species' success is extremely important for conservation and agricultural efforts across the globe (Theoharides and Dukes 2007; Gurevitch and Padilla 2004; Seebens et al. 2021). A prominent component of these studies in plant biology is discerning how various plant species will

be affected by both how well they thrive as the invader and how well they will thrive in response to threats such as novel herbivores in native plant environments (Vavra, Parks, and Wisdom 2007). The work in chapters 3 and 4 deal with these themes.

1.3 Agroecology of plant-biotic interactions

Improving our understanding of plant-biotic interactions and their dynamics will be critical for sustainable agriculture and predictive climate effect modeling efforts (Vandvik et al. 2020). Innovations such as industrial nitrogen-fixation and synthetic pesticides brought about major gains in crop yields and played a major part in international development over the last century. Unfortunately, these innovations have since demonstrated diminishing returns for agriculturalists (Grassini, Eskridge, and Cassman 2013; Ray et al. 2012). Past gains have come at the expense of soil health, water quality, atmospheric growth conditions, and biodiversity that provides resiliency against pests (Bindraban et al. 2012; McBratney, D. J. Field, and Koch 2014; Gomiero 2016). The resulting environmental degradation is often non-linear and recent work suggests that some of the most extreme changes in ecosystems result from the consequences of biotic interactions breaking down and their associated biological processes halting (Vandvik et al. 2020; Urban et al. 2016; Wisz et al. 2013; Kaarlejärvi, Eskelinen, and Olofsson 2017; Ettinger and HilleRisLambers 2017; Alexander, Diez, and Levine 2015).

One of the most common agricultural interventions, the application of chemical fertilizers, has continuously been shown to have negative effects on the functioning of plant nutritional mutualisms (Ahmed and Elsheikh 1998; Caballero-Mellado and Martinez-Romero 1998; Vitousek et al. 2010; Turrini et al. 2016; Ferrol, Azcón-Aguilar, and Pérez-Tienda 2019; Porter and Sachs 2020). Debate over the importance of considering soil symbionts like AMF for agriculture is still active in the literature (M. H. Ryan and J. H. Graham 2018), there exists a long history of evidence that colonization by AMF can result in yield losses under artificially high nutrient conditions (Thirkell et al. 2017) and references within). For rhizobia, evidence suggests losses in genetic diversity of rhizobial species under high fertilizer use (Caballero-Mellado and Martinez-Romero

1998) and evidence that modern legume cultivars are relaxing their defenses against poor quality partners in comparison to their older counterparts (Kiers, Hutton, and Denison 2007; Weese 2015; terHorst, Lennon, and Lau 2014). This growing collection of evidence for the loss of beneficial symbiotic traits in crop cultivars under agricultural selection pressures suggests an urgent need for researchers to consider the greater agroecosystem when assessing outcomes of modern practices and prescribing best practices for the future (Porter and Sachs 2020).

One such way of approaching crop yield improvement efforts is to embrace an increasingly transdisciplinary approach (Caporali 2011; Thrall et al. 2011). Many conventional agricultural interventions improve yields by attempting to remove dependence on biotic-interactions in lieu of more easily controlled abiotic alternatives (e.g. chemical fertilizers, tilling, pesticide application, etc.) (Shennan 2008). Though the reduction in complexity may seem useful, agricultural production systems are nearly always embedded within a broader natural ecosystem, rather than isolated from the dynamics of the natural environment. Since the early 2000s, calls to more thoroughly consider the ecological consequences of agricultural interventions on biotic interactions within agroecosystems have increased (Garnett et al. 2013; Shennan 2008; Gaba et al. 2014). As demonstrated with specific examples throughout this chapter, the current difficulties in the applications of quantitative methods at higher levels of biological organization remains as a barrier to the completion of such studies.

1.4 Conclusions

It's clear that facing the challenge of meeting the ever-growing agricultural needs while mitigating and repairing ecosystem damages to maintain agricultural productivity over time will require significant collaborative efforts. A consistent theme that emerges in predictive modeling approaches and other mechanistic investigations of ecological dynamics is that rigorous data collection at biologically-relevant scales is prohibitively difficult. Bridging methodological gaps between studies utilizing top down (i.e. ecological) and bottom up (i.e. genetic/ biochemical) will not be easy. Research practices such as eco-metabolomics– the characterization of biochemical

aspects of biotic-interactions across spatiotemporal scales (Peters et al. 2018; Villate et al. 2021)– demonstrate both the promises and challenges of such transdisciplinary studies; deploying methods traditionally used in highly controlled environments (and thus on small sample sizes) is often both cost- and time-prohibitive when asking questions at the level of populations and ecosystems (i.e. on very large sample sizes). To investigate mechanisms of biotic-interactions, researchers are limited in the number of variables that can be manipulated in a given experiment. However, development of tools that allow increasingly biologically-relevant experimental environments and reduce implementation barriers can be effective initial steps to asking mechanistic questions with more ecological relevance (Shennan 2008). In light of these challenges, the work presented in this dissertation is aimed at developing and deploying experimental tools and a theoretical framework that can be used to investigate changes in interspecific dynamics under different sets of nutritional conditions and their variations between populations.

BIBLIOGRAPHY

BIBLIOGRAPHY

Acevedo, Flor E et al. (Aug. 1, 2015). "Cues from Chewing Insects — the Intersection of DAMPs, HAMPs, MAMPs and Effectors". In: *Current Opinion in Plant Biology* 26, pp. 80–86.

Adami, Christoph (2012). "The Use of Information Theory in Evolutionary Biology". In: *Annals of the New York Academy of Sciences* 1256.1, pp. 49–65.

Afkhami, Michelle E. and John R. Stinchcombe (2016). "Multiple Mutualist Effects on Genomewide Expression in the Tripartite Association between Medicago Truncatula, Nitrogen-Fixing Bacteria and Mycorrhizal Fungi". In: *Molecular ecology* 25.19, pp. 4946–4962.

Ahmed, Ahlam Elsammani and Elsiddig Ahmed Elmustafa Elsheikh (1998). "Effects of Biological and Chemical Fertilizers on Growth and Symbiotic Properties of Faba Bean (Vida/Aha L.) Under Salt Stress". In: *University of Khartoum Journal of Agricultural Sciences* 6.1, pp. 150–164.

Alexander, Jake M., Jeffrey M. Diez, and Jonathan M. Levine (Sept. 2015). "Novel Competitors Shape Species' Responses to Climate Change". In: *Nature* 525.7570 (7570), pp. 515–518.

Antunes, Pedro M. and Michael J. Goss (2005). "Communication in the Tripartite Symbiosis Formed by Arbuscular Mycorrhizal Fungi, Rhizobia and Legume Plants: A Review". In: *Roots and Soil Management: Interactions between Roots and the Soil*. John Wiley & Sons, Ltd, pp. 199–222.

Apel, Klaus and Heribert Hirt (2004). "REACTIVE OXYGEN SPECIES: Metabolism, Oxidative Stress, and Signal Transduction". In: *Annual Review of Plant Biology* 55.1, pp. 373–399.

Appel, H. M. and R. B. Cocroft (Aug. 1, 2014). "Plants Respond to Leaf Vibrations Caused by Insect Herbivore Chewing". In: *Oecologia* 175.4, pp. 1257–1266.

Araya, Takao et al. (Feb. 4, 2014). "CLE-CLAVATA1 Peptide-Receptor Signaling Module Regulates the Expansion of Plant Root Systems in a Nitrogen-Dependent Manner". In: *Proceedings of the National Academy of Sciences* 111.5, pp. 2029–2034.

Archetti, Marco et al. (2011). "Economic Game Theory for Mutualism and Cooperation". In: *Ecology Letters* 14.12, pp. 1300–1312.

Austin, Amy T. and Carlos L. Ballaré (Oct. 1, 2014). "Plant Interactions with Other Organisms: Molecules, Ecology and Evolution". In: *New Phytologist* 204.2, pp. 257–260.

Baker, Barbara et al. (1997). "Signaling in Plant-Microbe Interactions". In: *Science* 276.5313, pp. 726–733.

Becerra, Judith X., Koji Noge, and D. Lawrence Venable (Oct. 27, 2009). "Macroevolutionary Chemical Escalation in an Ancient Plant–Herbivore Arms Race". In: *Proceedings of the National Academy of Sciences* 106.43, pp. 18062–18066.

Bednarek, Pawel and Anne Osbourn (May 8, 2009). "Plant-Microbe Interactions: Chemical Diversity in Plant Defense". In: *Science (New York, N.Y.)* 324.5928, pp. 746–748.

Bidartondo, Martin I. et al. (Aug. 23, 2011). "The Dawn of Symbiosis between Plants and Fungi". In: *Biology Letters* 7.4, pp. 574–577.

Bindraban, Prem S et al. (Nov. 1, 2012). "Assessing the Impact of Soil Degradation on Food Production". In: *Current Opinion in Environmental Sustainability*. Terrestrial Systems 4.5, pp. 478–488.

Bixenmann, Ryan J. et al. (2016). "High Herbivore Pressure Favors Constitutive over Induced Defense". In: *Ecology and Evolution* 6.17, pp. 6037–6049.

Boller, Thomas and Georg Felix (June 2009). "A Renaissance of Elicitors: Perception of Microbe-Associated Molecular Patterns and Danger Signals by Pattern-Recognition Receptors". In: *Annual Review of Plant Biology* 60.1, pp. 379–406.

Bond, W. J. (1994). "Do Mutualisms Matter? Assessing the Impact of Pollinator and Disperser Disruption on Plant Extinction". In: *Philosophical Transactions of the Royal Society B: Biological Sciences* 344.1307, pp. 83–90.

Bonfante, Paola and Andrea Genre (2010). "Mechanisms Underlying Beneficial Plant - Fungus Interactions in Mycorrhizal Symbiosis". In: *Nature Communications* 1.4, pp. 1–11.

Bronstein, Judith (2015). *Mutualism*. Oxford University Press, p. 320.

Caballero-Mellado, Jesus and Esperanza Martinez-Romero (1998). "Soil Fertilization Limits the Genetic Diversity of Rhizobium in Bean Nodules". In: p. 11.

Caporali, Fabio (2011). "Agroecology as a Transdisciplinary Science for a Sustainable Agriculture". In: *Biodiversity, Biofuels, Agroforestry and Conservation Agriculture*. Ed. by Eric Lichtfouse. Sustainable Agriculture Reviews. Dordrecht: Springer Netherlands, pp. 1–71.

Chamberlain, Scott A., Judith L. Bronstein, and Jennifer A. Rudgers (2014). "How Context Dependent Are Species Interactions?" In: *Ecology Letters* 17.7, pp. 881–890.

Cheynier, Véronique et al. (2013). "Plant Phenolics: Recent Advances on Their Biosynthesis, Genetics, Andecophysiology". In: *Plant Physiology and Biochemistry* 72, pp. 1–20.

Chisholm, Stephen T. et al. (2006). "Host-Microbe Interactions: Shaping the Evolution of the Plant Immune Response". In: *Cell* 124.4, pp. 803–814.

Clay, Keith and Paula X. Kover (1996). "The Red Queen Hypothesis and Plant/Pathogen Interactions". In: *Annual Review of Phytopathology* 34.1, pp. 29–50.

Coll, N. S., P. Epple, and J. L. Dangl (2011). "Programmed Cell Death in the Plant Immune System". In: *Cell Death and Differentiation* 18.8, pp. 1247–1256.

Dangl, Jeffery L. and Jonathan D. G. Jones (June 2001). "Plant Pathogens and Integrated Defence Responses to Infection". In: *Nature* 411.6839 (6839), pp. 826–833.

Day, Brad and Terry Graham (2007). "The Plant Host–Pathogen Interface". In: *Annals of the New York Academy of Sciences* 1113.1, pp. 123–134.

De de Bary, A. (1879). *Die Erscheinung der Symbiose: Vortrag*. De Gruyter.

Deising, H B, S Werner, and M Wernitz (Nov. 1, 2000). "The Role of Fungal Appressoria in Plant Infection". In: *Microbes and infection* 2.13, pp. 1631–1641.

De Wit, Mieke et al. (Sept. 4, 2012). "Plant Neighbor Detection through Touching Leaf Tips Precedes Phytochrome Signals". In: *Proceedings of the National Academy of Sciences* 109.36, pp. 14705–14710.

Dodds, Peter N. and John P. Rathjen (Aug. 2010). "Plant Immunity: Towards an Integrated View of Plant–Pathogen Interactions". In: *Nature Reviews Genetics* 11.8 (8), pp. 539–548.

Douglas, Angela E. (Aug. 10, 2021). *The Symbiotic Habit*. Princeton University Press. 216 pp.

Douglas, Susan E (1994). "Chloroplast Origins and Evolution". In: p. 28.

Dudareva, Natalia et al. (2013). "Biosynthesis, Function and Metabolic Engineering of Plant Volatile Organic Compounds". In: *New Phytologist* 198.1, pp. 16–32.

Edger, Patrick P. et al. (2015). "The Butterfly Plant Arms-Race Escalated by Gene and Genome Duplications". In: *Proceedings of the National Academy of Sciences* 112.27, pp. 8362–8366.

Ettinger, Ailene and Janneke HilleRisLambers (Sept. 2017). "Competition and Facilitation May Lead to Asymmetric Range Shift Dynamics with Climate Change". In: *Global Change Biology* 23.9, pp. 3921–3933.

Ferrol, Nuria, Concepción Azcón-Aguilar, and Jacob Pérez-Tienda (Mar. 2019). "Review: Arbuscular Mycorrhizas as Key Players in Sustainable Plant Phosphorus Acquisition: An Overview on the Mechanisms Involved". In: *Plant Science: An International Journal of Experimental Plant Biology* 280, pp. 441–447.

Field, Katie J. et al. (2019). "Functional Complementarity of Ancient Plant–Fungal Mutualisms: Contrasting Nitrogen, Phosphorus and Carbon Exchanges between Mucoromycotina and Glom-

eromycotina Fungal Symbionts of Liverworts". In: *New Phytologist* 223.2, pp. 908–921.

Franklin, Keara A., Victoria S. Larner, and Garry C. Whitelam (Sept. 1, 2004). "The Signal Transducing Photoreceptors of Plants". In: *International Journal of Developmental Biology* 49.5-6, pp. 653–664.

Fujita, Miki et al. (Aug. 1, 2006). "Crosstalk between Abiotic and Biotic Stress Responses: A Current View from the Points of Convergence in the Stress Signaling Networks". In: *Current Opinion in Plant Biology*. Biotic Interactions / Edited by Anne Osbourn and Sheng Yang He 9.4, pp. 436–442.

Gaba, Sabrina et al. (2014). "Managing Biotic Interactions for Ecological Intensification of Agroecosystems". In: *Frontiers in Ecology and Evolution* 2.

Garnett, T. et al. (July 5, 2013). "Sustainable Intensification in Agriculture: Premises and Policies". In: *Science* 341.6141, pp. 33–34.

Gilbert, Scott F., Thomas C. G. Bosch, and Cristina Ledón-Rettig (Oct. 2015). "Eco-Evo-Devo: Developmental Symbiosis and Developmental Plasticity as Evolutionary Agents". In: *Nature Reviews Genetics* 16.10 (10), pp. 611–622.

Glick, Bernard R. (2012). "Plant Growth-Promoting Bacteria: Mechanisms and Applications". In: *Scientifica* 2012, p. 963401.

Gomiero, Tiziano (Mar. 2016). "Soil Degradation, Land Scarcity and Food Security: Reviewing a Complex Challenge". In: *Sustainability* 8.3 (3), p. 281.

Grassini, Patricio, Kent M. Eskridge, and Kenneth G. Cassman (Dec. 17, 2013). "Distinguishing between Yield Advances and Yield Plateaus in Historical Crop Production Trends". In: *Nature Communications* 4.1 (1), p. 2918.

Gurevitch, Jessica and Dianna K. Padilla (2004). "Are Invasive Species a Major Cause of Extinctions?" In: *Trends in Ecology and Evolution* 19.9, pp. 470–474.

Hammerstein, Peter and Reinhard Selten (Jan. 1, 1994). "Chapter 28 Game Theory and Evolutionary Biology". In: *Handbook of Game Theory with Economic Applications*. Vol. 2. Elsevier, pp. 929–993.

Han, Guan-Zhu (2019). "Origin and Evolution of the Plant Immune System". In: *New Phytologist* 222.1, pp. 70–83.

Harris, Jeanne Marie, Katharina Pawlowski, and Ulrike Mathesius (Apr. 28, 2020). "Editorial: Evolution of Signaling in Plant Symbioses". In: *Frontiers in Plant Science* 11, p. 456.

Hasanuzzaman, Mirza, Susana Araújo, and Sarvajeet Singh Gill (2020). *The Plant Family Fabaceae*. Springer.

Haskell, Gordon (1949). "Some Evolutionary Problems Concerning the Bryophyta". In: *The Bryologist* 52.2, pp. 50–57.

He, Ping, Libo Shan, and Jen Sheen (2007). "Elicitation and Suppression of Microbe-Associated Molecular Pattern-Triggered Immunity in Plant–Microbe Interactions". In: *Cellular Microbiology* 9.6, pp. 1385–1396.

Hedges, S Blair et al. (Jan. 28, 2004). "A Molecular Timescale of Eukaryote Evolution and the Rise of Complex Multicellular Life". In: *BMC Evolutionary Biology* 4, p. 2.

Heil, Martin and Walter G. Land (2014). "Danger Signals – Damaged-Self Recognition across the Tree of Life". In: *Frontiers in Plant Science* 5.

Hoeksema, Jason D. and Emilio M. Bruna (Nov. 1, 2000). "Pursuing the Big Questions about Interspecific Mutualism: A Review of Theoretical Approaches". In: *Oecologia* 125.3, pp. 321–330.

Howard, R J et al. (Dec. 15, 1991). "Penetration of Hard Substrates by a Fungus Employing Enormous Turgor Pressures." In: *Proceedings of the National Academy of Sciences of the United States of America* 88.24, pp. 11281–11284.

Hoysted, Grace A. et al. (Oct. 1, 2019). "Mucoromycotina Fine Root Endophyte Fungi Form Nutritional Mutualisms with Vascular Plants1 [CC-BY]". in: *Plant Physiology* 181.2, pp. 565–577.

Indrasumunar, Arief et al. (Feb. 2010). "Inactivation of Duplicated Nod Factor Receptor 5 (NFR5) Genes in Recessive Loss-of-Function Non-Nodulation Mutants of Allotetraploid Soybean (Glycine Max L. Merr.)" In: *Plant & Cell Physiology* 51.2, pp. 201–214.

Jones, Melanie D and Sally E Smith (Aug. 1, 2004). "Exploring Functional Definitions of Mycorrhizas: Are Mycorrhizas Always Mutualisms?" In: *Canadian Journal of Botany* 82.8, pp. 1089–1109.

Kaarlejärvi, Elina, Anu Eskelinen, and Johan Olofsson (Sept. 4, 2017). "Herbivores Rescue Diversity in Warming Tundra by Modulating Trait-Dependent Species Losses and Gains". In: *Nature Communications* 8.1, p. 419.

Karban, Richard (2008). "Plant Behaviour and Communication". In: *Ecology Letters* 11.7, pp. 727–739.

Kiers, E. Toby, Mark G Hutton, and R. Ford Denison (Dec. 22, 2007). "Human Selection and the Relaxation of Legume Defences against Ineffective Rhizobia". In: *Proceedings of the Royal*

Society B: Biological Sciences 274.1629, pp. 3119–3126.

Kong, Chui-Hua et al. (Sept. 24, 2018). "Plant Neighbor Detection and Allelochemical Response Are Driven by Root-Secreted Signaling Chemicals". In: *Nature Communications* 9.1 (1), p. 3867.

Kumar, A., M. Memo, and A. Mastinu (2020). "Plant Behaviour: An Evolutionary Response to the Environment?" In: *Plant Biology* 22.6, pp. 961–970.

Langerhans, R. B. (Jan. 1, 2008). "Coevolution". In: *Encyclopedia of Ecology*. Ed. by Sven Erik Jørgensen and Brian D. Fath. Oxford: Academic Press, pp. 644–648.

Legris, Martina et al. (2017). "Perception and Signalling of Light and Temperature Cues in Plants". In: *The Plant Journal* 90.4, pp. 683–697.

Li, Dapeng et al. (2020). "Information Theory Tests Critical Predictions of Plant Defense Theory for Specialized Metabolism". In: *Science Advances* 6.24, eaaz0381.

Lopatkin, Allison J. and James J. Collins (Sept. 2020). "Predictive Biology: Modelling, Understanding and Harnessing Microbial Complexity". In: *Nature Reviews Microbiology* 18.9 (9), pp. 507–520.

López-García, Purificación, Laura Eme, and David Moreira (Dec. 7, 2017). "Symbiosis in Eukaryotic Evolution". In: *Journal of Theoretical Biology*. The Origin of Mitosing Cells: 50th Anniversary of a Classic Paper by Lynn Sagan (Margulis) 434, pp. 20–33.

Maffei, Massimo E., Gen-Ichiro Arimura, and Axel Mithöfer (Oct. 2012). "Natural Elicitors, Effectors and Modulators of Plant Responses". In: *Natural Product Reports* 29.11, pp. 1288–1303.

Maffei, Massimo E., Axel Mithöfer, and Wilhelm Boland (July 2007). "Before Gene Expression: Early Events in Plant–Insect Interaction". In: *Trends in Plant Science* 12.7, pp. 310–316.

Martín-Robles, Nieves et al. (2018). "Impacts of Domestication on the Arbuscular Mycorrhizal Symbiosis of 27 Crop Species". In: *New Phytologist* 218.1, pp. 322–334.

McBratney, Alex, Damien J. Field, and Andrea Koch (Jan. 1, 2014). "The Dimensions of Soil Security". In: *Geoderma* 213, pp. 203–213.

McFadden, Geoffrey I. (Jan. 1, 2001). "Chloroplast Origin and Integration1". In: *Plant Physiology* 125.1, pp. 50–53.

McNamara, John M. (Nov. 6, 2013). "Towards a Richer Evolutionary Game Theory". In: *Journal of The Royal Society Interface* 10.88, p. 20130544.

McNickle, Gordon G. and Ray Dybzinski (2013). "Game Theory and Plant Ecology". In: *Ecology Letters* 16.4, pp. 545–555.

Meier, Markus et al. (Sept. 2020). "Auxin-Mediated Root Branching Is Determined by the Form of Available Nitrogen". In: *Nature Plants* 6.9 (9), pp. 1136–1145.

Mello, O and Marcio Silva-Filho (Aug. 1, 2002). "Plant-Insect Interactions: An Evolutionary Arms Race between Two Distinct Defense Mechanisms". In: *Brazilian Journal of Plant Physiology* 14.

Mereschkowsky, Konstantin (1905). "Annotated English Translation of Mereschkowsky's 1905 Paper 'Über Natur Und Ursprung Der Chromatophoren Im Pflanzenreiche'". In: *European Journal of Phycology* 34.3, pp. 287–295.

Mylona, P, K Pawlowski, and T Bisseling (July 1995). "Symbiotic Nitrogen Fixation." In: *The Plant Cell* 7.7, pp. 869–885.

Nelson, Matthew S. and Michael J. Sadowsky (2015). "Secretion Systems and Signal Exchange between Nitrogen-Fixing Rhizobia and Legumes". In: *Frontiers in Plant Science* 6.

Nelsosn, David and Michael Cox (2012). *Lehninger: Principles of Biochemsitry*.

Newton, Adrian C. et al. (2010). "Pathogenesis, Parasitism and Mutualism in the Trophic Space of Microbe-Plant Interactions". In: *Trends in Microbiology* 18.8, pp. 365–373.

Nishad, Resna et al. (July 3, 2020). "Modulation of Plant Defense System in Response to Microbial Interactions". In: *Frontiers in Microbiology* 11, p. 1298.

Oldroyd, Giles E. D. (Apr. 2013). "Speak, Friend, and Enter: Signalling Systems That Promote Beneficial Symbiotic Associations in Plants". In: *Nature Reviews Microbiology* 11.4 (4), pp. 252–263.

Orozco-Cardenas, Martha and Clarence A. Ryan (May 1999). "Hydrogen Peroxide Is Generated Systemically in Plant Leaves by Wounding and Systemin via the Octadecanoid Pathway". In: *Proceedings of the National Academy of Sciences* 96.11, pp. 6553–6557.

Osakabe, Yuriko et al. (Jan. 1, 2013). "Sensing the Environment: Key Roles of Membrane-Localized Kinases in Plant Perception and Response to Abiotic Stress". In: *Journal of Experimental Botany* 64.2, pp. 445–458.

