# Ecological impacts of anthropogenic stress from a contemporary herbicide on species interactions involving plants

by

**Veronica Iriart**

Bachelor of Arts, Wake Forest University, 2018

Submitted to the Graduate Faculty of the

Dietrich School of Arts and Sciences in partial fulfillment

of the requirements for the degree of

Doctor of Philosophy

University of Pittsburgh

2024

UNIVERSITY OF PITTSBURGH

DIETRICH SCHOOL OF ARTS AND SCIENCES

This dissertation was presented

by

**Veronica Iriart**

It was defended on

May 13, 2024

and approved by

Dr. Kevin Kohl, Associate Professor, Department of Biological Sciences, University of Pittsburgh

Dr. Mart Turcotte, Assistant Professor, Department of Biological Sciences, University of Pittsburgh

Dr. Tera Levin, Assistant Professor, Department of Biological Sciences, University of Pittsburgh

Dr. Corlett Wood, Assistant Professor, Department of Biology, University of Pennsylvania

Dr. Regina Baucom, Associate Professor, Department of Ecology and Evolutionary Biology, University of Michigan

Thesis Advisor/Dissertation Director: Dr. Tia-Lynn Ashman, Distinguished Professor, Department of Biological Sciences, University of Pittsburgh

Copyright © by Veronica Iriart

2024

**Ecological impacts of anthropogenic stress from a contemporary herbicide on species interactions involving plants**

Veronica Iriart, Ph.D.

University of Pittsburgh, 2024

Global use of herbicides to meet food demands and its associated chemical pollution have surged in recent decades. While the effects of off-target herbicide exposures on some crop species are known, we know little of how wild plants in proximity to agriculture, i.e. at the 'agro-ecological interface', are impacted or how their species interactions tied to essential ecosystem services are likewise affected. Across three dissertation chapters, I investigated these important—yet previously undescribed—ecological consequences of herbicide use. In Chapter 1, I performed a greenhouse experiment to characterize the growth and flowering of 25 plant species common to agro-ecosystems following herbicide exposure via 'drift' (atmospheric chemical movement). In Chapter 2, I focused on a key member of these agro-ecosystems, the legume *Trifolium pratense*, and conducted a plant-microbe study to investigate the impacts of herbicide drift on the mutualism between plants and nitrogen-fixing bacteria (rhizobia). I further explored this topic in Chapter 3, where I simultaneously exposed *T. pratense* and rhizobia to herbicidal chemicals in the rhizosphere—the region surrounding roots where plants and microbes interact and herbicides can also contaminate—in a microcosm experiment. In all chapters, I used dicamba, a highly-used synthetic auxin herbicide known for off-target movement, to simulate exposures. Results conveyed that despite comprising only ~0.5-1% of what is typically applied in agriculture, off-target dicamba concentrations can significantly impact wild plants and their species interactions. Exposure to dicamba drift inhibited or in some cases enhanced plant growth, depending on the species, and it also had species-specific effects on flowering phenology. As these traits relate to plant-plant

competition for resources and pollination, these alterations would probably influence interactions between plant species in nature. Additionally, dicamba exposure either from drift or the rhizosphere reduced the growth promoting benefits of rhizobia, but the degree of reduction depended on rhizobial genotype, and other traits including nitrogen fixation were also mediated by interactions between rhizobial genotypes and the herbicide environment. Altogether, my dissertation relays timely insights into the ecological impacts of off-target herbicide exposures, the biological forces which mediate them, and the evolutionary trajectories that may follow them.

## Table of Contents

## List of Tables

## List of Figures

## Preface

The last chapter of my dissertation exemplifies the positive influence that the 'right' partner can have when an organism is faced with a stressful condition. In a way, I myself have appreciated this lesson throughout my Ph.D. journey. I have benefitted tremendously from the various partnerships I have made in this time, and without these mentors, colleagues, research assistants, friends, and family, this work would not have been possible. Firstly, I would like thank my advisor, Dr. Tia-Lynn Ashman. With her infectious energy, Tia-Lynn gave me the confidence to take on these large research projects, and I always knew I could depend on her to support my career goals and offer guidance when I needed it (which was often). I also must thank Tia-Lynn for molding such a collaborative and positive work environment throughout all of my years at Pitt. I learned so much from the diverse and inspiring scientists who have overlapped with me in the Ashman lab, including post-doctoral researchers Drs. Maria Rebolleda Gomez, Avery Russell, Na Wei, Rainee Kaczorowski, Sergio Ramos, Anne Sternberger, Thomas Anneberg, Nathália Streher, graduate students Dr. Nicole Forrester, Dr. Andrea Fetters, Dr. Nevin Cullen, Amber Stanley, Hannah Assour, and lab technicians Elizabeth O'Neill and Rebecca Hayes. In addition, there were several talented Ashman lab undergraduate researchers who were essential in helping collect the data presented in these dissertation chapters, namely Alex Blanco, Elizabeth Rarick, Mikaela Moore, Jennla Ruit, Trezalka Budinksi, Kayla Downs, Madison Jerome, Steffani O'Neill, Sara Parker, and Evie Perry. Beyond the Ashman Lab, I would also like to thank my outstanding dissertation committee—Drs. Kevin Kohl, Mart Turcotte, Tera Levin, Corlett Wood, and Regina Baucom—for consistently supporting me while simultaneously challenging me to become a better scientist. I thank our greenhouse manager, Laurie Follweiler, for teaching me about plant husbandry and for

always being so helpful in caring for my experimental plants. I thank Dr. Roslyn Gleadow, Dr. Scarlett Howard, and their labs for recently welcoming me into the vibrant academic community at Monash University while I call Melbourne my new home. I thank Philippa Carter for directing the Hot Metal Bridge Fellowship Program, which prepared me for graduate-level research, and Dr. Elizabeth Amarh for her mentorship during that period. I thank our Graduate Administrator Cathy Barr for always helping me stay on track with my administrative tasks and for always being a friendly face to visit in the main office of our department. Last but certainly not least, I thank my friends and family. I was fortunate that my cohort of fellow graduate students at Pitt was extremely supportive and fun – I will miss our evenings spent at Primanti Bros. together! I was also very fortunate to have grown up with exceptional role models, including my mother and father, Angeles and Ricardo, sister Belen, and brother Javier. They always encouraged me to be ambitious while also helping me to appreciate the things that truly matter in life. And of course, I thank my husband, JJ Dunn, for moving to Pittsburgh for me to pursue this degree back in 2018 and for being right by my side through all of the setbacks and all of the triumphs that followed.

# 1.0 Introduction

## 1.1 Herbicides as anthropogenic disruptors of species interactions at the agro-ecological interface

In writing *Silent Spring* in 1962, Pittsburgh-native Rachel Carson first called public attention to the danger of releasing toxic chemicals into the environment without prior knowledge of their ramifications for wildlife. Most notably, she explained how the indiscriminate use of the pesticide dichlorodiphenyltrichloroethane (DDT), was not only killing its target pests (insects), but also birdlife; thus, the book's title was itself a warning that the continued use of DDT could rid the world of its birdsongs. Despite the success of *Silent Spring* and the environmental movement it inspired, the release of anthropogenic toxins into the environment is still a pertinent threat to ecological communities of today and the ecosystem services they provide (Tosi et al. 2016; Miller et al. 2020). Pesticide usage rates, in particular, have risen to three million tons applied per year to meet global food demands, and among them herbicides are by far the most used class of pesticide both in the USA and globally (FAO 2024). As such, herbicides are also the most common type of pesticide residue found on land (Maggi et al. 2023).

However, knowledge of the downstream ecological consequences of herbicide exposure, which can occur through targeted application or unintentional environmental contamination, is still significantly lacking (Iriart et al. 2021). For instance, while considerable progress has been made in understanding the direct effects of herbicides on plant species and some other organisms likely to encounter them (e.g., pollinators [Motta et al. 2018], herbivores [Rainio et al. 2018], microbes

[Voos and Groffman 1997], etc.), we know little about how these direct effects on organisms scale up to indirectly affect the interactions that occur between them (reviewed in Iriart et al. 2021). In particular, the agro-ecological interface, i.e. where agricultural production makes contact with natural plant communities and ecosystems, is often the context for diverse forms of species interactions involving plants, including plant-plant, plant-insect, and plant-microbe interactions (Burdon and Thrall 2008).

Of the studies which have considered the effects of herbicides at ≤ field-realistic concentrations on species interactions, the results are noteworthy: e.g. exposure to glyphosate augmented competition between two grass species (Damgaard et al. 2014), dicamba reduced pollinator visitation to alfalfa and common boneset (Bohnenblust et al. 2016), and 2-4D reduced symbiotic investment of legumes to nitrogen-fixing rhizobial bacteria (Zaidi et al. 2005). As many of these ecological interactions result in important ecosystem services, such as habitat structuring, food and resource availability, nutrient cycling, and others (Morgan et al. 2005; Brooker et al. 2006; Klein et al. 2017), gaining a better understanding of the impacts of herbicides on these interactions, as well as identifying the forces that could mitigate them, is an important challenge of our time.

### 1.2 Evolutionary rescue theory and the potential for genetic variation among and within species to mediate the ecological impacts of herbicide exposures

Evolutionary rescue is the theory that a population could recover from exposure to an abrupt stressor—i.e. have its population growth restored and thereby avoid extinction—through

natural selection acting on heritable variation. Considering that herbicides can be one such stressor, a seminal paper on the topic once said "an evolutionary rescue experiment is launched whenever a field is sprayed with herbicide" (Bell 2017). The ability to predict whether this evolutionary rescue experiment would be successful or not, however, would depend upon several factors relating to the herbicide-exposed population, including its density, standing genetic variation, mutation rate, and dispersal rate. In particular, greater standing variation increases the likelihood of evolutionary rescue because it is immediately availability for selection to act on (Carlson et al. 2014), and indeed it has been emphasized as a key mechanism for herbicide resistance evolution (Baucom 2019; Kersten et al. 2023). Thus, another important topic that is currently lacking research is whether or not there is adequate standing genetic variation among or within species of the agro-ecological interface such that evolution could rescue these populations from the threat of extinction via the rise of herbicide-resistant genotypes.

If evolutionary rescue in response to herbicide exposure is possible, it is likewise important to consider its implications for ecological interactions among these species. For example, if the expression of herbicide resistance comes at a cost to other traits that are important for species interactions, then the rise of resistant genotypes could signify a breakdown of these interactions. Trade-offs such as this have been documented before: e.g. herbicide-resistant plants have been shown to have reduced vegetative biomass (Alcocer-Ruthling et al. 1992), smaller and delayed flowers (Gassman and Futuyma 2005; Bingham et al. 2017), and thinner or less developed roots (Hall and Romano 1995; Tardif et al. 2006), changes which could potentially disrupt competitive interactions between plant species as well as beneficial mutualisms between plants and pollinators or symbiotic microbes. To evaluate whether evolution in response to herbicides could result in significant alterations in species interactions in this manner, studies are currently needed which

use genetically diverse species or communities common to the agro-ecological interface which measure both fitness and species interaction traits across levels of environmental herbicide exposure. If there are significant genotype-by-environment (G × E) interactions for species' fitness, then this would suggest that there is genetic variation in resistance to herbicide exposure. Identifying trends between resistance and ecologically-relevant traits would shed light on whether or not herbicide resistance and species interactions (e.g. their quantity or quality) would trade-off with one another, thereby revealing the possible ecological consequences of species evolution in response to herbicide-driven selection.

### 1.3 Dissertation Aims: Uncovering the ecological costs and evolutionary implications of non-target exposures to a newly-rising herbicide on plant mutualisms

In my dissertation, I completed three research chapters to provide insights into the consequences of herbicide exposure for plant ecology and evolution at the agro-ecological interface. In each chapter, I used the synthetic auxin, dicamba, as the focal herbicide, because of its current relevance as a novel anthropogenic stressor. Although dicamba was invented in 1964, its use has only recently increased dramatically in the USA, e.g. by ~500% from 2017 to 2019, and in other countries (Rippy et al. 2017; Carbonari et al. 2022) since the development of genetically modified dicamba-resistant crops (Johnson et al. 2023; U. S. Geological Survey 2024). Additionally, dicamba has been strongly linked to off-target movement, most notably from wind carrying particles away from application sites via 'dicamba drift', which has caused millions of acres of damage to nontarget plants (US EPA 2021). However, as a pre- and post-emergent

herbicide, dicamba can be applied either to bare soil (to prevent weeds from germinating) or existing vegetation (to kill existing plants), thus other forms of off-target movement are also possible, such as 'run-off' where precipitation disperses the herbicide over the ground or soil (Watts and Hall 2000; Rippy et al. 2017). Yet despite all of these probable cases of dicamba contamination in the environment, the ecological impacts of dicamba exposures for off-target plants and their associates in the wild and the important ecosystem services they provide are understudied. This significant knowledge gap is what my dissertation aimed to address.

In Chapter 1, I investigated the effects of dicamba drift (~1% of the field application rate [Egan et al. 2012]) on the fitness and coflowering interactions of 25 plant species common to the agro-ecological interface in a greenhouse experiment. I was interested in coflowering interactions, specifically, because they reflect the timing and intensity by which plant species overlap in flowering; thus, they can not only affect plant-plant competition for pollination, but also plant-pollinator interactions via changes in floral resource availability. Specifically, I aimed to answer the questions: Is there interspecific genetic variation in fitness responses to dicamba drift exposure or in traits related to plant-plant interactions via flowering, e.g. flowering time, flower number and flower size? And if there are genotype-by-environment (i.e. species × dicamba drift) interactions for fitness, indicating interspecific variation in dicamba drift resistance/tolerance, are species' involvement in co-flowering interactions in the presence of drift related to their ability to resist/tolerate dicamba drift?

In Chapter 2, I researched the impacts of dicamba drift on the fitness of the model legume *Trifolium pratense* and its interactions with its microbial symbiont, the rhizobia *Rhizobium leguminosarum*. *T. pratense* is a common member of the agro-ecological interface and its symbiosis with *Rhizobium leguminosarum* bacteria provides the important ecosystem service of

nitrogen fixation, wherein rhizobia housed within root nodules transform atmospheric nitrogen into a plant-accessible form in exchange for photosynthates. In Chapter 1, I discovered that *T. pratense* was especially sensitive to dicamba: its fitness was the most negatively affected by drift exposure. However, we did not know whether this sensitivity could be mediated by intraspecific plant genetic variation (i.e. $G_P$), or even that of its rhizobial partners ($G_R$), given that ample studies in the field of legume-rhizobia interactions had indicated that $G_R \times E$ or $G_P \times G_R \times E$ interactions can determine legume fitness and trait expression across different environmental contexts (Heath and Tiffin 2007; Cauwnberghe et al. 2016; Batstone et al. 2020; Heath et al. 2020). Further, the ecological costs of drift-level dicamba exposure on this important mutualism had yet to be characterized. Therefore, in a greenhouse inoculation study, I paired 17 *T. pratense* genotypes with two *R. leguminosarum* genotypes, and asked: Does $G_P$ or $G_R$ mitigate the effects of dicamba drift on legume fitness or key traits related to the legume-rhizobia mutualism (e.g. nodule number, nodule size, and nitrogen fixation)? And if genotype-by-environment interactions are important, what inferences can be drawn about the broader ecological and evolutionary impacts of dicamba drift on legume-rhizobia symbioses?

In Chapter 3, I pursued further research questions that were inspired by results from Chapter 2. Because non-target plants and rhizobia can also be affected by dicamba via exposures in the rhizosphere (i.e. the area immediately surrounding plant roots) through off-target movement, in this chapter, I was interested in characterizing the impacts of rhizospheric exposure to dicamba on the legume-rhizobia mutualism. Thus, unlike in Chapters 1 and 2 where I applied a drift-level rate of dicamba to the aboveground portion of plants, here I applied a rate of dicamba similar to what has previously been detected in water samples affected by herbicide run-off (i.e. 0.5% of the field application rate [Vance and Kryzyszowka 1994; Ma et al. 1999; Rice et al. 2010]) to the plant

growing media, thereby exposing the belowground portion of the plant system. In this way, both plants and rhizobia directly encountered the herbicide. Furthermore, because in Chapter 2 I only considered two rhizobial genotypes but 17 plant genotypes, in this chapter I incorporated eight rhizobial and two plant genotypes to better understand how rhizobial variation could mitigate plant stress and mutualism responses to dicamba. Altogether, in a growth chamber microcosm experiment, I posed the questions: Does the presence of rhizospheric dicamba disrupt key traits related to the legume-rhizobia mutualism or legume fitness? Do plant or rhizobial genotypes mediate the effects of dicamba on mutualism traits or legume fitness via $G_P \times E$ or $G_R \times E$ interactions? And lastly, if genotype-by-environment interactions are important, what inferences can be drawn about the broader ecological and evolutionary impacts of *rhizospheric* dicamba on legume-rhizobia symbioses?

## 1.4 Significance

My dissertation confronted a "major challenge" of the 21st century: determining whether evolution could rescue natural systems from anthropogenic-imposed decline (Bell 2017). In each chapter, I considered the role of genetic variation, the 'raw material' for natural selection, in driving the response of plants and their interactions with other species to a novel environmental contaminant, the herbicide dicamba, in order to make some predictions about the evolutionary trajectories of species inhabiting the agro-ecological interface as well as the potential ecological consequences of dicamba as a selective agent. More broadly, this work aimed to provide insights into the threats facing wild plants and their ecological interactions, which provide essential

ecosystem services for human and planetary health. Therefore, as with Rachel Carson's *Silent Spring*, this research also aspired to promote more sustainable agricultural practices that would help us "reach a destination that assures the preservation of the earth" (Carson 1964).

# 2.0 Interspecific variation in resistance and tolerance to herbicide drift reveals potential consequences for plant community coflowering interactions and structure at the agro-eco interface

This chapter is a slightly modified version of the publication:

Iriart, V., R. S. Baucom, and T.-L. Ashman. 2022. Interspecific variation in resistance and tolerance to herbicide drift reveals potential consequences for plant community co-flowering interactions and structure at the agro-eco interface. Annals of Botany 130: 1015–28.

## 2.1 Introduction

Wild plant communities at the agro-ecological interface (Bernardo et al. 2018) are important reservoirs of plant diversity and support the maintenance of agro-ecosystems (Requier et al. 2015; Ouvrard et al. 2018). These assemblages of native and introduced species (Burdon and Thrall 2007) contribute to a range of ecosystem services, including nutrient cycling (Altieri et al. 1999), habitat structuring (Brooker, 2006), and food production (Marshall et al., 2003; Beismeijer et al. 2006). In particular, these communities produce critical floral resources that sustain pollinators, especially while crops are not in bloom (Holzaschuh et al. 2008; Karamaouna et al. 2019; Kati et al. 2021).

Anthropogenic stressors associated with farming such as agrochemical pollution, however, have led to a 50% decrease in wild plant diversity over the past 70 years (Bretagnolle and Gaba 2015). This loss combined with concurrent widespread declines in pollinator richness and

abundance (Kluser and Peduzzi 2007; Potts et al. 2010) threaten the sustainability of agro-ecosystems and thereby the services they provide for human health, including the pollination of up to 75% of the world's leading food crops (Klein et al. 2007). Thus, understanding the mechanisms by which agrochemical usage impacts wild plant communities is a key concern.

Non-target exposure to herbicides via drift pollution is a leading form of anthropogenic stress at the agro-ecological interface (Marshall et al. 2003; Boutin et al. 2014; Schütte et al. 2017). Despite drift-levels being typically very low (~0.5-10% of the field application rate [Grover et al. 1972; Egan et al. 2014; Olszyk et al. 2017]), they can significantly affect the growth and flowering of various plants. Depending on the herbicide, drift to susceptible plants can reduce vegetative size and growth as well as decrease or delay flower production (reviewed in Iriart et al. 2021; later Ramos et al. 2021 and Strandberg et al. 2021).

However, some plants may be resistant to herbicide drift, i.e. able to prevent or limit damage incurred; while others may be tolerant, i.e. unable to prevent damage but able to buffer negative effects on fitness. Although herbicide resistance and tolerance can be defined in other ways as well (e.g., Neve and Powles 2005; Devine 2005; Vieira et al. 2020), we follow the approach by Baucom and Mauricio (2004; 2008) and Baucom (2019) in this paper to define them within an evolutionary ecology framework (Table 1). In addition, at low concentrations, herbicides that mimic plant growth hormones such as auxin (e.g., 2,4-D, dicamba) may stimulate growth (Allender et al. 1997; Guardiola and García-Luis 2000; Belz and Duke 2014), leading to drift effects more akin to overcompensation than herbicide damage (Table 1, Garcia and Eubanks 2019). Plant-defense theory posits that because resources are finite, there will be a trade-off between resistance and tolerance (Fineblum and Rausher 1995; Debban et al. 2015; Mikaberidze and McDonald 2020). Thus, within a community, there could be considerable interspecific

variation in resistance or tolerance to herbicide drift, leading to community-level changes that affect higher-level processes.

These community-level repercussions could occur via changes in vegetative or flowering dynamics (reviewed in Iriart et al. 2021). Sensitive species may not survive whereas resistant or tolerant ones may achieve greater vegetative biomass – outcomes that can affect community structure, i.e., species evenness (Hald 1999; Mayerová et al. 2018). For example, Egan et al. (2014) found that applications of ~1% of the field rate of the herbicide dicamba led to declines in forbs but left grasses unaltered, thereby changing the evenness of field plant communities. But this metric alone may not reflect interspecific variation in the timing of, and investment in, flowering which could in-turn change floral community structure.

Previous studies have shown the utility of network analyses to assess the impact of anthropogenic stressors on multi-species interactions (e.g., Hoffmiester et al. 2015; Filipe-Lucia 2020); therefore, we propose that a network approach can be used to inform on how herbicide drift affects floral community structure by documenting changes in patterns of coflowering (Arceo-Gomez et al. 2018). For instance, if multiple plant species in a community are sensitive to drift and respond to exposure by shifting flowering phenology, then these changes could lead to less frequent and/or weaker coflowering interactions, indicating a reduction in the average diversity and abundance of floral resources available at a given time. In turn, this outcome may lead to decreased reproductive success among species due to reduced facilitation for pollinator visitation, especially when plant species share pollinators (Ghazoul 2006). Moreover, variation in flowering response to herbicide drift could alter the composition of coflowering modules, i.e. groups of species that are more likely to overlap in flowering (Olesen et al. 2007), or cause shifts in floral community dominance. Tolerant or overcompensated species may become novel network hubs

that, by producing flowers consistently and coflowering with many species, are important for community stability (Bader et al. 2007).

Altogether, these species-level impacts could scale up to affect community-level properties, especially connectance (the total number of links [coflowering interactions] relative to the total possible; Dunne et al. 2002) and modularity (the difference in the fraction of links occurring within groups of species and the expected amount if links were distributed randomly; Olesen et al. 2007; Brandes et al. 2008). On a larger scale, these fluctuations could disrupt patterns of plant-plant competition or facilitation for pollinators that are mediated through trends in coflowering (e.g., Waser 1979). Consequently, we propose a network perspective can provide a richer evaluation of the impacts of herbicide drift than separately examining components of coflowering, such as the timing, duration, or date of peak flowering (e.g., Poole and Rathcke 1979; Parra-Tabla and Vargas 2004; Forrest et al. 2010).