Ossler, J. N., C. A. Zielinski, and K. D. Heath (2015). "Tripartite Mutualism: Facilitation or Trade-Offs between Rhizobial and Mycorrhizal Symbionts of Legume Hosts". In: *American Journal of Botany* 102.8, pp. 1332–1341.

Otte, Daniel (Nov. 1974). "Effects and Functions in the Evolution of Signaling Systems". In: *Annual Review of Ecology and Systematics* 5.1, pp. 385–417.

Paini, Dean R. et al. (July 5, 2016). "Global Threat to Agriculture from Invasive Species". In: *Proceedings of the National Academy of Sciences of the United States of America* 113.27, pp. 7575–7579.

Parniske, Martin (Aug. 1, 2000). "Intracellular Accommodation of Microbes by Plants: A Common Developmental Program for Symbiosis and Disease?" In: *Current Opinion in Plant Biology* 3.4, pp. 320–328.

— (2008). "Arbuscular Mycorrhizae: The Mother of Plant Root Endosymbioses". In: *Nature reviews. Microbiology* 6.10, pp. 763+.

Peter, J., W. Young, and Kaisa E. Haukka (1996). "Diversity and Phylogeny of Rhizobia". In: *New Phytologist* 133.1, pp. 87–94.

Peters, Kristian et al. (May 6, 2018). "Current Challenges in Plant Eco-Metabolomics". In: *International Journal of Molecular Sciences* 19.5, E1385.

Pierik, Ronald, Carlos L. Ballaré, and Marcel Dicke (2014). "Ecology of Plant Volatiles: Taking a Plant Community Perspective". In: *Plant, Cell & Environment* 37.8, pp. 1845–1853.

Pierik, Ronald, Liesje Mommer, and Laurentius ACJ Voesenek (2013). "Molecular Mechanisms of Plant Competition: Neighbour Detection and Response Strategies". In: *Functional Ecology* 27.4, pp. 841–853.

Plett, Jonathan M. and Francis M. Martin (2018). "Know Your Enemy, Embrace Your Friend: Using Omics to Understand How Plants Respond Differently to Pathogenic and Mutualistic Microorganisms". In: *The Plant Journal* 93.4, pp. 729–746.

Poisot, Timothée et al. (2012). "The Dissimilarity of Species Interaction Networks". In: *Ecology Letters* 15.12, pp. 1353–1361.

Poland, Therese M and Deborah G McCullough (2006). "Emerald Ash Borer: Invasion of the Urban Forest and the Threat to North America's Ash Resource". In: *Journal of Forestry*, p. 7.

Porter, Stephanie S. and Joel L. Sachs (May 1, 2020). "Agriculture and the Disruption of Plant–Microbial Symbiosis". In: *Trends in Ecology & Evolution* 35.5, pp. 426–439.

Primieri, Silmar et al. (2022). "Perennial, but Not Annual Legumes Synergistically Benefit from Infection with Arbuscular Mycorrhizal Fungi and Rhizobia: A Meta-Analysis". In: *New Phytologist* 233.1, pp. 505–514.

Raina, Jean-Baptiste et al. (Feb. 22, 2018). "Symbiosis in the Microbial World: From Ecology to Genome Evolution". In: *Biology Open* 7.2, bio032524.

Rajendran, SenthilKumar et al. (Apr. 2014). "Differential Activation of Sporamin Expression in Response to Abiotic Mechanical Wounding and Biotic Herbivore Attack in the Sweet Potato". In: *BMC Plant Biology* 14.1, p. 112.

Ray, Deepak K. et al. (2012). "Recent Patterns of Crop Yield Growth and Stagnation". In: *Nature Communications* 3, p. 1293.

Raymond, Jason et al. (Mar. 1, 2004). "The Natural History of Nitrogen Fixation". In: *Molecular Biology and Evolution* 21.3, pp. 541–554.

Ryan, Megan H. and James H. Graham (2018). "Little Evidence That Farmers Should Consider Abundance or Diversity of Arbuscular Mycorrhizal Fungi When Managing Crops". In: *New Phytologist* 220.4, pp. 1092–1107.

Scott-Phillips, Thomas C. et al. (May 2012). "How Do Communication Systems Emerge?" In: *Proceedings of the Royal Society B: Biological Sciences* 279.1735, pp. 1943–1949.

Seebens, Hanno et al. (2021). "Projecting the Continental Accumulation of Alien Species through to 2050". In: *Global Change Biology* 27.5, pp. 970–982.

Shennan, Carol (Feb. 27, 2008). "Biotic Interactions, Ecological Knowledge and Agriculture". In: *Philosophical Transactions of the Royal Society B: Biological Sciences* 363.1492, pp. 717–739.

Shiu, Shin-Han and Anthony B. Bleecker (Dec. 18, 2001). "Plant Receptor-Like Kinase Gene Family: Diversity, Function, and Signaling". In: *Science's STKE* 2001.113, re22–re22.

Smith, John Maynard (Oct. 21, 1982). *Evolution and the Theory of Games*. Cambridge University Press. 244 pp.

Smith, Sally E. and David J. Read (July 26, 2010). *Mycorrhizal Symbiosis*. Academic Press. 815 pp.

Smith, Sally E. and F. Andrew Smith (2011). "Roles of Arbuscular Mycorrhizas in Plant Nutrition and Growth: New Paradigms from Cellular to Ecosystem Scales". In: *Annual Review of Plant Biology* 62, pp. 227–250.

Soran, Maria-Loredana et al. (Sept. 15, 2014). "Influence of Microwave Frequency Electromagnetic Radiation on Terpene Emission and Content in Aromatic Plants". In: *Journal of Plant Physiology* 171.15, pp. 1436–1443.

Srivastava, Vivek, Valentine Lafond, and Verena Griess (Apr. 24, 2019). "Species Distribution Models (SDM): Applications, Benefits and Challenges in Invasive Species Management". In:

CAB Reviews Perspectives in Agriculture Veterinary Science Nutrition and Natural Resources 14, pp. 1–13.

Stribley, D. P., P. B. Tinker, and J. H. Rayner (1980). "Relation of Internal Phosphorus Concentration and Plant Weight in Plants Infected by Vesicular-Arbuscular Mycorrhizas". In: *New Phytologist* 86.3, pp. 261–266.

Suzaki, Takuya, Emiko Yoro, and Masayoshi Kawaguchi (Jan. 1, 2015). "Chapter Three - Leguminous Plants: Inventors of Root Nodules to Accommodate Symbiotic Bacteria". In: *International Review of Cell and Molecular Biology*. Ed. by Kwang W. Jeon. Vol. 316. Academic Press, pp. 111–158.

Tafforeau, Marc et al. (2004). "Plant Sensitivity to Low Intensity 105 GHz Electromagnetic Radiation". In: *Bioelectromagnetics* 25.6, pp. 403–407.

Taylor, Jane E., Paul E. Hatcher, and Nigel D. Paul (Jan. 1, 2004). "Crosstalk between Plant Responses to Pathogens and Herbivores: A View from the Outside In". In: *Journal of Experimental Botany* 55.395, pp. 159–168.

terHorst, Casey P., Jay T. Lennon, and Jennifer A. Lau (June 22, 2014). "The Relative Importance of Rapid Evolution for Plant-Microbe Interactions Depends on Ecological Context". In: *Proceedings of the Royal Society B: Biological Sciences* 281.1785, p. 20140028.

Theoharides, K a and J S Dukes (2007). "Plant Invasion across Space and Time: Factors Affecting Nonindigenous Species Success during Four Stage of Invasion". In: *New Phytologist* 176.2, pp. 256–273.

Thirkell, Thomas J. et al. (2017). "Are Mycorrhizal Fungi Our Sustainable Saviours? Considerations for Achieving Food Security". In: *Journal of Ecology* 105.4, pp. 921–929.

Thrall, Peter H. et al. (2011). "Evolution in Agriculture: The Application of Evolutionary Approaches to the Management of Biotic Interactions in Agro-Ecosystems". In: *Evolutionary Applications* 4.2, pp. 200–215.

Tian, C. et al. (2010). "Regulation of the Nitrogen Transfer Pathway in the Arbuscular Mycorrhizal Symbiosis: Gene Characterization and the Coordination of Expression with Nitrogen Flux". In: *Plant Physiology* 153.3, pp. 1175–1187.

Tilman, David (1982). *Resource Competition and Community Structure. (MPB-17), Volume 17*. Vol. 107. Monographs in Population Biology.

Tisserant, E. et al. (2013). "Genome of an Arbuscular Mycorrhizal Fungus Provides Insight into the Oldest Plant Symbiosis". In: *Proceedings of the National Academy of Sciences* 110.50, pp. 20117–20122.

Tóth, Katalin and Gary Stacey (2015). "Does Plant Immunity Play a Critical Role during Initiation of the Legume-Rhizobium Symbiosis?" In: *Frontiers in Plant Science* 6.

Trewavas, Anthony (Aug. 21, 2014). *Plant Behaviour and Intelligence*. OUP Oxford. 305 pp.

Tsai, Allen Yi-Lun, Morihiro Oota, and Shinichiro Sawa (2020). "Chemotactic Host-Finding Strategies of Plant Endoparasites and Endophytes". In: *Frontiers in Plant Science* 11.

Turrini, A. et al. (Jan. 1, 2016). "Large Variation in Mycorrhizal Colonization among Wild Accessions, Cultivars, and Inbreds of Sunflower (Helianthus Annuus L.)". In: *Euphytica* 207.2, pp. 331–342.

Udvardi, Michael and Philip S. Poole (Apr. 29, 2013). "Transport and Metabolism in Legume-Rhizobia Symbioses". In: *Annual Review of Plant Biology* 64.1, pp. 781–805.

Urban, M. C. et al. (Sept. 9, 2016). "Improving the Forecast for Biodiversity under Climate Change". In: *Science* 353.6304, aad8466.

Van Valen, Leigh (July 1973). "A New Evolutionary Law". In: *Evol. Theory* 1, pp. 1–30.

Vandvik, Vigdis et al. (Sept. 15, 2020). "Biotic Rescaling Reveals Importance of Species Interactions for Variation in Biodiversity Responses to Climate Change". In: *Proceedings of the National Academy of Sciences* 117.37, pp. 22858–22865.

Vavra, Marty, Catherine G. Parks, and Michael J. Wisdom (July 16, 2007). "Biodiversity, Exotic Plant Species, and Herbivory: The Good, the Bad, and the Ungulate". In: *Forest Ecology and Management*. Biodiversity Management in Pacific Northwest Forests: Strategies and Opportunities. 246.1, pp. 66–72.

Villate, Aitor et al. (Feb. 1, 2021). "Review: Metabolomics as a Prediction Tool for Plants Performance under Environmental Stress". In: *Plant Science* 303, p. 110789.

Vitousek, Peter M et al. (2010). "Terrestrial Phosphorus Limitation : Mechanisms , Implications , and Nitrogen – Phosphorus Interactions". In: *Ecological Applications,* 20.1, pp. 5–15.

Wall, Diana H. and John C. Moore (Feb. 1, 1999). "Interactions Underground: Soil Biodiversity, Mutualism, and Ecosystem Processes". In: *BioScience* 49.2, pp. 109–117.

Wang, Ertao et al. (Dec. 4, 2012). "A Common Signaling Process That Promotes Mycorrhizal and Oomycete Colonization of Plants". In: *Current Biology* 22.23, pp. 2242–2246.

Wang, Guihua et al. (2016). "Allocation of Nitrogen and Carbon Is Regulated by Nodulation and Mycorrhizal Networks in Soybean/Maize Intercropping System". In: *Frontiers in Plant Science* 7 (December), pp. 1–11.

War, Abdul Rashid, Michael Gabriel Paulraj, Tariq Ahmad, et al. (Oct. 2012). "Mechanisms of Plant Defense against Insect Herbivores". In: *Plant Signaling & Behavior* 7.10, pp. 1306–1320.

War, Abdul Rashid, Michael Gabriel Paulraj, Mohd Yousf War, et al. (Nov. 2011). "Herbivore- and Elicitor- Induced Resistance in Groundnut to Asian Armyworm, Spodoptera Litura (Fab.) (Lepidoptera: Noctuidae)". In: *Plant Signaling & Behavior* 6.11, pp. 1769–1777.

Weese, Dylan (2015). "Long-term Nitrogen Addition Causes the Evolution of Less-cooperative Mutualists". In: *Evolution* 69.3, pp. 631–642.

Weibull, Jörgen W. (1997). *Evolutionary Game Theory*. MIT Press. 292 pp.

Werner, Gijsbert D. A. et al. (May 15, 2018). "Symbiont Switching and Alternative Resource Acquisition Strategies Drive Mutualism Breakdown". In: *Proceedings of the National Academy of Sciences* 115.20, pp. 5229–5234.

Wisz, Mary Susanne et al. (2013). "The Role of Biotic Interactions in Shaping Distributions and Realised Assemblages of Species: Implications for Species Distribution Modelling". In: *Biological Reviews* 88.1, pp. 15–30.

Xing, Xiaoke et al. (Mar. 22, 2012). "Mutualism Breakdown in Breadfruit Domestication". In: *Proceedings of the Royal Society B: Biological Sciences* 279.1731, pp. 1122–1130.

Yates, Katherine L. et al. (Oct. 1, 2018). "Outstanding Challenges in the Transferability of Ecological Models". In: *Trends in Ecology & Evolution* 33.10, pp. 790–802.

Zebelo, Simon A. and Massimo E. Maffei (Feb. 2015). "Role of Early Signalling Events in Plant–Insect Interactions". In: *Journal of Experimental Botany* 66.2, pp. 435–448.

Zhang, Jie and Jian-Min Zhou (Sept. 1, 2010). "Plant Immunity Triggered by Microbial Molecular Signatures". In: *Molecular Plant* 3.5, pp. 783–793.

Zhang, Shujuan et al. (2019). "Arbuscular Mycorrhizal Fungi Increase Grain Yields: A Meta-Analysis". In: *New Phytologist* 222.1, pp. 543–555.

Zipfel, Cyril and Silke Robatzek (Oct. 1, 2010). "Pathogen-Associated Molecular Pattern-Triggered Immunity: Veni, Vidi. . . ?" In: *Plant Physiology* 154.2, pp. 551–554.

Zu, Pengjuan et al. (June 19, 2020). "Information Arms Race Explains Plant-Herbivore Chemical Communication in Ecological Communities". In: *Science* 368.6497, pp. 1377–1381.

CHAPTER 2

COERCION IN THE EVOLUTION OF PLANT-MICROBE COMMUNICATION: A PERSPECTIVE

2.1 Abstract

Plants and microbes are dependent on chemical signals as a means of interkingdom communication. There are two predicted paths for the evolution of these signals. Ritualization is the oft-assumed pathway for the evolution of plant–microbe communication systems. In this process, chemical signals, which benefit both receiver and sender, evolve from chemical cues, which benefit only the receiver. However, plant–microbe signaling may evolve from coercive interactions as well, a process known as sensory manipulation. Here, we aim to highlight the prevalence of coercive interactions and discuss sensory manipulation in the context of plant–microbe interactions. We present two examples of stabilized coercion: microbial coercion of plants via the release of phytohormones and plant coercion of microbes via manipulation of quorum-sensing compounds. Furthermore, we provide an evolutionary framework for the emergence of signaling from coercive plant–microbe interactions through the process of sensory manipulation. We hope that researchers will recognize the relevance of coercive interactions in plant–microbe systems and consider sensory manipulation as a plausible evolutionary trajectory for the emergence of plant–microbe signaling.

2.2 Introduction

Communication systems in nature provide a foundation for both intra- and interspecific interactions (T. C. 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 2.1; note that the general communication theory we modify for specificity to plant-microbe interactions was originally presented by (T. C. Scott-Phillips 2008) and (Thomas C. 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 2.1). A recent review by (van't Padje, Whiteside, and Kiers 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 (H. N. Rasmussen et al. 2015). The prevalence of coercion, a formal definition of which is provided in Table 2.1, 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 2.1). 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 manipulationCoercion in the Evolution of Plant-Microbe Communication." (T. C. Scott-Phillips 2008; Thomas C. Scott-Phillips et al. 2012).

2.3 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.

Table 2.1: List of definitions specific to plant-microbe interactions dependent on chemical information*

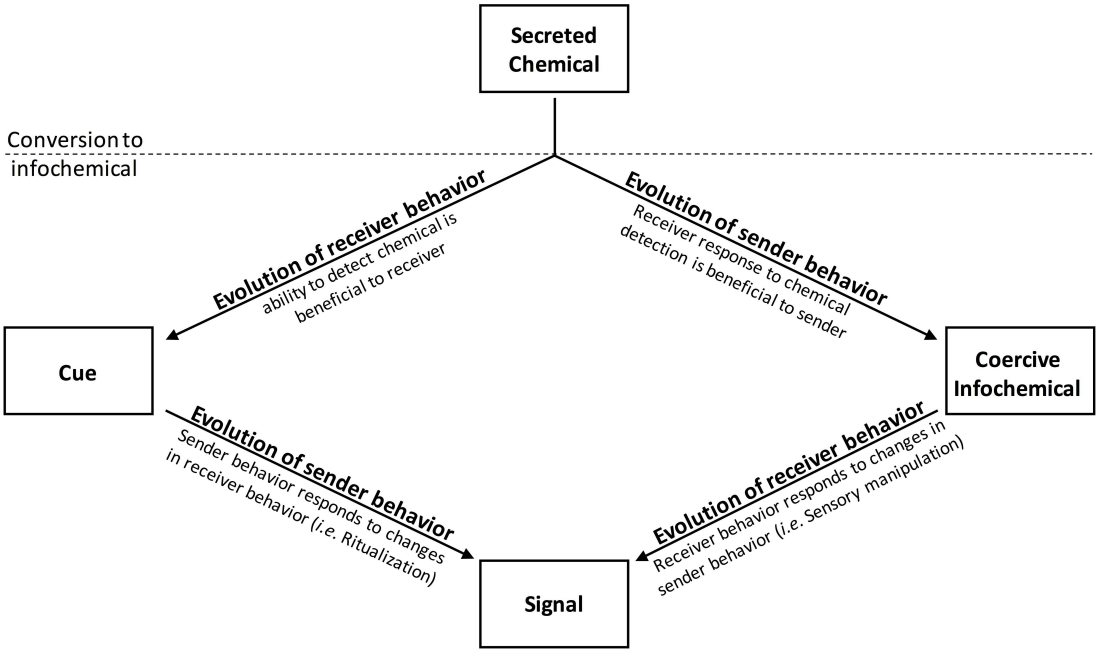
Term	Definition
Information	a reduction in uncertainty
Infochemical	a biologically-derived molecule that reduces uncertainty
Sender	organism that releases an infochemical
Receiver	organism that detects an infochemical
Cue	any infochemical that (i) affects the behavior of other organisms; (ii) which is 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).

2.3.1 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

Figure 2.1: 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, Whiteside, and Kiers 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.

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, Robinson, and Bauer 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, Rogozhin, and Deryabin 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, Robinson, and Bauer 2000), and have since been shown in garlic (*Allium sativum*) (T. B. 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, Tarzaban, and Khodaygan 2014) among others. Enzymatic degradation of autoinducers in the extracellular environment, a trait common to many bacterial taxa ((H. N. 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, Hare, and Friesen 2017)). Future work should seek explore the benefits of this form of coercion in natural plants in a field setting.

2.3.2 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, van Overbeek, and van Elsas 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, Cherdyntseva, and Netrusov 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, Satoh, and Minamisawa 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.

2.4 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. (Thomas C. 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, Whiteside, and Kiers 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 2.1.

2.4.1 Stabilized coercion

(Thomas C. 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.

2.4.2 Elimination of coercion

If the fitness of the receiver is negatively impacted by coercion, then (Thomas C. 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.

2.4.3 Sensory manipulation

Sensory manipulation is an evolutionary process by which coercion becomes communication (Figure 2.1). (Thomas C. 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, Tsavkelova, and Selitskaia 2014). 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 (Thomas C. Scott-Phillips et al. 2012) posit that ritualization, the evolution of signals from cues (Figure 2.1), 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.

2.5 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. Furthermore, 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 in-

herent 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.

Contribution Statement

This work was completed under the mentorship of JS Norman. I initiated the project and designed the argument, initially for the purposes of facilitating a group discussion with ML Friesen's research group. JS Norman encouraged me to transform the ideas into a publishable manuscript, which I did with his input and assistance. I addressed reviewer comments and handled the submission process for the manuscript. ML Friesen was my PhD adviser at the time and provided input on the manuscript. I am the first and corresponding author.

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BIBLIOGRAPHY

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Annapoorani, Angusamy et al. (Sept. 2012). "Computational Discovery of Putative Quorum Sensing Inhibitors against LasR and RhlR Receptor Proteins of Pseudomonas Aeruginosa". In: *Journal of Computer-Aided Molecular Design* 26.9, pp. 1067–1077.

Baker, Barbara et al. (1997). "Signaling in Plant-Microbe Interactions". In: *Science* 276.5313, pp. 726–733.

Barto, E. Kathryn et al. (Nov. 2012). "Fungal Superhighways: Do Common Mycorrhizal Networks Enhance below Ground Communication?" In: *Trends in Plant Science* 17.11, pp. 633–637.

Bassler, B. L. (Dec. 1999). "How Bacteria Talk to Each Other: Regulation of Gene Expression by Quorum Sensing". In: *Current Opinion in Microbiology* 2.6, pp. 582–587.

Bleecker, A. B. and H. Kende (2000). "Ethylene: A Gaseous Signal Molecule in Plants". In: *Annual Review of Cell and Developmental Biology* 16, pp. 1–18.

Blinkov, E. A., E. A. Tsavkelova, and O. V. Selitskaia (2014). "[Auxin production by Klebsiella planticola strain TSKhA-91 and Its Effect of development of cucumber (Cucumis sativus L.) Seeds]". In: *Mikrobiologiia* 83.5, pp. 543–551.

Chanclud, Emilie and Jean-Benoit Morel (2016). "Plant Hormones: A Fungal Point of View". In: *Molecular Plant Pathology* 17.8, pp. 1289–1297.

Chernin, L. S. et al. (Sept. 1998). "Chitinolytic Activity in Chromobacterium Violaceum: Substrate Analysis and Regulation by Quorum Sensing". In: *Journal of Bacteriology* 180.17, pp. 4435–4441.

Corral-Lugo, Andrés et al. (Jan. 2016). "Rosmarinic Acid Is a Homoserine Lactone Mimic Produced by Plants That Activates a Bacterial Quorum-Sensing Regulator". In: *Science Signaling* 9.409, ra1.

Danhorn, Thomas and Clay Fuqua (2007). "Biofilm Formation by Plant-Associated Bacteria". In: *Annual Review of Microbiology* 61.1, pp. 401–422.

Degrassi, Giuliano et al. (2007). "Oryza Sativa Rice Plants Contain Molecules That Activate Different Quorum-Sensing N-acyl Homoserine Lactone Biosensors and Are Sensitive to the Specific AiiA Lactonase". In: *FEMS Microbiology Letters* 269.2, pp. 213–220.

Desjardins, A.E. et al. (Apr. 2000). "Occurrence of Fusarium Species and Mycotoxins in Nepalese Maize and Wheat and the Effect of Traditional Processing Methods on Mycotoxin Levels". In: *Journal of agricultural and food chemistry* 48.4, pp. 1377–1383.

Dong, Y. H. et al. (June 2001). "Quenching Quorum-Sensing-Dependent Bacterial Infection by an N-acyl Homoserine Lactonase". In: *Nature* 411.6839, pp. 813–817.

Engebrecht, J and M Silverman (Dec. 1987). "Nucleotide Sequence of the Regulatory Locus Controlling Expression of Bacterial Genes for Bioluminescence." In: *Nucleic Acids Research* 15.24, pp. 10455–10467.

Gao, Mengsheng et al. (Sept. 2003). "Production of Substances by Medicago Truncatula That Affect Bacterial Quorum Sensing". In: *Molecular Plant-Microbe Interactions®* 16.9, pp. 827–834.

Glick, Bernard R. (2020). "Modulating Phytohormone Levels". In: *Beneficial Plant-Bacterial Interactions*. Ed. by Bernard R. Glick. Cham: Springer International Publishing, pp. 139–180.

Grandclément, Catherine et al. (Jan. 2016). "Quorum Quenching: Role in Nature and Applied Developments". In: *FEMS microbiology reviews* 40.1, pp. 86–116.