While previous work reviewed studies of herbicide exposure on plants and called for more holistic approaches (Iriart et al. 2021), it also emphasized the lack of a comprehensive understanding of how diverse plant species respond to sublethal herbicide levels and how these responses could influence community structure. To fill this gap in knowledge, in this study, we grew plants from 25 species collected from the agro-eco interface in a greenhouse environment and exposed half to a drift-level rate of an herbicide. We evaluated interspecific variation in herbicide drift tolerance and resistance and determined their impact on community metrics, such as species evenness or coflowering structure. We chose dicamba as our focal herbicide, a synthetic auxin, whose use to control eudicot plants has surged in the United States (Knezevic et al. 2018; U. S. Geological Survey 2021) and has been linked to unprecedented numbers of off-target exposures (US EPA 2021).

Specifically, we ask five questions: 1) Do species vary in resistance or tolerance to dicamba drift? 2) Is there a trade-off between resistance and tolerance across species? 3) Does dicamba drift alter the probability of flowering, day of first flower, duration of flowering, and/or flower size, and if so, do these flowering responses vary among species? 4) Does dicamba exposure lead to community-level changes in species evenness or in metrics of coflowering interaction for a greenhouse synthetic community? And finally, 5) can changes in community-level interactions due to dicamba drift be explained by resistance or tolerance among species?

## 2.2 Methods

### 2.2.1 Study Species

We collected seeds from 25 agro-eco species (described in Table 2) in 2018 from 1-3 populations growing near soybean or fallow fields in southwest Kentucky and northeast Tennessee, USA (Appendix A: Table 6). Species occurred at varying frequencies across all surveyed sites, with the rarest species (*G. canadense*, *S. canadensis*, and *A. theophrasti*) observed about 4% of the time and the most common (*S. spinosa*, *I. lacunosa*, and *E. serotinum*) about 55% of the time (Iriart, Baucom, and Ashman unpublished data). Species were mainly insect-pollinated (Table 2). Although three species were primarily wind-pollinated, insects can visit them when pollen sources are limited (Saunders 2018; Ashman pers. obs.). Dicamba use at the time of seed collection was estimated to be high (> 1.1 L km-1) according to the United States Geological Survey (U. S. Geological Survey 2021) and conversations with local farmers (Ashman and

Baucom pers. obs.). Although we did not acquire detailed information about the history of dicamba in the area, our framework for defining resistance and tolerance sought to characterize standing variation for resistance and tolerance, rather than previous evolutionary histories (Table 1).

### 2.2.2 Experiment Set-Up

For each species, we planted 3-10 seeds in each of 22 pots (11.4 cm × 11.4 cm × 10.2 cm) filled with a 3:1 mixture of unfertilized Old castle C/B soil (45% Canadian Sphagnum Peat Moss, 35% Aged Pine Bark, 15% Perlite, 5% Vermiculite; BFG Supply Co., Burton, OH) and Germination Mix (65% Canadian Sphagnum Peat Moss, 25% Perlite, 10% Vermiculite; BFG Supply Co., Burton, OH) in the University of Pittsburgh greenhouse. We transplanted some seedlings (66 out 479) to new pots to make up for those with zero germination and thinned pots with multiple seedlings to one seedling per pot. Final sample sizes were 22 plants per species except nine species which had 6-21 plants. The average daily temperature was 25.6℃ ± 1.6 and daylength ranged from 12-16 hours throughout the experiment (20 May – 8 Nov. 2019). We supplied water as needed and fertilized plants once with 0.2 g of Osmocote 14 N -14 P-14 K (ICL Specialty Fertilizers, Ltd., Dublin OH).

### 2.2.3 Herbicide Treatments

We divided 16-day old plants into two groups with 3-10 plants per species, then treated them with one of two levels of dicamba (3,6-dichloro-o-anisic acid, Albaugh, LLC, Ankeny, IA): 0% (‘control’) or 1% (‘drift’) of the field application rate of 561 g of active ingredient per hectare

(Albaugh 2018). The drift treatment represented a particle drift rate, i.e., when herbicidal particles travel away from application sites by wind (Felsot et al. 2011). While the control and drift treatments related directly to our research questions, we also treated a third group with 2 plants per species with 100% of the field application rate of dicamba to confirm the effectiveness of our dicamba stock and to identify any unaffected species (i.e., alive at 145 days post-treatment of this high dosage). All treatments included 'Preference' surfactant (non-ionic surfactant blend, WinField Solutions, St. Paul, MN) at 0.1% v/v and were applied with a handheld multi-purpose sprayer with an adjustable nozzle (Chapin International Inc., Batavia, NY, USA; Model #1002; operating pressure = 40-60 PSI; flow rate = 1.5-2.3 L minute-1) set to a medium-fine mist. Plants were sprayed until they were just wet. We randomized plants by treatment and species across each bench in the greenhouse.

### 2.2.4 Data Collection

Twenty-four hours prior to applying herbicide treatments, we counted the total number of leaves and measured the longest leaf with a digital caliper to the nearest 0.1 mm and used the product of these to estimate 'pre-treatment plant size.' We used this metric to estimate plant size because it could easily be standardized across our species set which included plants of various heights and life forms (Table 2). Forty-eight hours after treatment, we assessed damage by enumerating the number of leaves showing typical symptoms of dicamba injury, i.e., leaf cupping or twisting (Foster and Griffin 2018; Griffen et al. 2013). Given our general definition of resistance (Table 1), an instantaneous measure of damage ('proportion of undamaged leaves' = 1 - # damaged leaves 48 hours after treatment/total # leaves pre-treatment) is appropriate. This measure

also accounts for the fact that synthetic auxins may positively or negatively affect growth at low concentrations (Gianfagna 1995; Kelley and Riechers 2007; Grossman 2010) and initial leaf damage could impact downstream flower production (Mothershead and Marquis 2000; Jacobsen and Raguso 2018).

Since we could not measure reproductive success directly across all species given that the majority required insect pollination (Table 2), we measured two standard fitness proxies to assess tolerance (Table 1): 1) 'short-term growth' estimated from plant size at 21 days post-treatment and 2) 'final biomass' of shoots harvested at 145 days post-treatment, dried at 70°C for at least 48 hours, and weighed to the nearest 0.01 g (Mettler AE200 Analytical Balance, Mettler-Toledo International Inc., Columbus, OH). Plant size and biomass are known to positively correlate with fecundity, especially for plants of the same age as those in our study (reviewed in Younginger et al. 2017).

We recorded the 'day of first flower' and counted the total number of open flowers (i.e., 'floral display') per plant 2-3 times per week from 15 July to 8 Nov. Thus, 'flowering duration' reflected the count in days from the first day flowers were present to the last day flowers were present or at the end of experiment (for four species; see Appendix A**:** Table 7 for details). On each flowering plant, we collected 2-5 of the first 10 open flowers, dried them on silica gel and weighed each to the nearest 0.1 mg (Mettler AE200) to obtain dry 'biomass per flower.' For *E. annuus*, *P. lanceolata*, *P. virginica*, and *T. officionale*, which had extremely small (~3-25 mm long) flowers clustered into heads or spikes, we counted and sampled biomass at the flowerhead or spike-level.

### 2.2.5 Statistical Analyses

We performed all statistical analyses, unless otherwise specified, in R version 3.6.1 (R Core Team 2019), using linear, mixed-effects linear, and generalized linear models via the *lm*, *lmer*, and *glm* functions, respectively, from the *lme4* package (Bates et al. 2015; see Appendix A: Table 9 for a full list and description of all linear models). All models included data from the control and drift treatments only. We graphically inspected residuals of all response variables for normality using the *ggqqplot* function (*ggpubr* package; Kassambara 2019) and performed square-root or log-transformation as needed to meet model assumptions. If graphical assessments and kolmogorov-smirnov tests (*ks.test* function; R stats library) confirmed non-normality of both transformed and original scales of measurement, then we performed the nonparametric Wilcoxon Mann-Whitney Test (*wilcox.test* function; R stats library). We tested significance of fixed effects with type III sums of squares using the *Anova* function (*car* package; Fox and Weisberg 2019) and of correlations using Pearson's product moment correlation coefficients (Sharma 2005) using the cor function from the R stats library. All figures, unless otherwise specified, were created using the *ggplot2* package (Wickham 2016).

#### 2.2.5.1 Species-level analyses

To analyze the response variable 'proportion of undamaged leaves', we performed a linear model where only plants in the drift treatment were analyzed and species was the sole explanatory variable, because control-treated plants showed no evidence of leaf damage. For all other dependent variables, including short-term growth, final biomass, day of first flower, flowering duration, floral display, and biomass per flower, we ran linear models which contained the

explanatory variables: species, treatment, and the species × treatment interaction. Models assessing effects on short-term growth and final biomass included 'pre-treatment plant size' as a covariate. The short-term growth model additionally included the random effect 'transplanted' (a binary variable) to account for any effects of transplantation (see Methods).

We used a generalized linear model with the Poisson distribution (Katti and Rao 1968) to analyze herbicide effects on flowering duration. We also ran two models, with and without short-term growth as a covariate, for flowering duration as well as floral display, to determine the extent to which drift effects on flowering production and duration were dependent on growth effects. Some species were removed from some models due to leaf drop (short-term growth: *D. illinoensis*), or nonflowering/inadequate replication (see Table 2 for species that were removed for day of first flower, flowering duration, floral display, and biomass per flower analyses).

To characterize species-specific resistance, given species was a significant predictor of 'proportion undamaged leaves', we ran independent sample *t*-tests to determine whether species' estimated marginal means (Searle et al. 1980) significantly differed from one (Table 1). We used the *emmeans* (*emmeans* package; Lenth 2020) and *test* functions to calculate estimated marginal means and perform *t*-tests, respectively.

To characterize species-specific tolerances, given a significant species × treatment effect for tolerance variables (Table 1), we calculated contrast estimates for each species using the *contrast* function (Abdi and Williams 2010; *emmeans* package). Contrast estimates reflected the difference in the estimated marginal means for tolerance between treatments (e.g., growth in drift subtracted by that in control). Thus, we used the degree to which species estimates differed from zero to describe species' tolerances to drift (Table 1). We also used contrast estimates to detail species' responses to drift via flowering time, duration, floral display and biomass per flower.

To explore whether there was a tradeoff between resistance and tolerance on either time scale across species, we estimated correlations between standardized estimated marginal means for resistance and contrast estimates for tolerance variables using z-scores. To elucidate whether long-term responses to dicamba drift could be predicted from short-term responses, we regressed z-scores of short-term tolerance on long-term tolerance.

To test whether dicamba drift affected the probability of flowering and whether it varied by species, we performed a chi-squared test of independence and a chi-squared test of homogeneity (Stuart 1955), respectively. Some species were excluded from this analysis due to low replication (see Table 2 for details).

To account for shared evolutionary histories (Felsenstein 1983), we created a phylogenetic tree (Appendix A: Figure 11) with the *phytools* (Revell 2012) and *V. PhyloMaker* (Jin and Qian 2019) packages using phylogenetic information extracted from the mega-phylogenetic tree 'GBOTB' for seed plants (Smith and Brown 2018) and used the tree to conduct associated phylogentic models with the *phylolm* function (*phylolm* package; Ho and Ane 2014) and the *pglmm_compare* function (*phyr* package; Ives et al. 2020) for all response variables. For models including <25 species, we reconstructed the phylogenetic tree accordingly. We used Akaike Information Criterion (AIC) model selection to determine which models, phylogenetically-corrected or not, demonstrated the lowest AIC values and thus were the best-fit (Akaike 1973). We found that phylogenetically-controlled models performed worse than models which did not account for phylogeny (AIC values were two or more units higher; Appendix A: Table 8); thus, we present results from the latter models only.

#### 2.2.5.2 Community-level analyses

To assess the effects of dicamba drift at the community scale, we considered data from all plants in the drift treatment as one synthetic community and all control plants as another.

To compare the control community against the drift for species evenness, we used species mean final biomass data to estimate each community's Shannon's Equitability Index ($E_H$; Kent 1992):

$$E_{\mathrm{H}} = [\ \sum_{i=1}^{S} p_i \ln(p_i)\ ] \ / \ln(S)\ .$$

Here, $S$ is the number of species in the community and $p_i$ is the relative proportion of species $i$.

To evaluate dicamba drift effects on community-wide patterns of coflowering, we estimated a coflowering index for every pair of plant species within each community using the daily number of open flowers. This index was adapted from Schoener's index (SI) of niche overlap (Schoener 1970) as applied to flowering (following Arceo-Gomez et al. 2018):

$$SI = 1 - \frac{1}{2} \sum_k \left| p_{ik-} p_{jk} \right|$$

where $p_{ik}$ and $p_{jk}$ are the proportion of open flowers by species $i$ and $j$, respectively, occurring on day $k$. SI ranges from 0 (no flowering overlap, i.e. the absence of potential interaction) to 1 (complete flowering overlap, i.e. maximum potential interaction). By inputting SI values into the program Gephi, version 9.2 (Bastian et al. 2009), we constructed weighted, unipartite networks for both communities (see Figure 1 for a schematic that contrasts two hypothetical coflowering networks).

We characterized several species-level network properties, including degree (the average number of times that plant species interact with each other by coflowering; Figure 1), strength

(average SI value for coflowering, i.e. the intensity and duration of flowering overlap; Figure 1), weighted degree (degree weighed by strength; i.e., the mean sum of interaction strengths across species), and betweenness centrality (the relative importance of species to network stability as measured by the average percentage of shortest paths in the coflowering network that must go through a species; Figure 1) and assessed how these were impacted by community type (control and dicamba drift) using Wilcoxon Mann-Whitney tests. We conducted each of these analyses twice, once where each community included the 'full' set of 22 species that flowered in at least one treatment and again where each network included only the 'subset' 19 species that had at least one flowering plant in both treatments (see Table 2 for species that were removed from full and subset network analyses). In this way, we gauged whether network differences were due to species-level differences in flowering propensity in full networks or due to changes in flowering pattern alone in subset networks.

Further, we identified community-level flowering properties by estimating network connectance and modularity using Gephi (the Blondel et al. [2008] optimization algorithm at a 1.0 resolution estimated modularity).

To test whether observed differences in species evenness, connectance, and modularity between communities were significant, we simulated two random communities of 25 species by taking random samples of datapoints without replacement from our mean final biomass data or SI data for flowering. We then counted the number of times out of at least 100 iterations that the difference in $E_H$, connectance, or modularity between two random communities was greater than or equal to the actual difference between the control and drift community.

To address whether changes in critical species-level coflowering metrics (Figure 1) that occurred between the drift and control communities are related to resistance or tolerance to

dicamba drift, we calculated the change in these metrics between the two full networks (i.e., metric value in drift network subtracted by that in control) for each species. We then estimated correlations between these changes and resistance, short-term tolerance, and long-term tolerance (Table 1).

## 2.3 Results

### 2.3.1 Do species vary in resistance or tolerance to dicamba drift?

We found that species was a significant predictor of resistance to dicamba drift ($F_{24,188}$, $P < 0.001$; Appendix A: Table 9). Most species (21 out of 25) showed significant signs of dicamba-related injury as the proportion of undamaged leaves 48 hours after treatment ranged from 0.85 to 0.26 (Figure 2A; Appendix A: Figure 12A, Table 10). Remarkably, four species showed no signs of damage: *S. spinosa*, *C. virginica*, *A. theophrasti*, and *I. lacunosa* (Figure 2A).

Dicamba drift did not have a uniform effect on growth or biomass across species ($P > 0.7$ for both; Appendix A: Figure 13). Rather, its effect on both measures was highly influenced by species (treatment × species interaction: all $P < 0.001$; Appendix A: Table 9). Species-level variation in tolerance occurred at both time scales. One quarter of the species were intolerant at 21 days post-treatment (Figure 2B; Appendix A: Table 11). On the longer timeframe, however, most species showed tolerance; but several still showed significant reductions in biomass (by 13-39%; Figure 2C; Appendix A: Table 12). Interestingly, two species overcompensated in response to drift exposure — drift-treated *P. philadelphica* plants grew significantly larger (by 50%) in the short

term than controls and *P. virginica* (by 25%) in the long term (Figure 2B-C; Appendix A: Figure 12B-C, Figure 14A-B).

All species except four were killed by 100% of the field application rate of the herbicide dicamba (three eudiocots: *O. stricta*, *P. lanceolata*, and *P. virginica*; and one monocot: *C. virginica*).

### 2.3.2 Is there a trade-off between resistance and tolerance?

Resistance and long-term tolerance were not significantly correlated across species ($r$ = 0.26, df = 23, $P$ = 0.22; Appendix A: Figure 15A). However, short-term tolerance did predict long-term tolerance ($r^2$ = 0.25, df = 22, $P$ = 0.01; Appendix A: Figure 15B).

### 2.3.3 Does dicamba drift affect flowering?

Flowering time was marginally significantly delayed due to drift (by 8 days, $F_{1,224}$ = 3.31, $P$ = 0.07, Appendix A: Figure 16A). It was also affected by species ($F_{16,224}$ = 26.70, $P$ < 0.001) and its interaction with treatment ($F_{16,224}$ = 7.38, $P$ <0.001; Appendix A: Table 9). Two out of the 17 species were significantly delayed in producing their first flower relative to controls (Figure 2D; Appendix A: Table 13), *T. officinale* and *T. pratense* (41 and 47 days later, Figure 2D); while one, *I. lacunosa*, was significantly accelerated in flowering (11 days earlier, Figure 2D; Appendix A: Figure 17A). All others showed only modest or no effects. Beyond flowering initiation, species ($X^2$ = 2152.34, df = 16, $P$ < 0.001) and treatment ($X^2$ = 6.38, df = 1, $P$ = 0.012) were significant predictors of flowering duration. On average, drift shortened flowering duration by six days, but a

significant treatment × species interaction ($X^2$ = 335.72, df = 16, $P$ < 0.001) also suggested that this result varied significantly in intensity and direction depending on species identity (Appendix A: Figure 16B, Figure 17B, Table 9). Contrast analyses revealed that about 50% of species flowered for a shorter period of time (by 4 – 41 days) in the drift treatment relative to the control, while a small portion flowered for longer (by 8 –12 days), and the remainder were unchanged (Figure 2E; Appendix A: Table 7). These results were mostly unaffected when short-term growth was included as a covariate in the analysis (Appendix A: Figure 18). In this case, short-term growth was likewise a significant predictor of flowering duration ($X^2$ = 38.52, df = 1, $P$ < 0.001) along with treatment *(*$X^2$ = 6.33, df = 1, $P$ = 0.012), species ($X^2$ = 2189.94, df = 16, $P$ < 0.001) and their interaction ($X^2$ = 361.95, df = 16, $P$ < 0.001). For two species *(I. lacunosa and E. serotinum),* differences between treatments became significant after accounting for short-term growth. The fact that significant changes in flowering duration for species were maintained in this way suggests that the drift effect went beyond what was mediated by plant size.

Neither drift, nor its interaction with species, significantly affected the probability of flowering (treatment effect: $X^2$ = 1.03, df = 1, $P$ = 0.31; species × treatment effect: $X^2$ = 0.65, df = 13, $P$ = 1.0) or size of floral display (treatment effect: $F_{1,203}$ = 0.341, $P$ = 0.56; species × treatment effect: $F_{16,\ 203}$ = 0.734, $P$ = 0.76). Floral display, however, did vary among species ($F_{16,203}$ = 76.02, $P$ < 0.001; Appendix A: Table 9). These results remained consistent when short-term growth was a covariate in the model, as it was not a strong predictor of floral display ($F_{1,202}$ = 1.38, $P$ = 0.24).

Species ($F_{16,197}$ = 204.62, $P$ < 0.001) and its interaction with treatment ($F_{16,197}$ = 1.82, $P$ < 0.05), but not treatment alone ($F_{1,197}$ = 2.79, $P$ = 0.10; Appendix A: Figure 16C) affected biomass per flower (Appendix A: Table 9). One species (*A. palmeri*) responded to drift by producing 50%

smaller flowers ($P < 0.01$), but all other species were not significantly affected (Figure 2F; Appendix A: Table 14, Figure 19).

### 2.3.4 Does dicamba exposure lead to community-level changes in species evenness or in metrics of coflowering interaction for a greenhouse synthetic community?

Potential community-level effects of drift exposure were assessed by assembling control and dicamba drift-treated plants into two separate 'synthetic' communities. Evenness based on biomass of control and dicamba drift-treated synthetic communities was not affected by dicamba drift ($P = 0.98$; Appendix A: Figure 20).

In contrast, dicamba drift significantly decreased average degree (by 23%), strength (by 32%), and weighted degree (by 30%) of coflowering community networks (Table 3; Figure 3A-B). These shifts resulted in a reduction in overall connectance (23% less) and increase in modularity (49% more) of the drift-exposed flowering community (Table 3). These changes were larger than expected by chance alone ($P < 0.01$). Analyses constrained to have at least one flowering plant per species in both treatments (i.e., subset networks with $n$ =19 species) showed similar results although slightly fewer statistically significant differences (Appendix A: Figure 21A-B, Table 15). Not only was connectivity reduced by dicamba drift, but the identity of the most important species in the community shifted (Figure 3C-D)—while the control network contained numerous species with the highest betweenness centrality value of 3 (*T. pratense*, *C. halicacabum*, *D. carota*, and *P. pennsylvanica*), the drift network only contained two species with high betweenness centrality values of 16 (*C. halicacabum*) and 11 (*A. theophrasti*), and all other species

values were at least 66% lower than that (Figure 3C-D). Although less extreme, the subset network showed similar trends (Appendix A: Figure 21C-D).

## 2.4 Discussion

This study demonstrated considerable variation in resistance and tolerance to dicamba drift among species common to the agro-ecological interface. Few of the species even showed overcompensation in response to this herbicide. Drift effects extended to flowering traits by impacting day of first flower, flowering duration, and flower size for some species but not others. These variable species-level effects transcended to community-level impacts, especially for coflowering structure in our greenhouse communities. Specifically, dicamba drift significantly decreased and weakened coflowering interactions and the direction of change in species roles within the community could be predicted from species' degree of tolerance.

Among-species variation in resistance and tolerance to dicamba at a very low, drift-level rate is consistent with previous findings of interspecific variation in LD50 for dicamba (Boutin et al. 2014; Olszyk et al. 2015). However, we identified new species with potential resistance to dicamba drift. For example, while we expected *C. virginica* to show resistance (Figure 2A), because it is a monocot and dicamba is designed to target eudicot species, we did not anticipate finding resistance for four additional species (*S. spinosa*, *A. theophrasti*, *I. lacunosa*, and *P. lanceolata*; Figure 2A). In addition, while the majority of species showed significant signs of dicamba damage at 48 hours post-treatment, only one-quarter of species demonstrated significant fitness losses in the short or long term, i.e. decreases in size at 21 days post-herbicide treatment

and final biomass (Figure 2B-C). This result suggests that some species may be capable of recovering from initial damage due to dicamba drift exposure over time. Such an outcome has been documented before with sub-lethal levels of herbicides, including dicamba (Carpenter and Boutin 2010; Ramos et al. 2021).