Hardoim, Pablo R., Leo S. van Overbeek, and Jan Dirk van Elsas (Oct. 2008). "Properties of Bacterial Endophytes and Their Proposed Role in Plant Growth". In: *Trends in Microbiology* 16.10, pp. 463–471.

Karnwal, Arun (Mar. 2009). "Production of Indole Acetic Acid by Fluorescent Pseudomonas in the Presence of L-tryptophan and Rice Root Exudates". In: *JOURNAL OF PLANT PATHOLOGY* 91.

Köhler, T. et al. (Nov. 2000). "Swarming of Pseudomonas Aeruginosa Is Dependent on Cell-to-Cell Signaling and Requires Flagella and Pili". In: *Journal of Bacteriology* 182.21, pp. 5990–5996.

Lambrecht, Mark et al. (July 2000). "Indole-3-Acetic Acid: A Reciprocal Signalling Molecule in Bacteria–Plant Interactions". In: *Trends in Microbiology* 8.7, pp. 298–300.

Mäe, Andres et al. (Sept. 2001). "Transgenic Plants Producing the Bacterial Pheromone N-Acyl-Homoserine Lactone Exhibit Enhanced Resistance to the Bacterial Phytopathogen Erwinia Carotovora". In: *Molecular Plant-Microbe Interactions®* 14.9, pp. 1035–1042.

Mahmoudi, E., S. Tarzaban, and P. Khodaygan (July 2014). "DUAL BEHAVIOUR OF PLANTS AGAINST BACTERIAL QUORUM SENSING: INHIBITION OR EXCITATION". in: *Journal of Plant Pathology* 96.2, pp. 295–301.

McClean, Kay H. et al. (1997). "Quorum Sensing and Chromobacterium Violaceum: Exploitation of Violacein Production and Inhibition for the Detection of N-acylhomoserine Lactones". In: *Microbiology (Reading, England)* 143.12, pp. 3703–3711.

Morris, R O (1986). "Genes Specifying Auxin and Cytokinin Biosynthesis in Phytopathogens". In: *Annual Reviews Plant Physiology*, p. 30.

Norman, Jeffrey S., Jake R. Hare, and Maren L. Friesen (2017). "Comment: Isolation and Screening of Bacteria for Their Diazotrophic Potential and Their Influence on Growth Promotion of Maize Seedlings in Greenhouses". In: *Frontiers in Plant Science* 8, p. 212.

Palmer, Andrew G. et al. (Aug. 2014). "Plant Responses to Bacterial N-Acyl l-Homoserine Lactones Are Dependent on Enzymatic Degradation to l-Homoserine". In: *ACS Chemical Biology* 9.8, pp. 1834–1845.

Penmetsa, R. Varma and Douglas R. Cook (Jan. 1997). "A Legume Ethylene-Insensitive Mutant Hyperinfected by Its Rhizobial Symbiont". In: *Science (New York, N.Y.)* 275.5299, pp. 527–530.

Pérez-Montaño, Francisco et al. (Sept. 2013). "Rice and Bean AHL-mimic Quorum-Sensing Signals Specifically Interfere with the Capacity to Form Biofilms by Plant-Associated Bacteria". In: *Research in Microbiology* 164.7, pp. 749–760.

Rasmussen, Hanne N. et al. (Sept. 2015). "Germination and Seedling Establishment in Orchids: A Complex of Requirements". In: *Annals of Botany* 116.3, pp. 391–402.

Rasmussen, Thomas B. and MichaelYR 2006 Givskov (2006). "Quorum Sensing Inhibitors: A Bargain of Effects". In: *Microbiology (Reading, England)* 152.4, pp. 895–904.

Ratcliff, William C. and R. Ford Denison (July 2009). "Rhizobitoxine Producers Gain More Poly-3-Hydroxybutyrate in Symbiosis than Do Competing Rhizobia, but Reduce Plant Growth". In: *The ISME Journal* 3.7, pp. 870–872.

Redfield, Rosemary J. (Aug. 2002). "Is Quorum Sensing a Side Effect of Diffusion Sensing?" In: *Trends in Microbiology* 10.8, pp. 365–370.

Scott-Phillips, T. C. (2008). "Defining Biological Communication". In: *Journal of Evolutionary Biology* 21.2, pp. 387–395.

Scott-Phillips, Thomas C. et al. (May 2012). "How Do Communication Systems Emerge?" In: *Proceedings of the Royal Society B: Biological Sciences* 279.1735, pp. 1943–1949.

Sun, Tai-ping (2010). "Gibberellin Signal Transduction in Stem Elongation & Leaf Growth". In: *Plant Hormones: Biosynthesis, Signal Transduction, Action!* Ed. by Peter J. Davies. Dordrecht: Springer Netherlands, pp. 308–328.

Ta, Chieu Anh Kim and John Thor Arnason (Jan. 2016). "Mini Review of Phytochemicals and Plant Taxa with Activity as Microbial Biofilm and Quorum Sensing Inhibitors". In: *Molecules (Basel, Switzerland)* 21.1, p. 29.

Teplitski, Max, Jayne B. Robinson, and Wolfgang D. Bauer (June 2000). "Plants Secrete Substances That Mimic Bacterial N-Acyl Homoserine Lactone Signal Activities and Affect Pop-

ulation Density-Dependent Behaviors in Associated Bacteria". In: *Molecular Plant-Microbe Interactions®* 13.6, pp. 637–648.

Tolmacheva, Anna A., Eugene A. Rogozhin, and Dmitry G. Deryabin (May 2014). "Antibacterial and Quorum Sensing Regulatory Activities of Some Traditional Eastern-European Medicinal Plants". In: *Acta Pharmaceutica* 64.2, pp. 173–186.

Tsavkelova, E. A., T. A. Cherdyntseva, and A. I. Netrusov (Jan. 2005). "Auxin Production by Bacteria Associated with Orchid Roots". In: *Microbiology (Reading, England)* 74.1, pp. 46–53.

Van't Padje, Anouk, Matthew D Whiteside, and E Toby Kiers (Aug. 2016). "Signals and Cues in the Evolution of Plant–Microbe Communication". In: *Current Opinion in Plant Biology*. Biotic Interactions 32, pp. 47–52.

Yasuta, Tsuyoshi, Shigeru Satoh, and Kiwamu Minamisawa (Feb. 1999). "New Assay for Rhizobitoxine Based on Inhibition of 1-Aminocyclopropane-1-Carboxylate Synthase". In: *Applied and Environmental Microbiology* 65.2, pp. 849–852.

Yuhashi, Ken-Ichi et al. (June 2000). "Rhizobitoxine Production by Bradyrhizobium Elkanii Enhances Nodulation and Competitiveness onMacroptilium Atropurpureum". In: *Applied and Environmental Microbiology*.

CHAPTER 3

A HIGH-THROUGH METHOD OF MULTIPLE PLANT DEFENSIVE COMPOUNDS IN MINIMIZED SAMPLE MASS

3.1 Abstract

PREMISE OF THE STUDY: Current methods for quantifying herbivore-induced alterations in plant biochemistry are often unusable by researchers due to practical constraints. 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 less than 10 mg. Our protocol takes two people 6 h 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.

3.2 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, Arimura, and Mithöfer 2012; A. R. War, Paulraj, Ahmad, 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 (A. R. War, Paulraj, Ahmad, 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; A. R. War, Paulraj, Ahmad, et al. 2012). PPO is up-regulated directly by the presence of herbivore-associated signaling compounds such as methyl-jasmonate (Koussevitzky, Ne'eman, and Harel 2004). PPO breaks down diphenolic compounds to produce more reactive phenolic compounds that have anti-insect activity once consumed (A. R. War, Paulraj, Ahmad, 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 A.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.

3.3 Methods and results

3.3.1 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 A.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.

3.3.2 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

Table 3.1: 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: (H_2O_2) production prior to herbivory was negligible.

Table 3.2: Comparison of technical replicate standard errors between spectrophotometer and microplate assays for POD and PPO of *Solanum lycopersicum*

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%

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 A.1.

3.3.3 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.

3.3.4 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.

3.3.5 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.

3.3.6 Protease inhibition (PI) Activity

Protease inhibition activity was quantified using an adapted method from (Orians, Pomerleau, and Ricco 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.

3.3.7 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, Yordanov, and Edreva 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.

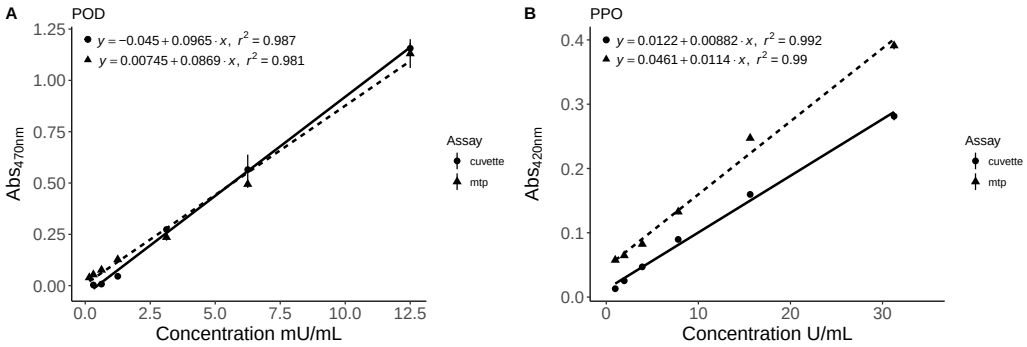
3.3.8 Protocol Validation

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, Pomerleau, and Ricco 2000; A. R. War, Paulraj, M. Y. 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 (Figure 3.1). 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,

Figure 3.1: Absorbance value comparisons for POD & PPO

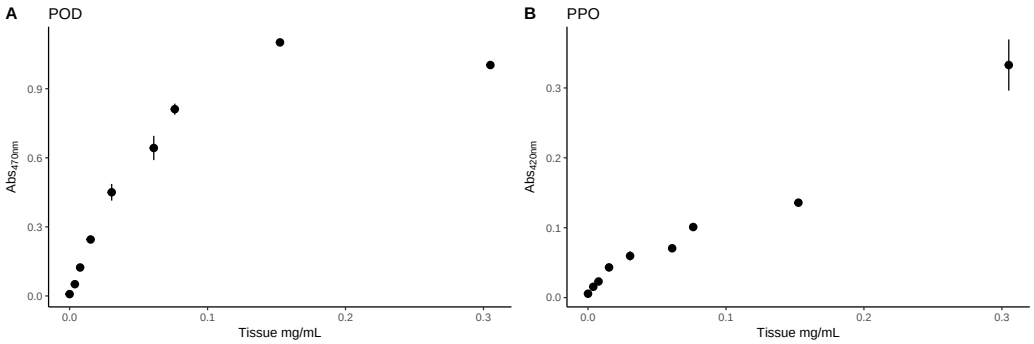


Figures 3.1A and 3.1B compare the respective absorbance values of POD and PPO when measured using either a spectrophotometer (cuvette) or microplate reader (mtp) to generate a standard curve using horseradish peroxidase for POD (measured at 470 nm) and mushroom polyphenol oxidase for PPO (measured at 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.

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 (Figure 3.1). 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 A.4 and Table 3.1). Table 3.1 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 3.2). Pooling tissue samples, such as what is required to get adequate tissue masses for spectrophotometric assays, increases variability due

Figure 3.2: 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 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 (Figure 3.2). 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 A.5).

3.4 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

(A. R. War, Paulraj, M. Y. 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, Pomerleau, and Ricco 2000; A. R. War, Paulraj, M. Y. 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.

Contribution Statement

CN Jack and I conceived and designed the study. I built the initial protocol set, developed the extraction buffers, selected equipment used, provided parameters, and familiarized CN Jack with the equipment and protocols where necessary. CN Jack and I performed the experiments. CN Jack analyzed the data. CN Jack and I co-wrote the manuscript and developed figures. ML Friesen provided feedback and revisions to the manuscript. CN Jack, SL Rowe, SS Porter, and ML Friesen gave final approval of the version to be published. SS Porter and ML Friesen provided funding for the materials and other resources used to conduct the study. I am co-first author on this publication.

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BIBLIOGRAPHY

BIBLIOGRAPHY

Bolnick, Daniel I. et al. (Apr. 2011). "Why Intraspecific Trait Variation Matters in Community Ecology". In: *Trends in Ecology & Evolution* 26.4, pp. 183–192.

Bradford, Marion M. (May 1976). "A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein-Dye Binding". In: *Analytical Biochemistry* 72.1, pp. 248–254.

Cavalcanti, Fabio Rossi et al. (2004). "Superoxide Dismutase, Catalase and Peroxidase Activities Do Not Confer Protection against Oxidative Damage in Salt-Stressed Cowpea Leaves". In: *New Phytologist* 163.3, pp. 563–571.

Fitzpatrick, Connor R. et al. (2015). "The Importance of Plant Genotype and Contemporary Evolution for Terrestrial Ecosystem Processes". In: *Ecology* 96.10, pp. 2632–2642.

Goud, Prashanth B. and Manvendra S. Kachole (Oct. 2012). "Antioxidant Enzyme Changes in Neem, Pigeonpea and Mulberry Leaves in Two Stages of Maturity". In: *Plant Signaling & Behavior* 7.10, pp. 1258–1262.

Greenbaum, Dov et al. (2003). "Comparing Protein Abundance and mRNA Expression Levels on a Genomic Scale". In: *Genome Biology* 4.9, p. 117.

Grimplet, Jérôme et al. (2009). "Proteomic and Selected Metabolite Analysis of Grape Berry Tissues under Well-Watered and Water-Deficit Stress Conditions". In: *PROTEOMICS* 9.9, pp. 2503–2528.

Junglee, Sanders et al. (2014). "Optimized Assay for Hydrogen Peroxide Determination in Plant Tissue Using Potassium Iodide". In: *American Journal of Analytical Chemistry* 05.11, p. 730.

Kerwin, Rachel et al. (Apr. 2015). "Natural Genetic Variation in Arabidopsis Thaliana Defense Metabolism Genes Modulates Field Fitness". In: *eLife* 4. Ed. by Merijn R Kant, e05604.

Koussevitzky, Shai, Emma Ne'eman, and Eitan Harel (July 2004). "Import of Polyphenol Oxidase by Chloroplasts Is Enhanced by Methyl Jasmonate". In: *Planta* 219.3, pp. 412–419.

Maffei, Massimo E., Gen-Ichiro Arimura, and Axel Mithöfer (Oct. 2012). "Natural Elicitors, Effectors and Modulators of Plant Responses". In: *Natural Product Reports* 29.11, pp. 1288–1303.

Mithöfer, Axel and Wilhelm Boland (2012). "Plant Defense Against Herbivores: Chemical Aspects". In: *Annual Review of Plant Biology* 63.1, pp. 431–450.

Olson, Bradley J.S.C. and John Markwell (2007). "Assays for Determination of Protein Concentration". In: *Current Protocols in Protein Science* 48.1, pp. 3.4.1–3.4.29.

Orians, Colin M., Jason Pomerleau, and Rafael Ricco (Feb. 2000). "Vascular Architecture Generates Fine Scale Variation in Systemic Induction of Proteinase Inhibitors in Tomato". In: *Journal of Chemical Ecology* 26.2, pp. 471–485.

Orozco-Cardenas, Martha and Clarence A. Ryan (May 1999). "Hydrogen Peroxide Is Generated Systemically in Plant Leaves by Wounding and Systemin via the Octadecanoid Pathway". In: *Proceedings of the National Academy of Sciences* 96.11, pp. 6553–6557.

Pande, S. V. and M. S. R. Murthy (Aug. 1994). "A Modified Micro-Bradford Procedure for Elimination of Interference from Sodium Dodecyl Sulfate, Other Detergents, and Lipids". In: *Analytical Biochemistry* 220.2, pp. 424–426.

Quan, Li-Juan et al. (2008). "Hydrogen Peroxide in Plants: A Versatile Molecule of the Reactive Oxygen Species Network". In: *Journal of Integrative Plant Biology* 50.1, pp. 2–18.

Rajalingam, Dakshinamurthy et al. (2009). "Trichloroacetic Acid-Induced Protein Precipitation Involves the Reversible Association of a Stable Partially Structured Intermediate". In: *Protein Science* 18.5, pp. 980–993.

Velikova, V, I Yordanov, and A Edreva (Feb. 2000). "Oxidative Stress and Some Antioxidant Systems in Acid Rain-Treated Bean Plants: Protective Role of Exogenous Polyamines". In: *Plant Science* 151.1, pp. 59–66.

War, Abdul Rashid, Michael Gabriel Paulraj, Tariq Ahmad, et al. (Oct. 2012). "Mechanisms of Plant Defense against Insect Herbivores". In: *Plant Signaling & Behavior* 7.10, pp. 1306–1320.

War, Abdul Rashid, Michael Gabriel Paulraj, Mohd Yousf War, et al. (Nov. 2011). "Herbivore- and Elicitor- Induced Resistance in Groundnut to Asian Armyworm, Spodoptera Litura (Fab.) (Lepidoptera: Noctuidae)". In: *Plant Signaling & Behavior* 6.11, pp. 1769–1777.

Whitham, THOMAS G. (Jan. 1983). "Host Manipulation of Parasites: Within-Plant Variation as a Defense against Rapidly Evolving Pests". In: *Variable Plants and Herbivores in Natural and Managed Systems*. Ed. by ROBERT F. Denno and MARK S. McCLURE. Academic Press, pp. 15–41.

Winn, Alice A. (1996). "Adaptation to Fine-Grained Environmental Variation: An Analysis of Within-Individual Leaf Variation in an Annual Plant". In: *Evolution; international journal of organic evolution* 50.3, pp. 1111–1118.

Zebelo, Simon A. and Massimo E. Maffei (Feb. 2015). "Role of Early Signalling Events in Plant–Insect Interactions". In: *Journal of Experimental Botany* 66.2, pp. 435–448.

Zhang, Shu-Dong and Timothy W. Gant (Dec. 2005). "Effect of Pooling Samples on the Efficiency of Comparative Studies Using Microarrays". In: *Bioinformatics (Oxford, England)* 21.24, pp. 4378–4383.

CHAPTER 4

RAPID EVOLUTION OF HERBIVORE INTERACTIONS DESPITE CONSERVED TRADE-OFFS IN *MEDICAGO POLYMORPHA*

4.1 Abstract

The invasion of novel environments by plants opens up opportunities for access to previously unavailable resources while also exposing the individuals to novel environmental threats. One such example of these types of threats are insect herbivores with which an introduced plant population has not co-evolved. The shifting defense hypothesis (SDH) predicts that selection pressures on defensive traits will change over the course of an invasion; specifically, it predicts a shift away from more energetically expensive compounds that deter generalists and more specialized enemies to cheaper compounds that garner more broad spectrum protection. We tested these predictions by comparing feeding preferences of two herbivores on burr clover (*Medicago polymorpha*) genotypes from *M. polymorpha*'s native range around the Mediterranean and invasive range in the North and South America–neither herbivore occurs in the host plant's native range but both are common in North and South America. We also compared the activity of peroxidase, polyphenol oxidase, and total protein production for host plant genotypes from the two ranges. Our study found that both herbivores preferred *M. polymorpha* tissue from the native range genotypes (representing the unfamiliar range). However, when tissues had their herbivory defense responses induced prior to feeding, we found that deterrence by the defenses of the invasive (familiar) range genotypes was stronger than that of the native (unfamiliar) range genotypes. The apparent induction response was most significant for the more specialized herbivore. Thus, our study documents a shift in herbivore preference between constitutive and previously induced tissue between native and invaded host plant ranges, thus supporting the shifting defense hypothesis.

4.2 Introduction

Rapid evolution of invasive species can result from selection pressure differences between the native and invaded environments. These changes may stem from differences in biotic stress due to novel enemy and pathogen pressures (Baker 1974; Blossey and Notzold 1995; Bossdorf, Prati, et al. 2004; Bossdorf, Auge, et al. 2005) or changes in abiotic stresses such as differences in water availability or soil salinity (Ozaslan et al. 2016; Richards et al. 2008). Plants colonizing a novel environment lack the niche-specific evolutionary benefits they may have gained as a consequence of sharing space with specific organisms over evolutionary time. This absence of co-evolution results in novel interactions with species such as insect herbivores (Keane and Crawley 2002; Maron and Vilà 2001; Verhoeven et al. 2009). Because resistance to herbivores is costly, so their absence may lead to rapid evolutionary changes in anti-herbivore defense traits (Joshi and K. Vrieling 2005; Koricheva, Nykänen, and Gianoli 2004).

Many hypotheses are available to make predictions about the effects of plant invasion on host-herbivore relationships. If the probability of insect attack is low, selection will favor reduced allocation to plant defense, as predicted by the Evolution of Increased Competitive Ability (EICA) Hypothesis (Blossey and Notzold 1995), and favor inducible over constitutive defenses (Zangerl and Rutledge 1996). These factors predict that populations in the invaded range will have lower levels of constitutive defense to herbivores in the native range compared to native range populations of a plant. However, if the probability of insect attack is high, high levels of constitutive plant defenses will be favored over inducible defenses (Bixenmann et al. 2016; Rasmann et al. 2015). To encompass aspects and arguments from both these positions, the Shifting Defense Hypothesis (SDH) recognizes that the likelihood of insect attack in a novel environment is high but bases its predictions on the demographic change of herbivores between the native and invasive ranges. Specifically, SDH argues that the lack of specialist herbivores in a novel environment would lead to the rapid increases in cheaper, broad-spectrum compounds that are effective against generalists while simultaneously resulting in a reduction of more expensive compounds evolved in response to co-evolved specialists (Doorduin and Klaas Vrieling 2011). Changes like what are predicted by the

SDH can further be described as a trade-off in resource allocation between constitutive expression of general herbivore deterrents and inducibility of more specialized herbivore deterrents.

Since insects can experience evolutionary changes over shorter time-scales relative to most plants, it can be useful to think about the interacting partners as familiar or unfamiliar rather than native or invasive. Further, there are old world plants for which naturalists have been documenting for hundreds of years. For instance, that annual legume Burr Clover (*Medicago polymorpha*), moved from its native range in the Mediterranean via human-assisted migration over 500 years ago. Consequently, *M. polymorpha's* native range represents an unfamiliar population to herbivores that have long been found in the Americas while the invaded range represents the familiar range. It can be assumed that *M. polymorpha* has become well-adapted to its new world range based on its continued spread and well-established populations in both North and South America (Helliwell et al. 2018). This establishment also implies the potential for adaptations to local herbivores. Because developing theory about how plants are affected by changes in herbivores from their previous biogeographical location to the next, the concepts of "native" and "invasive" are only useful if the herbivores in question are novel to the plants in the invaded range. Thus, when considering different hypotheses of evolutionary changes in this context, it's important to remember that length of shared evolutionary history is the key variable for consideration.

In recent work by Jack and Friesen (2019), it was found that a herbivore, the soybean looper (*Chrysodeixis includens*), prefers native range *Medicago polymorpha* (an unfamiliar population) over co-occurring, invasive-range genotypes, supporting the SDH. Also in line with the SDH, an increased presence of generalists over specialists should lead to a decrease in the production of digestibility reducers and increase toxin production. However, this work did not aim to investigate changes to specific defense responses by *M. polymorpha* in response to co-occurring or novel herbivores. Future studies are needed to further comment on the changes that may or may not be occurring in the inducible defenses of both populations (Liu et al. 2020). We begin the process of investigating that here.

The aim of this study was to test for rapid evolutionary changes in defense responses in plants

by comparing populations of *M. polymorpha* from its native and invaded range. In particular, this study examined trade-offs between constitutively expressed and inducible defensive traits. To assess the extent of herbivory-related defense responses in plants, we quantified two commonly observed oxidative enzymes, polyphenol oxidase (PPO) and peroxidase (POD). PPO and POD both break down phenolic compounds into toxic quinones and interfere with insect digestive activity once consumed (Ali and Agrawal 2012; Constabel et al. 2000; Verhoeven et al. 2009; War et al. 2012). POD and PPO are both expressed by *M. polymorpha* (Jack and Friesen 2019) and their activity correlates with aphid resistance in a closely related species, *Medicago sativa* (H. Wei, Zhikuan, and Qingfang 2007). Additionally, total soluble protein was measured as a metric for biochemical differences that are likely observable, but not captured by the limited set of assays used (Kessler and Baldwin 2002; Chen 2008; Kerchev et al. 2012). Finally, we select and deploy two herbivores native to the invaded range of *M. polymorpha* to asses differences in the feeding habits of specialists vs. generalists as a metric for the changes in defense strategies expected under the SDH.

Specifically, we ask:

1. Are invaded/familiar range plants constitutively less palatable and more chemically defended than native/unfamiliar range plants?