To our knowledge, our study is also the first to demonstrate that dicamba drift can have significant positive effects on growth for some species (Figure 2B-C). This response has previously been observed with low-dose applications of other synthetic auxins on crops to stimulate growth (Agustí et al. 2002; Gianfagna 1995). We suspect these species may have overcompensated in response to the moderate stress induced by drift exposure, similar to what can be caused by other herbicides or herbivory (Agrawal 2000; Belz and Duke 2014; Vieira et al. 2020). Alternatively, since auxins are known to stimulate cell elongation in shoots and initiate the formation of new leaves, these species might use low doses of dicamba to increase growth (Liscum et al. 2014; Xiong and Jiao 2019). Thus, while sublethal herbicide stress is typically expected to affect plants in a neutral or negative manner (reviewed in Iriart et al. 2021), this finding suggests that exposure to dicamba drift might enhance fitness for at least a handful of species, potentially influencing competitive dynamics in agro-ecosystems.

Despite the noted interspecific variation in resistance and tolerance to dicamba drift and past research showing that shifts in evenness from sensitive species to tolerant ones can occur in herbicide-exposed communities (reviewed in Iriart et al. 2021; later Qi et al. 2020), we did not detect such a change in our synthetic communities (Appendix A: Figure 20). Instead, we found that the drift treatment, while strong enough to significantly affect some species' growth, was not potent enough to affect species evenness based on biomass in the greenhouse environment where plants were not competing for resources.

Additionally, while previous work showed trade-offs between resistance and tolerance to stressors such as herbicides (Baucom and Mauricio 2008), we did not find this negative correlation in response to dicamba drift across species (Appendix A: Figure 15A). The lack of a trade-off could potentially be explained by variation in mechanisms of resistance. While resistance evolution to herbicides like glyphosate are commonly attributed to mutations in herbicide-targeted biosynthetic pathways, synthetic auxins do not target a specific pathway. Consequently, resistance evolution to auxinic herbicides is more complex and multiple resistance mechanisms have been found (Mithila et al. 2011; Goggin et al. 2016; Goggin et al. 2018). Thus, if experimental plants varied in resistance mechanisms, this variation may have traded off with other life-history traits beyond tolerance (e.g. reduced seed set, mutualistic interactions, or increased disease susceptibility; Vila-Aiub et al. 2009 and Baucom 2019; Cousens and Fournier-Level 2018). The lack of a relationship between resistance and tolerance may also suggest that instantaneous measures of damage do not reflect impacts on plant fitness, although tolerance reflected in short-term growth is a good proxy for long-term tolerance, i.e. final biomass (Appendix A: Figure 15B).

Our results fill the gap in knowledge of the effects of sublethal herbicide exposure on floral traits and reveal striking effects of interspecific variation on these outcomes. While Bohnenblust et al. (2016) found that dicamba drift decreased flower production and delayed flowering in two agro-eco species (*Medicago sativa* and *Eupatorium perfoliatum L.*), we did not detect an overall trend in flower production. Our results do, however, corroborate Bohnenblust et al. (2016) in terms of drift delaying flowering for some species.

The most striking result was the wide range of flowering phenological responses to dicamba drift, including flowering initiation and duration. As expected, the species that experienced the largest decrease in flowering duration under drift conditions were also those that

were the most delayed in day of first flower and vice versa (Figure 2D-E). The four-month 'season' in our greenhouse community is analogous to what these species experience in nature. Therefore, these detected shifts in flowering phenology are likely to have important ecological implications. For instance, extreme delays in flowering onset may lead to reduced pollination or insufficient time to accumulate resources and maximize investment in seed production following pollination. Meanwhile, accelerations in flowering can cause phenological mismatch between flowering period and pollinator emergence (e.g., Kudo and Ida 2013). Further, while an increase in flowering duration could benefit plants by increasing the potential for reproduction if pollinators are present (Barber et al. 2015), a decrease in flowering duration could have the opposite effect, leading to a decrease in reproductive output (Jin et al. 2015).

In our synthetic communities, we uncovered that interspecific variation in the deployment of flowers following herbicide exposure can lead to profound changes in coflowering network properties. In particular, simulations showed that the dicamba drift community was significantly less connected but more modular than the control community, meaning drift exposure resulted in less flowering overlap and more exaggerated differences in flowering time among species. Moreover, drift reduced the frequency of flowering time overlaps and the quantity of open flowers overlapping (network degree and strength; Figure 3; Table 3). Most important perhaps is that the identities of species within modules was changed. For example, species whose flowering durations were significantly lengthened or shortened due to drift (e.g., *A. palmeri* and *C. virginica*'s; Figure 2E) experienced a drastic change in the composition of their interacting module partners between networks (Figure 3). If these patterns hold under field conditions, then they could impact heterospecific pollen transfer, pollen limitation, and/or resources for pollinators (Ashman et al.

2004, Ashman and Arceo-Gómez 2013; Fang and Huang 2013; Vitt et al. 2020; Arceo-Gómez 2021).

Species roles within the two communities also changed and these were correlated with their tolerance to dicamba drift (Figure 4). Specifically, the most tolerant species either increased or maintained their ability to provide strong and plentiful connections (i.e., high weighted degree values) under dicamba drift, whereas the least tolerant species incurred the greatest devaluation in coflowering interactions between communities (Figure 4A). By the same token, the species that experienced the largest increase in their role as network hubs (i.e., greatest change in betweenness centrality due to drift), were all tolerant whereas the least tolerant species decreased considerably in importance from the control to the drift community (Figure 4B). Thus, it is possible that wild coflowering networks affected by dicamba drift may likewise experience shifts in flowering dominance in favor of more drift-tolerant species.

These results add significantly to the growing body of novel work employing network analysis to characterize complex ecological interactions and monitor anthropogenic impacts on natural communities (Gray et al. 2014; Watts et al. 2016; Leite et al. 2018). Specifically, our findings support previous work describing human-mediated impacts on connectance (e.g., Doré et al. 2020), modularity (e.g. Larson et al. 2016), or the identities of dominant species (e.g., O'Gorman et al. 2012). Thus, we argue that by providing a rich characterization and evaluation of an herbicide-stressed plant community, network analysis allowed us to make refined predictions about the consequences of herbicide drift for pollinator-mediated plant-plant interactions.

One potential outcome of plant communities becoming more modular and less connected due to herbicide drift may be decreased facilitation, especially if plant species jointly attract shared pollinators (Moeller 2004; Ghazoul 2006; Mitchel et al. 2009). On the contrary, if pollinators are

limited, then plant species in less connected, more modular communities may experience less competition for pollinators, since they are able to occupy different flowering niches (i.e., modules) and therefore more evenly engage with pollinators (Waser 1978; Rathcke 1988; Liao et al. 2011; Albor et al. 2019). The network perspective also allows for identification of species key to community structure and stability under herbicide-stressed and control communities (Figure 3). Specifically, our results suggest herbicide-stressed communities are more vulnerable to breakdown, since species that can produce flowers consistently throughout the growing season and thereby serve as pollination bridges while other species are not flowering (Arceo-Gomez et al. 2018) would be scarce relative to unstressed communities. Further, the extinction of these species could be detrimental (Bascompte and Jordano 2007; Martín Gonzàlez et al. 2010).

Beyond pollinator-mediated plant-plant interactions, the consequences of herbicide drift on plant communities have important implications for pollinators as well, because patterns of coflowering reflect nectar and pollen resource availability. Thus, if herbicide drift results in less connected plant communities with limited key flowering species, then pollinators will have less abundant and diverse floral resources available to them on average as well as less plant species to utilize as resource bridges during significant resource gaps (Timberlake and Memmot 2019).

In conclusion, our study provides strong evidence that herbicide pollution, even at extremely low drift concentrations, can have significant consequences for agro-eco plant species, the coflowering interactions between them, and potentially the pollinators that would visit them. However, it is important to note that plant species may respond differently to herbicide exposure depending on the context: for example, *A. theophrasti* showed higher sensitivity to dicamba drift in a recent field study than what we report here, and these results also varied by year (Johnson and Baucom 2022). Moreover, while we gained insight into how interspecific variation in response to

dicamba drift could affect communities using 'synthetic' communities, these differed from real plant communities in important ways that could affect outcomes. Unlike natural communities, our plants were approximately the same age, grown in pots, and randomly distributed throughout a constant greenhouse environment. Our controlled design, however, enabled us to isolate and characterize the effects of dicamba drift on broad ecological phenomena, particularly biomass accumulation and coflowering interactions, thereby allowing us to make predictions that now can be tested in natural plant communities.

In particular, our work highlights both unanswered questions and prompts new ones concerning drift in the wild, such as: does variation in resistance or tolerance lead to persistent shifts in plant community composition over time (e.g. Baucom 2009)? And do shifts in coflowering interactions caused by herbicide drift significantly affect pollinator visitation patterns? The adoption of our multi-species (≥ 25 species) community model into long-term field experiments with opportunities for direct plant-plant and plant-pollinator interactions would provide these key insights.

## 2.5 Acknowledgements

We thank former University of Pittsburgh undergraduate students G. Blanco, and S. O'Neill for their help in data collection, L. Follweiler for administering herbicide treatments, and anonymous reviewers and the editor for comments that improved the paper. V. I., R. S. B., and T.-L. A. conceived the study, and V. I. and T.-L. A. designed it. V. I. performed the study under supervision by T.-L. A. V. I. analyzed the data, and wrote the first draft of the manuscript, wherein

R. S. B. and T.-L. A. contributed revisions. This work was supported by NSF DEB-1834496 and USDA 2017-09529/1016564 to T.-L. A. and R. S. B. V. I. was additionally supported by the University of Pittsburgh Dietrich School of Arts and Sciences First-Year and K. Leroy Irvis Fellowships.

**Table 1. Key terms for defining plant responses to dicamba drift across the 25 species in this study.**

Under 'Functional Definition', see 'statistical analysis' for information about how estimated marginal means, contrast estimates, and significance were determined.

| Term | General Definition | Functional Definition |
|---|---|---|
| Resistance | The ability to inhibit or rapidly reduce immediate damage caused by a stressor. | The estimated marginal mean of the proportion of undamaged leaves (1 - the number of damaged leaves divided by the total number of leaves) at 48 hours post-treatment of dicamba drift is not significantly different than 1. |
| Tolerance | The ability to minimize damage caused by a stressor on fitness. | The contrast estimate of the difference in either short-term growth (plant size 21 days post-treatment) and/or final biomass (dry shoot biomass 145 days post-treatment) between dicamba drift- and control-treated plants is not significantly different than 0, i.e., $\text{growth/biomass}_{\text{drift}} - \text{growth/biomass}_{\text{control}} = 0$. |
| Overcompensation | The ability to utilize dicamba drift exposure to enhance fitness in the short-term or long-term. | The contrast estimate of the difference in either short-term growth or final biomass between dicamba drift- and control-treated plants is significantly greater than 0, i.e., $\text{growth/biomass}_{\text{drift}} - \text{growth/biomass}_{\text{control}} > 0$. |

**Table 2. Twenty-five agro-eco species used in the greenhouse experiment.**

'Life cycle' relates to the United States Department of Agriculture official PLANTS database characterization as annual (A), biennial (B), and/or perennial (P; USDA 2018). 'Pollination' (insect or wind) is based on Mulligan (1979) and Hilty (2019). 'Traits not analyzed' identifies species that were excluded from analyses on flowering time (i.e. day of first flower and flowering duration; FT), floral display (FD), probability of flowering (PF), biomass per flower (BF), 'full' (FN) and/or 'subset' (SN) coflowering networks or none of the above (indicated by a dash). In 'Category', eudicots are considered susceptible to synthetic auxin herbicides such as 2,4-D or dicamba whereas monocots are not. Taxonomic source for species names: Plants of the World Online. Facilitated by the Royal Botanic Gardens, Kew (http://www.plantsoftheworldonline.org, accessed 26 September 2022).

| **Species** | **Code** | **Family** | **Category** | **Life Cycle** | **Pollination** | **Traits not analyzed** |
|---|---|---|---|---|---|---|
| *Amaranthus palmeri* | AMPA | Amaranthaceae | Eudicot | A | Wind | - |
| *Daucus carota* | DACA | Apiaceae | Eudicot | B | Insect | PF |
| *Asclepias syriaca* | ASSY | Apocynaceae | Eudicot | P | Insect | FT, FD, PF, BF, FN, SN |
| *Erigeron annuus* | ERAN | Asteraceae | Eudicot | A | Insect | FT, FD, PF, BF, SN |
| *Eupatorium serotinum* | EUSE | Asteraceae | Eudicot | P | Insect | - |
| *Solidago canadensis* | SOCN | Asteraceae | Eudicot | P | Insect | FT, FD, PF, BF, SN |
| *Taraxacum officinale* | TAOF | Asteraceae | Eudicot | P | Insect | - |
| *Lepidium virginicum* | LEVI | Brassicaceae | Eudicot | A, B, P | Insect | FT, FD, PF, BF |
| *Commelina virginica* | COVI | Commelinaceae | Monocot | P | Insect | - |

| | | | | | | |
|---|---|---|---|---|---|---|
| *Ipomoea hederacea* | IPHE | Convolvulaceae | Eudicot | A | Insect | - |
| *Ipomoea lacunosa* | IPLA | Convolvulaceae | Eudicot | A | Insect | - |
| *Desmanthus illinoensis* | DEIL | Fabaceae | Eudicot | P | Insect | PF |
| *Senna obtusifolia* | SEOB | Fabaceae | Eudicot | A, P | Insect | - |
| *Trifolium pratense* | TRPR | Fabaceae | Eudicot | P | Insect | - |
| *Abutilon theophrasti* | ABTH | Malvaceae | Eudicot | A | Insect | - |
| *Sida spinosa* | SISP | Malvaceae | Eudicot | A | Insect | - |
| *Oxalis stricta* | OXST | Oxalidaceae | Eudicot | P | Insect | - |
| *Plantago lanceolata* | PLLA | Plantaginaceae | Eudicot | A, B, P | Wind | BF, PF, |
| *Plantago virginica* | PLVI | Plantaginaceae | Eudicot | A, B | Wind | FT, FD, PF, BF, SN |
| *Persicaria pensylvanica* | PEPE | Polygonaceae | Eudicot | A | Insect | - |
| *Rumex crispus* | RUCR | Polygonaceae | Eudicot | P | Wind | FT, FD, PF, BF, FN, SN |
| *Geum canadense* | GECA | Rosaceae | Eudicot | P | Insect | FT, FD, PF, BF, FN, SN |
| *Cardiospermum halicacabum* | CAHA | Sapindaceae | Eudicot | A, B, P | Insect | - |
| *Physalis philadelphica* | PHPH | Solanaceae | Eudicot | A | Insect | - |
| *Solanum carolinense* | SOCA | Solanaceae | Eudicot | P | Insect | - |

**Table 3. Estimated values for each coflowering network metric in the control and dicamba drift synethetic communities when all species that produced at least one flower in both communities were included in the analysis.**

*W* and *P*-values were obtained from Wilcoxon Mann-Whitney Tests for significant differences between the control and drift communities, except for connectance and modularity where *P*-values were obtained by comparing observed data against null models.

| **Network Metric** | **Community** | | ***W*** | ***P*** |
|---|---|---|---|---|
| | **Control** | **Dicamba Drift** | | |
| Degree | 16.55 | 12.73 | 367 | <.01 |
| Strength | 0.22 | 0.15 | 32700 | <.001 |
| Weighted Degree | 4.56 | 3.20 | 327 | <.05 |
| Connectance | 0.788 | 0.606 | - | <.01 |
| Modularity | 0.109 | 0.212 | - | <.01 |

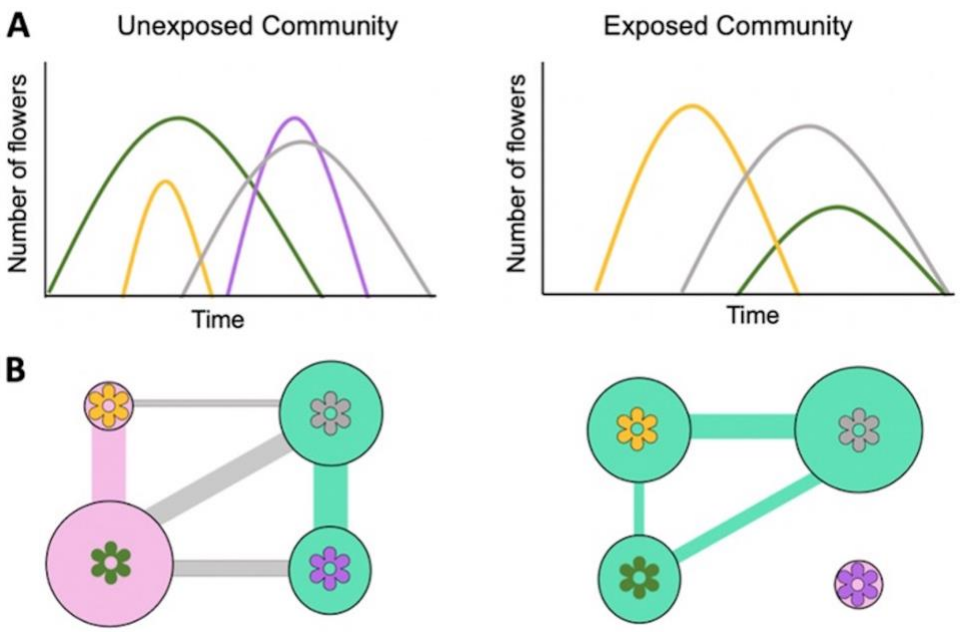



**Figure 1. Conceptual framework for assessing the impact of herbicide drft on coflowering interaction networks.**

A: Hypothetical flowering phenologies for four plant species (different colored lines) in an herbicide drift unexposed (left) and exposed (right) community over a growing season. B: Corresponding coflowering interaction networks for the four hypothetical plant species (different colored flower icons) based on flowering deployment shown in A. Links between species represent coflowering interactions (flowering overlap between species). The thickness of the lines reflects the strength of interactions (duration and intensity of flowering overlap). Different colored filled circles represent different modules (groups of species that interact more strongly, i.e. are more likely to coflower with each other than with other species); different colored lines indicate when species within modules (green or pink) or from different modules (grey) are interacting. The size of the circles reflects species betweenness centrality (the average percentage of shortest paths in the coflowering network that must go through a species, i.e. the relative importance of a species to network stability).

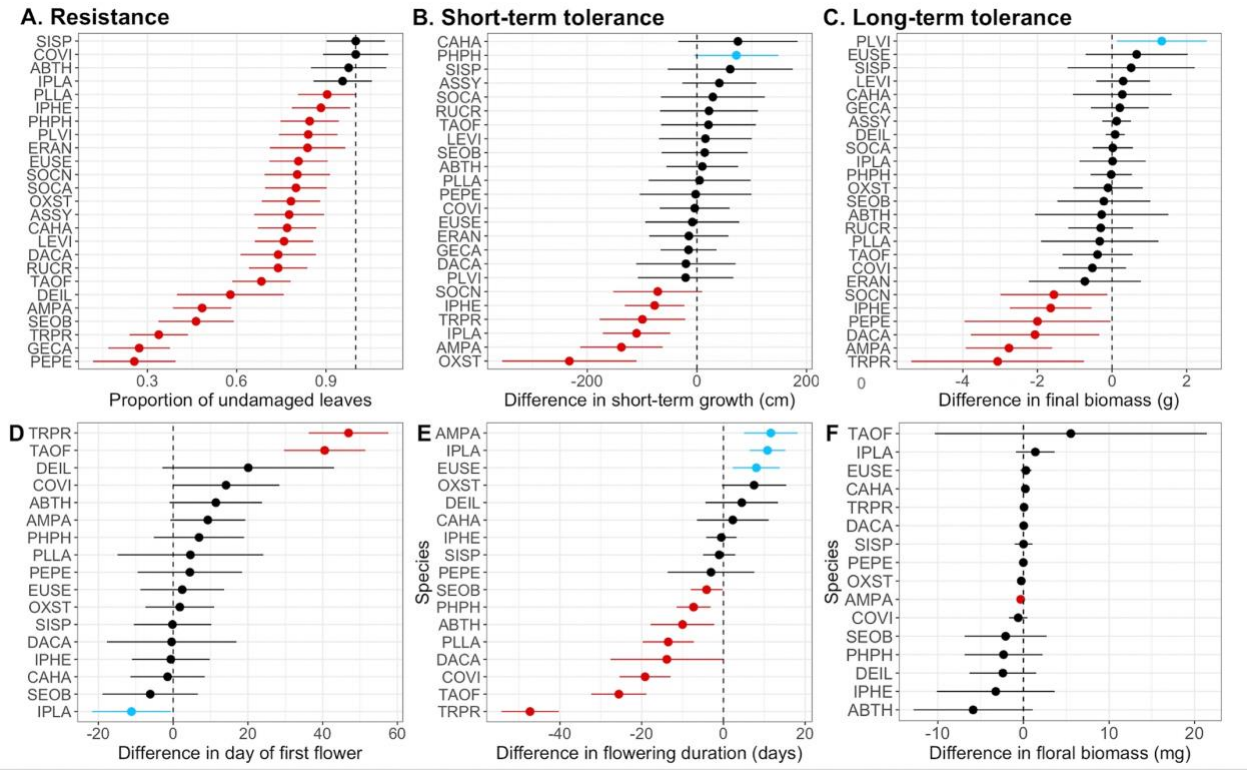



**Figure 2. Plant species vary in resistance and tolerance to dicamba drift and in how drift affects floral traits.** A: Estimated marginal means ± 95% confidence intervals show the proportion of undamaged leaves 48 hours after dicamba drift treatment by species, i.e. resistance scores. The vertical dashed line at 1 is a reference for no damage. B-F: Contrast estimates ± 95% confidence intervals show the difference between dicamba drift-treated plants and control plants, relative to control plants, in short-term tolerance (i.e., plant size at 21 days post-treatment; B), long-term tolerance (i.e., final biomass at 145 days post-treatment; C), day of first flower (D), flowering duration (E), and biomass per flower (F). Red denotes species that (A-C) were significantly negatively impacted by dicamba drift, (D) dicamba drift delayed the day of first flower, (E) shortened flowering duration, or (F) decreased biomass per flower. Light blue shows significant effects in the opposite direction and black indicates no significant change. See Appendix A: Tables 7, 10-14 for results of tests of significance. Species are designated by four-letter codes as in Table 2. Values plotted are back-transformed (see Appendix A: Figure 14 for transformed data used in statistical models).