2. Do invaded/familiar range plants show a reduction in inducible defenses?

3. Does there exist a trade-off between constitutive and inducible defenses and can the effects of such a trade-off be observed by differences in preferences of generalist herbivores relative to more specialized herbivores?

4.3 Methods

4.3.1 *Medicago polymorpha* field collection

M. polymorpha is an annual legume that is indigenous to the Mediterranean basin but has spread worldwide. It forms a symbiotic relationship with the rhizobium *Ensifer medicae*. A field collection of wild lineages was established from 29 populations in the native range throughout Europe and from 11 populations in the North American invaded range collected between May and September 2015.

4.3.2 *Medicago polymorpha* growth

270 genotypes (123 from the invaded range and 190 from the native range; Appendix A.2) were chosen at random from the field collection. Seeds were scarified with 600 grit sandpaper and planted into 158-ml D-pots (Stuewe & Sons, Tangent OR) filled with Sungro Sunshine Mix #1. Three seeds were planted into each cone 5 mm below the soil surface in the Washington State University, Vancouver greenhouse (45.732679, –122.635799) on April 8-9, 2016. Plants were fertilized with slow release fertilizer (Osmocote Plus Outdoor & Indoor fertilizer pellets) and inoculated a week after planting with a rhizobium strain mixture of 107 cells of equal parts *E. medicae* strain WSM419 and *E. meliloti* strain 1021. During germination, seeds were mist irrigated twice a day for 20 minutes, and then mist-irrigated daily as needed. The plants were grown for 8 weeks before use in the experiments. *M. polymorpha* is an annual legume that is indigenous to the Mediterranean basin but has spread worldwide. It forms a symbiotic relationship with the rhizobium *E. medicae*. A field collection of wild lineages was established from 29 populations in the native range throughout Europe and from 11 populations in the North American invaded range collected between May and September 2015.

4.3.3 Herbivore species

To assess variation between populations of *M. polymoprrpha* from the invaded and native ranges and to assess the effect of constitutive and induced resistance within each range, we used two caterpillar species that differed in the breadth of their diets. Species exist on a continuum from highly specialized diets to extreme generalist behavior (Finlay-Doney and Walter 2012; Dapporto and Dennis 2013). Further, insects have the ability of to rapidly adapt to novel conditions due to short generation times and high reproductive rates (Hawkins et al. 2019; Carroll et al. 2005). Thus, our selected herbivores will be categorized by their relative dietary breadths as of the time of this publication. Our first herbivore represented the more generalist species and was the larvae of the Soybean Looper, *Pseudoplusia includens* (Lepidoptera: Noctuidae). Although it has a preference for soybeans, it is a polyphagous species that is known to feed on 174 plants across 39 families and includes wild, agricultural, and floricultural species (Herzog 1980). Our second and more specialized herbivore was the larvae of the oligophagous Velvetbean caterpillar, *Anticarsia gemmatalis* (Lepidoptera: Noctuidae). It is a oligophagous species that feeds primarily on leguminous plants (Panizzi 2013; Carner and Turnipseed 1977). Larvae were obtained from Benzon Research (Carlisle, PA) and fed a multi-species artificial diet from Southland Products Inc. until they were used in the experiment.

4.3.4 Biochemical tissue analysis

Insect preparation– 24 hours before tissue collection from experimental plants, a soybean looper and velvetbean caterpillar of approximately the same size were fed a mix of native and invaded range *M. polymorpha* leaf tissue. The day of the experiment these two larvae were homogenized together with a mortar and pestle. The homogenate was mixed with 0.5ml of DI water kept on ice.

Tissue collection– 95 genotypes were selected for the biochemical assays. Scissors dipped in the insect homogenate were used to cut off a trifoliate (Time 0) and induce production of herbivore chemical defense compounds. Trifoliates were similarly collected at 4 and 24 hours. Each trifoliate

was placed in a separate 96 deep well plate on ice. Once all plants were sampled, the plates were flash frozen in liquid nitrogen and cryogenically stored at -80°C until analysis.

Extraction and homogenization– Frozen leaf tissue was quickly removed from tubes and weighed before thawing. The tissue was returned to tubes and again flash frozen in liquid nitrogen. The tissue was homogenized for 15 minutes at 300 rpm in a tissuelyser (QIAGEN TissueLyser II) with a 2mm steel bead. For each time point, two trifoliates were collected of each genotype in separate plates. Half of the tubes received 1ml of 0.1% TCA buffer, while the other received 1 ml of PE buffer (1mM EDTA, 88mM Trizma Base, 10% glycerol). The tubes were centrifuged at 4°C for 10 minutes at 4500 rpm and the supernatant transferred into clean microcentrifuge tubes. The PE buffer tubes were then diluted to 1/10X.

Protein Quantification, Peroxidase (POD) Activity, and Polyphenol Oxidase (PPO) Activity– Protein quantification was performed using the Thermo Scientific PierceTM BCA Protein Assay Kit (Product number: 23337) according to manufacturer instructions for microplate samples. POD and PPO activity were measured following the protocol outlined in Jack et al (2019).

4.3.5 Plant range preference assays

We used 69 native and 69 invaded range *M. polymorpha* genotypes in unique pairings to assess preference for either native or invaded range tissue. For each pair, a single trifoliate leaf from native native range and a single trifoliate leaf from the invaded range genotypes were weighed and placed on opposite sides of a 95 mm petri dish divided down the center with a thin plastic ridge. One caterpillar was placed in the middle of the petri dish and left to feed for 24 hours. After 24 hours, the remaining leaf tissue was weighed to calculate how much of each genotype was eaten. Each genotype pairing was tested in two separate assays: one with the soybean looper and one with the velvetbean caterpillar.

4.3.5.1 Plant inducible defense preference assays

We used 40 native and 40 invaded range *M. polymorpha* genotypes to test plasticity of herbivore preference. Within each range, half of the genotypes were induced using insect homogenate prepared as above. After 24 hours, the induced genotypes were paired with a different non-induced genotype from the same range to assess preference for either induced or non-induced leaf tissue within a range. As above, tissue was placed on opposite sides of a petri dish with a caterpillar for 24 hours and weighed before and after the feeding trial. This assay was conducted tested using both the soybean looper and velvetbean caterpillar in separate petri dishes. Four pairs were removed from later analysis due to discrepancies between weights before and after feeding trials.

4.3.6 Statistical analysis

The data was analyzed using R version 3.5.2 using packages lme4 (Bates et al. 2014), glmmTMB (Brooks et al. 2017), emmeans (Lenth 2017), and cocor (Diedenhofen and Musch 2015). Normality and heteroskedasticity were checked using DHARMa (Hartig 2019). Figures were created using ggplot2 3.1.1 (Wickham 2009), cowplot (Wilke 2015), and corrplot (T. Wei 2016). We provide a summaries of the statistical methods used for analysis of presented results below.

Preference Assays: An herbivore's preference for one tissue sample over another was quantified as the amount of tissue of the focal sample that was consumed divided by the total amount of tissue consumed by the herbivore across both samples present in a Petri dish. Higher values for this metric indicate a stronger preference for one sample over another. A generalized linear model with a beta distribution was used to analyze the data because the data was defined as a proportion. Genotype and Pair were used as random effects in both the Range and Defense model.

Biochemical Assays: The response variable for each assay was log transformed to bring the residuals closer to normal before running the data analysis using a linear mixed model.

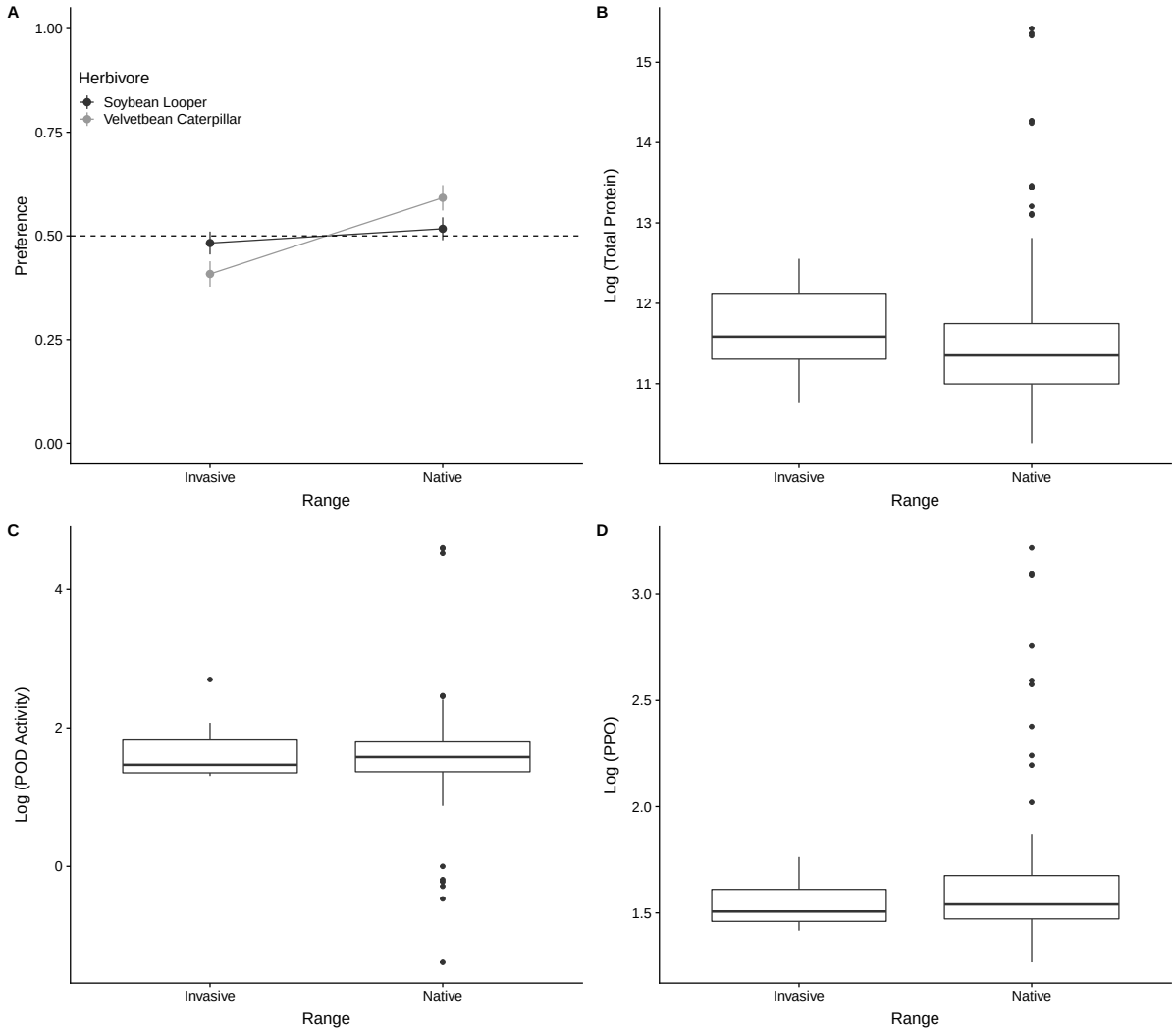
Defensive Compound Production Correlations: Values from the biochemical assays at the three time points were log transformed to give a normal approximation and tested for significant Pearson correlations after adjusting for multiple comparisons using Holm's method.

Constitutive Expression vs Inducibility Trade-offs: For each biochemical assay, we used Kendall's Tau on untransformed data to look for significant monotonic correlations between constitutive expression and degree of inducibility. We calculated inducibility using the difference between measured values at 0 and 24 hours. We analyzed each range separately in addition to analyzing the entire dataset. Then, to determine if the correlations between ranges were different from each other, the data was log transformed and analyzed using Fisher's Z-test. To ensure that the correlations between the variables were not spurious, since they were calculated from the same underlying data, we used an R implementation of the simulation procedure proposed by Morris, Traw, and Bergelson (2006).

4.4 Results

Herbivores demonstrated a preference for native/unfamiliar range plants. When given a choice between uninduced tissue from the familiar and unfamiliar range, our herbivores showed a preference for uninduced unfamiliar range plants over uninduced familiar range plants (Figure 3.3A). Both the soybean looper and the velvetbean caterpillar preferred native range *M. polymorpha* genotypes over invaded range genotypes (Figure 3.3A, Table 3.3). Post-hoc contrasts of the significant interaction between range and herbivore found that the preference was significant for the velvetbean caterpillar but not the soybean looper; however, in line with our results, a previous study with soybean loopers and *M. polymorpha* demonstrated a significant preference for the unfamiliar range. Our results indicate a stronger response by the velvetbean caterpillar, the relatively specialized herbivore in this study. This result is what one would predict under the assumptions of the Shifting Defense Hypothesis (SDH). More specifically, our results demonstrate that for the familiar range, the constitutive defenses of the host are far more successful at deterring the specialist than those of the unfamiliar range hosts. We also observe, and have previously observed (Jack and

Figure 4.1: Experimental outcomes for invasive and native herbivores



Herbivore preference for leaf tissues with constitutive expression of herbivore defenses (A); constitutive levels of total soluble protein content (log transformed to correct for skewness) compared between invasive(familiar) and native (unfamiliar) ranges (B); constitutive levels of total soluble peroxidase (POD) levels (log transformed to correct for skewness) compared between invasive(familiar) and native (unfamiliar) ranges (C); constitutive levels of polyphenol oxidase (PPO) levels (log transformed to correct for skewness) compared between invasive (familiar) and native (unfamiliar) ranges (D). Bars in all panels show the mean ± standard error for three technical replicates.

Friesen 2019), a slight preference for the unfamiliar range for our generalist species. These findings suggest a change in anti-herbivore defenses of *M. polymorpha* after invasion of a novel range.

Herbivore preference was not related to POD or PPO induction. The apparent preference for the unfamiliar range appears to not be a result of either peroxidase (POD) or polyphenol oxidase (PPO) induction. Constitutive levels of total protein, POD, and PPO did not significantly differ between the ranges. For both ranges, soluble total protein decreased significantly, while both POD and PPO showed significant increases after induction. Similar to the findings for constitutive defensive levels, both ranges had indistinguishable levels of induced leaf chemistry values from one another (Table 3.3). POD and PPO are both enzymes that catalyze the oxidation of phenols; it's believed that these responses reduce the digestibility of consumed proteins by the covalent linking of proteins to quinones formed after oxidation (War et al. 2012). POD and PPO comprise a small fraction of the total number of characterized herbivory-induced defense (Ali and Agrawal 2012). The total reduction in protein content for both plant ranges indicates a reallocation of resources to defense strategies not directly measured in this study. Though both ranges show a decrease, here it cannot be determined if the decreases in soluble protein content are indicating identical or highly similar biochemical changes because of the wide variety of changes that could be captured in this change. Given the preference change between ranges and the increased variability in the unfamiliar range, it suggests that is a difference in biochemical defenses between ranges that was not directly captured by our assays (Figure 3.2A-D).

Herbivores demonstrated greater sensitivity to induced defenses in the familiar range. When given a choice between consumption of leaf tissue expressing constitutive or herbivore-induced defenses, both herbivores showed a strong aversion for the induced tissue from the familiar range genotypes while the response to the induced tissue from unfamiliar range genotypes was less robust (Figure 3.4A, Table 3.3). Post-hoc analysis of the individual herbivores found that the shift was, again, significant for the velvetbean caterpillar but not the soybean looper (Table 1). This result further suggests a change in defense responses between familiar (invaded) and unfamiliar (native) range genotypes.

Additionally, we again observed a stronger effect on the more specialized species. Interestingly,

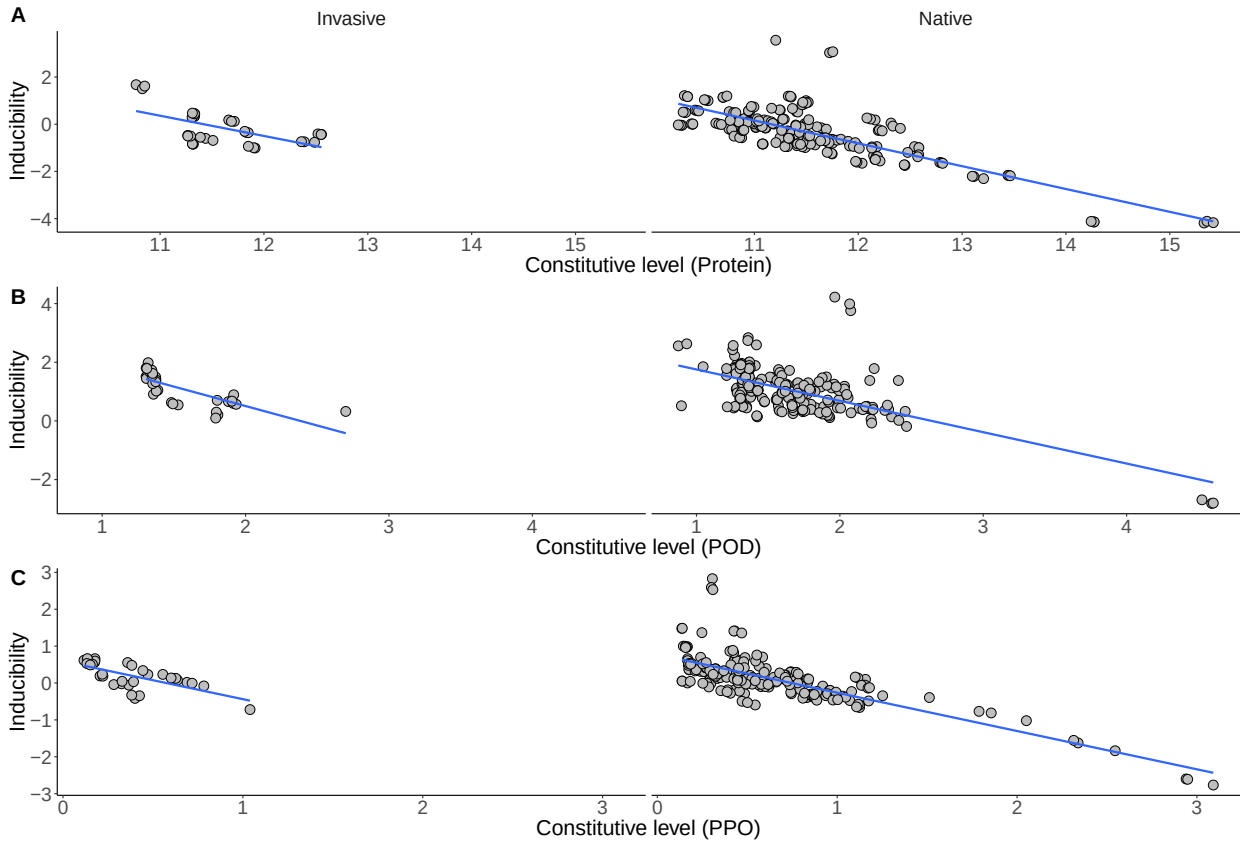
Table 4.1: Summary of Results from Statistical Tests

Assay	Assay subgroup	Figure	F-stat	p-value
Range Preference	Velvetbean	1A	$F_{1,243} = 20.896$	<0.001
	Soybean L.	1A	$F_{1,243} = 0.915$	0.3396
Constitutive vs. Induced	Protein	1B	$F_{1,472} = 23.45$	<0.001
	POD	1C	$F_{1,472} = 446.62$	<0.001
	PPO	1D	$F_{1,472} = 11.72$	<0.001
Range Diff. (Constitutive)	Protein	2B	$F_{1,129} = 0.165$	0.201
	POD	2C	$F_{1,127} = 0$	1
	PPO	2D	$F_{1,132} = 2.39$	0.125
Range Diff. (Induced)	Protein	2B	$F_{1,129} = 0.595$	0.442
	POD	2C	$F_{1,127} = 0.001$	0.98
	PPO	2D	$F_{1,132} = 0.1.72$	0.191

our specialist (velvetbean) had opposite preference for induced leaf tissues for genotypes from both ranges. When given choice between constitutive and induced tissues from the familiar range, the velvetbean showed a preference for the constitutive tissues. For the unfamiliar range, the velvetbean had a stronger preference for the induced tissue than for the constitutive tissue (Figure 3.4A). As before, this change in preference appears to not be driven by differences in the specific defense responses measured over the course of this study (Figure 3.4B-D). Inline with the data from the constitutive biochemical assays, we observed more variability for all biochemical metrics in our unfamiliar genotypes than those from the familiar range (Figure 3.4B-D).

Constitutive expression and inducibility of measured defense responses were negatively correlated, indicating a trade-off. To further investigate possible changes to herbivory responses between ranges, we investigated the degree to which any given change was induced pre- and post-herbivory. In some plant species, inducibility of defenses has been found to be negatively correlated with the constitutive levels present in a given population (Cipollini et al. 2005). Since there was not a significant difference between data from our herbivore species at either 0 hours (constitutive) or 24 hours (induced), we collapsed the data from both herbivores to look for correlations over time, between compounds, and differences in the correlations between ranges. We found a significant, negative relationship between constitutive expression of both defensive proteins and their inducibility upon herbivore attack (Figure 3.5B-C, Table 3.3). This finding is inline with previous results (Cipollini et al. 2005) and supports the theoretical emphasis on changes in herbivore pressures as suggested by the SDH. Further, this correlation undercuts assumptions posited by those of the Evolution of Increased Competitive Ability (Blossey and Notzold 1995) which suggests that host plants are released from herbivores and the corresponding pressures when invading a new range. This correlation was also present for the drop in soluble protein levels observed post-induction by herbivore secretions (Figure 3A), further supporting the claim that the changes we observe in total soluble protein content is a result of defense responses. The correlations between ranges were not significantly different from each other indicating that the trade-off was conserved (Figure 3.5A-C).

Figure 4.2: Tradeoffs between constitutive express and inducibility of measured defenses responses of invasive and native herbivores



Correlation plot of constitutive total soluble protein content (x-axis) plotted against the net change (i.e. inducibility) in total soluble protein content between the constitutive and induced tissues (y-axis) (A); Correlation plot of constitutive POD levels (x-axis) plotted against the net change (i.e. inducibility) in POD levels between the constitutive and induced tissues (y-axis) (B); Correlation plot of constitutive PPO levels (x-axis) plotted against the net change (i.e. inducibility) in PPO levels between the constitutive and induced tissues (y-axis) (C).

4.5 Discussion

The aims of this study were to determine if *Medicago polymorpha* underwent rapid evolutionary change resulting in a shift in defensive traits as a result of populations migrating out of their native range and establishing stable populations with novel pests. Using two herbivores with different diet breadths (reflecting a generalist and a specialist), we investigated differences in constitutively expressed and inducible defenses as well as evidence for trade-offs in investments between the two. We used changes in herbivore preference and plant defense activity as indicators of evolutionary

change resulting from invasion in *M. polymorpha*.

Herbivore resistance traits are costly and the fitness consequences of plant investment in them are context dependent (Bixenmann et al. 2016; Koricheva, Nykänen, and Gianoli 2004; Rasmann et al. 2015; Zangerl and Rutledge 1996). Thus, the absence or low abundance of an exotic plant's natural enemies in a new environment could lead to selection for reduced anti- herbivore defenses (Blossey and Notzold 1995). However, if only specialist herbivores are absent, then there may be selection for increased qualitative defenses like toxins that are cheap to produce and effective only against generalist herbivores (Joshi and K. Vrieling 2005; Müller-Schärer, Schaffner, and Steinger 2004). Based on previous results, we expected the generalist soybean looper to show a slight preference for the native/unfamiliar range genotypes (Jack and Friesen 2019). Though slightly outside of the significance range in this study, the trend found here matches what was observed by Jack & Friesen. Moreover, our finding that velvetbean caterpillars show a preference for the native/ unfamiliar range further adds to the evidence of the previous study (Figure 3.4A).

We found that insect interactions indicated that invasive range genotypes have undergone a shift in their defense strategy as compared to native genotypes, but that herbivore preference is not correlated with the defensive biochemical traits we measured. Theory predicts that a specialist herbivore (e.g. the velvetbean caterpillar) will likely be more affected by herbivory-induced defenses by familiar hosts (e.g. the invaded range) than a more generalist species (e.g. the soybean looper). Our results support this prediction a all of our experiments resulted in more robust responses in our more specialized herbivore.

Most curiously, the specialist herbivore showed a slight preference for induced native/unfamiliar range tissues relative to the constitutive tissue. The generalist species seemed generally unaffected by the pre-induction of defense responses in the native/unfamiliar range (Figure 3.5A). Though surprising, the result has been observed in at least two other studies (Harrison and Karban 1986; Hartley and Lawton 1987). One hypothesis for this is that increased consumption can reflect compensatory feeding in response to decreased nutritional quality (Steppuhn and Baldwin 2007).