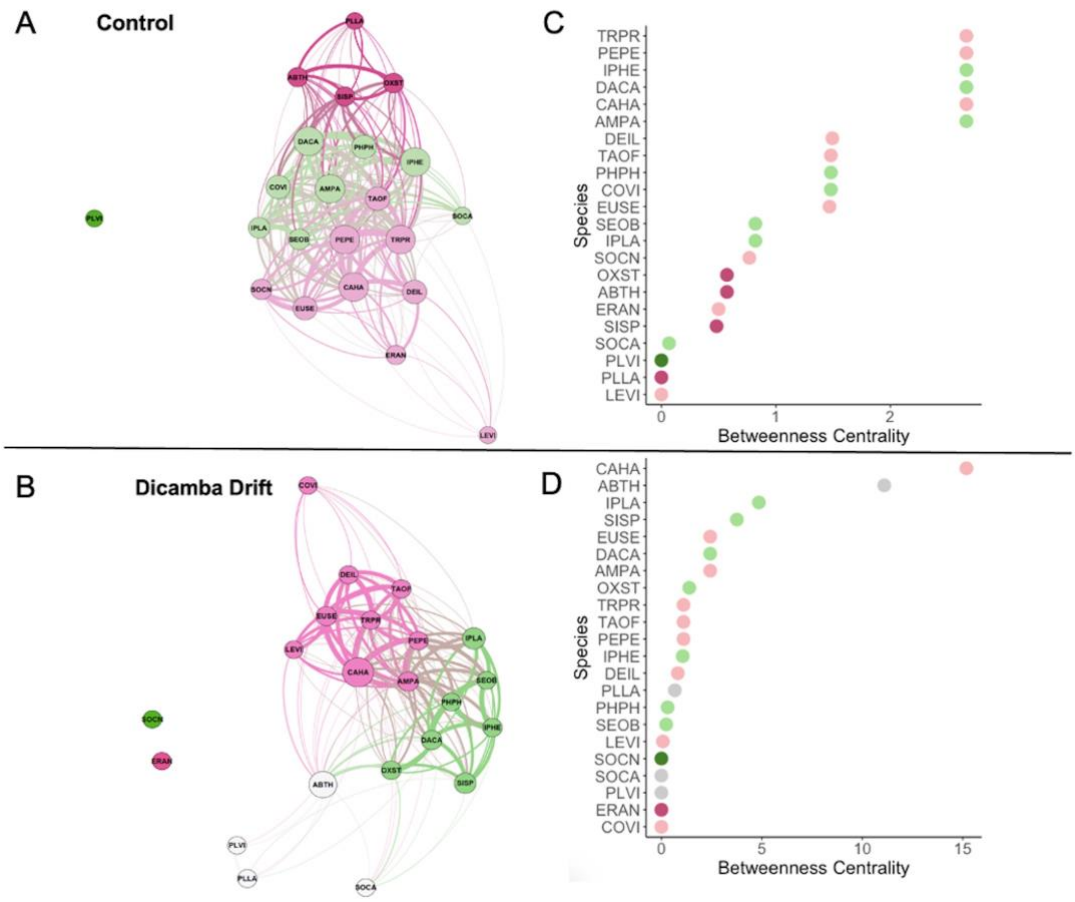



**Figure 3. Coflowering networks of control and dicamba drift exposed synthetic greenhouse communities.** A-B: Full networks when all flowering species ($n$ = 22) are represented in the control (A) and drift (B) synthetic plant community. Each plant species is represented as a circle, and links between them represent coflowering interactions. The thickness of the lines reflects the strength of coflowering overlap (duration and intensity), and circle size reflects species betweenness centrality (the relative importance of species for network stability). C-D: Betweenness centrality for each species according to the full networks in rank order for the control (C) and drift (D) community. High values reflect higher relative importance in the network. A-D: Different colors represent different modules (groups of species that coflower more strongly with each other than with other species). See Table 2 for species codes noted in circles (A-B) and on y-axes (C-D), and Appendix A: Figure 21 for results of subset networks that only show species that flowered in both communities.

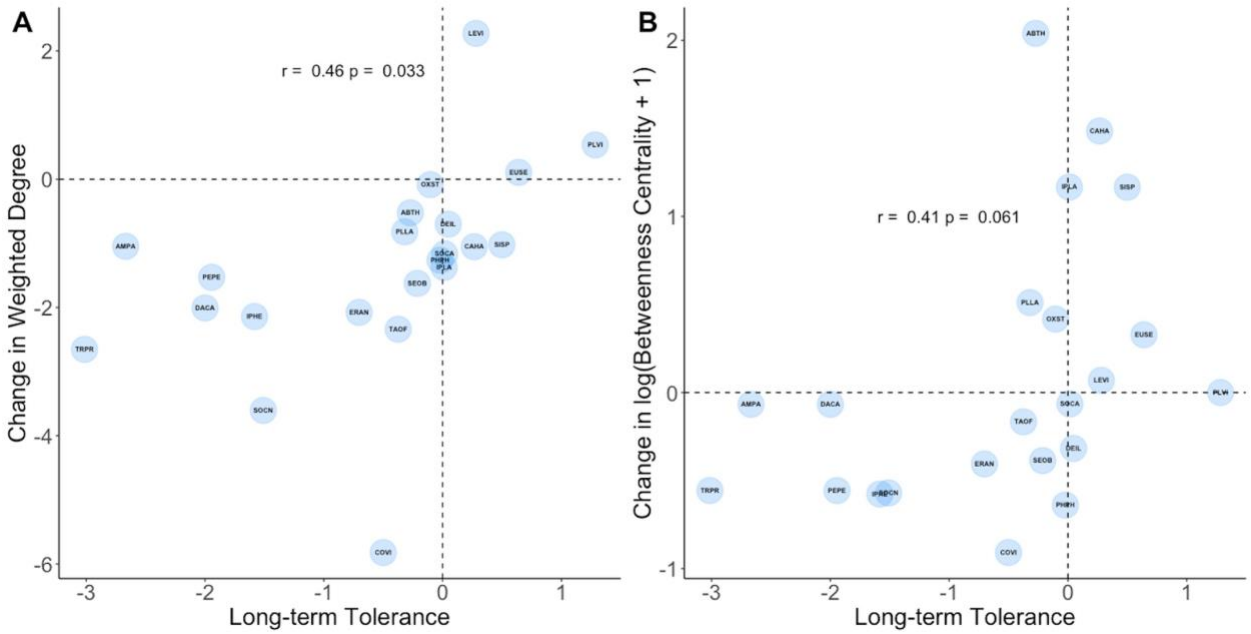



**Figure 4. Species-level tolerance is correlated with a change in coflowering interactions between dicamba drift- exposed and control synthetic communities.**

Species (blue points labeled with four-letter codes; Table 2) and long-term tolerance scores (Table 1; Figure 2C) correlated with the change in (drift subtracted by control) weighted degree (A; Table 3) and log-transformed betweenness centrality (B) between the dicamba drift and control greenhouse communities.

# 3.0 Rhizobial variation, more than plant variation, mediates plant symbiotic and fitness responses to herbicide stress

This chapter is a slightly modified version of the following research article that is currently under review in *Ecology*:

Iriart, V., E. M. Rarick, and T.-L. Ashman. *In review*. Rhizobial variation, more than plant variation, mediates plant symbiotic and fitness responses to herbicide stress.

## 3.1 Introduction

Virtually every ecosystem on earth benefits from the services provided by mutualistic interactions (Janzen 1985; Bronstein 2001; Christian 2001; Ollerton et al. 2011). However, of all species interactions, mutualisms are predicted to be the least stable—meaning that, under altered biotic and/or abiotic conditions, mutualistic partners may not maintain fitness benefits from interacting (Chamberlain et al. 2014). Given this context-dependency, the rise of strong anthropogenic stressors such as agriculture intensification, urbanization, climate change, and the spread of invasive species may result in severe mutualism disruptions. Specifically, anthropogenic stressors could reduce the frequency of interactions between mutualistic partners (Chittka and Schürkens 2001; Ashman et al. 2005) or induce a breakdown in the mutualism altogether, wherein mutualists cease interacting, become parasitic, or go extinct (e.g. Brown 1997; Sachs and Simms 2006). Thus, understanding how mutualisms are changing throughout the world as a result of

contemporary sources of stress represents a grand and timely challenge (Kiers et al. 2010; Bell 2017; Teixido et al. 2022).

The plant-rhizobia symbiosis is an ecologically and agriculturally important mutualism between nitrogen-fixing bacteria known as rhizobia and leguminous plants. Within root nodules, rhizobia transform atmospheric nitrogen (N) into a plant-accessible form in exchange for photosynthates. Stressful abiotic conditions such as exposure to agrochemicals can negatively affect plant-rhizobia interactions by interfering with nodulation (e.g. inhibiting nodule formation or decreasing nodule number or size), and/or reducing symbiotic N fixation (reviewed in Karmakar et al. 2014). However, genotypes of either partner, i.e. plants ($G_P$) or rhizobia ($G_R$), can vary in stress tolerance (Thrall et al. 2008; Porter and Rice 2013; Burquero et al. 2022; Zhang et al. 2023), as well as the benefits they provide each other, i.e. 'partner quality' (Heath and Tiffin 2007; Sachs et al. 2010; Batstone et al. 2016). In turn, variation in stress tolerance and/or partner quality could influence how symbiotic traits are impacted by stress, yet this hypothesis has rarely been addressed (Gopalakrishnan et al. 2015; O'Brien et al. 2018; Porter et al. 2020).

Genetic variation in partner interactions may vary with the environment and can impact outcomes of plant-rhizobia symbioses under stress in several ways. First, genetic variation in one partner type ($G_P$ or $G_R$), can additively mediate how mutualism-related traits are affected by a stressful environment (E); i.e. there can be $G_{(P\ or\ R)} + E$ effects on mutualisms (Figure 5a). For example, Wang et al. (2018) showed that, although high levels of salinity stress decreased plant dependence on arbuscular mycorrhizal fungi, the magnitude of decrease was significantly less with the more active (i.e. 'higher quality') fungal strain than with the less active strain. Additionally, Ashraf and Iram (2005) found that a more-drought tolerant plant species incurred a smaller percent reduction in nodule number, mass, and N fixation activity in the presence of water stress than a

more-drought sensitive plant species. In these cases, variation in plant or microbial partner gives rise to rank differences in the abilities of mutualists to interact regardless of environmental stress.

Second, genetic variation in plant or rhizobial partner can modulate how mutualisms are impacted by stress via significant $G_{(P \text{ or } R)} \times E$ interactions (Figure 5b). For instance, the presence of a stressor can alter which partner shows the highest value mutualistic traits (e.g., in Figure 5b, Partner a loses first rank to Partner b when the stressor herbicide is present). For example, plant genotypes with the highest nodule number in one environment can differ from those with highest nodule numbers in another (Vaidya and Stinchcombe 2020). Additionally, there is some evidence that symbionts with greater stress tolerance can better maintain mutualistic services under stress than more sensitive ones (such as Partner b in Figure 5b; Ahemad and Khan 2010; Maslennikova et al. 2022). Alternatively, differences in rank among symbionts could also become less extreme as the environment shifts from optimal to more stressful or depleted (see Zheng et al. 2014), thereby making symbionts more equivalent in terms of partner quality (e.g. the difference in mutualism trait value between Partners a and c diminishes in the presence of a stressor in Figure 5b).

Finally, *both* plant and rhizobial genotypes could contribute to the expression of symbiotic traits under stress in additive (e.g. $G_P + G_R + E$; Figure 5c) or interactive (e.g. $G_P \times G_R \times E$; Figure 5d) ways or a combination of the two (e.g. $G_P \times G_R + E$ [Figure 5e] or $G_{P \text{ or } R} + G_{P \text{ or } R} \times E$ [Figure 5f]). In particular, $G \times G \times E$ effects in mutualisms have largely been studied in the context of local adaptation; i.e. when the fitness of both partners is higher when they are both local rather than foreign (Thompson 2005; Hoeksema et al. 2008; Barrett et al. 2012). By extension, certain combinations of plant and rhizobial genotypes could mitigate the way mutualistic traits are expressed across gradients of stress. Johnson et al. (2010), for example, demonstrated that when

resources were limited, various plant ecotypes received the greatest growth benefits from fungal symbionts that they were coadapted to as opposed to novel ones, but when resources were abundant, this symbiosis shifted towards commensalism or parasitism, regardless of plant-fungal combination. Moreover, it is well-known that anthropogenic N addition can modulate nodule number and plant growth benefits conferred from rhizobia depending on the legume and rhizobial genotypic pairing (e.g. Heath and Tiffin 2007; Heath et al. 2010; Weese et al. 2016). However, we currently lack studies which examine the interactions between genetically variable plant and microbial partners and their response to many other modern human-mediated environmental changes.

Accordingly, we investigated whether genetic variation in plants and/or rhizobia could mediate the consequences of herbicide exposure on the plant-rhizobia symbiosis. Herbicides are the most-used class of pesticides globally (FAO 2020); thus they frequently contaminate the air, water, and soil of ecosystems near agricultural fields and other areas where they are sprayed such as managed lawns, landscapes, or forests (Pimentel 1995). And while herbicide exposures at high, field application rates will kill on-site plants, exposure at lower, 'drift' levels relevant to off-site movement in the atmosphere (~1% of the field rate; Egan et al. 2012) can still have damaging impacts on nontarget plants, such as vegetative deformities and reductions in growth/fitness (reviewed in Iriart et al. 2021). As leguminous plants and their rhizobial symbionts are common members of ecological communities throughout the world (Crews 1999), including those that border agricultural areas (i.e. along the 'agro-eco interface'; Burdon and Thrall 2008; Iriart et al. 2022), and are also cultivated for food, forage, and N inputs (Goyal et al. 2021), herbicide pollution represents a relevant and widespread threat to the stability of plant-rhizobia interactions. Presently, it is known that herbicide exposures at field rates can negatively impact nodulation (Ahemad and

Khan 2012; Patil et al. 2012), yet it is unknown whether drift levels also negatively affect aspects of the plant-rhizobia symbiosis or whether plant or rhizobia genetic variation could mitigate these impacts.

Here, we used the herbicide 'dicamba' to address these questions. Dicamba is currently one of the most commonly-used herbicides in the United States (U. S. Geological Survey 2022) and it has frequently been associated with drift pollution (Bohnenblust et al. 2016; Olzyk et al. 2017; Iriart et al. 2022). Rather than inhibiting a specific molecular target, dicamba's mode of action involves mimicking the key plant growth hormone auxin to cause abnormal growth in dicot plants (Gleason et al. 2011). Thus, variation among plant and/or rhizobial genotypes in the utilization and metabolizing of auxin, which is also necessary for nodule initiation/formation (Gomes and Scortecci 2021) and is synthesized by rhizobia (Spaepen and Vanderleyden 2011), could result in phenotypic variation of plant-rhizobia symbiotic traits in response to dicamba drift exposure. Therefore, dicamba is an especially useful model to elucidate relationships between anthropogenic stress, genetic variation, and outcomes of the plant-rhizobia mutualism.

We grew 17 families of red clover (*Trifolium pratense*), a species sensitive to dicamba drift (Iriart et al. 2022), in the greenhouse in combination with one of two rhizobial genetic strains or without rhizobia, and exposed half to a drift-level rate of the herbicide. We evaluated whether: 1) plant or rhizobial genetic variation ($G_P$ or $G_R$) mediated the damage plants incurred immediately following herbicide exposure; 2) herbicide exposure (E), $G_P$, $G_R$, or their interactions significantly impacted key aspects of the plant-rhizobia mutualism (nodule number, nodule size, and symbiotic N fixation); and 3) herbicide treatment, plant and/or rhizobial variation, or their interactions ultimately influenced the fitness (biomass) benefit plants gained from interacting with rhizobia.

## 3.2 Methods

### 3.2.1 Study Species and Genetic Variation

Red clover (*Trifolium pratense*, Fabaceae) is an herbaceous, perennial legume with a broad geographic range, present throughout all of Europe and in large parts of the other six continents excluding Antarctica (Dias et al. 2008; Nay et al. 2023). To capture $G_P$, we obtained a globally diverse set of accessions from the United States Department of Agriculture National Genetic Resources Program (https://npgsweb.ars-grin.gov/, accessed 27 January 2020) and through direct collection to encompasses the natural range of the species and grew them in the greenhouse at the University of Pittsburgh at 21.3℃ ± 2.9 under supplemental lighting (16:8 light:dark). Plants were in 11.4 cm × 11.4 cm ×10.2 cm pots and fertilized every two months with Osmocote 14N – 14P – 14K (ICL Specialty Fertilizers, Ltd., Dublin, OH). Under these common conditions, we created 17 full-sibling families by performing reciprocal crosses between two plants sourced from the same accession (Appendix B: Table 16).

To capture $G_R$, we obtained two strains of rhizobial symbionts of red clover (*Rhizobium leguminosarum* biovar *trifolii*): one from the American Type Culture Collection (Manassas, VA, USA; strain ATCC 14479, hereafter: '14479'), and one from the Northern Regional Research Lab (Peoria, IL, USA; strain NRRL B-4386, hereafter: '4386'). Both strains can nodulate red clover but are genetically distinct at the 16S rRNA region and were originally isolated from different regions in the USA (Appendix B: Table 16).

### 3.2.2 Experimental Planting Conditions

We surface-sterilized seeds from each full-sib family with ethanol and bleach, and planted 1-3 into sterile soil (twice-autoclaved 3:1 mixture of Old Castle C/B soil [even mix of peat moss, bark, perlite, and vermiculite] and Germination Mix [65% peat moss, 25% perlite, 10% vermiculite]; BFG Supply Co., Burton, OH, USA) without any initial nutrient or N fertilizer in 656 mL pots in trays (D40 and D20T; Stuewe & Sons, Inc., Tangent, OR, USA). To avoid microbial contamination, prior to planting, we bleach sterilized pots and covered bottom holes with 0.45 $\mu$m Nylon membrane filter paper (Tisch Scientific, Cleves, OH, USA), which allows water but limits microbial flow-through (Petipas et al. 2020).

We planted seeds in three spatiotemporal blocks in the greenhouse then thinned pots to one plant/pot once seedlings germinated. Planting event 'A' occurred on 21-28 April ($n$ = 91 plants), 'B' on 13-21 May ($n$ = 148 plants), and 'C' on 21-28 May 2021 ($n$ = 140 plants). Each occupied a different bench in the greenhouse. Planting events had on average of eight plants per family (range= 1-32), except for planting event A where seeds from two families (NOR39 and SRB91) were in short supply thus were not planted. Greenhouse conditions were 24.4°C $\pm$ 2.2 and the light cycle was 16:8 light:dark. After planting, we top-watered plants as needed and fertilized them weekly with 10mL of N-free Fahräeus media (Barker et al. 2007; modified from Vincent 1970) that was supplemented with 0.5mM of $KNO_3$ to provide a low amount of N. A drench of broad-spectrum fungicide (Banrot, Everris NA Inc., Dublin, OH, USA) was applied twice.

### 3.2.3 *Rhizobium* Inoculation and Herbicide Treatments

We inoculated plants twice (at five and six weeks post-germination) with 1mL of rhizobial culture containing either strain of *R. leguminosarum* diluted in liquid tryptone yeast media to 1 x $10^8$ CFU $mL^{-1}$ or media alone ('uninoculated'). To dilute rhizobial cultures, we measured their optical density at 600nm (OD600) and used the relationship between OD600 and CFU $mL^{-1}$ for each strain (Appendix B: Figure 22) to dilute them to an OD600 reflective of ~1 x $10^8$ CFU $mL^{-1}$. To prevent contamination between inoculation treatments (hereafter, IT), we applied the same IT to every plant within a tray of 8-12 plants from 4-6 full-sib families. Trays within each planting block were stratified across the greenhouse benches to prevent inter-treatment contamination.

We exposed 7-week old plants to one of two levels of the herbicide treatment (hereafter, HT): 1% ('drift') of the field application rate of dicamba (3,6-dichloro-2-methoxybenzoic acid; Albaugh, LLC, Ankeny, IA), i.e. 561 g of active ingredient per hectare (Albaugh 2018), or 0% ('control') using a handheld sprayer as in Iriart et al. (2022). To confirm the efficacy of our dicamba product, we exposed additional plants ($n$ = 12 from different families) to 100% of the field application rate at the same time as the other HTs were applied and confirmed 100% death by 21 days post-HT. All treatments included 0.1% v/v 'Preference' surfactant (non-ionic surfactant blend, WinField Solutions, St. Paul, MN) and were conducted in a separate room. After HT, we repopulated trays in the greenhouse such that every tray had a 1:1 ratio of drift:control plants. Altogether, there were 1-2 plants/family/HT per tray and 13 trays per IT.

### 3.2.4 Characterization of Plant and Symbiosis Responses to Herbicide Drift

Prior to HT, we enumerated the total leaves and measured the length of the longest leaf with a digital caliper to the nearest 0.1 mm and estimated pre-HT plant size as their product as in Iriart et al. (2022). To quantify instantaneous damage, at 48 hours post-HT, the time when dicamba injury first becomes apparent (Huang et al. 2016), we recounted leaves and recorded those with symptoms characteristic of dicamba damage (e.g., cupping or twisting; Griffin et al. 2013; Foster and Griffin 2018) as in Iriart et al. (2022). To assess the effect of HT on plant biomass as a proxy for fitness (Younginger et al. 2017), we harvested shoots at 45 days post-HT, dried them at 70℃ for >48 hours, and weighed them to the nearest $\mu$g (Ohaus SPX223 portable scale, Ohaus corp., Parsippany, NJ, USA).

To assess plant-rhizobia mutualism, we scored nodule number and size per plant. For plants from the two larger planting events, B and C, we scored these traits for a random subset (11/17) of the families. Specifically, at harvest, we photographed cleaned roots on a light box. We randomly selected 10 nodules/plant by forming a transect across the length of roots and removing nodules at regular intervals to mitigate bias for larger or smaller nodules. Then we collected, dried and weighed them to the nearest ng (Cahn Model 31 Microbalance, Thermo Fisher Scientific Corp., Waltham, MA, USA) and estimated mean dry biomass per nodule (i.e. 'size') per plant by dividing the total mass of the selected nodules by 10. Finally, we manually counted all nodules per plant from the photographs of roots using the 'cell counter' tool within the application Fiji (Schindelin et al., 2012; Appendix B: Figure 23). We dried and weighed roots for use as a covariate in our analyses. We added the total mass of removed nodules to root biomass estimates. Only a small percentage (7%; 6/89) of uninoculated plants had any nodules and these were insignificant

in number relative to inoculated plants (< 5 vs hundreds), indicating negligible contamination between inoculation treatments.

We estimated symbiotic N fixation using foliar $\delta^{15}$N data (the isotopic ratio of $^{15}$N:$^{14}$N in a sample relative to atmospheric air). Plants which receive high amounts of symbiotically-fixed N are enriched in $^{14}$N and have lower foliar $\delta^{15}$N content compared to those receiving little or no fixed N (Craine et al. 2015; Lindström and Mousavi 2020). Dry leaf tissue from plants inoculated with rhizobia (mean = 3 plants/family across all families; *n*= 174) were analyzed by the Cornell University Stable Isotope Laboratory (Ithaca, NY, USA) for $\delta^{15}$N quantification via a Thermo Delta V isotope ratio mass spectrometer (IRMS) interfaced to a NC2500 elemental analyzer. A subset of uninoculated plants in each HT (mean = 4 plants/ family for 6 families, *n* = 43 samples) was also analyzed to confirm that rhizobial inoculation led to decreased $\delta^{15}$N.