Our results demonstrated a net reduction in the fraction of total soluble protein upon induction

with no significant difference between populations. Though POD and PPO (both proteins) increased post-induction, these again were not quantitatively different between populations. This is likely attributed to the fact that we measured a small subset of representative biochemical responses that was in no way a comprehensive investigation. Still, this result indicates that expression of constitutively produced proteins was likely reduced in response to the attack. This can happen as a result of reallocation of energy and matter resources away from growth and reproduction processes and towards defense processes (Kerchev et al. 2012). It's possible that the resulting tissue composition was nutritionally less beneficial to the caterpillars tested here. The shift for the specialist could represent the host having defense responses more specialized for herbivores with which it has a history of coexistence. Regardless, the changes in herbivore preferences between the populations and the significant drop in net protein content pre- and post-induction suggest a change in strategies. Further investigations of a broader range of defensive responses would be needed to tease apart the specific nature of this observed change.

The Shifting Defense Hypothesis (SDH) predicts that induced defenses to herbivores will be more effective against specialists compared to generalists in familiar or co-occurring range. Our result showing a shift in preference for constitutive leaf tissue in one range to induced leaf tissue in the other was the greatest indication of rapid evolution between the native and invasive ranges, supporting the Shifting Defense Hypothesis (SDH). Invading plant populations encounter a suite of novel biotic and abiotic stresses in their new environments that may apply different selective pressures than what they are subjected to in their home ranges. Rapid adaptation prompted by these interactions is likely to be key for successful spread after establishment. In plants especially, the presence or absence of co-evolved herbivores can have a profound effect on survival probability short-term and can lead to evolutionary changes in not only defensive compound production but also competitive ability long-term. Our biogeographic comparison of *Medicago polymorpha* found that genotypes in the invasive range have undergone rapid evolutionary changes that make them less preferred over their native range conspecifics. Since we found that constitutive and inducible defense levels of the measured proteins were conserved between the ranges were conserved, we

cannot rule out that there have been changes in traits not directly related to anti-herbivore defense production, for example a change in nutritional status or plant structure (Ho and Pennings 2013; Morrison and Hay 2011). However, the herbivores' shift in preference between the overall levels of defense compounds produced constitutively and those induced after herbivore attack align with components of the Shifting Defense Hypothesis and are evidence of rapid evolution between native and invaded range populations of *Medicago polymorpha*.

Contribution Statement

CN Jack conceived the preliminary study and designed it in discussion with ML Friesen and SS Porter. I expanded on the study design and I designed and modified the assays used to test biochemical herbivory responses. CN Jack, Z Lopez, C Sackos, and D Ross conducted and collected data from the first set of greenhouse experiments. CN Jack and I designed the second set of greenhouse experiments and CN Jack conducted and collected data. I conducted the biochemical response experiments with CN Jack's help. CN Jack analyzed the caterpillar choice assay data and CN Jack and I analyzed the biochemical response data. I co-wrote the manuscript with CN Jack and we produced figures with feedback from ML Friesen and SS Porter. CN Jack, ML Friesen, and SS Porter provided funds and resources to complete the necessary experiments and analyses.

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BIBLIOGRAPHY

BIBLIOGRAPHY

Ali, Jared G. and Anurag A. Agrawal (May 2012). "Specialist versus Generalist Insect Herbivores and Plant Defense". In: *Trends in Plant Science* 17.5, pp. 293–302.

Baker, Herbert G. (1974). "The Evolution of Weeds". In: *Annual Review of Ecology and Systematics* 5.1, pp. 1–24.

Bates, Douglas et al. (June 23, 2014). "Fitting Linear Mixed-Effects Models Using Lme4".

Bixenmann, Ryan J. et al. (2016). "High Herbivore Pressure Favors Constitutive over Induced Defense". In: *Ecology and Evolution* 6.17, pp. 6037–6049.

Blossey, Bernd and Rolf Notzold (1995). "Evolution of Increased Competitive Ability in Invasive Nonindigenous Plants: A Hypothesis". In: *Journal of Ecology* 83.5, pp. 887–889.

Bossdorf, Oliver, Harald Auge, et al. (June 1, 2005). "Phenotypic and Genetic Differentiation between Native and Introduced Plant Populations". In: *Oecologia* 144.1, pp. 1–11.

Bossdorf, Oliver, Daniel Prati, et al. (2004). "Reduced Competitive Ability in an Invasive Plant". In: *Ecology Letters* 7.4, pp. 346–353.

Brooks, Mollie E. et al. (Dec. 2017). "glmmTMB Balances Speed and Flexibility among Packages for Zero-Inflated Generalized Linear Mixed Modeling". In: *The R journal* 9.2, pp. 378–400.

Carner, G. R. and S. G. Turnipseed (Oct. 1, 1977). "Potential of a Nuclear Polyhedrosis Virus for Control of the Velvetbean Caterpillar in Soybean12". In: *Journal of Economic Entomology* 70.5, pp. 608–610.

Carroll, Scott P. et al. (2005). "And the Beak Shall Inherit – Evolution in Response to Invasion". In: *Ecology Letters* 8.9, pp. 944–951.

Chen, Ming-Shun (2008). "Inducible Direct Plant Defense against Insect Herbivores: A Review". In: *Insect Science* 15.2, pp. 101–114.

Cipollini, Don et al. (July 1, 2005). "Expression of Constitutive and Inducible Chemical Defenses in Native and Invasive Populations of Alliaria Petiolata". In: *Journal of Chemical Ecology* 31.6, pp. 1255–1267.

Constabel, C. Peter et al. (Sept. 1, 2000). "Polyphenol Oxidase from Hybrid Poplar. Cloning and Expression in Response to Wounding and Herbivory1". In: *Plant Physiology* 124.1, pp. 285–296.

Dapporto, Leonardo and Roger L. H. Dennis (Jan. 1, 2013). "The Generalist–Specialist Continuum: Testing Predictions for Distribution and Trends in British Butterflies". In: *Biological Conservation* 157, pp. 229–236.

Diedenhofen and Musch (2015). *Cocor*.

Doorduin, Leonie J. and Klaas Vrieling (Mar. 1, 2011). "A Review of the Phytochemical Support for the Shifting Defence Hypothesis". In: *Phytochemistry Reviews* 10.1, pp. 99–106.

Finlay-Doney, Mary and Gimme H. Walter (Dec. 1, 2012). "The Conceptual and Practical Implications of Interpreting Diet Breadth Mechanistically in Generalist Predatory Insects". In: *Biological Journal of the Linnean Society* 107.4, pp. 737–763.

Harrison, Susan and Richard Karban (June 1, 1986). "Effects of an Early-Season Folivorous Moth on the Success of a Later-Season Species, Mediated by a Change in the Quality of the Shared Host, Lupinus Arboreus Sims". In: *Oecologia* 69.3, pp. 354–359.

Hartig, F (2019). *DHARMa: Residual Diagnostics for Hierarchical (Multi-Level / Mixed) Regression Models.*

Hartley, S. E. and J. H. Lawton (1987). "Effects of Different Types of Damage on the Chemistry of Birch Foliage, and the Responses of Birch Feeding Insects". In: *Oecologia* 74.3, pp. 432–437.

Hawkins, Nichola J. et al. (Feb. 2019). "The Evolutionary Origins of Pesticide Resistance". In: *Biological Reviews of the Cambridge Philosophical Society* 94.1, pp. 135–155.

Helliwell, Emily E. et al. (2018). "Rapid Establishment of a Flowering Cline in Medicago Polymorpha after Invasion of North America". In: *Molecular Ecology* 27.23, pp. 4758–4774.

Herzog, Donald C. (1980). "Sampling Soybean Looper on Soybean". In: *Sampling Methods in Soybean Entomology*. Ed. by Marcos Kogan and Donald C. Herzog. Springer Series in Experimental Entomology. New York, NY: Springer, pp. 141–168.

Ho, Chuan-Kai and Steven C. Pennings (Mar. 22, 2013). "Preference and Performance in Plant–Herbivore Interactions across Latitude–A Study in U.S. Atlantic Salt Marshes". In: *PLOS ONE* 8.3, e59829.

Jack, Chandra N. and Maren L. Friesen (2019). "Rapid Evolution of Medicago Polymorpha during Invasion Shifts Interactions with the Soybean Looper". In: *Ecology and Evolution* 9.18, pp. 10522–10533.

Joshi, J. and K. Vrieling (2005). "The Enemy Release and EICA Hypothesis Revisited: Incorporating the Fundamental Difference between Specialist and Generalist Herbivores". In: *Ecology Letters* 8.7, pp. 704–714.

Keane, Ryan M. and Michael J. Crawley (Apr. 1, 2002). "Exotic Plant Invasions and the Enemy Release Hypothesis". In: *Trends in Ecology & Evolution* 17.4, pp. 164–170.

Kerchev, Pavel I. et al. (2012). "Plant Responses to Insect Herbivory: Interactions between Photosynthesis, Reactive Oxygen Species and Hormonal Signalling Pathways". In: *Plant, Cell & Environment* 35.2, pp. 441–453.

Kessler, André and Ian T. Baldwin (June 2002). "PLANT RESPONSES TO INSECT HERBIVORY: The Emerging Molecular Analysis". In: *Annual Review of Plant Biology* 53.1, pp. 299–328.

Koricheva, Julia, Heli Nykänen, and Ernesto Gianoli (2004). "Meta-analysis of Trade-offs among Plant Antiherbivore Defenses: Are Plants Jacks-of-All-Trades, Masters of All?" In: *The American Naturalist* 163.4, E64–E75.

Lenth, R (2017). *Mmeans: Estimated Marginal Means, Aka Least-Squares Means.* R Package.

Liu, Shao-Lun et al. (2020). "Comparative Genome Analysis Reveals Cyanidiococcus Gen. Nov., A New Extremophilic Red Algal Genus Sister to Cyanidioschyzon (Cyanidioschyzonaceae, Rhodophyta)". In: *Journal of Phycology* 56.6, pp. 1428–1442.

Maron, John L. and Montserrat Vilà (2001). "When Do Herbivores Affect Plant Invasion? Evidence for the Natural Enemies and Biotic Resistance Hypotheses". In: *Oikos* 95.3, pp. 361–373.

Morris, William F., M. Brian Traw, and Joy Bergelson (2006). "On Testing for a Tradeoff between Constitutive and Induced Resistance". In: *Oikos* 112.1, pp. 102–110.

Morrison, Wendy E. and Mark E. Hay (Mar. 4, 2011). "Herbivore Preference for Native vs. Exotic Plants: Generalist Herbivores from Multiple Continents Prefer Exotic Plants That Are Evolutionarily Naïve". In: *PLOS ONE* 6.3, e17227.

Müller-Schärer, Heinz, Urs Schaffner, and Thomas Steinger (2004). "Evolution in Invasive Plants: Implications for Biological Control". In: *Trends in Ecology & Evolution* 19.8, pp. 417–422.

Ozaslan, Cumali et al. (Oct. 14, 2016). "Invasion Potential of Two Tropical Physalis Species in Arid and Semi-Arid Climates: Effect of Water-Salinity Stress and Soil Types on Growth and Fecundity". In: *PLOS ONE* 11.10, e0164369.

Panizzi, A. R. (Apr. 1, 2013). "History and Contemporary Perspectives of the Integrated Pest Management of Soybean in Brazil". In: *Neotropical Entomology* 42.2, pp. 119–127.

Rasmann, Sergio et al. (May 1, 2015). "Trade-off between Constitutive and Inducible Resistance against Herbivores Is Only Partially Explained by Gene Expression and Glucosinolate Production". In: *Journal of Experimental Botany* 66.9, pp. 2527–2534.

Richards, Christina L. et al. (2008). "Plasticity in Salt Tolerance Traits Allows for Invasion of Novel Habitat by Japanese Knotweed s. l. (Fallopia Japonica and F.×bohemica, Polygonaceae)". In: *American Journal of Botany* 95.8, pp. 931–942.

Steppuhn, Anke and Ian T. Baldwin (2007). "Resistance Management in a Native Plant: Nicotine Prevents Herbivores from Compensating for Plant Protease Inhibitors". In: *Ecology Letters* 10.6, pp. 499–511.

Verhoeven, Koen J. F. et al. (2009). "Plant Invaders and Their Novel Natural Enemies: Who Is Naïve?" In: *Ecology Letters* 12.2, pp. 107–117.

War, Abdul Rashid et al. (Oct. 2012). "Mechanisms of Plant Defense against Insect Herbivores". In: *Plant Signaling & Behavior* 7.10, pp. 1306–1320.

Wei, Huang, Jia Zhikuan, and Han Qingfang (June 1, 2007). "Effects of Herbivore Stress by Aphis Medicaginis Koch on the Malondialdehyde Contents and the Activities of Protective Enzymes in Different Alfalfa Varieties". In: *Acta Ecologica Sinica* 27.6, pp. 2177–2183.

Wei, Taiyun (2016). *Package 'Corrplot'*.

Wickham, H (2009). *Ggplot2: Elegant Graphics for Data Analysis.* Springer Science & Business Media.

Wilke, C. O. (2015). *Cowplot: Streamlined Plot Theme and Plot Annotations for "Ggplot2."*.

Zangerl, Arthur R. and Claire E. Rutledge (Apr. 1, 1996). "The Probability of Attack and Patterns of Constitutive and Induced Defense: A Test of Optimal Defense Theory". In: *The American Naturalist* 147.4, pp. 599–608.

CHAPTER 5

DESIGN OF A ROBUST SYSTEM FOR THE INVESTIGATION OF HOST–ARBUSCULAR MYCORRHIZAL FUNGI RELATIONSHIPS AND APPLICATIONS TO FUNGI-MEDIATED PLANT-TO-PLANT NUTRIENT EXCHANGE

5.1 Abstract

Nearly 80% of terrestrial plants form symbiotic associations with a group of fungi known as arbuscular mycorrhizal fungi (AMF). These fungi are widely beneficial with their most-notable benefit being the uptake and transfer of soil phosphorus (P) to host plants. In exchange, hosts provide AMF with carbon such as hexoses and fatty acids. AMF are also known to uptake and transfer inorganic nitrogen (N) and other nutrients from the soil environment to the host plant, improve resilience of hosts under various forms of environmental stress, and potentially move signaling and nutritional compounds between hosts via common mycorrhizal networks. However, current methods for the study of plant-AMF mutualism have proven to be insufficient for comprehensive characterization of many proposed functions of AMF and exchange dynamics with plant hosts. To begin to address this methodological barrier, I have developed a divided microcosm system that allows differential nutrient uptake by roots and AMF hyphae to enable the study of nutrient transfer between AMF and whole host plants. The divided microcosms were rigorously tested and experimentally validated to demonstrate their effectiveness. This system, paired with isotopic labeling experiments, will allow for numerous high-throughput studies to be conducted to investigate aspects of host nutrient acquisition and interplant transfer.

5.2 Introduction & Background

Plants develop numerous relationships with organisms across the tree of life to help meet their reproductive, defense, and nutritional needs (Wall and Moore 1999; Herre et al. 1999; Newton et al. 2010; Delavaux, Smith-Ramesh, and Kuebbing 2017). Arbuscular mycorrhizal fungi (AMF) are a collection of fungal species from the phylum Glomeromycota that associate with ~70% of

all land plants; these species are considered nutritional mutualists (Neuhauser and Fargione 2004; Parniske 2008; N. C. Johnson and Graham 2013) and are able to transport water and various micro- and macronutrients, most famously phosphorus (Baylis 1959; Hayman and B. Mosse 1971; Pearson and Jakobsen 1993). Arbuscular mycorrhiza (AM) are likely the most ubiquitous terrestrial symbiosis and have been credited as a major factor in the colonization of dry land by plants over 400 million years ago (Remy et al. 1994). AMF are considered the most ecologically influential plant mutualists due to their ubiquity and net benefit to hosts. Their name is derived from a highly branched structure, the arbuscule, that forms inside host root cells and serves as a site of nutrient exchange during symbiosis (Parniske 2008). There, AMF transfer nutrients from the soil environment to their hosts in exchange for photosynthetically fixed carbon sources (Barea 1991; Augé and Duan 1991; Roth and Paszkowski 2017). Symbiotic interactions with AMF increase the surface area and physical reach of root systems resulting in improved water and nutrient uptake as well as communication with other organisms (Harrison 2005; M. Chen et al. 2018). Despite their long-shared history with land plants, their ubiquitous nature, and their obvious ecological importance for plant success, AMF are poorly-studied in comparison to some other agriculturally important symbioses, such as the relationship between legumes rhizobia (van der Heijden et al. 2015; Ferrol, Azcón-Aguilar, and Pérez-Tienda 2019).

AMF form an intimate, obligate relationship with their hosts and have no sustainable free-living state outside of the symbiosis (Bécard and Piché 1989); consequently, physically isolating the AMF environment from the host's root environment, which is extensively colonized by AMF when present, is extremely difficult. Further complicating investigations is the unique and notably mysterious genetic and cellular make-up of AMF. Having poorly defined divisions between cells, AMF species tend to be mildly heterokaryotic (having multiple, diverse nuclei within individual cells) and demonstrate an ability to move nuclei between cells up and down their hypha (Bago et al. 1999). Also unclear is the division between individual organisms; a single AMF, or genet, is nearly impossible to pinpoint and there is extensive evidence that individuals can fuse and share genetic material (Croll et al. 2009). Though admirable efforts to genetically transform species of

AMF have been made (Harrier and Millam 2001), the vast majority of modern molecular biology tools used for genetic and functional characterizations are not well-suited for studying species of the Glomeromycota. These difficulties are reflected as gaps in our knowledge of mycorrhizal symbioses. In 1948, J. L. Harley wrote, referring to ectomycorrhizas, "A great majority of observers agree that under some conditions the growth of tree seedlings bearing mycorrhizal roots greatly surpasses those without." Nearly 75 years later, research on mycorrhizal fungi research oft resembles the qualitative, black-box style investigations as a result of limited appropriate technologies. Relative to similar relationships, such as that between leguminous plants and nitrogen-fixing rhizobia, our understanding of the genetics and physiology of arbuscular mycorrhizae is far more limited. Although many researchers have made and continue to make contributions to the field, our inability to knockout well-characterized functions or even effectively alter the environment of the AMF in isolation has limited our exploration into the specifics of metabolic pathways. Some work has involved calculation-based techniques based on estimated deviations in baseline levels of (^{13}C / ^{12}C) and conversion of ^{14}C (Bq) to ^{14}C content (mg) for the purpose of determining assessing C exchange between mycorrhizally-connected (specifically ectomycorrhizae) plants (Simard et al. 1997). Such approaches come to big conclusions based on variations not beyond what can be found in natural environments for stable isotopes and are thus limited in their ability investigate mechanisms of transfer and regulation. Others have attempted to use specialized substrates such as the use of quantum dot technology (i.e. semiconductor particles with variable optical and electronic properties that differ from larger particles due to quantum mechanics) (van't Padje, Werner, and Kiers 2021) to track uptake of phosphorus. Unfortunately, the mechanism of quantum dot uptake is unknown and the implementation of such a technique is both expensive and has the opportunity to introduce additional variation.

The examples above, though innovative, are apparent outliers in the history of AMF research; to date, the vast majority of systems deployed for experimental AM investigations that require isolation of the fungal partner have involved fine mesh divisions and a wide array of compartmented microcosm systems. In order to study metabolic exchange between host plants and AMF, a plant

growth system that allows the two organisms to maintain intimate contact while also allowing AMF to access a physically separated and distinct pool of nutrients is necessary (Mosse 1973; Kafle et al. 2021). To achieve this, researchers have used various strategies such as mesh divisions to exploit the smaller diameter of hyphae relative to plant roots (e.g. (Frey and Schüepp 1992; Schweiger, Thingstrup, and Jakobsen 1999)) and the addition of soil buffer zones to take advantage of AMF hyphae penetrating further into the soil than their hosts (e.g. (Schweiger, Thingstrup, and Jakobsen 1999; Pearson and Jakobsen 1993; X. Chen et al. 2021)). These strategies trace their roots back to the 1970s, during which many researchers were heavily reliant on the use of buried perforated or mesh bags (e.g. (Daniels and Trappe 1980; Haystead, Malajczuk, and Grove 1988)) (Figure 4.1E). These systems were flexible, affordable, field deployable (Rosling et al. 2003) and reproducible thus allowing for key insights into nutrient uptake and regulation dynamics as well as variations between populations of mycorrhizas (e.g. (Jakobsen, Gazey, and Abbott 2001)). Unfortunately, these systems are not suitable for studies involving the uptake and transfer of water mobile compounds due to diffusion throughout the interconnected soil compartments (Schweiger, Thingstrup, and Jakobsen 1999).

One of the most commonly cited and replicated versions of such systems was presented by Hodge (2001). Hodge (2001) created a 6 compartment, mesh-divided system that allowed AMF access to a litter patch and an additional uninoculated plant through a double-layered 20-μm mesh. The system, while effectively excluding most or all roots, cannot inhibit mass flow between compartments and thus required additional controls to attempt to quantify the effect of solute diffusion across the mesh barrier (Figure 4.1A). Though this system allowed the authors to observe nitrogen acquisition from an organic litter patch and draw interesting conclusions, the results demonstrated apparent differences between their inoculated and uninoculated controls despite the measures taken (Figure 3B in Hodge (2001). Additional issues include possibly cumbersome microcosm construction and maintenance as suggested by the low total number of replicated, lack of drainage, and limited durability as demonstrated by the observed 10% failure rate with plant roots breaching the mesh barriers in their systems (S1). Additional versions of the system have been implemented by

this group and others with some improvements such as the addition of drainage (e.g. (Thirkell, Cameron, and Hodge 2016)), use of a mesh-lined patch compartment (e.g. (Thirkell, Cameron, and Hodge 2016), and earlier by (F. A. Smith, Jakobsen, and S. E. Smith 2000)), physical disruption by trimming or twisting (e.g. (D. Johnson, Leake, and Read 2001)) (Figure 4.1F), or the addition of soil buffer zones to attempt to control for solute diffusion (e.g. (X. Chen et al. 2021), also seen earlier in work by (Pearson and Jakobsen 1993) and (Bethlenfalvay et al. 1991)). However, none of these systems demonstrate an ability to successfully minimize complications due to factors related to bulk solute movement between compartments, root intrusion through a barrier, or an insufficiently large number of replicates. Such complications increase variation in measurements and reduce the quantitative power of a given experiment making them less than ideal for investigations reliant on quantitative measurements.