### 3.2.5 Statistical Analyses

All analyses were performed in R (version 4.0.5; R Core Team 2021). We ran mixed-effects general and generalized linear models using the *(g)lmer* function from the *lme4* package (Bates et al. 2015). We performed natural log-transformations on all response variables except instantaneous damage to meet model assumptions for normality. For the foliar $\delta^{15}$N data, which contained negative values, we added a small constant before performing the log-transformation so that the minimum $\delta^{15}$N value equaled one. Covariates were also log-transformed as doing so improved model fit, assessed via AICc (Akaike Information Criterion, adjusted for small sample sizes; Akaike, 1973) using the *AICcmodavg* package (Mazerolle 2023). We validated the best-fitting candidate models with the lowest AICc for each response variable by visually inspecting

residual vs fitted plots and histograms of residuals to confirm linearity and normality, respectively. All models included planting event as a random effect. The additional random effect of tray (nested within IT) did not affect model performance and explained little if not zero variance, therefore we excluded it from final models.

To examine whether red clover families differed in their immediate vegetative response to HT (question 1; Table 4) and whether rhizobia influenced this, we constructed generalized linear mixed-effects models with a binomial distribution on plants treated with the drift HT only. The response variable was a two-column matrix where the first column was the number of leaves showing immediate damage post-HT (i.e. number of 'successes') and the second was the number of leaves that were not damaged (i.e. number of 'failures'; total # leaves – # damaged leaves) (Muschelli et al. 2014). The explanatory variables were family, IT (i.e. rhizobial strain 14479, 4386, or uninoculated), and the family × IT interaction.

To analyze variation in how dicamba drift impacted the plant-rhizobia mutualism and plant fitness (questions 2 & 3; Table 4), we ran mixed-effects linear models with nodule number, nodule size, symbiotically-fixed N (estimated as $-1 \times$ foliar $\delta^{15}$N), and shoot biomass as response variables. We compared candidate models which included family, IT, HT, and all possible two-way and three-way interactions as explanatory variables. Family was a fixed effect because these were selected specifically to broadly capture plant genetic variation. Models analyzing symbiotic traits included only plants that were inoculated with rhizobia and confirmed to have nodules. Root biomass was used as a covariate in models analyzing nodule number and size to relativize nodule biomass production per g of root tissue. Nodule number and pre-HT size were covariates in models analyzing symbiotically fixed nitrogen and shoot biomass, respectively.

We tested for significant fixed effects by running ANOVAs with type III sum of squares using the *Anova* function from the *car* package (Fox and Weisberg 2011). We determined estimated marginal means (EMMs) of factor levels and performed planned *post hoc* pairwise comparisons to test for significant differences between them with Tukey adjustments using the *emmeans* function from the *emmeans* package (Lenth 2020). Specifically, if there was a plant family × rhizobial IT interaction for immediate vegetative response to dicamba drift, then for each category of family, we compared EMMs according to IT category to assess how IT mediated damage incurred by plant families. If there was a family × HT or IT × HT interaction for the other responses (nodule number/size, symbiotically fixed N, and shoot biomass), then we conducted two types of comparisons. First, for each respective family, strain, or IT category, we compared EMMs according to HT, i.e. dicamba drift v. control. Second, for each HT category, we compared EMMs according to family or IT category. While the first method elucidates how plant/rhizobial genotypes modulate the effects of dicamba drift on a given trait, the second reveals how dicamba drift could potentially affect genotypic rank differences in trait values. If there was a three-way interaction, then for each plant family category, we planned to compare EMMs according to both IT and HT to evaluate how interactions between plant and rhizobial genotypes were affected by dicamba drift.

Limited seed production by some plant families led to low replication, so we assessed the possible impact of these imbalances on our conclusions by constructing models based on the full but imbalanced dataset and on a reduced, completely balanced dataset (only families with $\geq 2$ plants/treatment included; Appendix B: Table 16). We then assessed whether the best fitting model for each response variable differed (i.e. by 2 or more AICc; Zuur et al. 2009) or if their results were conflicting. The best-fitting models and the majority of significant fixed effects derived from

the full dataset were equivalent to those from the reduced dataset, thus we present results from best-fitting models on the full dataset in the main text (see Appendix B: Table 17 for all models; Appendix B: Table 18 for results of analyses of reduced datasets).

Figures were created using the ggplot2 package (Whickham 2012). Results are reported as mean ± SE throughout.

## 3.3 Results

### 3.3.1 $G_P \times G_R$ Determined Immediate Damage from Herbicide

On average, dicamba-related injury was apparent on about half (46% ± 2.4%) of the leaves of plants 48 hours after the drift HT was applied. However, the degree of damage was highly dependent on a family × IT interaction ($p$ <.0001; Table 4a; Figure 6). *Post hoc* analyses revealed that, for about 40% of families (AUT57, CAN66, HUN71, NZL47, SRB91, SWE49, and TUR37), plants showed significantly more herbicide damage when inoculated with rhizobial strain 14479 than when uninoculated (Appendix B: Table 19). In particular, those that incurred the maximum damage were SWE49 plants inoculated with strain 14479: 76% ± 8.7% of their leaves were damaged, which was 44% more than uninoculated SWE49 plants (Appendix B: Table 20). Plants from just one family (NZL77) had significantly less damage (by 37%) when inoculated with strain 14479 than when uninoculated. Conversely, all families incurred similar levels of immediate damage when inoculated with strain 4386 than when uninoculated, except for family SRB91, which incurred significantly more damage when inoculated with either strain (54% greater damage

with 4386 v. uninoculated; 35% greater damage with 14479 v. uninoculated). For a few families (AUT57, NZL47, and TUR37), the rhizobial strain modified damage intensity. For example, AUT57 plants inoculated with strain 4386 only exhibited injury on 33% $\pm$ 5.4% of leaves, which was 30% less than the injury shown on AUT57 plants inoculated with 14479. The remaining families showed no impact of inoculation treatments on immediate damage expression.

### 3.3.2 $G_P + G_R \times E$ Determined Nodule Number, $G_R \times E$ Determined Nodule Size

Of the plants that we scored for nodule traits, 94% (80/85) of those inoculated with rhizobial strain 14479 and 80% (57/72) of those inoculated with strain 4386 had nodules, indicating that the vast majority of plants invested in interacting with both rhizobial genotypes. Both main effects of plant and rhizobial genetic variation significantly influenced the number of nodules (per g of root biomass: both $p$ <0.001; Table 4b; Appendix B: Figure 24a) on red clover roots. Overall, inoculation with strain 14479 resulted in more nodules than with 4386; however, dicamba drift exposure increased the magnitude of rhizobial differences in nodule number (strain $\times$ HT: $p < 0.0001$; Table 4b; Figure 7a). Herbicide treatment did not affect nodule number when plants were inoculated with 14479 ($t = 0.473$, df = 157, $p = 0.64$), but those with strain 4386 made significantly less (by 59%) nodules when treated with herbicide compared to controls ($t = 3.82$, df = 157, $p < 0.001$). Thus, the percent difference in nodule number between strains increased from 125% in control conditions (14479 v. 4386: $t = 4.50$; $p = <.001$) to 400% in response to drift (14479 v. 4386: $t = 3.82$, $p <.001$) (Figure 7a; Appendix B: Table 21).

Rhizobial strains additionally influenced nodule size, but their effect depended on herbicide exposure ($p = 0.0010$); meanwhile, plant genetic variation did not have a significant

effect on this symbiotic trait (Table 4b). When plants were untreated (control), nodule sizes between plants inoculated with strain 14479 v. 4386 were similar ($t$ = 0.380, df = 162, $p$ = 0.71). When treated with herbicide, plants with 14479 made significantly smaller nodules (by 34%; $t$ = 2.97, df = 159, $p$ = 0.0035) but those with 4386 made larger ones (by 27%), although this difference was not statistically significant ($t = -1.35$, df = 158, $p$ = 0.18; Figure 7b; Appendix B: Table 22).

### 3.3.3 $G_R$ + E Determined Symbiotically-Fixed N

Confirming expectations, plants inoculated with rhizobia had significantly lower foliar $\delta^{15}$N than uninoculated plants, indicating the plants received symbiotically fixed N ($\delta^{15}$N with rhizobia = 0.26 $\pm$ 0.13 v. without rhizobia = 2.27 $\pm$ 0.21; $X^2$ = 71.7, df = 1, $p$ < .0001). Among inoculated plants, rhizobial strain ($p$ = 0.0011) and HT ($p$ < 0.001) each significantly and independently influenced foliar $\delta^{15}$N (controlling for nodule number; Table 4b). Strain 14479 was more efficient, i.e. fixed 55% more N per nodule, than strain 4386 ($\delta^{15}$N with strain 14479 = $-0.34$ $\pm$ 0.37 v. strain 4386 = 0.15 $\pm$ 0.46; $t = -3.00$, $p$ = 0.017). However, herbicide treatment decreased the symbiotic N plants received from either rhizobia: foliar $\delta^{15}$N increased by 258% on average when plants were treated with herbicide compared to controls ($\delta^{15}$N in control = $-0.44$ $\pm$ 0.35 v. drift = 0.28 $\pm$ 0.49; $t = -4.44$, $p$ = <.001; Figure 7c; Appendix B: Table 23). Plant family did not have a significant main effect ($p$ > 0.05) nor did it interact with herbicide treatment or rhizobial strain to affect these results (Table 4b).

### 3.3.4 $G_P + G_R \times E$ Determined Plant Fitness

Herbicide exposure directly decreased shoot biomass in a way that was inoculation-dependent (inoculation × HT interaction: $p = 0.011$; Table 4c; Figure 7d). After accounting for pre-HT size, plants inoculated with rhizobial strain 14479 were the most negatively affected by dicamba drift, showing a 60% reduction in shoot biomass compared to their control-treated counterparts ($t = 6.51$, df = 301, $p < .0001$), followed by plants inoculated with strain 4386 (36% reduction; $t = 2.93$, df = 301, $p = 0.0036$) and finally uninoculated plants (30% reduction; $t = 2.55$, df = 301, $p = 0.011$). Consequently, herbicide exposure altered the magnitude of fitness benefit plants received from symbionts. Without exposure to herbicide, compared to uninoculated plants, those growing with strain 14479 were 248% larger ($t = 9.47$, df = 301, $p = <.0001$) and with 4386 were 105% larger ($t = 5.28$, df = 301, $p <.0001$) and the difference between strains was also significant ($t = 3.94$, df = 303, $p = 0.0003$). Conversely, with herbicide exposure, the benefit of rhizobia was still significant but weaker: plants grown with strains 14479 and 4386 were 100% ($t = 4.66$, df = 300, $p = <.0001$) and 87% ($t = 3.74$, df = 300, $p = 0.0006$) larger, respectively, than uninoculated plants; however the difference between strains disappeared (Figure 7d; $t = 0.499$, df = 300, $p = 0.87$; Appendix B: Table 24). While plant family also explained variation in fitness ($p = 0.010$; Appendix B: Figure 24b), we did not detect a significant family × IT × HT interaction, suggesting that families responded similarly to the inoculation and herbicide environments.

## 3.4 Discussion

Our results provide first insight into the consequences of drift-level synthetic auxin herbicide exposure on belowground mutualisms between plants and beneficial soil rhizobia. Importantly, we demonstrate that plant and rhizobial genetic variation moderate the consequences for plants, but that more often than not herbicide effects depend on *Rhizobium* strain. While herbicide exposure immediately resulted in leaf damage, it also significantly affected all of the relevant symbiotic traits measured and decreased plant fitness, and the magnitude and/or direction of these impacts were consistently dependent on rhizobial variation and often plant variation. Thus, these findings highlight the significance of genetic variation in both plant and rhizobial partners ($G_P$ and $G_R$) in response to anthropogenic environmental changes (E), as well as how interactions between them shape the efficacy of the plant-rhizobia mutualism.

### 3.4.1 Effects of Rhizobial Symbiosis on Plants in Herbicide-Exposed Contexts

Although it is known that inter- and intraspecific variation for herbicide sensitivity in plants is significant, the drivers of this variation are often undetermined (Espeby et al. 2011; Olszyk et al. 2015; Iriart et al. 2022). Our findings shed light on ecological interactions with rhizobial symbionts as one such driver. By the time the inoculated plants in our study were treated with herbicide (2 weeks post-inoculation), they had likely already initiated interactions with their rhizobial partners: red clover typically forms nodules 1-3 weeks after contact with rhizobia (Sprent 2007; Malinda Sameera Thilakarathna 2013; Iriart, pers. obs.), and even free-living rhizobia in soil produce phytohormones, including auxin, that typically stimulate plant growth (Sarkar and Laha

2013) but in the context of synthetic auxin exposure might have varying effects on plants. Thus, differences in initial interactions between plant and rhizobial genotypes and/or genetic mechanisms for how plants and rhizobia at first respond to an overabundance of auxin (Bhat et al. 2015; Ristova et al. 2018) could explain the plant family × rhizobial strain interaction for immediate damage post- dicamba drift treatment discovered here and would be important to test for in future research. More generally, following the geographic mosaic theory of coevolution (Nuismer et al. 2000; Thompson 2002), this $G_P \times G_R$ effect suggests that natural selection may act on plants (legumes) in herbicide-exposed contexts to influence their coevolution with rhizobia. Specifically, the immediate effects of herbicide contamination on a legume population would depend on the genotypes of both the plants and the local rhizobia, and certain combinations of plants and rhizobial genotypes would be advantaged over others, at least in the short term. If however, this advantage is great enough to affect legume fitness, then herbicides may be an important driver of genotypic frequencies among legumes and rhizobia via their symbiotic interactions, especially in areas where exposures are frequent (e.g. near agriculture; Gomulkiewicz and Kirkpatrick 1992).

However, our result that only $G_R$ interacted with the herbicide environment to determine shoot biomass (a more long-term estimate of fitness) at several weeks post herbicide treatment indicates that *rhizobial* genotype, i.e., the biotic ecological context, might in some cases actually be more important than plant genotype for predicting the ultimate consequences of herbicide stress on legume and rhizobial evolution. In particular, we found evidence that herbicide exposure diminishes the fitness benefit that plants gain from interacting with rhizobia, such that rhizobial partners that would otherwise differ substantially in partner quality become equals (Figure 7a). If these findings relate to real-world outcomes, it is possible that legumes affected by herbicide

pollution in agro-ecosystems experience less selection pressure to interact with different strains than those in unpolluted environments. By extension, this relaxed selection may allow rhizobial genotypes that are lower quality, in terms of N fixation ability, to rise in frequency, similarly to how rhizobia in the field evolved to be less effective at promoting plant growth following long-term anthropogenic N addition (Weese et al. 2016). And indeed, previous models have predicted that rhizobial strains that invest more resources in N fixation exhibit lower fitness than strains that invest less in the absence of legume-imposed selection (West et al. 2002; Denison 2021). In addition to the knowledge that rhizobial strain variation in auxin production can result in plant growth variation (Lebrazi et al. 2020), it is well-known that rhizobial variation in symbiosis genes affects nodulation rates and nitrogen fixation, thereby ultimately affecting plant growth and biomass (Provorov and Tikhonovich 2003; Remans et al. 2008; Spaepen and Vanderleyden 2011; Wang et al. 2018). To uncover whether polymorphisms in symbiosis genes could explain the rhizobial-mediated ecological responses of plants across levels of dicamba exposure discussed here, more thorough genetic sequencing (e.g. whole-genome, as in Aguilar et al. 2018) and analysis of rhizobial strains such as the ones used in this study is warranted.

### 3.4.2 Herbicide Disruption of Mutualistic Resource Exchange

We additionally found that $G_R$ significantly interacted with the herbicide environment to mediate key traits related to the plant-rhizobia mutualism, including nodule number and nodule size, and also influenced symbiotic N fixation; meanwhile, $G_P$ contributed only additive or negligeable effects (Figure 7). This latter finding was surprising, considering that dicamba, as an auxin mimic, affects many critical plant biochemical pathways. Possibly, high conservation in red

clover for genes regulating auxin receptors, metabolism, and/or transporter proteins—which have all been proposed as potential mechanisms driving plant sensitivity/resistance to synthetic auxins (reviewed in Busi et al. 2017)—explains this result, although this degree of genetic analysis was outside of the scope of our study. According to resource mutualism theory as applied to plant-rhizobia interactions, plants derive net fitness benefits from interacting with rhizobia when mutualism costs, i.e. energetic investment in nodule production and development, are outweighed by mutualism benefits, i.e. symbiotic N fixation (Kiers et al. 2003; Sachs et al. 2018), which depend on the interacting strain as well as the environment (Heath et al. 2020; Westhoek et al. 2021). Thus, our results not only support the idea that rhizobial variation is a strong driver of symbiotic resource exchange in this mutualism, but also that herbicide exposure may disrupt the way in which rhizobial strains differentially inflict cost or add benefit to plants, thereby altering the overall fitness advantage that plants gain from rhizobial interactions.

For example, our results on the effects of herbicide exposure on indicators of mutualism costs were conflicting based on rhizobial partner: whereas herbicide treatment led to similar numbers but smaller nodules in 14479 plants, it resulted in fewer but bigger nodules (on average) in 4386 plants (Figure 7a-b). Although it is unclear whether making many small nodules is more costly for plants than making few large ones or vice versa, it has been shown that mutant legumes that are unable to regulate nodulation produce ‘supernumerary’ nodules that are smaller in size compared to wildtype and these plants experience substantial reductions in biomass yield (Ferguson et al. 2014; Mohd-Radzman and Drapek 2023). Similarly, our results suggested that changes in nodule number better explained the potential shift in costs that led to the observed strain-specific differences in plant fitness in response to herbicide exposure. While nodule number costs for 14479 plants was unchanged by herbicide treatment, the fixed N benefit received was

reduced (Figure 7c). In contrast, herbicide-treated plants paired with strain 4386 displayed a reduction in nodule number costs (Figure 7a) as well as in N benefits (Figure 7c); and the resulting decrease in fitness gain due to rhizobial inoculation between herbicide-treated 4386 plants and controls was less pronounced (Figure 7d). A valuable next step would be to track nodule respiration in legumes affected by sublethal levels of herbicide(s), e.g. via $^{13}C$ analysis of nodulated roots as in Hansen et al. (1993), in addition to conducting foliar $\delta^{15}N$ analysis, to more directly evaluate how this stress influences carbon allocation to versus amount of fixed N received from symbiotic rhizobia.

In contrast to plants, rhizobia are expected to derive fitness benefits from the number and size of nodules from the symbiotic interaction, because these traits correlate with the release of reproductive bacteria back into the soil during nodule senescence (Burghardt et al. 2018; Granada Agudelo et al. 2023). In turn, rhizobia incur metabolic costs of N fixation (Denison and Kiers 2004). From this perspective, our results suggest that plant exposure to herbicides could affect the balance of mutualism costs and benefits for rhizobia, but the extent depends on the strain. For instance, strains such as 14479 when interacting with herbicide-stressed plants, might be able to reduce N fixation costs while simultaneously maintaining benefits from occupying an abundance of nodules, but others such as 4386 might not, instead leading to a reduction in rhizobial fitness for them (Figure 7a,c). Similarly, strains could differ in fitness benefits derived from the size of nodules they promote in herbicidal conditions (Figure 7b); however direct measurements of rhizobial fitness such as the counting of reproductive rhizobial units per nodule will be necessary to clarify the impacts of herbicide exposure on rhizobial fitness as mediated by plant interactions (Oono et al. 2011). This would be an exciting future research direction.

### 3.4.3 Implications for Plant-Rhizobia Mutualism Ecology, Agriculture, and Future Research

Our findings support the growing body of research showing that alterations in the costs and benefits of symbioses may result in changes in symbiotic outcomes, and the environment can affect these cost-benefit continuums (i.e. resource mutualism theory; Mortier et al. 2011; Wyatt et al. 2014; Sachs et al. 2018; Quides et al. 2021). For example, Heath et al. (2020) recently found that significant variation among rhizobial strains in the carbon costs and N benefits they exchanged with legumes affected the net growth benefit legumes gained from interacting with them. However, whereas they found that a change in the (light) environment did not cause different strains to change rank in terms of plant growth benefit, we found that exposure to herbicide stress equalized ranks between rhizobia in net growth benefits. More broadly, the finding that dicamba drift exposure inhibited the benefits that rhizobia provided conflict with the general pattern described by Porter et al. (2020) that beneficial microbes such as rhizobia ameliorate abiotic stress in plants by providing more benefits in stressful conditions. Notably, the authors did not include herbicides as a source of abiotic stress in their meta-analysis; therefore our results emphasize that microbial stress mediation may be more dependent on the type of stress than previously thought. Yet, the fact that the herbicide-exposed plants in our study still received a fitness advantage from rhizobial inoculation with either strain likewise suggests that rhizobial associations in general would remain beneficial (mutualistic) for plants even in the presence of drift-level herbicide stress. It is important to note, however, that the partnerships of plant and rhizobial genotypes in our study may be novel, given their varying geographic origins. Therefore, while our work was a necessary first step in demonstrating that genotype-by-environment interactions, mediated primarily by rhizobia, are

relevant in the context of herbicide stress, future studies should consider using partners from the same as well as different localities to determine if plant-rhizobial coevolutionary histories could alter these symbiotic outcomes.

Finally, our work also conveys important implications for the N cycle and for agriculture. With the predicted continual use of dicamba (EPA 2021; U. S. Geological Survey 2021), our findings suggest that drift onto non-target legume crops could reduce not only their biomass yields but also potentially the amount of N they provide to the soil for other plants and crops to utilize. This issue would be especially pertinent to sustainable agriculture-focused programs which attempt to optimize biological N fixation as a source of N fertilizer over the use of synthetic chemicals (Goyal et al. 2021). However, our study also highlights important research gaps. While our study was limited to single rhizobial monocultures, in nature, various rhizobial strains compete to form nodules, and this inter-strain competition can affect both N fixation and plant performance (Mendoza-Suarez et al. 2021; Batstone et al. 2023; Burghardt and DiCenzo 2023; Rahman et al. 2023). Therefore, it would be valuable to consider whether providing plants with a community of rhizobia would influence their response to herbicide stress in ways that could not be predicted from their interactions with individual community members. Furthermore, while our results suggested that herbicide drift exposure impacts resource exchange between plants and rhizobia, additional measurements of symbiotic costs and benefits from both the plant and rhizobial perspective (e.g. plant carbon supply to nodules, N fixation rates, rhizobial population sizes within nodules, etc.) would quantify the finer points of this interaction. Altogether, future work should aim to expand our understanding of the impacts of current anthropogenic forces on beneficial plant-microbe interactions and the potential genetic and ecological mechanisms that mediate them.

## 3.5 Acknowledgements

We thank C. Wood, M. Rebolleda-Gómez, and N. Forrester for their guidance on microbiology techniques. We thank J. Ruit, M. Moore, J. Simmons, and E. O'Neill for their assistance in processing root samples, L. Follweiler for maintaining the UPitt greenhouse, and anonymous reviewers and the editor for comments that improved the manuscript. Our research was made possible through generous funding provided by the Graduate Women in Science National Fellowship Program, Botanical Society of America Graduate Student Research and Karling Awards, and the Phipps Conservatory and Botanical Gardens Botany in Action Fellowship Program. V. I. was supported by the National Science Foundation Graduate Research Fellowship (#1747452).