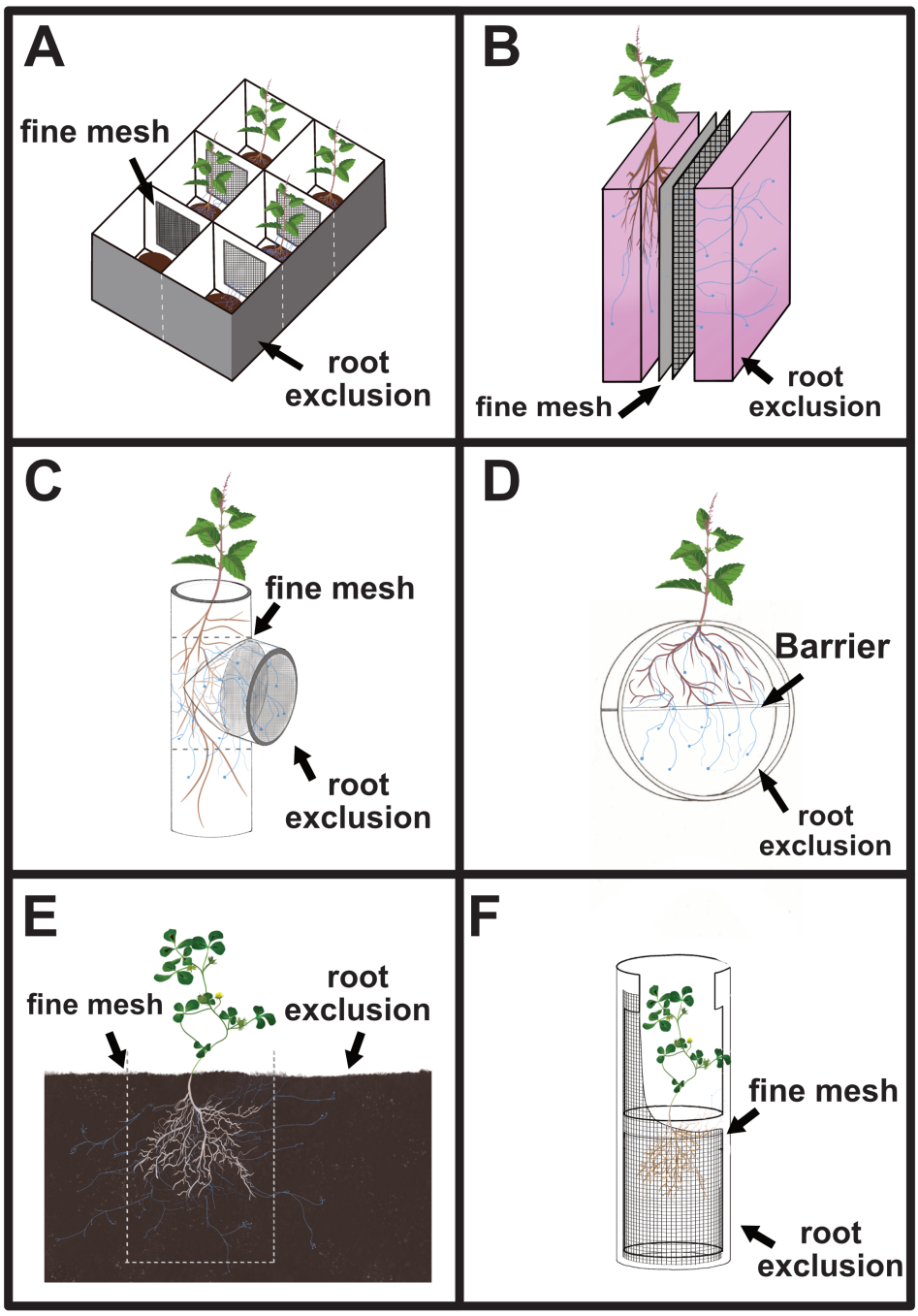
To better tackle the issue of bulk solute diffusion between compartments, some research has involved the use of a slim air-gap between two mesh-lined compartments (e.g. (Tanaka and Yano 2005)). A particularly effective version of this was demonstrated by (Tanaka and Yano 2005) (Figure 4.1B). To test N delivery to maize via AMF, the authors separated their fungal-access and plant compartments by a mesh-lined air gap that resulted from placing 1.5 mm thick wire mesh between the mesh lining of each compartment (Tanaka and Yano 2005). Although the innovative system was far from a biologically-relevant growth environment due to its size and sterility requirements, the researchers experimentally demonstrated vastly reduced diffusion of ^{15}N-containing solutes with the implementation of an air gap; the difference was significant for NH_4^+ but far less pronounced than the 40-fold reduction of NO_3^- transfer from the mycorrhizal compartment to the host with the addition of a gap. This result demonstrates the strong effect that solute diffusion across the dividing barrier can have on the experimental results. The wire separator strategy has been used with soil-based microcosms by Fellbaum et al. (2014). Though this system potentially avoids the growth and sterility issues of the previous system, the implemented separation strategy– a flat spiral of wire between two nylon mesh sheets flanked by soil– seems unlikely to guarantee an air gap and the authors provide no data to verify that their system successfully blocked solute

diffusion between compartments. Further, the system relies on distally located patch compartments (such as the horizontal extension pictured in Figure 4.1C). F. A. Smith, Jakobsen, and S. E. Smith (2000) encountered experimental problems as a consequence of implementing distal fungal-access compartments resembling those implemented in the study by Fellbaum et al. (2014); they ultimately concluded that AMF demonstrated preferential uptake of phosphorus from zones in closer proximity to the host roots.

Finally, some researchers have side-stepped issues of solute diffusion by fully separating AMF-access compartments from plants compartments. At least one study has used a "split-root" system in which researchers sever the root tip of seedlings and effectively groom plants to develop a bifurcated root system (Augé and Duan 1991; Vierheilig et al. 2000; Kafle et al. 2021). A more common approach to strict separation techniques is a modified divided Petri dish system for growing whole plants (Cooper and Tinker 1978; St-Arnaud et al. 1996). Often used for experiments involving neoplastic root cultures (St-Arnaud et al. 1996), this system uses divided Petri dishes (Sigma Aldrich) that are divided with a rigid plastic barrier. By pouring media slightly lower in the fungal compartment relative to the plant compartment, the ability of AMF to bridge gaps is again effectively exploited for spatially separating hyphae and roots. This is nicely demonstrated by recent work by Cardini et al. (2021) in which they grew whole *Medicago truncatula* plants with sterile root systems and protruding shoot tissues (Figure 4.1D). Despite being innovative and well-designed, these systems also suffer from limitations and potentially phenotype altering features. First, both systems necessitate root damage and significant on-going stress to the host roots with unknown consequences for the symbiosis (e.g. (Jasper, Abbott, and Robson 1989)). Second, the divided Petri dish system is another that severely restricts root growth and has strict sterility requirements making maintenance significantly more complicated and less biologically relevant.

Previous plant-AMF microcosm systems have undoubtedly enabled significant progress in our understanding of plant-AMF mutualisms. Unfortunately, numerous technological barriers to further enable rigorous study of plant-AMF relational dynamics still remain and our current understanding of mycorrhizal biology in areas such as regulation of nitrogen uptake by plants in the presence

Figure 5.1: Comparison of relevant microcosm systems demonstrating the use of barriers for the purpose of root exclusion



Arrows are drawn to show the presence of a fine mesh barrier or, in the instance of panel D a solid plastic barrier, and the location of the root exclusion zone/ fungal compartment. A) Adapted from (Hodge 2001), B) adapted from (Tanaka and Yano 2005), C) adapted from (Fellbaum et al. 2014), D) adapted from (Cardini et al. 2021) , E) adapted from (Jakobsen and Rosendahl 1990) , and F) adapted from (D. Johnson, Leake, and Read 2001).

of AMF, sulfur metabolism, interplay between distinct nutrient uptake and transfer pathways, and others (Bücking and Kafle 2015). Bridging these knowledge gaps will develop of technologies that are reliable, accessible, and able to be standardized across various research groups. Future microcosm systems developed to quantitatively study shared metabolism and regulation of exchange should focus on optimizing features that allow for: 1) reliable and consistent separation of plant and fungal-access compartments, 2) creation and maintenance of biologically-relevant plant growth conditions, 3) durable materials for better reusability and between-experiment sterilization, and 4) sufficiently easy construction and maintenance to allow for both larger sample sizes and multi-factor experiments. Improving the reliability, relevance, consistency, and sample sizes will very likely help researchers looking to quantitatively investigate metabolic and regulatory questions in the plant-AMF mutualism.

To this end, we present a novel microcosm system that is 1) reliably leak-proof as demonstrated by subsequent planting and harvest cycles with minimal bulk nutrient flow, 2) developed with biologically-relevant root compartment sizes, 3) built for reusability with durable, autoclave-safe materials for ease of use and cleaning purposes, and 4) sufficiently uncomplicated in construction and maintenance to allow for experiments with hundreds of plants. The system described here, constructed inexpensively and straight-forwardly, builds on the work of those before it by implementing a stainless-steel, 40 μM mesh-lined air gap maintained by frames (rather than spacers) as the divider between 2 separate compartments. The all-metal insert is fixed in place using silicone gaskets filled and lined with a silicone adhesive sealant for leak prevention. The entire pot is bound together with a PVC pipe coupling, plastic-bonding adhesive, and staples to maintain the integrity of the microcosms through robust harvesting and autoclave cycles. The system's properties were validated with multiple plant species and isotopes to ensure and demonstrate its broad applicability.

5.3 Methods

5.3.1 Microcosm materials and price

Table 5.1: Price per microcosm breakdown

Component	Quantity used	Price per microcosm in $USD (2021)
Large conetainers	1 pot	$0.34
Metal inserts	1 insert	$4.95
Wire mesh	45 sq. in.	$1.77
Steel epoxy	0.13 oz	$0.09
Silicone gasket	2 ft	$1.80
Silicone sealant	0.33 oz	$0.43
Silicone adhesive	0.15 oz	$1.89
PVC pipe coupling	$\frac{1}{2}$ coupling	$0.50
Superglue	0.05 g	$0.01
Support staples	2 staples	$0.01
Total		**$11.77**

5.3.2 Microcosm construction protocol

With the materials listed in Table 3.1, microcosm units were constructed as follows. The microcosm construction is described and graphically depicted in Figure 5.2. The protocol and provided dimensions are provided in inches (United States customary units) due to the original insert fabrication. For future replication purposes, additional details can be found in Appendix B, Tables B.1 and B.3.

1) Fabrication of metal frame for air gap insert (Figure 5.2A)

i) The insert was designed to to be 0.2 in. less in width than the diameter of the containing conetainer. This width was selected to leave space for a sealing gasket to be snugly fitted between the inner wall of the conetainer and the edges of the insert. The depth of the metal insert was selected to be 0.08 in. order to provide a suitable air gap while also

not inhibiting crossing over by fungal hyphae. The height of the insert was the height of the conetainer with 1 in of metal extended above and below. Four rectangular windows were cut to maximize open air gap space, minimize connecting surfaces to water to wick across, and maintenance of a rigid frame. Design was created using computer-aided design software (CAD) and then laser cut into stainless steel by General Filters, Inc. in Novi, MI, USA.

2) Assemble air gap insert (Figure 5.2B)

i) 40 micron stainless steel wire mesh was cut into trapezoids to be fixed on each side of the insert. The edge of the resulting trapezoid was 0.25 in. from both the edge of the frame and the each of the windows. Each insert required two mesh trapezoids.

ii) A thin, continuous bead of steel reinforced epoxy was applied around each window and a layer of mesh was adhered. Inserts were separated with parchment paper and stacked while epoxy was still setting thus allowing each mesh layer to sit flush against the insert. Epoxy was allowed to set for 24 hours.

iii) Two strips of silicone U-channel gasket material was cut to fit along the edges of the insert. Each gasket strip was filled with silicone sealant and applied to vertical edges of the metal frame. Inserts were placed into conetainers while sealant was wet.

3) Fixing insert inside conetainer (Figure 5.2C)

i) A continuous bead of silicone adhesive was laid along the full length of both sides of the outer edge of the silicone gaskets. To insert, the conetainer was gently compressed on it's vertical axis and the insert was carefully set into the container with care taken to not remove the adhesive with the edges of the conetainer until the insert was in place.

ii) Once the bottom tab of the insert was through the main drainage hole of the container, the conetainer was decompressed.

iii) Each coupling conetainer was gently dropped through $\frac{1}{2}$ of a PVC pipe coupling. The conetainer and the loosely placed coupling were placed on a large conetainer rack (Stuewe & Sons, Inc.). A continuous bead of rubber glue was placed in a circle around the conetainer at the junction with the top edge of the PVC coupling.

iv) The conetainer was then hammered in place using a large rubber mallet until the conetainer was visibly compressed by the coupling.

v) Freshly glued microcosms were left in racks and covered with approximately 20 lbs worth of weight to ensure that adhesives set without components shifting.

4) Inspection and soaking to remove residues from construction process

i) After 24 hours, each microcosm was inspected for gaps along the seal, lifting of the insert out of the container during drying, or any damage that could potentially impair the functionality of the microcosm.

ii) Microcosms were submerged in RT dH_2O for 24 hours to wash away remaining debris and to leech out acetic acid present in the silicone sealant that deterred mycorrhizal crossing over.

iii) After soaking, microcosms were autoclaved for 20 minutes at 121°C and 15 psi. Microcosms were allowed to air dry for 24 hours before assembled for experiments.

5) Microcosm assembly for plant growth (Figure 5.2D)

i) Microcosms were evenly spaced in conetainer racks with 10 pots to a 20 pot rack to prevent leaf crowding once plants matured.

ii) For each conetainer, two 15 in. lengths of rope were cut and an overhand knot was tied 5 in. from one end to form rope wicks. Wicks were placed on both sides of each microcosm and pulled through an appropriate drainage hole so that 5 in. of the wick protruded from the bottom.

iii) Reservoirs were constructed using 4oz foam cups and lids. Each lid was painted black to inhibit algal growth and had a 1 cm diameter hole punched through the center. Protruding rope wicks were fed through the hole of the reservoir lids.

5.3.3 Growth conditions

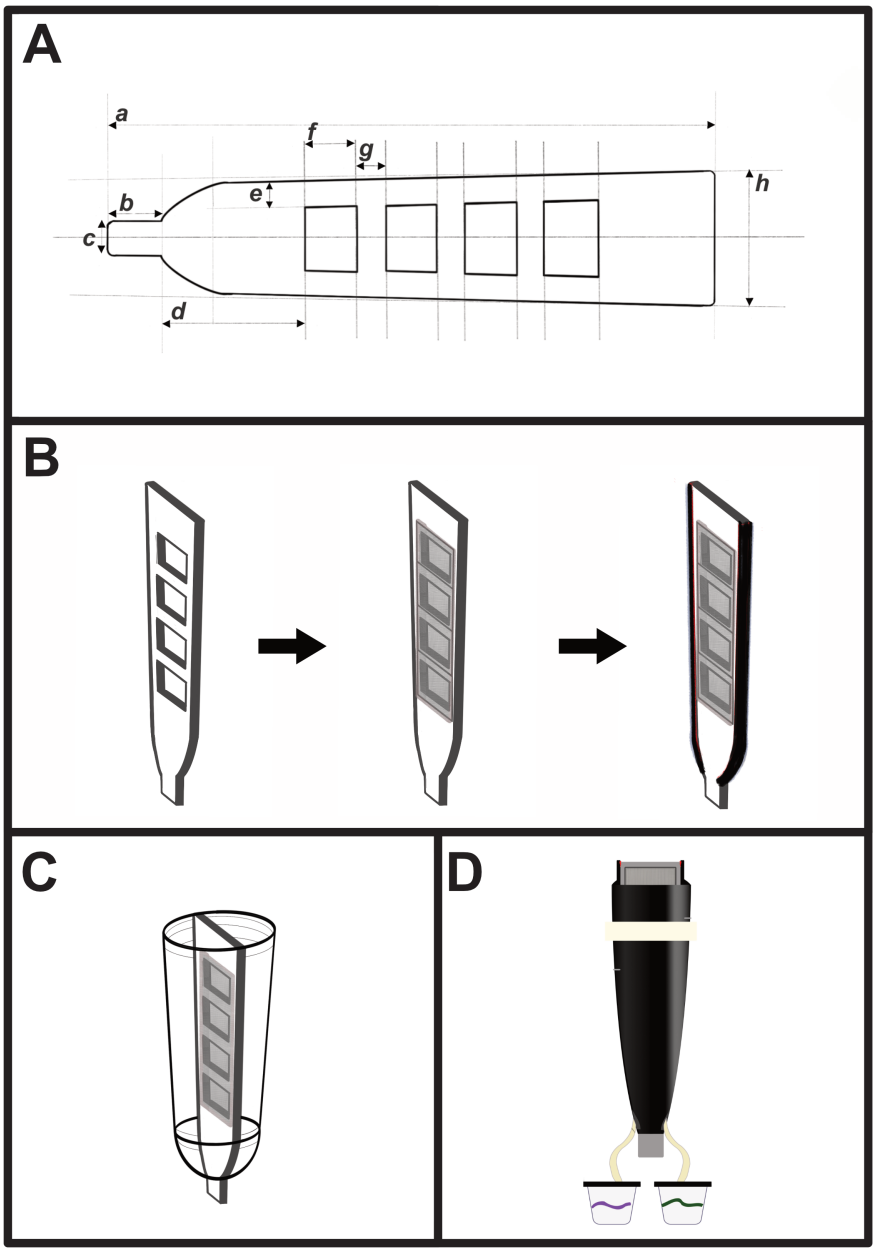
All plants were grown under a 16hr/8hr day/night cycle in climate-controlled chambers. Temperature was maintained at between 19°C and 21°C, relative humidity of 40%, and light levels of 350 μmol/m^2/s. Unless otherwise noted, plants were grown on a twice autoclaved, 3:1 mixture of SureMix Perlite (Mixture of peat, perlite, wetting agent, and starter nutrient mix by Michigan Grower Products, Inc.) and silica-based Kolorscape Washed Play Sand (Oldcastle Lawn & Garden, Inc. Atlanta, GA, USA). The appropriate nutrient levels for the fertilizer mix were experimentally determined with *Medicago trucatula* to optimize colonization by AMF and repress nodule formation by any rhizobia that may have been present in the soils (Appendix B, Table B.2).

5.3.4 Biological materials

Medicago truncatula A17 seeds were obtained from the Noble Foundation and bulked in lab. Seed pods were crushed and scarified for 8 minutes in 5 mL of concentrated Sulfuric Acid. Seeds were washed 6 times with dH_2O and sterilized with 6% Sodium Hypochlorite for 1 minute. Sterilized seeds were rinsed 6 times with sterile dH_2O and left to vernalize for 24 hours at 4°C. Seeds were placed on clean Petri dishes and remaining water was removed. Seeds were spaced evenly and the Petri dish was sealed with parafilm before inverting (seeds adhered to top the side of the Petri dish) and allowed to germinate in the dark at RT for 4 days. Germinated seeds were carefully transferred to plug trays (with and without fungal inoculum) and allowed to establish for 10 days before transfer to the final growth vessel.

The AMF used in all experiments were *Rhizophagus irregularis* cultured from a clean but non-sterile suspension of spores and hyphae obtained from Premier Tech Horticulture - CANADA

Figure 5.2: Construction of microcosm units



A) Recreation of CAD illustration for fabrication. Measurements are in inches and can be found in Appendix B, Table B.1; B) assembly process for the stainless steel air gap insert. From left to right the process begins first with the naked steel frame, second the 40 micron mesh is fixed with epoxy to both side of the frame, and third the silicone gaskets are fixed with silicone sealant; C) the assembled insert receives a solid bead of silicone adhesive along the outer edge of the gaskets and is placed inside the container; and D) a fully assembled microcosm with rope wicks and labeled reservoirs ready for planting.

(Riviere-du-Loup, QC, Canada). Non-sterile cultures were maintained at the conditions described above on *Paspalum notatum* (Pensacola Bahiagrass, Tractor Supply Co. USA) grown in 5 in. greenhouse pots. Briefly, the soil was autoclaved for 20 minutes and moistened. Bahiagrass seeds were scattered in a layer across the soil surface and just covered with additional soil. Seeds were allowed to germinate under a plastic cover for approximately 7 days. Pots were bottom watered as needed alternating between water and a P-limited, N-limited modified Hoagland's solutions (Appendix B, Table B.2). Colonization was assessed at approximately 28 days after planting (described below).

5.3.5 Colonization assessment

To visualize AMF colonization of root tissues, a modified version of the protocol detailed by Giovannetti and Mosse, 1980 was followed. Briefly, root colonization by AMF was determined by visual assessment using a compound light microscope at 10x (Leica DM2000). Root samples were taken *Medicago truncatula* planted and colonized at the same time with the same stocks as the experimental plants. Roots were thoroughly washed of debris using dH_2O and a 2 inch sub-sample was taken beginning 1 inch from the top of the root system. Roots were cleared in 10% w/v KOH for 15 minutes (Smith et al 2000) at 120°C in an autoclave. KOH was removed and roots were washed in 5% HCl for 1 minute. Roots were stained with a Trypan Blue solution (250 mL glycerol, 235 mL dH_2O, 12.5 mL glacial acetic acid, 0.05 g trypan blue stain) overnight and allowed to destain overnight in an acetic acid and glycerol solution (250 mL glycerol, 235 mL dH_2O, 12.5 mL glacial acetic acid) before visualizing with at 10x. Roots were surveyed for the presence of arbuscules, hyphae, and spores.

5.3.6 Nutrient limitation tests in microcosms for *M. truncatula*

Three N levels (Full = 13 mM, med = 8 mM, and low = 4 mM) and three P levels (Full = 1 mM, med = 20 μM, and low = 15 μM) were tested for effects on plant biomass and colonization by AMF. Eight different fertilizer solutions were developed by modifying the Hoagland's solution

(Hoagland 1950) and tested on both colonized and uncolonized *M. truncatula*. After 6 weeks of treatment, plants were harvested and root and shoot tissues were separated and dried at 60°C for 2 weeks. Biomass was determined by dry weight measurements. Fertilizer recipes can be found in Appendix B, Table B.2.

5.3.7 Tissue sample preparation and metabolite extraction for liquid scintillation counting

To assess uptake of radioisotopes by host plants, polar metabolites were triple extracted and then analyzed by liquid scintillation counting (LSC). First, dried tissue samples were placed in 50 mL conical tubes with two 6mm glass beads and shaken for 30 minutes or until powdered at 4000 RPMs in a high-speed benchtop centrifuge at 4°C (15 Amp Centrifuge 5810 R, Eppendorf). Next, 2 mL of a cold 80% methanol, 20% dH_2O were added to each sample and vortexed for approximately 1 minute and solution appeared homogeneous. Samples were centrifuged for 10 minutes at 4000 RPMs (max speed) and the supernatant was collected in a new tube. After 3 extractions, the remaining pellet in the original conical tubes was allowed to dry overnight in a laminar flow hood and the previously collected supernatant samples were evaporated under N_2 gas to reduce their volume by approximately 80%. Following evaporation, samples were decolorized with 1 mL of 30% H_2O_2 at 50°C for 2 hours. Following decolorization, 0.5 mL of each sample was mixed with 2 mL of Safety Solve Liquid Scintillation Cocktail (RPI) in 4 mL plastic LSC vials. After at least 1 hour of dark adjustment, samples were processed on a PerkinElmer 1450 MicroBeta TriLux Microplate Scintillation and Luminescence Counter for 5 minutes per sample. Each sample was read twice separated by a 2 hour waiting period; readings were compared for relative similarity to detect for delayed chemiluminescence and to ensure sufficient dark adjustment for the first samples read. Controls included untreated plants, positive control plants grown in non-divided pots which received an equivalent radiation dose directly to the roots, and process controls with no plant tissue to test for solvent contamination or machinery issues. Tissue samples were compared against controls to check for both color-quenching and chemiluminescence.

5.3.8 Between-compartment leak to determine extent of bulk nutrient flow

Initially, pots were assessed for leaks by growing AMF-free *M. truncatula* in 20 divided microcosm pots for 3 weeks. At the beginning of week four, 4 μCi of ^{35}S in the form of $Na_2^{35}SO_4$ (PerkinElmer) was added to the fungal (no-plant) compartment of each pot. A 1 cm^3 cotton wool trap submerged 1 inch below the surface of the growth substrate and perpendicular from the metal insert was added during pot construction. Traps were retrieved at harvest and soaked for 24 hours in 5 mL of dH_2O. For subsequent tests, each pot was assigned a unique identifier and tracked through each cycle. *Camelina sativa* (a Brassica) was used for leak testing because of its fast growth rate, larger root systems relative to *Arabidopsis thaliana*, and inability to form associations with AMF which guards against any accidental colonization. *Camelina sativa* seeds were started in 1.6 cu. inch propagation plugs with autoclaved SureMix and grown under a transparent dome for 1 week before transfer to the microcosms. For each cycle, microcosms were filled with a 2:1 mixture of SureMix soil and washed play sand in both compartments. A standardized cotton wool trap was placed approximately 1.5 inches below the surface of the soil in each compartment. 1 week old *C. sativa* was transplanted to one side (plant compartment) of the fully constructed microcosms and allowed 3 days to establish. On day 4, each microcosm received 4 mL of dH_2O containing 4 μCi of ^{35}S. Radiation was slowly added to the second (fungal) compartment perpendicular to and fully opposite of the divider using a 5 mL pipette as to minimize the risk of flooding the the air gap with a sudden influx of fluid. Watering via reservoirs continued for 1 week after the addition of the radioisotope solution. On day 7, cotton traps were retrieved and soaked in 3 mL of dH_2O overnight. Cotton traps were pressed to the bottom of their respective tubes and the resulting supernatant was collected and mixed with RPI Safety-Solve Liquid Scintillation Cocktail. After a dark adjustment period of approximately 1 hour, samples were analyzed for counts per a minute (CPM) for 5 minutes per sample with a PerkinElmer TriLux 3000. The samples were measured twice. Each pot was emptied, washed, and autoclaved for 20 minutes between cycles. The testing was completed over 5 cycles. A pot was determined to have passed a cycle of testing if the sample from the compartment treated with radiation was at least 500 CPM and the untreated compartment was within a factor of

4 of the background in the unlabeled plant control LSC samples. These thresholds were set based on variation observed in machine measurements over the course of radioisotope decay and based on the experimentally observed bounds and degree of seperation between background samples and positive samples in pilot studies. Samples with sufficiently low untreated compartment samples but treated compartment samples below the 500 CPM threshold were given a NA score. Before each cycle, microcosms were inspected for root intrusion across the air-gap barrier and separation along the silicone seal (Appendix B, Table B.4).

5.3.9 Determining crossing of air gap barrier by AMF

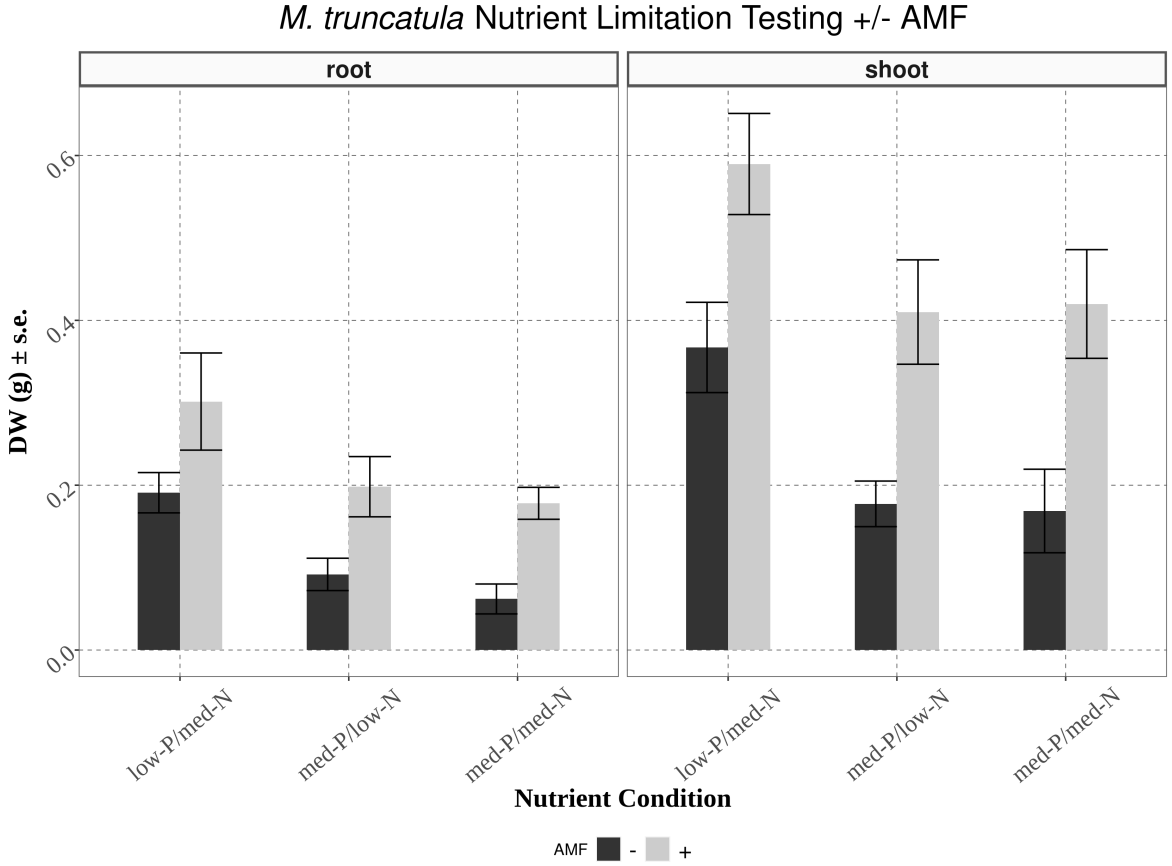
Inoculated and uninoculated *M. truncatula* plants were germinated on Petri dishes (described above) and established in 1.6 cu. in. plugs for 1 week before transfer to microcosms. Plants allowed to grow for 3 weeks in the microcosms. As with the previous leak tests, cotton wool traps were buried ~1.5 inches below the surface of the growth substrate for leak testing. Plants were starved of phosphorus during the first round of watering (approximately 3 days) after the 3 week growth period. At the time of the next watering (also without phosphorus), 4 μCi of ^{32}P in the form of $KH_2{}^{32}PO_4$ (PerkinElmer) in 4 mL of dH_2O was carefully supplied to the top of the fungal compartment (as described above) to avoid flooding of the air gap barrier. Plants then received their regular med-P/med-N fertilizer solution (Appendix Table B.2) for the remainder of the testing period.

Plants were harvested after 10 days post-treatment with ^{32}P. Four samples were collected from each pot: #1) sand sample for fungal compartment radiation leak test, #2) soil sample for plant compartment radiation leak test, #3) root sample for biomass and CPM measurements, and #4) shoot sample for biomass and CPM measurements. Liquid scintillation counting was performed as described previously.

5.4 Results

5.4.1 Establishing nutrient limiting conditions for *M. truncatula*

Figure 5.3: Nutrient limitation and growth enhancement tests for *Medicago truncatula* grown in divided microcosms



Plants were 8 weeks old at harvest. Roots and shoots were separated and tissues were dried and weighed. 2 samples in the "+AMF med-P/low-N" treatment developed nodules and were excluded. 2 samples from the "-AMF med-P/low-N" treatment were senescent before the end of the treatment period and were also excluded. n = 8 except where otherwise noted.

A solution for plant fertilization was selected by experimentally determining the appropriate levels of N and P for optimal colonization and AMF benefit with *M. truncatula* in the microcosms. Each microcosm root compartment can hold a soil volume of approximately 150 mL. In combination with the soil mix (3:1 (v/v) SureMix and washed play sand), the optimal nutrient conditions for the system were selected based on plant survival rate, suppression of colonization by unintentionally

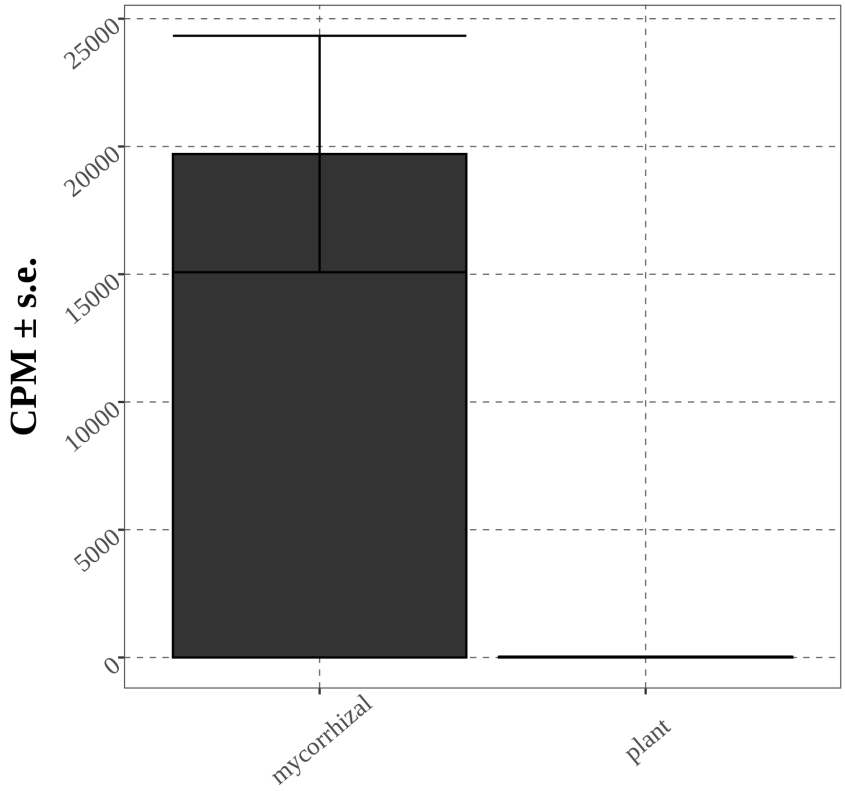
present rhizobial bacteria, and a clear growth benefit from colonization by AMF (Figure 5.3). Using these criteria, the nutrient solution was modified to have "med" levels for both phosphorus and nitrogen with the final recipe as follows: 9 mM KCl, 5 mM $CaCl_2$, 2 mM $MgSO_4$, 4 mM NH_4NO_3, 20 μM KH_2PO_4, 200 μM Fe-EDTA, and 1x Micronutrient Solution as described in Hoagland (1950).

5.4.2 Air gap barrier integrity and microcosm reusability demonstrated with repeated leak testing

The elimination of nutrient flow via the bulk diffusion of water between compartments is crucial for the separation of fungal and the root compartments. By ensuring predictable and consistent separation of the two zones, uptake by the extraradical mycelium can be effectively investigated by preventing the roots from directly accessing the substrates available in the fungal compartment. Microcosms were first assessed by determining the extent of bulk flow across the mesh-lined air gap barrier. This was achieved by sampling both compartments 1 week after the addition of $Na_2{}^{35}SO_4$. Durability and reusability were assessed on 10 randomly selected microcosms constructed exactly as described above (Figure 4.4A).

Repeated testing indicated that microcosms were able to undergo at least 5 consecutive construction, experiment, harvest, and cleaning cycles without compromised barriers (Figure 5.4a; Appendix B, Table B.4). Repeated testing of the microcosms enabled greater confidence in the source of labeled substrates delivered to plant tissues in experiments by eliminating ambiguity surrounding the degree of bulk nutrient flow between compartments. Further, in the event of between-compartment leaks, the leak-testing process serves as a check against drawing erroneous conclusions on isotopic uptake via fungal partners that may have been derived from a contaminated root compartment.

Figure 5.4: Testing for movement of water across air gap barrier using ^{35}Sulfur



Pot ID	Iteration	CPM: plant	CPM: myc.	Pass/Fail?
	1	36	6049	Pass
	2	56	8878	Pass
301	3	74	871	Pass
	4	29	448	Pass
	5	30	354	Pass

In Figure 5.4A, we are testing for movement of ^{35}S between compartments when grown with uninoculated *M. truncatula*. Results are shown in counts per minute (CPM) and error bars represent standard errors. Background ^{35}S counts were determined to be approximately 26 CPM. n=10 and $p<0.001$ for roots and shoots (paired t-test). In Figure 5.4B, a single microcosm is presented. Results were generated by liquid scintillation counting and are again measured in counts per minute (CPM). CPM of control samples representing background count levels for iterations 1-5 were established to be 31, 58, 59, 44, and 24 CPM (respectively). Each interaction was checked for movement of radioisotope across barrier and assigned a result of "pass" or "fail" to indicate status of test (further described in methods). Full results for all 10 microcosms tested can be found in Appendix B, Table B.4

5.4.3 Transfer of ^{32}Phosphorus via AMF from the fungal compartment to the host plant

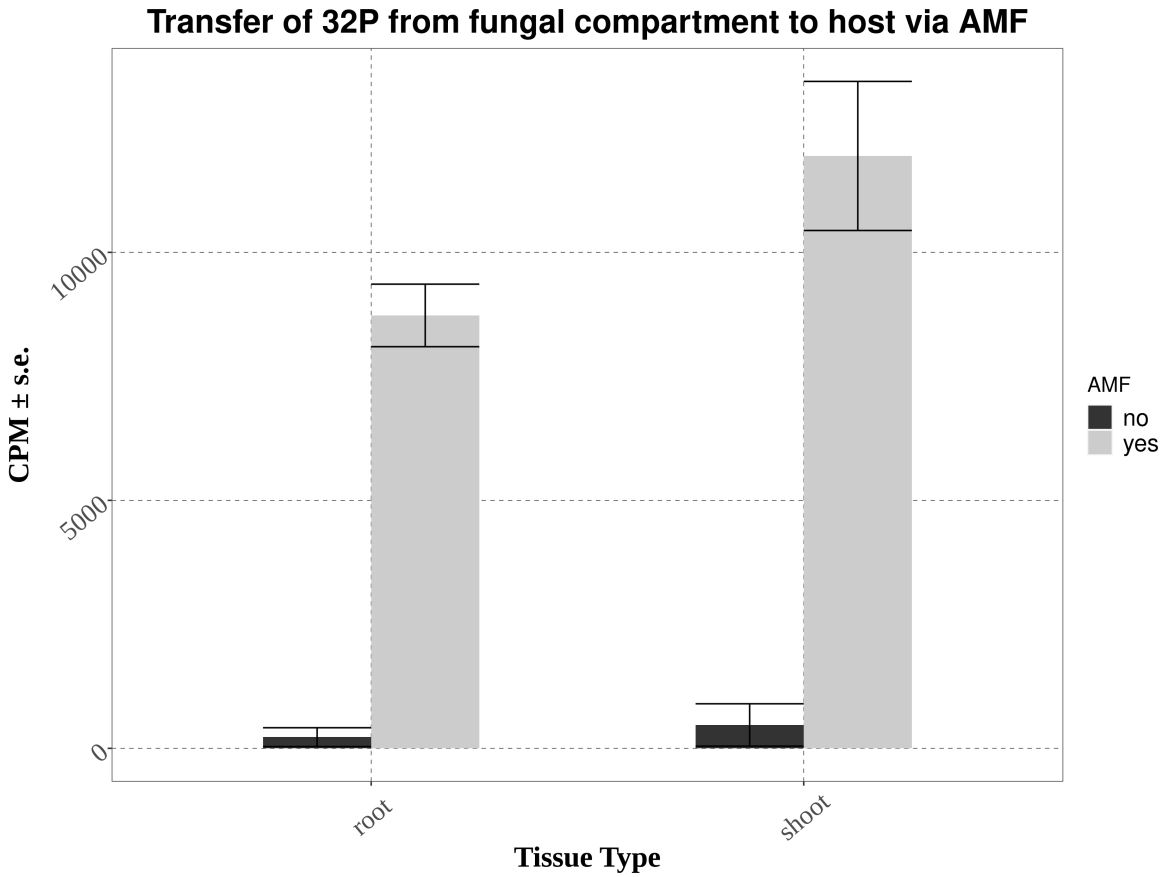
To test if AMF were able to successfully cross over the mesh-lined air gap barrier, AMF-colonized and uncolonized plants were assessed for their ability to transfer ^{32}Phosphorus from the fungal compartment to their host plant. This was verified by direct observation of fungal material in the distal compartment after inoculating in the plant compartment by wet-sieving for fungal structures.

Medicago truncatula accumulated significant quantities of ^{32}P from the fungal compartment over the duration of the treatment. The results were strongly signifiant in both roots and shoots with $p<0.001$ for both tissue types. The results presented here contain an "outlier" that resulted from an apparent leaking event (verified with cotton wool trap sampling as described previously). While most non-AMF plants showed nearly background levels of ^{32}P accumulation in their tissues, one non-AMF plant yielded CPM values of 2503 for the roots and 5599 for the shoots. These highly elevated CPM levels strongly indicate a leak. Without the exclusion of the leaky pot, the significant difference between AMF and non-AMF plants remained at the $p<0.001$ level (Figure 5.5).

5.5 Conclusions & Future Directions

The goals of this work were primarily to develop a simple, reusable, and effective divided microcosm system that would promote quantitative whole-plant investigations of plant-AMF symbioses. First and foremost, it was critical to create a reliably leak-proof barrier for the selective exclusion of plant roots while allowing the hyphae of associated AMF to pass. The rigidly constructed air gap barrier was largely sufficient for this purpose (Figure 5.4; Appendix B, Table B.4). Further, the durability of this barrier over repeated use (Appendix B, Table B.4) demonstrated an apparent impermeability to the roots of *M. truncatula* and *C. sativa*. The rigid frame and mesh material prevented the air gap from collapsing under the external pressures exerted on it over the course of multiple experiments. Unfortunately, there was some evidence of leaks likely as a consequence of flooding during watering of host plants (Figure 5.5). Those future applications of divided microcosm systems would benefit from establishing a rigid barrier such as the one here, modifications to

Figure 5.5: Transfer of ^{32}P to host *M. truncatula* via AMF in divided microcosms



Results are expressed in Counts Per a Minute (CPM) with error bars showing standard error. ^{32}P was applied to the distal (non-plant) compartment of each microcosm and radioactivity in plant tissues show differences between inoculated and uninoculated host plants. n=15 for both treatment groups and $p<0.001$ for roots and shoots (paired t.test). Controls can be found in Appendix B, Figure B.1.

further reduce the chances of flooding would be welcome.

In addition to the establishment of a durable and reliable leak-proof barrier, it was important for plants grown in this system to demonstrate a growth benefit from AMF colonization. Given the large body of evidence that suggests plant-AMF associations do not strictly function as mutualisms (Hammer et al. 2011), intentionally creating such an environment is necessary under artificial conditions. By testing this system with various fertilizers solutions, I was able to recreate the dynamic needed for symbiotic establishment (Figure 5.3). Future studies aimed at investigating a feature of the mutualism would be benefited by establishing optimal conditions in advance. These conditions undoubtedly contributed to the strength of the ^{32}P uptake response by colonized host

plants observed during the validation efforts presented here (Figure 5.5). Finally, the ease of initial and repeated construction allows a single researcher to reasonably handle hundreds of plants for a given experiment. This scale is essential for developing sufficient statistical power in whole-plant studies and in multi-species studies. Future methodological developments must consider ease of access and use in hopes of promoting investigations of plant-biotic interactions under multiple pressures.

Our current ability to interrogate changes in metabolic exchange and regulation in plant-AMF symbioses under variable environmental conditions is severely limited due to prohibitive methodology gaps (Parniske 2008). Challenges in manipulating plant-AMF must be overcome to in order to meaningfully ask questions about how mechanisms of exchange vary under different environmental conditions. To this end, I have demonstrated the conception, construction, and rigorous testing of a divided microcosm system for the study of plant-AMF symbioses. Future work with similar goals would be benefited by the adoption and use of the strategies used here.

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We extend a special thank you to Larry Scott for his CAD expertise and his assistance with the fabrication the metal frames used for the microcosms. This research was supported by a fellowship from Michigan State University under the Training Program in Plant Biotechnology for Health and Sustainability (T32-GM110523 to S.L.R.). This material is based in part upon work supported by the National Science Foundation under Cooperative Agreement No. DBI-0939454. 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.

BIBLIOGRAPHY

BIBLIOGRAPHY

St-Arnaud, M. et al. (Mar. 1, 1996). "Enhanced Hyphal Growth and Spore Production of the Arbuscular Mycorrhizal Fungus Glomus Intraradices in an in Vitro System in the Absence of Host Roots". In: *Mycological Research* 100.3, pp. 328–332.

Augé, Robert M. and Xiangrong Duan (Oct. 1, 1991). "Mycorrhizal Fungi and Nonhydraulic Root Signals of Soil Drying". In: *Plant Physiology* 97.2, pp. 821–824.

Bago, B. et al. (Mar. 1, 1999). "Nuclei of Symbiotic Arbuscular Mycorrhizal Fungi as Revealed by in Vivo Two-Photon Microscopy". In: *Protoplasma* 209.1, pp. 77–89.

Barea, J. M. (1991). "Vesicular-Arbuscular Mycorrhizae as Modifiers of Soil Fertility". In: *Advances in Soil Science: Volume 15*. Ed. by B. A. Stewart. Advances in Soil Science. New York, NY: Springer, pp. 1–40.

Baylis, G. T. S. (1959). "Effect of Vesigular-Arbuscular Mycorrhizas on Growth of Griselinia Littoralis (Cornaceae)". In: *New Phytologist* 58.3, pp. 274–278.

Bécard, G. and Y. Piché (1989). "New Aspects on the Acquisition of Biotrophic Status by a Vesicular—Arbuscular Mycorrhizal Fungus, Gigaspora Margarita". In: *New Phytologist* 112.1, pp. 77–83.

Bethlenfalvay, Gabor J. et al. (1991). "Nutrient Transfer between the Root Zones of Soybean and Maize Plants Connected by a Common Mycorrhizal Mycelium". In: *Physiologia Plantarum* 82.3, pp. 423–432.

Bücking, Heike and Arjun Kafle (Dec. 2015). "Role of Arbuscular Mycorrhizal Fungi in the Nitrogen Uptake of Plants: Current Knowledge and Research Gaps". In: *Agronomy* 5.4 (4), pp. 587–612.

Cardini, Alessio et al. (2021). "Direct Transfer of Zinc between Plants Is Channelled by Common Mycorrhizal Network of Arbuscular Mycorrhizal Fungi and Evidenced by Changes in Expression of Zinc Transporter Genes in Fungus and Plant". In: *Environmental Microbiology* 23.10, pp. 5883–5900.

Chen, Min et al. (Sept. 4, 2018). "Beneficial Services of Arbuscular Mycorrhizal Fungi – From Ecology to Application". In: *Frontiers in Plant Science* 9, p. 1270.

Chen, Xue et al. (2021). "Nitrogen Uptake, Not Transfer of Carbon and Nitrogen by CMN, Explains the Effect of AMF on the Competitive Interactions Between Flaveria Bidentis and Native Species". In: *Frontiers in Ecology and Evolution* 9, p. 103.

Cooper, Karen M. and P. B. Tinker (1978). "Translocation and Transfer of Nutrients in Vesicular-Arbuscular Mycorrhizas". In: *New Phytologist* 81.1, pp. 43–52.

Croll, Daniel et al. (2009). "Nonself Vegetative Fusion and Genetic Exchange in the Arbuscular Mycorrhizal Fungus Glomus Intraradices". In: *New Phytologist* 181.4, pp. 924–937.

Daniels, B. A. and J. M. Trappe (May 1980). "Factors Affecting Spore Germination of the Vesicular-Arbuscular Mycorrhizal Fungus, *Glomus Epigaeus*". In: *Mycologia* 72.3, pp. 457–471.

Delavaux, Camille S., Lauren M. Smith-Ramesh, and Sara E. Kuebbing (2017). "Beyond Nutrients: A Meta-Analysis of the Diverse Effects of Arbuscular Mycorrhizal Fungi on Plants and Soils". In: *Ecology* 98.8, pp. 2111–2119.

Fellbaum, Carl R. et al. (2014). "Fungal Nutrient Allocation in Common Mycorrhizal Networks Is Regulated by the Carbon Source Strength of Individual Host Plants". In: *New Phytologist* 203.2, pp. 646–656.

Ferrol, Nuria, Concepción Azcón-Aguilar, and Jacob Pérez-Tienda (Mar. 2019). "Review: Arbuscular Mycorrhizas as Key Players in Sustainable Plant Phosphorus Acquisition: An Overview on the Mechanisms Involved". In: *Plant Science: An International Journal of Experimental Plant Biology* 280, pp. 441–447.

Frey, Beat and Hannes Schüepp (1992). "Transfer of Symbiotically Fixed Nitrogen from Berseem (Trifolium Alexandrinum L.) to Maize via Vesicular—Arbuscular Mycorrhizal Hyphae". In: *New Phytologist* 122.3, pp. 447–454.

Hammer, Edith C. et al. (May 2011). "Tit for Tat? A Mycorrhizal Fungus Accumulates Phosphorus under Low Plant Carbon Availability: P Accumulation in an AMF under Low Plant C Status". In: *FEMS Microbiology Ecology* 76.2, pp. 236–244.

Harrier, Lucy A. and Steve Millam (May 1, 2001). "Biolistic Transformation of Arbuscular Mycorrhizal Fungi". In: *Molecular Biotechnology* 18.1, pp. 25–33.

Harrison, Maria J. (2005). "Signaling in the Arbuscular Mycorrhizal Symbiosis". In: *Annual Review of Microbiology* 59.1, pp. 19–42.

Hayman, D. S. and B. Mosse (1971). "Plant Growth Responses to Vesicular-Arbuscular Mycorrhiza". In: *New Phytologist* 70.1, pp. 19–27.

Haystead, A., N. Malajczuk, and T. S. Grove (1988). "Underground Transfer of Nitrogen between Pasture Plants Infected with Vesicular-Arbuscular Mycorrhizal Fungi". In: *New Phytologist* 108.4, pp. 417–423.

Herre, E. A. et al. (Feb. 1, 1999). "The Evolution of Mutualisms: Exploring the Paths between Conflict and Cooperation". In: *Trends in Ecology & Evolution* 14.2, pp. 49–53.

Hoagland, Arnon (1950). "The Water-Culture Method for Growing Plants without Soil". In: *Circular. California Agricultural Experiment Station* 347.2, p. 32.

Hodge, Angela (2001). "Arbuscular Mycorrhizal Fungi Influence Decomposition of, but Not Plant Nutrient Capture from, Glycine Patches in Soil". In: *New Phytologist* 151.3, pp. 725–734.

Jakobsen, I., C. Gazey, and L. K. Abbott (2001). "Phosphate Transport by Communities of Arbuscular Mycorrhizal Fungi in Intact Soil Cores". In: *New Phytologist* 149.1, pp. 95–103.