**Table 4. Effects of inoculation treatment (IT) or rhizobial strain (RS), plant family (F), herbicide treatment (HT), their interactions, and covariates (pre-HT size, root biomass, nodule number) on red clover traits related to herbicide injury (a), the plant-rhiozbia symbiosis (b), and plant fitness (c).**

All trait data was log-transformed prior to analysis except for instantaneous leaf damage data. Covariates (pre-HT size, root biomass, nodule number) were also log-transformed. See Methods for how traits were measured. Fixed effects were determined based on the best-fitting statistical model (see Appendix B: Table 17).

| | **Trait** | **Fixed Effects** | $\chi^2$ | **df** | **P** |
|---|---|---|---|---|---|
| a. Plant Herbicide Injury | Instantaneous Leaf Damage | **Inoculation Treatment** | 26.93 | 2 | **<.0001** |
| | | **Family** | 53.53 | 16 | **<.0001** |
| | | **IT × F** | 98.24 | 29 | **<.0001** |
| b. Symbiosis | Nodule No. | **Rhizobial strain** | 23.99 | 1 | **<.0001** |
| | | **Family** | 55.19 | 16 | **<.0001** |
| | | Herbicide Treatment | 0.26 | 1 | 0.61 |
| | | **Root Biomass** | 317.99 | 1 | **<.0001** |
| | | **RS × HT** | 8.80 | 1 | **0.0030** |
| | Nodule Size | Rhizobial Strain | 0.18 | 1 | 0.68 |
| | | Family | 23.11 | 16 | 0.11 |
| | | **Herbicide Treatment** | 10.43 | 1 | **0.0012** |
| | | **Root Biomass** | 8.07 | 1 | **0.0045** |
| | | **RS × HT** | 10.91 | 1 | **0.0010** |
| | Symbiotically Fixed N | **Rhizobial Strain** | 10.65 | 1 | **0.0011** |
| | | Family | 7.62 | 16 | 0.96 |
| | | **Herbicide Treatment** | 23.17 | 1 | **<.0001** |
| | | Nodule No. | 3.15 | 1 | 0.076 |

| | | | | | |
|---|---|---|---|---|---|
| c. Plant Fitness | Shoot Biomass | **Inoculation Treatment** | 97.83 | 2 | **<.0001** |
| | | **Family** | 31.94 | 16 | **0.010** |
| | | **Herbicide Treatment** | 46.03 | 1 | **<.0001** |
| | | **Pre-HT Size** | 956.08 | 1 | **<.0001** |
| | | **IT × HT** | 9.034 | 2 | **0.011** |

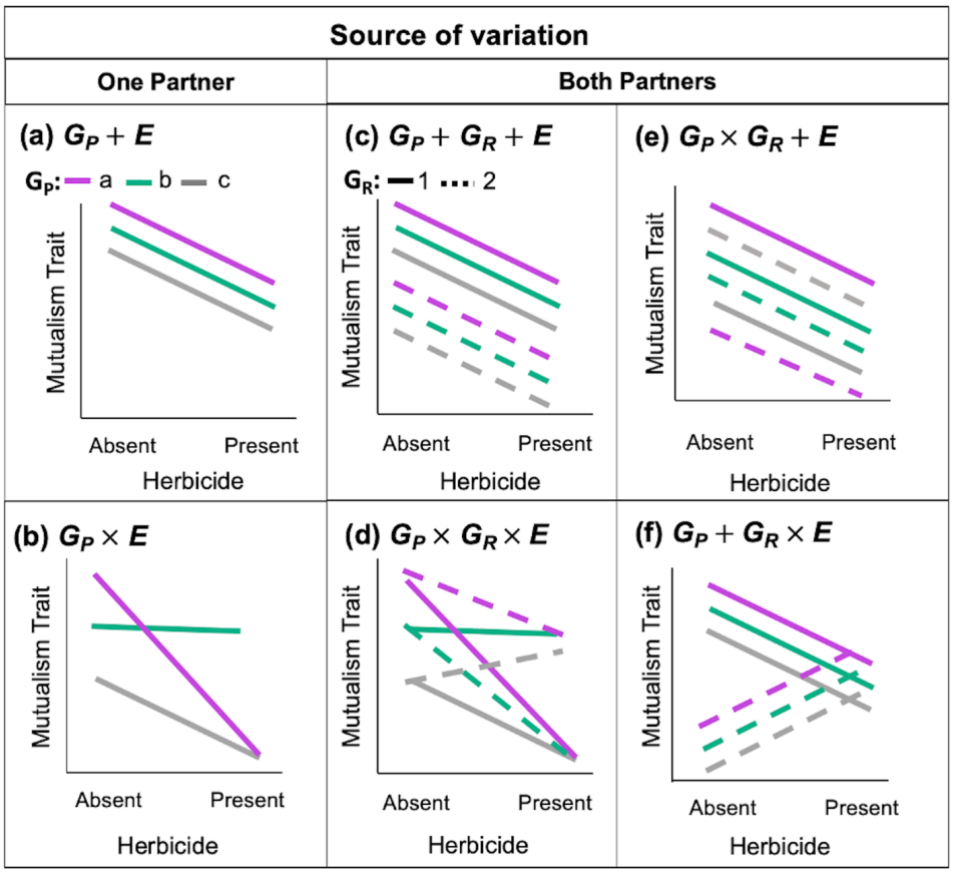



**Figure 5. Hypotheses of the effects of a stressor (e.g. an herbicide) and genetic varation in plant ($G_P$) and rhizobial ($G_R$) mutualist partners on mutualisms.**

(a & c) Herbicide presence affects the direction in which a trait related to a mutualism ('mutualism trait'; e.g. nodule no. in the plant-rhizobia mutualism) is expressed, while genetic variation in one (a) or both (c) partner(s) additively affects the magnitude of expression. (e) Herbicide presence affects the direction in which a mutualism trait is expressed, while genetic variation in both partners non-additively affects the magnitude of expression. (b & d) Herbicide presence and one (b) or both (d) partner(s) affect the magnitude and direction in which a mutualism trait is expressed. (f) Herbicide presence and genetic variation in one partner affects the direction in which a mutualism trait is expressed, while genetic variation in the other partner additively affects the magnitude of expression.

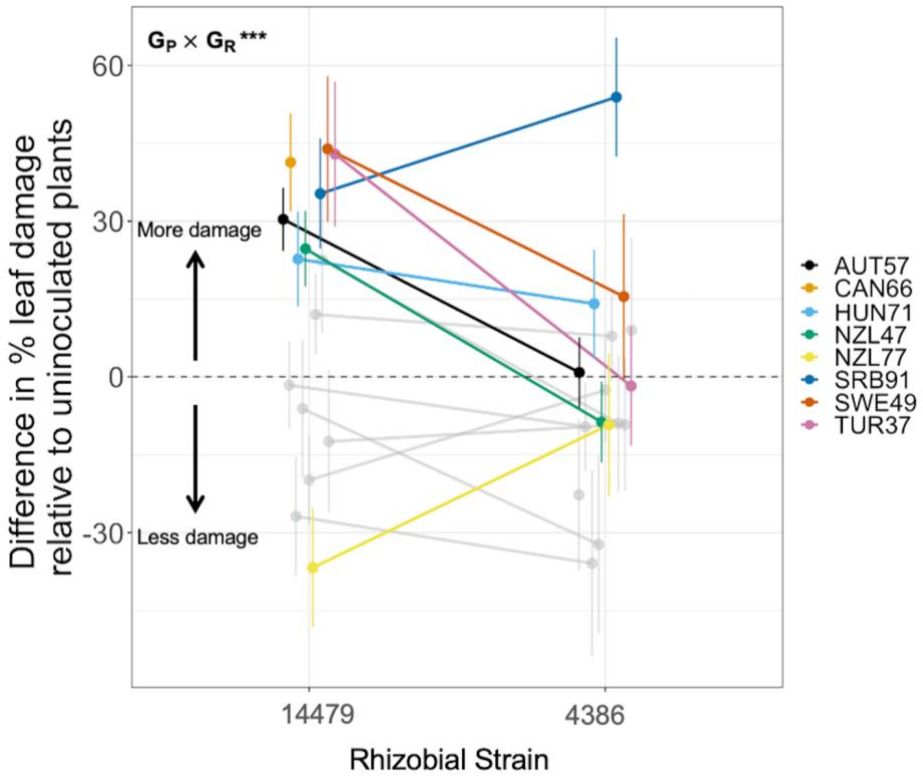



**Figure 6. Rhizobial inoculation influenced immediate damage plants incurred following herbicide exposure.** Points are contrast estimates ± SE of the difference between inoculated and uninoculated red clover plants in the estimated marginal means of the percentage of leaves which showed symptoms of herbicide-related damage 48 hours after treatment with dicamba drift. Plant families which showed significant differences in damage according to inoculation treatment are represented by different colors; all others are plotted in grey. The x-axis shows outcomes when inoculated with the two rhizobial strains (see Appendix S1: Table S3 for contrast analysis details). A missing reaction norm (solid line) indicates replication was lacking for a given combination of plant family and rhizobial strain inoculation due to limited seed numbers. ANOVA results for the effect of plant-rhizobial genotype-by-genotype interactions ($G_P \times G_R$) on damage response are noted at the top-left (***$p < .001$).

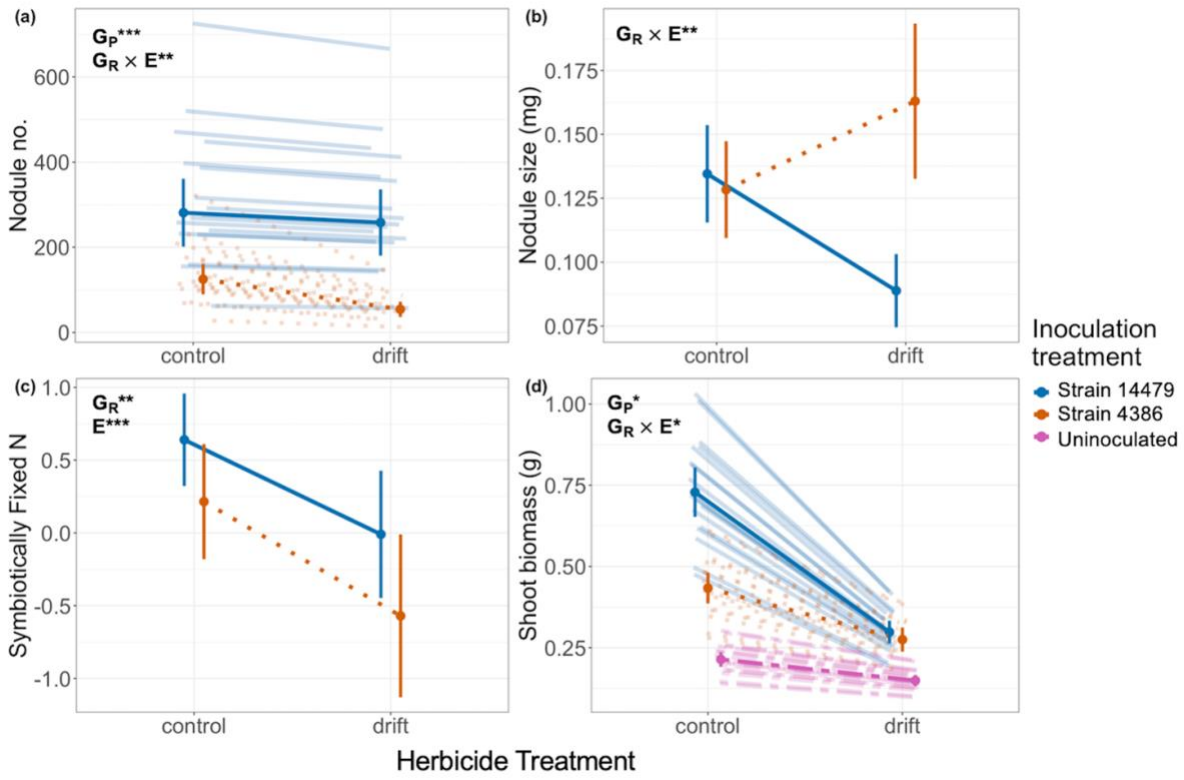



**Figure 7. Rhizobial inoculation and herbicide treatment influenced plant-rhizobia interactions and plant fitness.**

Points are estimated marginal means (back-transformed to the response scale) ± SE for nodule number (a), nodule size (b), symbiotically fixed N (c; estimated via foliar δ15N analysis) and shoot biomass (d) for red clover plants across herbicide treatment conditions (control and drift) and inoculated with rhizobial strain 14479 (blue solid line), rhizobial strain 4386 (orange dotted line), or uninoculated (pink dashed line; only shown in d since uninoculated plants did not interact with rhizobia). Dark lines show the effect of rhizobial inoculation averaged over plant families while more transparent lines show the effects of individual plant families, except in b-c where the main effect of plant family was not significant. ANOVA results for the effects of plant genotype ($G_P$), rhizobial genotype ($G_R$), herbicide treatment (E), and/or their interactions are noted at the top-left (*$p$ < .05, ** $p$ < .01, ***$p$ < .001).

# 4.0 Can the right partner mitigate harm? Rhizobial strains vary in their mediation of the effects of herbicide exposure in a plant-rhizobia mutualism

## 4.1 Introduction

Leguminous plants (legumes) and their symbiotic interactions with *Rhizobium* bacteria (rhizobia) constitute one of the most important mutualisms on Earth. Through biological nitrogen fixation or BNF, rhizobia housed within root nodules transform atmospheric nitrogen ($N_2$) into a form that plants can access ($NH_3$) in exchange for carbon, thereby increasing soil nitrogen availability in terrestrial ecosystems (Gou et al. 2023). In addition to BNF, free-living rhizobia produce phytohormones that enhance plant stress tolerance and growth (Brockwell et al. 1995; Jaiswal et al. 2021). Thus, maximizing BNF and other benefits from the legume-rhizobia mutualism has also been proposed as a major goal in sustainable agriculture to improve crop yields amidst the growing human population (Peoples et al. 1995; Mng'ong'o et al. 2023). At the same time however, anthropogenically-released chemicals such as pesticides can contaminate the rhizosphere—the region surrounding plant roots where symbionts coexist, engage in chemical cross-talk, and exchange resources—resulting in reductions in the efficacy of the legume-rhizobia mutualism (e.g. Mårtensson 1992; Fox et al. 2007). Therefore, focus on the factors that mitigate these stressors especially via lessening their impact on mutualism outcomes is key.

The effects of herbicides in the rhizospheric environment are particularly concerning and understudied. As the most-used class of pesticides globally (FAO 2024), herbicides can enter the rhizosphere either through direct application at full effective strength or through unintentional

movement away from target sites, e.g. ‘off target’ exposure at lower strengths (Boutin et al. 2014). Recently, the synthetic auxin, dicamba, surged in use (Riter et al. 2021; U. S. Geological Survey 2024). Dicamba is applied to bare soil (to prevent weeds from germinating) or to vegetation (to kill existing weeds), thereby creating opportunities for off-target exposures both in the rhizosphere as well as above ground. In particular, dicamba damage caused by ‘drift’ (movement via wind) has caused concern surrounding the ecological ramifications of this herbicide for wild plants and their animal associates, including pollinators and herbivores (Oseland et al. 2020; Iriart et al. 2022; Johnson et al. 2023). Indeed, above ground effects can be dramatic, resulting in leaf deformities and stunted growth (Foster and Griffin 2018), decreased pollinator visitation, and increased herbivory (Bohnenblust et al. 2016; Johnson and Baucom 2022) in wild and managed plants. Yet, the less visible effects of dicamba exposure in the rhizosphere, and especially on the legume-rhizobia symbiosis, has yet to be explored. Some work has shown that exposure to other synthetic auxins such as 2,4-D at $<$ full strengths can impede the chemical signaling necessary for initiating nodulation in rhizobia (Fox et al. 2004), and reduce nodule production or BNF in crops such as soybean and greengram (Saraf et al. 1999; Zaidi et al. 2005), suggesting that off-target dicamba exposure at lower concentrations in the rhizosphere could also inhibit symbiotic chemical signaling and aspects of the symbiotic relationship beyond the agricultural setting. However, we presently lack evidence to suggest whether wild legume-rhizobia partnerships, such as those which occur at the agro-ecological interface, likely to also be exposed to off-target dicamba (Burdon 2008; Iriart et al. 2022), would likewise be negatively affected by this stressor. Additionally, the biological forces which could mediate symbiotic costs of herbicides such as dicamba are largely unknown.

The potential harm that dicamba represents in the environment to symbiotic outcomes may be mitigated by genetic variation in either leguminous plant ($G_P$) or rhizobial ($G_R$) partners or their interaction. Specifically, plant or rhizobial genotypes could differ in their expression of mutualism-related traits depending on whether a novel chemical (e.g. dicamba) is present or absent in the environment. Such plasticity, or genotype-by-environment ($G \times E$) interaction, in the legume-rhizobia mutualism has been observed in other settings, especially under varied levels of nitrogen and light availability (e.g. Batstone et al. 2020 found $G_P \times E_{light}$, Regus et al. 2014 found $G_R \times E_{nitrogen}$, and van Cauwenberghe et al. 2016 found $G_P \times G_R \times E_{nitrogen}$). Yet in the context of herbicide exposures in the rhizosphere, there could likewise be pertinent, yet currently undescribed, $G_P \times E$, $G_R \times E$, and/or $G_P \times G_R \times E$ interactions at play.

With respect to $G_P \times E$, interactions may mediate the quantity of legume-rhizobia interactions (e.g. number of nodules produced or the timing of nodulation) across levels of rhizospheric herbicide, especially if herbicide-tolerant genotypes can invest more in the mutualism than sensitive ones. For example, Vaidya and Stinchcombe (2020) found that shade-tolerant plant genotypes maintained/increased their investment in nodules while most other less tolerant genotypes did not. And while intraspecific genetic variation for dicamba tolerance in plants has scarcely been characterized, it may be a potential mechanism determining mutualism trait expression in the rhizosphere. In above ground applications of drift-level dicamba, for example, Iriart et al. (*In review*) found that the amount of herbicide damage incurred by red clover depended on plant genotype but also whether plants were inoculated with rhizobia or not, and the two rhizobial genotypes (strains) used led to different responses. Likewise in soybean, aerial exposure of ~drift-level dicamba had negative effects on growth and yield, but some cultivars were more negatively affected than others (France et al. 2022; McCown et al. 2022). In neither case was

rhizosphere exposure studied, and when rhizobial effects were considered, only a few strains were used, therefore the inferences concerning the importance of $G_P \times E$ in this context are still quite limited.

Nevertheless, genetic variation in *rhizobial* symbionts and the symbiosis traits they engender may be altered in important ways under dicamba exposure. In particular, rhizobial strains can show context-dependency in the benefits they provide to legume hosts (e.g. fixed N) relative to their carbon costs, which can ultimately affect the net growth benefit plants gain from the symbiosis (Sachs et al. 2018). Athar and Johnson (1996) inoculated alfalfa with seven rhizobial strains and observed similar increases in shoot N and biomass under control conditions, but variable ones under water stress. If rhizobial strains likewise vary in the benefit they provide to legume hosts under dicamba exposure, then $G_R \times E$ interactions may drive symbiotic outcomes under changing rhizospheric conditions. Lastly, because the legume-rhizobia mutualism is a product of coevolution, their interactions can vary greatly depending on the interaction between plant host and rhizobial genotypes, in addition to the environment, resulting in $G_P \times G_R \times E$ interactions (Burghardt et al. 2022). Particular combinations of legume and rhizobial genotypes could thus dictate quantity and/or quality of legume-rhizobia interactions in the presence of rhizospheric dicamba, similar to how they shaped nodule traits such as number and size in response to environmental N availability in Heath and Tiffin (2007) and Heath et al. (2010). Yet how this complex interaction is affected by dicamba in the rhizosphere has never been addressed.

Here, we conducted a microcosm experiment where we exposed pairings of the model legume *Trifolium pratense* (two genotypes: $G_P$) with different genetic strains of *Rhizobium leguminosarum* (eight strains: $G_R$) to rhizosphere dicamba or not (E), and asked: (1) Does the presence of dicamba in the rhizosphere disrupt (a) key traits of the legume-rhizobia mutualism,

particularly the timing of nodulation initiation, the number of nodules formed, and BNF or (b) plant growth response? And (2) Does $G_P$ or $G_R$ mediate the effects of dicamba on mutualism traits or plant growth via (a) $G_P \times E$, (b) $G_R \times E$, or (c) $G_P \times G_R \times E$ interactions?

## 4.2 Methods

### 4.2.1 Study system

*Trifolium pratense* (Fabaceae) is an herbaceous, perennial plant that associates with N-fixing *Rhizobium leguminosurum* bacteria. It is native to Europe, Western Asia, and northwest Africa, but is cultivated (Evers 2011) and naturalized in many other regions, especially in the wild plant communities at the argo-eco interfaces across the globe (De Cauwer 2005; Nay et al. 2023). *T. pratense* is sensitive to direct foliar exposure to low-strength dicamba via drift (Iriart et al. 2021; Iriart et al. *In review*). However, the impact on the plant when dicamba exposure occurs in the rhizosphere and the extent that rhizobial strain variation influences this lacks exploration. Thus, we paired two cultivars ('genotypes') of *T. pratense* ('Kenland' and 'Mammoth'; Ernst Conservation Seeds; Meadville, PA, USA) with eight strains of *R. leguminosarum.* Strains were either acquired from the U.S. Department of Agriculture (USDA) Soybean Genomics and Improvement Laboratory (Beltsville, MD, USA) (accessions 2063, 2087, 2141, 2214, and 2220), the American Type Culture Collection (Manassas, VA, USA) (ATCC 14479, hereafter: '14479'), or the Northern Regional Research Lab (Peoria, IL, USA) (NRRL B-4386, hereafter '4386'). We selected strains 14479 and 4386 because previous work showed that these differ in symbiotic

outcomes in *T. pratense* (Iriart et al. *In review*). The other strains were selected because they were classified as genetically distinct and known to nodulate *T. pratense* or other *Trifolium* species.