Jakobsen, I. and L. Rosendahl (1990). "Carbon Flow into Soil and External Hyphae from Roots of Mycorrhizal Cucumber Plants". In: *New Phytologist* 115.1, pp. 77–83.

Jasper, D. A., L. K. Abbott, and A. D. Robson (1989). "Soil Disturbance Reduces the Infectivity of External Hyphae of Vesicular—Arbuscular Mycorrhizal Fungi". In: *New Phytologist* 112.1, pp. 93–99.

Johnson, D., J. R. Leake, and D. J. Read (2001). "Novel In-Growth Core System Enables Functional Studies of Grassland Mycorrhizal Mycelial Networks". In: *New Phytologist* 152.3, pp. 555–562.

Johnson, Nancy Collins and James H. Graham (Feb. 1, 2013). "The Continuum Concept Remains a Useful Framework for Studying Mycorrhizal Functioning". In: *Plant and Soil* 363.1, pp. 411–419.

Kafle, Arjun et al. (Nov. 17, 2021). "Split down the Middle: Studying Arbuscular Mycorrhizal and Ectomycorrhizal Symbioses Using Split-Root Assays". In: *Journal of Experimental Botany*, erab489.

Mosse, B (Sept. 1973). "Advances in the Study of Vesicular-Arbuscular Mycorrhiza". In: *Annual Review of Phytopathology* 11.1, pp. 171–196.

Neuhauser, Claudia and Joseph E. Fargione (Oct. 1, 2004). "A Mutualism–Parasitism Continuum Model and Its Application to Plant–Mycorrhizae Interactions". In: *Ecological Modelling* 177.3, pp. 337–352.

Newton, Adrian C. et al. (2010). "Pathogenesis, Parasitism and Mutualism in the Trophic Space of Microbe-Plant Interactions". In: *Trends in Microbiology* 18.8, pp. 365–373.

Parniske, Martin (2008). "Arbuscular Mycorrhizae: The Mother of Plant Root Endosymbioses". In: *Nature reviews. Microbiology* 6.10, pp. 763+.

Pearson, J. N. and I. Jakobsen (1993). "The Relative Contribution of Hyphae and Roots to Phosphorus Uptake by Arbuscular Mycorrhizal Plants, Measured by Dual Labelling with 32P and 33P". in: *New Phytologist* 124.3, pp. 489–494.

Remy, W. et al. (Dec. 6, 1994). "Four Hundred-Million-Year-Old Vesicular Arbuscular Mycorrhizae." In: *Proceedings of the National Academy of Sciences* 91.25, pp. 11841–11843.

Rosling, A. et al. (2003). "Vertical Distribution of Ectomycorrhizal Fungal Taxa in a Podzol Soil Profile". In: *New Phytologist* 159.3, pp. 775–783.

Roth, Ronelle and Uta Paszkowski (Oct. 1, 2017). "Plant Carbon Nourishment of Arbuscular Mycorrhizal Fungi". In: *Current Opinion in Plant Biology*. 39 Cell Signalling and Gene Regulation 2017 39, pp. 50–56.

Schweiger, P. F., I. Thingstrup, and I. Jakobsen (Jan. 8, 1999). "Comparison of Two Test Systems for Measuring Plant Phosphorus Uptake via Arbuscular Mycorrhizal Fungi". In: *Mycorrhiza* 8.4, pp. 207–213.

Simard, Suzanne W. et al. (Aug. 1997). "Net Transfer of Carbon between Ectomycorrhizal Tree Species in the Field". In: *Nature* 388.6642 (6642), pp. 579–582.

Smith, F. A., I. Jakobsen, and S. E. Smith (Aug. 2000). "Spatial Differences in Acquisition of Soil Phosphate between Two Arbuscular Mycorrhizal Fungi in Symbiosis with Medicago Truncatula". In: *The New Phytologist* 147.2, pp. 357–366.

Tanaka, Yoko and Katsuya Yano (2005). "Nitrogen Delivery to Maize via Mycorrhizal Hyphae Depends on the Form of N Supplied". In: *Plant, Cell & Environment* 28.10, pp. 1247–1254.

Thirkell, Tom J., Duncan D. Cameron, and Angela Hodge (2016). "Resolving the 'Nitrogen Paradox' of Arbuscular Mycorrhizas: Fertilization with Organic Matter Brings Considerable Benefits for Plant Nutrition and Growth". In: *Plant, Cell & Environment* 39.8, pp. 1683–1690.

Van der Heijden, Marcel G A et al. (2015). "Mycorrhizal Ecology and Evolution: The Past, the Present, and the Future". In: *New Phytologist* 205.4, pp. 1406–1423.

Van't Padje, Anouk, Gijsbert D. A. Werner, and E. Toby Kiers (2021). "Mycorrhizal Fungi Control Phosphorus Value in Trade Symbiosis with Host Roots When Exposed to Abrupt 'Crashes' and 'Booms' of Resource Availability". In: *New Phytologist* 229.5, pp. 2933–2944.

Vierheilig, H et al. (May 1, 2000). "Systemic Suppression of Mycorrhizal Colonization of Barley Roots Already Colonized by AM Fungi". In: *Soil Biology and Biochemistry* 32.5, pp. 589–595.

Wall, Diana H. and John C. Moore (1999). "Interactions Underground: Soil Biodiversity, Mutualism, and Ecosystem Processes". In: *BioScience* 49.2, pp. 109–117.

APPENDICES

APPENDIX A

CHAPTER 3 APPENDIX

A.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 4°C

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.

Table A.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

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_2O_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 express 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 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 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 potassium 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_2O, 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 components by the total number of reactions + 1. Reaction components are as follows: 100 μL of enzyme source, 166.6 μL of Trizma base buffer (Sigma-Aldrich), and 83.3 μL of 2% azocasein 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.

A.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)

A.3 Additional figures

Figure A.1: The standard curve generated to quantify hydrogen peroxide production. Absorbance values were measured at 390 nm

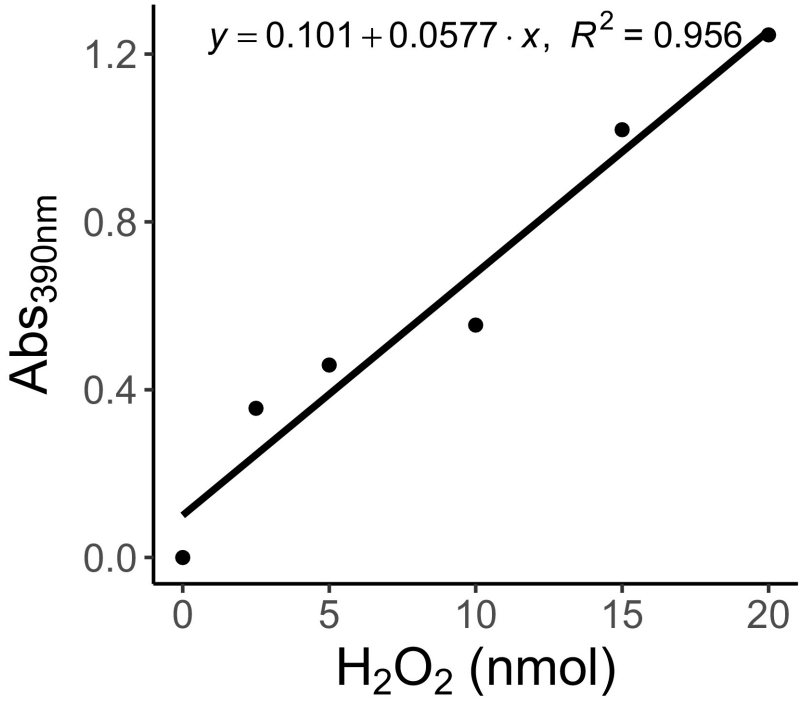
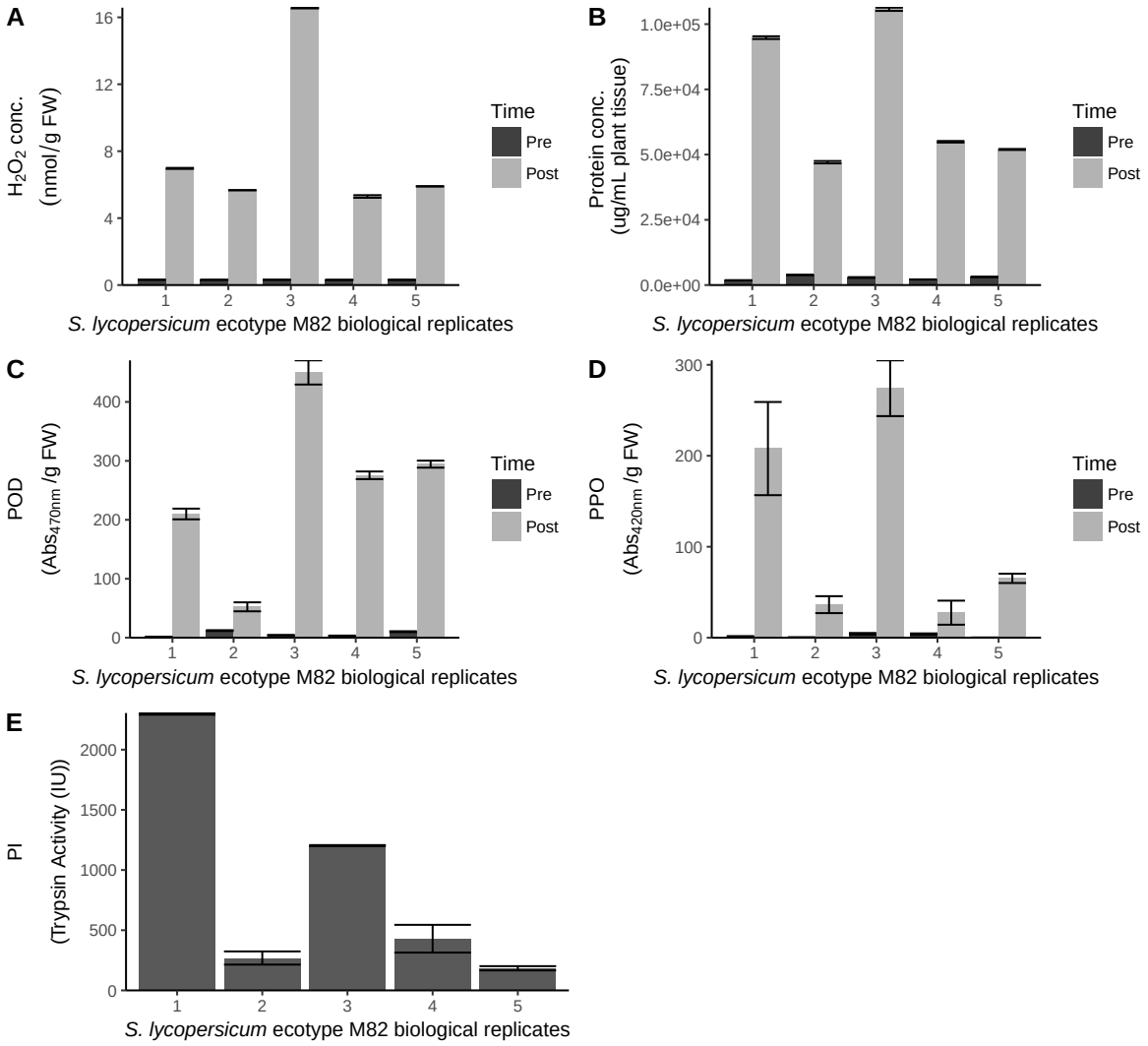
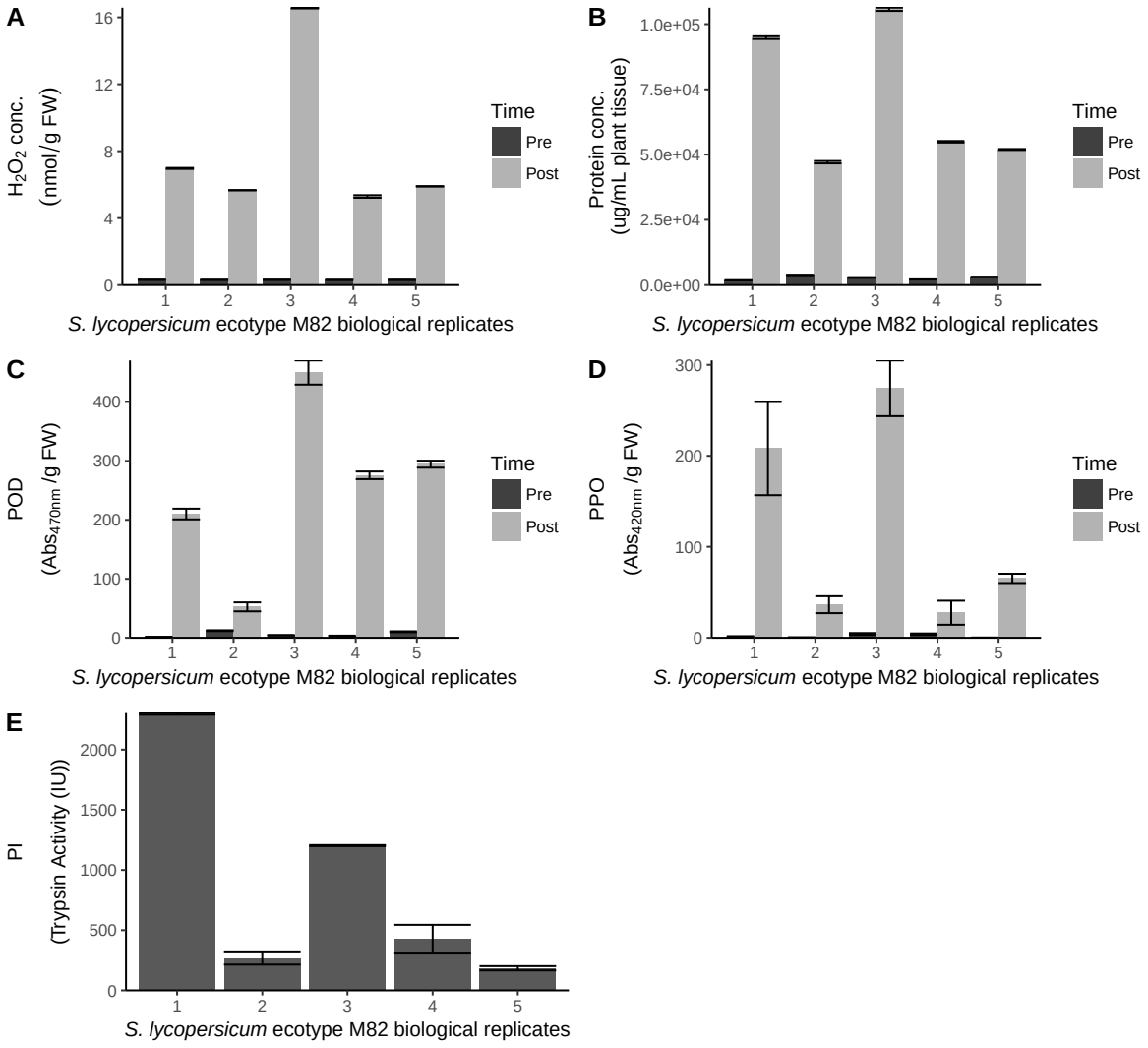


Figure A.2: Results of our microplate-based protocols using *Solanum lycopersicum* (ecotype M82)



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 pre- and post-herbivory. Trypsin activity is determined by the difference in inhibition pre- and post- herbivory, 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.

Figure A.3: Assays tested on *Medicago polymorpha*



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 pre- and post- mechanical wounding with regurgitant to simulate herbivory. Trypsin activity is determined by the difference in inhibition pre- and post-mechanical 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.

APPENDIX B

CHAPTER 5 APPENDIX

B.1 Construction of divided microcosms

Table B.1: Measurements (in.) of microcosm insert for fitting the conetainers used in this publication. CAD drawing in Figure 5.2A

Diagram label	Description	Measurement (in.)
a	Height of frame	11.41
b	Bottom extension length	1.00
c	Bottom extension width	0.64
d	Bottom of window to bottom of the pot	2.71
e	Window to edge	0.50
f	Window height	1.00
g	Gap between windows	0.50
h	Width at top	2.52

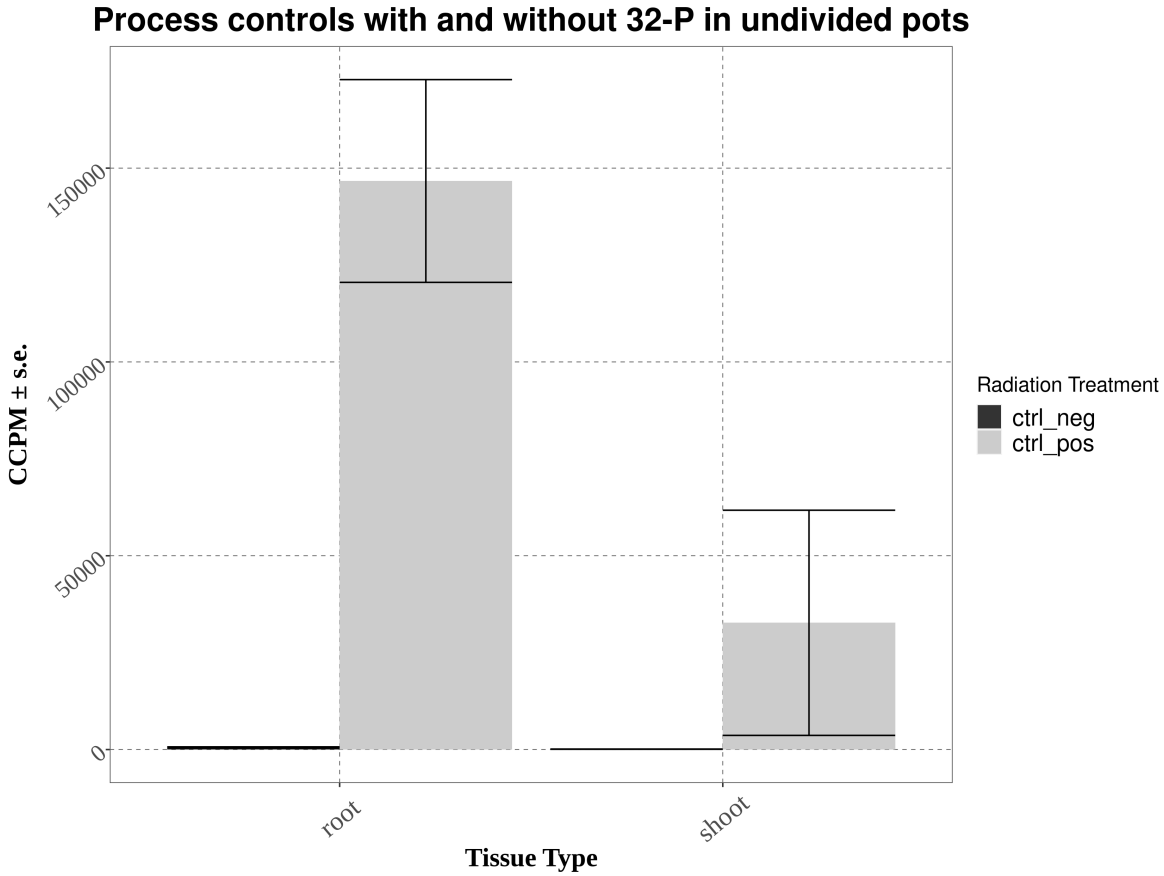
Table B.2: Fertilizer solutions used for nutrient limitation tests in microcosms with *M. truncatula*

Nutrient	Full	low-P/med-N	med-P/low-N	med-P/med-N
KNO_3	4mM	–	–	–
KCl	5 mM	9 mM	9 mM	9 mM
$Ca(NO_3)_2$	2.5 mM	–	–	–
$CaCl_2$	2.5 mM	5 mM	5 mM	5 mM
$MgSO_4$	2 mM	2 mM	2 mM	2 mM
NH_4NO_3	2 mM	4 mM	2 mM	4 mM
KH_2PO_4	1 mM	15 μM	20 μM	20 μM
Micronutrients	1x	1x	1x	1x
Fe-EDTA	200 μM	200 μM	200 μM	200 μM

Table B.3: Microcosm component sourcing information

Component	Item information	Supplier	Location
Large conetainers	D25L	Stuewe & Sons, Inc.	Corvallis, OR, USA
Metal inserts	custom fabrication	General Filters Inc	Novi, MI, USA
Wire mesh	Mesh T316 Micronic Stainless .0055"x.0045" Wire Dia.	TWP Inc	Berkeley, CA, USA
Silicone gasket	Dimensions: W = 0.335" H = 0.335" S = 0.110" D = 0.250"	Elastostar Rubber Corporation	Columbus, OH, USA
Steel epoxy	J-B Weld 8281 Professional Size Steel Reinforced Expoxy	J-B Weld	via Amazon (USA)
Silicone sealant	J-B Weld 31914 Red High Temperature RTV Silicone Gasket Maker and Sealant	J-B Weld	via Amazon (USA)
Silicone adhesive	SIL-poxy Rubber Silicone Adhesive	Smooth-On, Inc.	Macungi, PA, USA
PVC pipe coupling	2"H x H PVC DWV Repair Coupling 4801-RP	Ferguson.com	Newport News, VA, USA
Support staples	T50 17/32 in. Leg x 3/8 in. Crown 3/8 Gauge Galvanized Steeel Ceiltile Staples	Home Depot, Inc.	Okemos, MI, USA
Super glue	20g Rubber Glue	YUE	via Amazon (USA)
Styrofoam cups	Dart 4J4 4 oz. White Foam - 1000/Case	WebstaurantStore	Lancaster, PA, USA
Cup lids	Dart 4JL Translucent Vented Lid - 1000/Case	WebstaurantStore	Lancaster, PA, USA
5/16 in. braided rope	5/16 in. x 400 ft. Polyester Braided Outdoor Clothesline, White	Everbuilt	via Home Depot (USA)
Flat Black Spray Paint	12 oz cans	Rust-Oleum	via Home Depot (USA)

Figure B.1: Process controls for ^{32}P transfer experiment



Process controls were in the form of uninoculated plants grown in large conetainer pots (undivided) that either received a direct aliquot of radiation to the root compartment or were a no-radiation control. The positive control received 4 μCi of ^{32}P at the same time as the experimental plants in the microcosms. Controls were more nutrient stressed relative to their colonized and pot-bound experimental counterparts and thus reflect less growth and uptake at later experimental stages. n=3 and error bars represent standard error.

Table B.4: Microcosm durability testing results over 5 iterations. CPM measurements of cotton traps to asses radiation transfer between microcosm compartments

Pot ID	*testing iteration*	CPM: plant compartment	CPM: myc. compartment	pass/fail
301	*1*	36	6049	pass
	2	56	8878	pass
	3	74	871	pass
	4	29	448	NA
	5	30	354	NA
302	*1*	35	1359	pass
	2	43	2465	pass
	3	63	1978	pass
	4	34	17408	pass
	5	26	804	pass
303	*1*	38	56	NA
	2	120	1044	pass
	3	65	1937	pass
	4	27	2891	pass
	5	27	787	pass
304	*1*	87	5164	pass
	2	74	4361	pass
	3	79	2475	pass
	4	90	5772	pass
	5	32	1005	pass
305	*1*	42	944	pass
	2	69	3529	pass
	3	71	10623	pass
	4	67	51197	pass
	5	29	4314	pass
306	*1*	41	8082	pass
	2	127	2300	fail
	3	69	4856	pass
	4	79	3178	pass
	5	28	1972	pass
307	*1*	47	219	NA
	2	63	4141	pass
	3	69	1826	pass
	4	101	15057	pass
	5	28	741	pass
308	*1*	39	1521	pass
	2	38	1208	pass
	3	76	3259	pass
	4	91	100392	pass
	5	31	1323	pass
309	*1*	34	422	NA
	2	53	1645	pass
	3	78	395	pass
	4	102	2116	pass
	5	32	161	NA
310	*1*	43	1782	pass
	2	69	2901	pass
	3	99	1316	pass
	4	103	1806	pass
	5	40	534	pass

BIBLIOGRAPHY

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Bradford, Marion M. (May 1976). "A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein-Dye Binding". In: *Analytical Biochemistry* 72.1, pp. 248–254.

Peterson, Gary L. (Dec. 1977). "A Simplification of the Protein Assay Method of Lowry et al. Which Is More Generally Applicable". In: *Analytical Biochemistry* 83.2, pp. 346–356.