### 4.2.2 Experimental set-up: Rhizospheric herbicide treatment and rhizobial inoculation

A microcosm experiment was performed in two temporal blocks: 1) six weeks from June 13-25, 2023 (N = 288); 2) four weeks from September 12 – October 10, 2023 (N = 180). We germinated 30 surface-sterilized *T. pratense* seeds of each genotype in 100mm diameter petri dishes (N = 10 seeds/plate) containing 1.5% agar in a growth chamber (22℃, 50% relative humidity, 16:8 hours light:dark, 100-160 $\mu$M/m$^2$ light intensity). From these, we randomly selected 18 individual seedlings per genotype for transfer to single-plant 'microcosms' composed of a Jensen's agar medium as in Jones et al. (2013) but prepared using a slightly modified protocol (Appendix C). Half of the microcosms were pre-treated with dicamba herbicide to simulate off-target exposure in the rhizosphere (wherein both plant roots and rhizobia would grow and interact). Specifically, we used sterile glass beads to spread 150 $\mu$L of dicamba (3,6-dichloro-o-anisic acid; Albaugh, LLC, Ankeny, IA, USA), sterilized via a 0.22 $\mu$m syringe filter, at a concentration of 15 mg of active ingredient [a.i.]/L (i.e., 0.5% of the field application rate [FAR] of 3 g a.i./L [Albaugh, 2018]) over the surfaces of the agar of microcosms. This concentration is within the range of dicamba levels previously detected in soil water samples affected by herbicide run-off (0.1-20% of applied rate; Hall and Mumma 1992; Vance and Kryzyszowka 1994; Ma et al. 1999; Rice et al. 2010). The other 50% of microcosms were treated with 150 $\mu$L of filter-sterilized DI water in the same way; hereafter the 'no dicamba' treatment. To confirm the efficacy of our dicamba stock, we

transferred six additional seedlings (3 per plant genotype) individually to separate microcosms pre-treated with 100% of the field application rate of dicamba and monitored their survival.

After transferring seedlings, we inoculated roots of one plant genotype per microcosm type (i.e. with/without dicamba) with 100 $\mu$L of rhizobial cells (~$1 \times 10^8$ CFU/mL) of one of the eight *R. leguminosarum* strains. Cells were prepared by culturing strains in liquid Modified Arabinose Gluconate (MAG) media (in g/L: 1.3 HEPES, 1.1 MES, 1.0 Yeast Extract, 1.0 L-arabinose, 1.0 D-Glucomic Acid, 0.22 $KH_2PO_4$, 0.25 $NA_2SO_4$; 32.0 $NH_4Cl$, 0.67 $FeCl_3$, 1.5 $CaCl_2$, 18 $MgSO_4$). After ~2 days of bacterial growth, we centrifuged the cultures, resuspended the pellets in autoclaved DI water, measured the optical density at 600nm (OD600), and used the relationship between OD600 and CFU for each strain (Appendix B: Figure 22; Appendix D: Figure 25) to dilute the CFU count. To monitor rhizobial contamination between rhizobial inoculation treatments and obtain a baseline estimate for the effect of rhizobia on plants, we also treated one seedling per plant genotype per microcosm type with 100 $\mu$L of autoclaved DI water only (uninoculated treatment).

Finally, we sealed microcosms with parafilm and organized one replicate (i.e., 2 plant genotypes × 2 herbicide treatments × [8 rhizobial strain inoculations + 1 uninoculated group] = 36 microcosms) into a tray (transparent, plastic, 41 × 58 × 15 cm). In total, there were 13 trays across the two time blocks, 8 in the first and 5 in the second. Within a tray, microcosms were placed randomly and held up vertically by black Styrofoam sheets which covered the lower half of microcosms to promote downwards root growth and protect roots from light (Appendix D: Figure 26). Trays were stored in a growth chamber as above. The six seedlings exposed to 100% of the field application rate of dicamba (not inoculated with rhizobia) were stored in a separate section of one of the trays in the growth chamber.

### 4.2.3 Plant growth and mutualism metrics

We photographed microcosms weekly to record plant-rhizobia interactions and plant growth. To characterize timing of nodulation and nodule number, we recorded the presence/absence of nodules each week post-inoculation up to Week 4 and counted nodules at Week 4. We estimated plant size at two time points because the effects of microbial symbiosis and dicamba exposure can be time-dependent (Johnson et al. 1997; Ramos et al. 2021). From Week 4 photos, we used the application Fiji (Schindelin et al. 2012) to count the number of leaflets and measure the length of the longest leaflet from photographs. The product of these estimates plant size (cm) at Week 4. In Week 6 of the first temporal block, we also harvested shoots, dried them at 70℃ for at least 48 hours, and weighed them to the nearest pg (Cahn Model 31 Microbalance, Thermo Fisher Scientific Corp., Waltham, MA, USA). To assess BNF, we quantified foliar $\delta^{15}$N (the isotopic ratio of $^{15}$N:$^{14}$N in sample relative to atmospheric air) via an elemental analyzer at the Washington State University Stable Isotope Core Laboratory (Pullman, WA, USA). $\delta^{15}$N estimates BNF as plants with lower $\delta^{15}$N content have received high amounts of symbiotically-fixed N (enriched in $^{14}$N) compared to those receiving little or no fixed N (higher $\delta^{15}$N content), due to the observation that the nitrogenase enzyme discriminatorily utilizes the lighter of the two N isotopes during BNF (Craine et al. 2015; Lindström and Mousavi 2020). To obtain this data, we submitted leaf samples from five randomly-selected plants per genotype, herbicide treatment, and rhizobial inoculation (N = 160 total) as well as one uninoculated plant per genotype and herbicide treatment (N = 4 total) to confirm that rhizobial inoculation decreased $\delta^{15}$N. To account for the effects of nodule production on BNF, we also counted nodules on this set of plants at Week 6 to use as a covariate when analyzing $\delta^{15}$N data. Some plants (N = 75; 16% of the experiment) showed

signs of a minor fungal infection (slight discoloration of roots and/or leaves) at Week 4, so we additionally recorded the presence/absence of these symptoms. Data from plants with severe symptoms (N = 6; 1% of the experiment) was not analyzed.

### 4.2.4 Statistical analyses

We conducted all analyses in R version 4.2.2 (R Core Team 2022) and created figures using the *ggplot2* package (Wickham 2016). We built several statistical models to analyze the response variables: timing of nodulation, nodule number, $\delta^{15}$N, Week 4 plant size, and Week 6 plant size, and performed model selection on them using Akaike Information Criterion with correction for small sample sizes (AICc; Akaike 1973). Models included the covariates tray (random effect factor) and, if applicable, temporal block (fixed effect factor), to account for the time and location in which plants grew, and the extraneous variable minor fungal infection (fixed effect factor), unless it worsened model fit (i.e. increased AICc). We checked model assumptions using the *DHARMa* package (Hartig 2022).

Specifically, to analyze the timing of nodulation, we conducted a time-to-event analysis using the *survival* package (Therneau and Grambsch 2000). We used data of nodule presence/absence for the first four weeks post-inoculation and true/false data of whether a plant was still lacking nodules by Week 4 to calculate Kaplan-Meier curves (Rich et al. 2010), showing the probability that nodules would be absent among plants at each week post-inoculation. We then built mixed effects Cox proportional hazards regression models (Cox 1972) to assess which explanatory variables (defined below) influenced nodulation curves using the *coxme* function and calculated means for the time until nodulation (i.e., nodule initiation), by taking the area under the

nodulation curve from Week 0-4 (i.e. the 'restricted mean'; Han and Jung 2022) using the *survfit* function. When visualizing results (e.g., Figure 8), we plotted the probability of nodulation (1− Kaplan-Meier probability of nodule absence) over time for ease of interpretation.

To analyze whether plants differed in nodule number once nodulation commenced, we ran mixed-effects linear models on Week 4 nodule count data. Nodule number was natural log-transformed to achieve normality and plants without nodules were excluded. We also used mixed-effects linear models to analyze $\delta^{15}$N and plant size at Week 4 and Week 6 (square-root transformed shoot biomass). However, to analyze $\delta^{15}$N and Week 6 plant size, only data from the first temporal block was available.

We ran Type III sums of squares ANOVAS using the *car* package (Fox and Weisberg, 2019) to evaluate the effects of the explanatory variables (fixed effect factors) –plant genotype, rhizobial strain, herbicide treatment, and all two-way and three-way interactions between them on these models. A significant negative herbicide effect on the timing of nodulation, nodule number, or $\delta^{15}$N would indicate that the presence of dicamba in the rhizospheric environment disrupted important elements of the legume-rhizobia mutualism, such as by reducing or shortening opportunities for resource exchange via nodule production or by inhibiting BNF (Question 1a). A negative herbicide effect on plant size at Week 4 or 6 would indicate that dicamba impaired legume growth at early or later stages in development, respectively (Question 1b). However a significant plant genotype × herbicide (i.e. $G_P \times E$) interaction for traits related to nodulation, BNF, or plant size would suggest that mutualism/plant growth outcomes were driven by the plant genetic response to dicamba (Question 2a). Meanwhile a rhizobial strain × herbicide (i.e. $G_R \times E$) interaction would suggest that the response to dicamba was mediated by the rhizobial partner with which plants were inoculated (Question 2b), and a plant × strain × herbicide interaction (i.e. $G_P \times$

$G_R \times E$) would imply that this was instead mediated by the interaction between plant and rhizobial genotypes.

Furthermore, if we detected a significant $G_P \times E$ or $G_R \times E$ interaction for a given response variable (e.g. nodulation traits, BNF, plant size), we calculated the estimated marginal means (EMMs) for plant genotypes/rhizobial strains within each herbicide treatment environment using the *emmeans* package (Lenth et al. 2018). Then we conducted planned *post hoc* pairwise comparisons between EMMs with Dunnett's *P*-value adjustments to identify significant effects of rhizospheric dicamba exposure at the plant genotype/rhizobial strain-level. Further, to discern whether the $G_R \times E$ effect resulted in a change in rhizobial genotypic rank order, we calculated Spearman rank correlations between the rhizobial strain EMMs in the presence vs. absence of dicamba. A weak correlation (i.e. $r < 0.5$) would suggest that the rankings of inoculum in each herbicide environment were unrelated, therefore a shift in rank order had occurred. When we found a significant $G_P \times G_R$ or $G_P \times G_R \times E$ interaction, we compared strain EMMs among legume host genotypes and (if applicable) the herbicide environment to evaluate trait variation according to particular combinations of plant and rhizobial genotypes and how interactions between genotypes were altered by dicamba.

To further understand how dicamba affected plant growth and to address whether dicamba disrupted the predicted beneficial effects of rhizobia on plant growth, we calculated the difference in size between inoculated plants and uninoculated ones for each genotype, strain, herbicide treatment, and replicate (tray). Thus, if the mean rhizobial effect of strain A was > 0, then strain A's effects were beneficial for plant growth. If < 0, they were costly. We assessed significance by performing a paired *t*-test on the mean rhizobial effect of strains in the presence vs. absence of herbicide at Week 4 and Week 6.

During model selection, if a model which included the plant × strain × herbicide or plant × strain interaction had a greater AICc (by ≥ 2 units) than models which excluded them, indicating that these variables worsened the model's fit and ability to explain the data (Zuur et al. 2009), we dropped them and only tested the plant × herbicide and strain × herbicide interactions. In analyzing $\delta^{15}$N, we also considered how controlling for nodule number influenced results by running models with and without Week 6 nodule number as a covariate. We also validated the $\delta^{15}$N abundance method for estimating BNF by calculating the raw mean $\delta^{15}$N value per inocula. If we found that the mean $\delta^{15}$N of uninoculated plants was higher than that of most rhizobial strain inocula, then this would confirm our expectations for measuring BNF.

## 4.3 Results

Rhizobial inoculation treatments and herbicide treatments were effective: uninoculated plants did not form nodules, and plants within microcosms treated with 100% of the field application rate of dicamba died (N = 11/12) or did not grow (N = 1/12). Additionally, uninoculated plants had greater foliar $\delta^{15}$N values than plants inoculated with most (6/8) rhizobial strains, confirming expectations of the $\delta^{15}$N isotope abundance method for estimating BNF (Appendix D: Table 25). Across all analyses, models performed substantially worse (ΔAICc ≥ 8) when the three-way plant × strain × herbicide variable was included whereas best fit models

included two-way interactions between symbiont genotypes and the rhizospheric environment and these are presented below (see Appendix D: Table 26 for all models).

### 4.3.1 The presence of herbicide in the rhizosphere delayed nodulation

Herbicide treatment and rhizobial variation independently affected the timing of nodulation (Table 5A; Figure 8). Dicamba exposure increased the mean time until nodulation by 30% (Figure 8A; herbicide treatment effect: $P$ <.001; Table 5A; see Appendix D: Table 27A). Additionally, the time until nodulation differed among rhizobial strain inocula by 0.4 – 15% (Figure 8B; rhizobial strain effect: $P$ <.0001; Table 5A; see Appendix D: Table 27B). However, neither the strain × herbicide ($G_R \times E$) nor the plant × herbicide ($G_P \times E$) interaction influenced nodulation, instead nodulation was similarly delayed by dicamba exposure across rhizobial strains and plant genotypes (Table 5A).

### 4.3.2 Rhizobial strains mediated the effect of herbicide exposure on nodule number and BNF

Once plants began nodulating, they produced up to 32 nodules (raw mean = 3.62 $\pm$ 0.25) by Week 4. However, mean nodule number varied among rhizobial strain inocula ($G_R$: $P$ < .01; Table 5B). Unlike our results for nodulation timing, dicamba did not independently affect nodule number (E: $P$ > 0.05); rather, the effect of dicamba on this trait was mediated by rhizobial strains ($G_R \times E$: $P$ < 0.01; Table 5B; Figure 9A). In the presence of dicamba, plants inoculated with strains

4386, 2220, 2087, and 2316 made significantly or marginally significantly fewer nodules (by 48-67%; $P \leq 0.061$; Appendix D: Table 28A). Yet, dicamba had negligeable effects on nodule number for the other four strain inocula. Moreover, the Spearman rank correlation among strains for nodule number between herbicide environments was nonsignificant ($r = -0.40$, $P = 0.33$, N = 8), indicating that the strains which resulted in the most nodules without dicamba (e.g. 4386 and 2087) were often not the same as those that produced the most nodules with exposure to dicamba (e.g. 14479 and 2063). Additionally the quantity of nodules formed was also affected by the rhizobial and plant genotype interaction ($G_P \times G_R$; $P < 0.05$; Table 5B). Most strains resulted in similar nodule numbers on *T. pratense* genotypes, except that when paired with strain 2220, Kenland plants made significantly more (by 48%) nodules than Mammoth plants, whereas strain 2087 led Mammoth to make more nodules (by 38%) than Kenland (Appendix D: Figure 27, Table 29). The lack of a $G_P \times G_R \times E$ (see above) or significant $G_P \times E$ interaction ($P > 0.5$; Table 5B), however, indicates that these genotype-by-genotype patterns remained consistent across herbicide environments and that $G_R$ more so than $G_P$ drove the response of this mutualism trait to dicamba exposure.

Rhizobial strains also varied significantly in BNF as estimated by foliar $\delta^{15}N$ abundance ($G_R$: $P < .05$; Table 5C). As with our results for nodule number, dicamba did not have a main effect on BNF (E: $P > .05$), but it did significantly modify strain-specific BNF outputs ($G_R \times E$: $P < .05$; Table 5C; Figure 9B). Interestingly, the significant $G_R \times E$ effect and strain-specific $\delta^{15}N$ patterns in response to dicamba exposure were consistent even when nodule number was controlled for in a separate analysis (Appendix D: Table 30, Figure 28), suggesting that BNF activity, despite being a product of nodulation, was not directionally related to the number of nodules produced out of symbiosis with rhizobia from this study. Overall, the greatest BNF activity was observed when

plants were paired with strain 2220 in the absence of rhizospheric dicamba ($\delta^{15}$N = −0.321‰ ± 0.45) and the least occurred with strain 2316 in the presence of dicamba ($\delta^{15}$N = 2.060‰ ± 0.44), but both values fell within the range of BNF estimates previously recorded in wild and cultivated *T. pratense* populations via $\delta^{15}$N (Trněný et al. 2019). *Post hoc* tests did not detect statistically significant differences in BNF between dicamba-exposed vs. unexposed plants within any inoculum category (Appendix D: Table 28B), suggesting that the $G_R \times E$ effect was more so driven by differences in the directional effects of dicamba on strain-specific BNF output rather than differences in the magnitude of these effects in any one direction. For example, half of the strain inocula showed slightly reduced BNF activity under dicamba (2316, 2220, 2214, and 2087: $\delta^{15}$N increased by 0.404-1.222‰) and the remainder showed slightly increased BNF activity (4386, 14479, and 2141: $\delta^{15}$N decreased by 0.468-0.974‰) or zero change (2063). Additionally, genotypic rank order of rhizobial strains for foliar $\delta^{15}$N in the absence vs. presence of dicamba were not strongly or significantly related (Spearman's $r = -0.26$, $P = 0.54$, N = 8), suggesting that herbicide exposure affected the identity of strains which resulted in the most BNF (without dicamba: 2220 vs. with dicamba: 14479) or the least BNF (without dicamba: 2214 vs. with dicamba: 2316).

### 4.3.3 Rhizobia mediated the effect of rhizospheric herbicide exposure on legume growth

On average, the presence of rhizospheric dicamba reduced plant size by 46% at Week 4 (E: $P$ <.0001) and 37% at Week 6 ( $P$ < .01; Table 5D-E). In both instances, the effect on plant size, however, depended on rhizobial strain (both $G_R \times E$ effect: $P < 0.05$; Table 5D-E). Significant

size reductions from dicamba exposure were seen for all plants except those inoculated with strain 2214 or 2087 at Week 6 (Appendix D: Table 28C-D).

The correlation between rhizobial benefit to plants in terms of size across strains, in the presence vs. absence of dicamba was low and nonsignificant (Week 4 size, Spearman's $r = -0.10$, $P = 0.84$, N = 8; Week 6 size, Spearman's $r = -0.29$, $P = 0.50$, N = 8), suggesting that rhizospheric dicamba caused a rank shift in the inoculum which produced the largest plants. For example, without rhizospheric dicamba, the strains that were most beneficial for plant growth (i.e. highest ranking) were 2316 and 2220, but with rhizospheric dicamba, they became neutral, (Figure 9C-D), whereas strain 2214, which was among the lowest ranking inoculum without dicamba, became the highest ranking with dicamba, suggesting that this strain tended to ameliorate the effects of dicamba on plant growth. In contrast, inoculation with strains 2063, 14479 and especially 4386 tended to worsen the effect of the herbicide—although these strains promoted average growth without dicamba, in the presence of dicamba these strains led to even smaller plants (Figure 10).

Although dicamba depressed plant growth on the whole, the effect of rhizobial inoculation (i.e., difference in size of inoculated vs uninoculated plants) on plant growth was age- and herbicide -dependent. At Week 4, in the absence of dicamba, interactions with rhizobial strains tended to reduce plant size (Appendix D: Figure 29A), suggesting they were on average costly for plant growth (mean rhizobial effect = $-0.25$ cm). However, in the presence of dicamba, these interactions were virtually neutral (mean rhizobial effect = +0.04), and this change in costs was marginally significant ($t = -1.83$, df = 15, $P = 0.087$). This trend was reversed at Week 6, where in the absence of dicamba, the average rhizobial effect on plant size was positive (mean rhizobial effect = +0.75 mg; Appendix D: Figure 29B) as predicted by mutualism theory. Yet in the presence

of dicamba at Week 6, turned costly (mean rhizobial effect = −0.26 mg; $t$ = 3.82, df = 15, $P$ = 0.0016), contradictory to theoretical predictions.

Finally, plant size was also impacted by $G_P \times E$ interactions, but only at Week 4 ($P < 0.05$; Week 6 $P$ = 0.11; Table 5D-E). Without herbicide exposure plant genotypes were similar in size, but herbicide reduced size in Mammoth more than Kenland (showing a 12% greater decrease) (Appendix D: Figure 30, Table 31), indicating the former was more sensitive to rhizospheric dicamba exposure at life earlier stages but not later (Week 6).

## 4.4 Discussion

A rising challenge of ecological research is to determine how mutualisms and the ecosystem services they provide are affected by human-mediated change and to identify which countermeasures mitigate them (Kiers et al. 2010). In the ecologically and agriculturally important symbiosis between legumes and N-fixing rhizobia, ample research has shown that mutualism outcomes are sensitive to changes in the environment in ways that are often dependent on the genotypes of symbiotic partners and/or their genotype-by-genotype interactions (Heath and Tiffin 2007; Barrett et al. 2015; Sachs et al. 2018; Vaidya and Stinchcombe 2020; Burghardt and DiCenzo 2023); yet no study has previously investigated the impacts of herbicide presence in the rhizosphere on the legume-rhizobia mutualism in light of these complex genotypic variables. Here, our results provide a new insight that genotype-by-environment ($G \times E$) interactions—driven by rhizobial genetic variation—can mitigate mutualism consequences. Specifically, we showed that rhizospheric exposure to the widely-used herbicide dicamba, at a level relevant to present-day off-

target exposures, universally delayed rhizobial colonization of plants via root nodule formation, but its effects on the number of nodules produced and BNF depended on rhizobial partner. Concordantly, we provide a novel example for how microbial symbionts could serve as both an 'extended genotype' for their hosts (Carthey et al. 2018) and as a potential defense mechanism in a stressful environment (Mehlferber et al. 2022): while dicamba exposure was detrimental for plant size on average, the degree of this effect was mediated by rhizobial strains. Below, we discuss our findings, their potential causes, and implications for natural and agricultural systems and mutualism evolution.

### 4.4.1 Herbicide-driven disruptions in initiation of symbiosis

An interference of symbiotic signal exchange by dicamba molecules could explain the overall delay in nodulation that we observed in *T. pratense* following rhizospheric exposure to dicamba. Although dicamba has yet to be specifically tested, other chemicals which mimic hormones, including synthetic auxins, can interfere with rhizobial *nod* gene expression, which is essential for cross talk between plants and rhizobia (Fox et al. 2001; Fox et al. 2004, Checcucci and Marchetti 2020). In an ecological context, as nodules are the precursor to BNF, this effect on the timing of nodulation implies that dicamba-exposed ecosystems might experience a delay in receipt of fixed N, which could alter competitive dynamics between plant species with varying nutritional developmental requirements (Benner and Bazzaz 1987; Wilson and Tilman 1993; Blacksaw et al. 2004). Moreover, because legume phenology can be highly sensitive to N availability (Thies et al. 1995; Cafaro La Menza et al. 2020) nodulation delays could impact other

aspects of *Trifolium* biology including flowering and thus resources for pollinators (David et al. 2019). From an agricultural perspective, this nodulation/BNF delay suggests that dicamba presence in the soil where legume crops are grown could experience delays until harvest as predicted by models in Fox et al. (2007). Moreover, if our short-lived microcosm experiments mimic real world outcomes including those of wild legumes in natural settings, then our results suggest these knock-on effects related to the initiation of legume-rhizobia interactions will be broadly seen, regardless of genetic variation among plants or rhizobia.

### 4.4.2 G × E effects on mutualism outcomes driven by rhizobia

In contrast to our results on the timing of legume-rhizobia interactions, our study provides rare yet convincing evidence that *rhizobial* genetic variation, potentially more so than plant genetic variation, is a prominent genetic driver of other key mutualism factors in response to rhizospheric dicamba exposure. Nodule number, BNF activity, and legume growth were highly dependent on the interaction between rhizobial genotypes and the rhizospheric environment, $G_R \times E$. This finding was particularly transformative because although other studies have indicated that some rhizobia can mitigate legume stress caused by anthropogenic toxins in the rhizosphere, such as heavy metals or agrochemicals (e.g. Märtensson et al. 1992; Ahemad and Khan 2010; Wani and Khan 2013; Bianucci et al. 2018; Shahid and Khan 2018), to our knowledge, our study is the first to provide evidence for this ability while also accounting for biologically-relevant $G_P$ and $G_P \times G_R \times E$ effects. Likewise, in other contexts where G × E in the legume-rhizobia mutualism has been more heavily researched, such as in varying soil N or light availability, the majority of these studies

varied genotypes of only one partner, $G_P$ (e.g. Batstone et al. 2020; Vaidya and Stinchcombe 2020; Millar et al. 2023) or $G_R$ (e.g. Esfahani and Mostajeran 2011; Regus et al. 2015; Heath et al. 2020), thereby making it difficult to assess each one's contribution to the mutualism response across environments. Of the limited G × E studies which have manipulated variation in both plants and rhizobia, most found that *plant* genetic variation was driving mutualism responses (e.g. $G_P \times E_{nitrogen}$ in Porter and Simms 2014 and Burghardt et al. 2022; $G_P \times E_{salt}$ in Thrall et al. 2008) or its interaction with rhizobial variation (e.g. $G_P \times G_R \times E_{nitrogen}$; Heath and Tiffin 2007; Heath et al. 2010). Here, our finding of rhizobia as a prominent genetic driver of the symbiotic responses to dicamba exposure is robust, even though our plant genetic variation was necessarily limited to two genotypes because: 1) we never found a significant $G_P \times G_R \times E$ interaction, and 2) in our previous study investigating the effects of dicamba drift to above ground plant parts, we also found a strong $G_R \times E_{dicamba}$ effect across a greater number (N = 17) of plant genotypes (Iriart et al. *In review*). More broadly, this result provides a case for how microbial symbionts serve not only as an 'extended genotype' for their hosts (Carthey et al. 2018; Bruessow and Brüssow 2020) but also how their microbial associates contribute to their defense phenotype (Dawkins 1982; Qin et al. 2016; Mehlferber et al. 2022) —although *T. pratense* genotypes on their own lacked variation in response to dicamba exposure (possibly because of high conservation in genes related to auxin proteins and metabolism [Busi et al. 2018]), genetic variation among their rhizobial partners gave rise to phenotypic variation in which selection could potentially act upon to influence legume evolution and that of the legume-rhizobia mutualism.

### 4.4.3 Implications for mutualism ecology and evolution in herbicide-exposed contexts

In particular, our results can be deconstructed into three key findings with important implications for the ecology and evolution of legumes and their mutualism with rhizobia. Firstly, rhizospheric dicamba shifted the genotypic rank order of rhizobial strains which resulted in the most nodules, greatest BNF output, or the largest plants (Figure 9). If these shifts also occur in natural rhizospheres after dicamba exposure, then the evolution of the legume-rhizobia mutualism could be affected. For instance, as nodule number is positively related to the quantity of rhizobia released back into the environment, it estimates a component of rhizobial fitness (Burghardt et al. 2022). Thus a change in genotypic rank order for nodule number suggests that natural selection acting on rhizospheres commonly exposed to dicamba could favor rhizobial strains that produce the most nodules under dicamba. On the other hand, vegetative size/biomass relate to plant fitness (Younginger et al. 2017), therefore a shift in rhizobial ranking for legume growth benefit in this context would result in selection pressure on legumes to associate with rhizobia that minimize the negative growth effects of dicamba. Mutualism evolution could be affected because, for the symbiosis to remain mutualistic, rhizobial and plant fitness must be positively aligned (reviewed in Friesen 2012). Thus if rhizobial genotypes which are high-nodulating do not correlate with high plant growth promotion in dicamba conditions, evolution in response to long-term dicamba exposure, as it did in response to long-term N deposition (Weese et al. 2015), could cause the mutualism to breakdown. Additionally, for genes promoting BNF in rhizobia to be maintained, BNF must also correlate positively with rhizobial fitness. To better predict pesticide-mediated evolutionary trajectories and their consequences for mutualism and BNF service stability, however, future work should elucidate the genetic correlations between rhizobial fitness, BNF,

and plant fitness under varying levels of exposure to dicamba in the rhizosphere. This would require a study with many more rhizobial genotypes (i.e. ~50; Wood and Brodie 2015).

Secondly, patterns regarding the significant main effect of dicamba exposure coupled with the $G_R \times E$ effect on legume growth indicated that dicamba exposure negatively affected legume productivity overall, but the magnitude of its effect was modified by rhizobial strain (Figure 9C-D). Additionally, we found that rhizobial inoculation was in large part costly for the growth of dicamba-exposed plants by the end of the experiment (Appendix D: Figure 29B). The biological market theory of mutualisms (Schwartz and Hoeksema 1998) predicts that legume-rhizobia associations can lead to this generally negative result if rhizobial energetic demands for carbon exceed the growth-promoting benefits returned. Surprisingly, herbicide treatment did not have equivalently strong, negative effects on BNF across rhizobial inocula (Table 5C, Figure 9B); therefore, a reduction in rhizobial N benefits could not explain this outcome. However, considering that dicamba's mode of action reduces photosynthesis and causes abnormal shoot and root growth (Gleason et al. 2011; Figure 10), it is likely that the exposed plants in our microcosm study were lacking in energetic reserves, which would make nodulation as well as plant facilitation of BNF, more costly (Minchin and Witty 2005; Chaulagain and Frugoli 2021). The finding that beneficial rhizobial strains turned costly in this way is similar to that of Regus et al. (2017), where a high N-fixing strain that was beneficial for legume growth at low rates of N deposition became detrimental at high rates. And more precisely, it expands upon recent results from Iriart et al. (*In review*) which showed that exposure to dicamba drift diminished the growth promoting benefits that two rhizobial strains provided to their legume partners. Thus, it may be that under field conditions, legumes would generally be disadvantaged under rhizospheric dicamba exposure, despite maintaining

symbiotic interactions of similar quantity and quality with rhizobia that would otherwise be advantageous for their productivity.

Lastly, the particulars of the $G_R \times E$ result for legume growth revealed that one strain (4386) exacerbated the negative effect of dicamba while another (2214) ameliorated it (Figure 9C-D; Figure 10). Although strain 4386 was not a high-nodulating strain under rhizospheric dicamba, it was in the absence of it (Figure 9A). Furthermore, BNF activity of this strain was unchanged if not slightly enhanced by dicamba exposure (Figure 9B); therefore it is possible that heightened investment in nodule formation upon recognition of this symbiont and/or enhanced associated metabolic costs for BNF explain why it was especially detrimental to exposed plants. Interestingly, 2214 was neither a high-nodulating strain nor highly effective at BNF in either rhizospheric environment (Figure 9A-B); therefore, the ameliorating effects of this strain for dicamba-exposed legumes could be a result of reduced symbiotic costs, and potentially enhanced benefits unrelated to nodulation. For example, some rhizobia produce the enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase (reviewed in Nascimento et al. 2016), which decreases levels of ethylene, a phytohormone that is augmented in response to dicamba exposure and contributes to vegetative decay (Gleason et al. 2011). To clarify which mechanisms are causing these strain-specific differences, additional work measuring carbon metabolism in plants (as in Kleinert et al. 2017) as well as rhizobial metabolism in the context of rhizospheric dicamba is needed.

### 4.4.4 Conclusions

In sum, our results represent a critical first step in understanding the role of genetic variation in determining outcomes of the legume-rhizobia mutualism when both symbionts are

simultaneously exposed to herbicide at a realistic level and location. The use of a closed microcosm system in a growth chamber allowed us to both control the concentration of herbicide that plants and rhizobia were exposed to and minimize extraneous variables, especially other microbes that could have confounded rhizobial strain effects. However, in the field, the effects of herbicides can depend on various environmental factors, such as soil type, weather, and local microbial diversity (Burul et al. 2022). Thus, an important next step is to replicate our methodologies in the field, such as by growing legumes outdoors in soil that has been modified to include common rhizospheric microbes but lacking in rhizobia, so that when rhizobial strain inocula and herbicide treatments are applied, their effects can be evaluated in light of these other relevant abiotic and biotic variables. Moreover, our work could be expanded by investigating the effects of exposures of synthetic auxins such as dicamba on more diverse symbiotic pairings, including those with previously described coevolutionary histories or from different legume and rhizobial species, to test whether the $G_R \times E$ interaction is still the predominant driver of mutualism outcomes in this context. Finally, future studies could link herbicide-mediated delays in BNF or reductions in legume growth to other ecologically and agriculturally meaningful variables such as flowering, seed production, and pollination. Ultimately, these avenues would give us greater power to define the ecological ramifications and ecosystem service costs associated with continued herbicidal interference in the rhizosphere and greater knowledge as to what extent rhizobia can be biotic mediators of them.

## 4.5 Acknowledgements

We thank H. Assour for her help in experimental set-up, and S. Parker and E. Perry for their help in prepping plant samples for weighing and isotope analysis. We also thank members of the Ashman and Turcotte labs at the University of Pittsburgh for their guidance and support. Our research was made possible through generous funding provided by the Phipps Conservatory and Botanical Gardens Botany in Action Fellowship Program and the Sigma Xi Grants in Aid of Research Program, and the DSA&S funding to T.-L. A. V. I. was supported by the National Science Foundation Graduate Research Fellowship (#1747452).

**Table 5. Type III sums of squares ANOVAs for traits related to plant-rhizobia interactions (A-B) and plant growth (C-D).**

Rows correspond to fixed effect factors ('fixed effect') and their degrees of freedom ('df'), $\chi^2$, and *P*-values from the highest performing models for each response variable (A-D). Time until nodulation was analyzed with a mixed effects Cox proportional regression hazards model; all other response variables were analyzed with mixed effects linear models. Plant size was estimated from leaflet number and length at 4 weeks post rhizobial inoculation and from shoot biomass at 6 weeks post-inoculation. Dashes indicate that the fixed effect was not included in the analysis because it was either not applicable or it worsened model fit (see Methods).

| | | **A. Time Until Nodulation** | | **B. Nodule Number** | | **C. BNF ($\delta^{15}$N)** | | **D. Week 4 Plant Size** | | **E. Week 6 Plant Size** | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **Fixed Effect** | **df** | $\chi^2$ | ***P*** | $\chi^2$ | ***P*** | $\chi^2$ | ***P*** | $\chi^2$ | ***P*** | $\chi^2$ | ***P*** |
| Herbicide Treatment (E) | 1 | 11.97 | **0.0005** | 0.46 | 0.50 | 2.39 | 0.12 | 36.95 | **<.0001** | 7.44 | **0.0064** |
| Rhizobial Strain ($G_R$) | 7 | 33.51 | **<.0001** | 23.63 | **0.0013** | 14.49 | **0.043** | 5.56 | 0.59 | 9.33 | 0.23 |
| Plant Genotype ($G_P$) | 1 | 2.39 | 0.12 | 0.16 | 0.69 | 0.38 | 0.54 | 0.23 | 0.63 | 0.07 | 0.79 |
| Temporal Block | 1 | 0.05 | 0.48 | 31.26 | **<.0001** | - | - | 1.68 | 0.2 | - | - |
| Minor Fungal Infection | 1 | - | - | 9.45 | **0.0021** | 6.11 | **0.013** | 3.96 | **0.047** | - | - |
| $G_R \times E$ | 7 | 8.65 | 0.28 | 20.51 | **0.0046** | 16.98 | **0.018** | 16.74 | **0.019** | 19.86 | **0.0059** |
| $G_P \times E$ | 1 | 2.22 | 0.14 | 0.22 | 0.64 | 0.19 | 0.66 | 6.4 | **0.011** | 2.49 | 0.11 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| $G_P \times G_R$ | 7 | - - | 17.15 **0.016** | - - | - - | - - | |

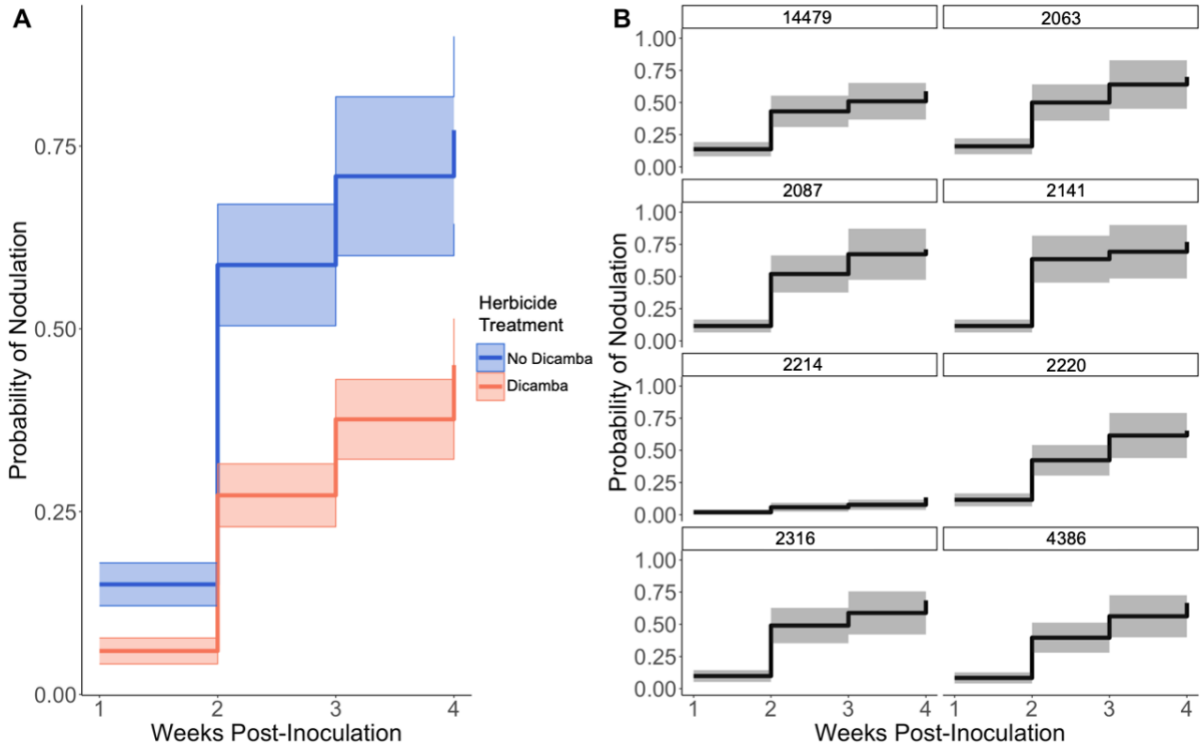



**Figure 8. Rhizospheric herbicide exposure and rhizobial strain independently affect the timing of nodulation.** Kaplan-Meier curves show the mean + SE (bold line and shading) probability of nodulation on *T. pratense* following inoculation with rhizobia according to herbicide treatment (A**;** dicamba present in orange, dicamba absent in blue) and rhizobial strain used in inoculation (B).

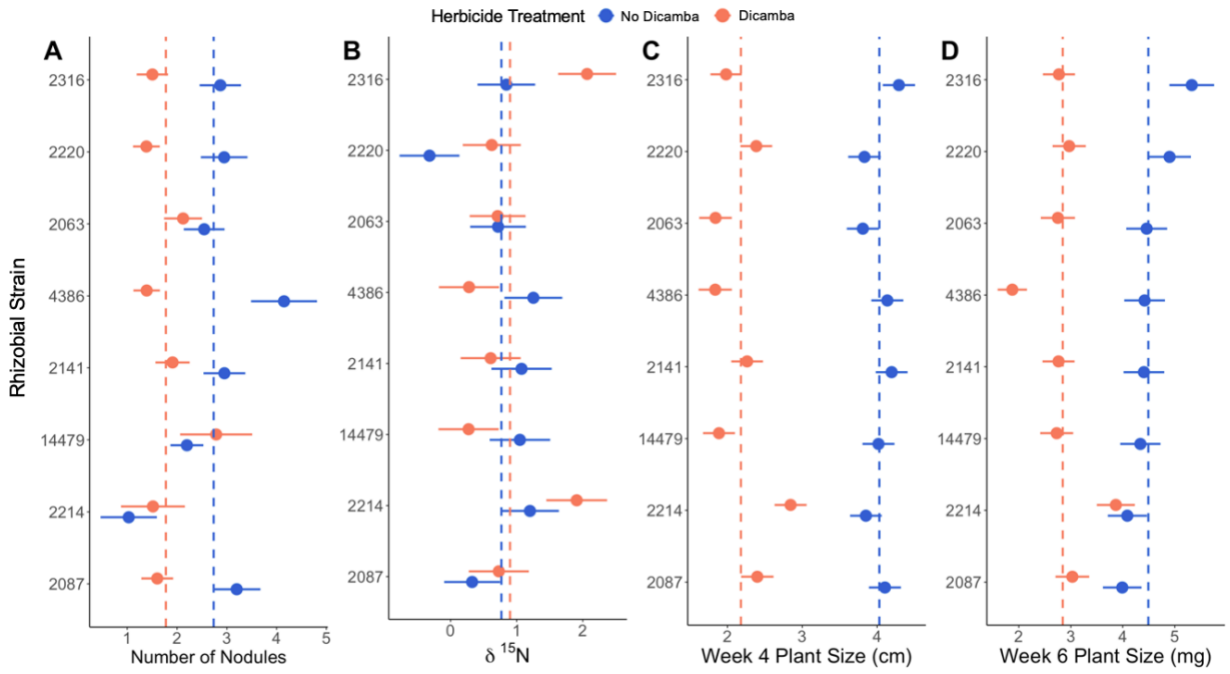



**Figure 9. Herbicide exposure and rhizobial strain interacted to determine a key trait of the legume-rhizobia mutualism and legume growth.**

EMMs (± SE) for nodule number (A), foliar δ15N abundance (B), size at 4 weeks post-inoculation (C; leaflet number × longest leaflet length in cm), and size at 6 weeks post-inoculation (D; shoot biomass in mg) in *T. pratense* when grown in the presence (orange) or absence (blue) of rhizospheric dicamba. Dashed lines represent herbicide treatment means across inocula.

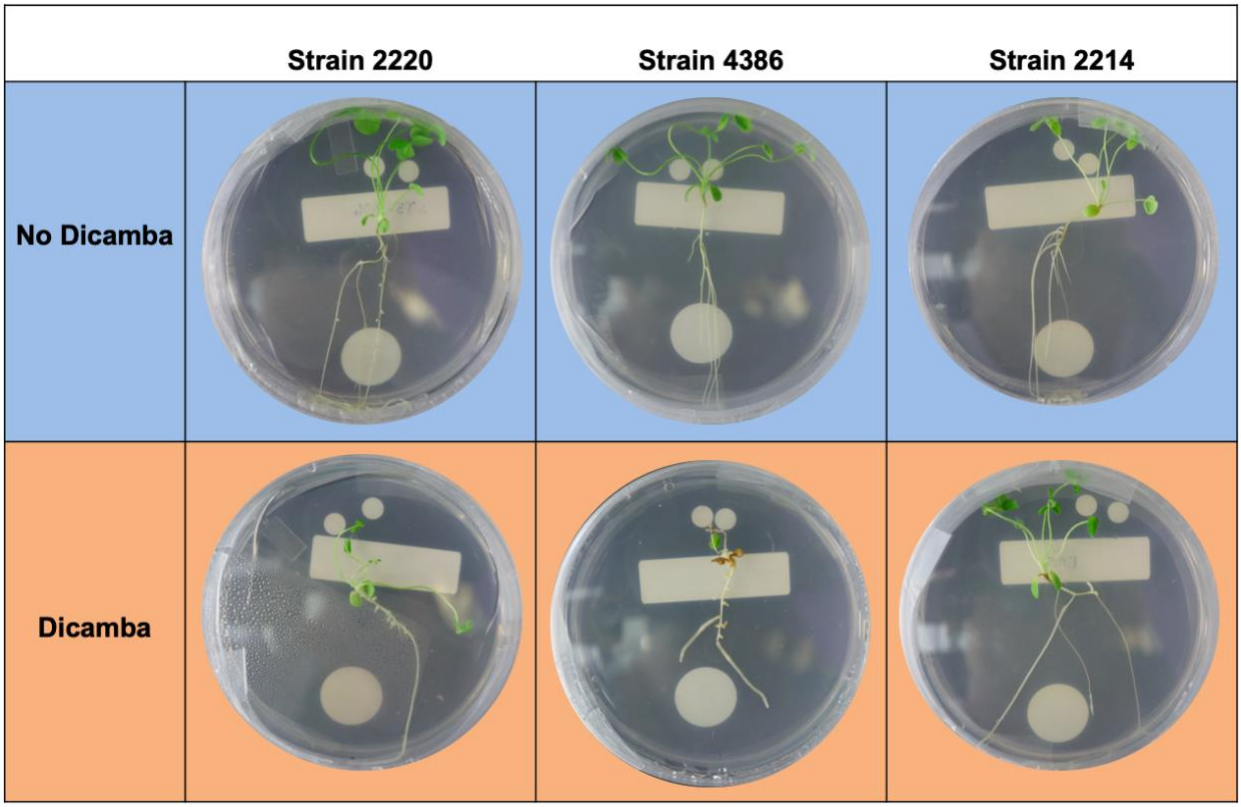



**Figure 10. Rhizospheric dicamba exposure altered rhizobial genotypic rank order for greatest legume growth benefit.**

Photographs of six representative experimental microcosms with and without rhizospheric exposure to dicamba, taken one week prior to harvest at 6 weeks post-inoculation. *T. pratense* plants were inoculated with different strains (2220, 4386, 2214) of rhizobia from left to right in order of greatest to least plant growth in the absence of dicamba herbicide.

# 5.0 Conclusion

As with climate change, invasive species, land-use change, and the over-exploitation of resources, chemical pollution is considered one of the five most pervasive anthropogenic threats to biodiversity (Pelletier and Coltman 2018). In particular, herbicide pollution caused by the movement of herbicidal particles away from application sites, mainly agricultural fields, threatens natural plant communities that occur along agro-ecological interfaces across the globe (Burden and Thrall 2008; FAO 2024). Possible ecological impacts of herbicide exposures, especially from highly-used herbicides known for off-target movement such as dicamba, include the indirect effects on species interactions such as plant-plant, plant-insect, and plant-microbe interactions and their related ecosystem services (e.g. habitat structuring, nutrient cycling, and pollination), which are mediated by the direct effects of these chemicals on non-target plants and/or their ecological partners. However, these knock-on ecological consequences of herbicides, especially at low rates relevant to off-target movement, have been largely understudied due to their complexity (Brühl and Zaller 2021). Thus, to shed light on the ecological consequences associated with herbicide pollution, in my dissertation chapters, I researched the direct and indirect effects of off-target herbicide exposures on plants and their species interactions through greenhouse and microcosm experiments with dicamba. Here, I synthesize the main findings acquired from this work and highlight key avenues for future research.

## 5.1 Impacts of Herbicide Exposures on Plant-*Plant* and Plant-*Insect* Interactions

Plant-plant interactions include competition for resources, both abiotic (e.g. nutrients, water, light) and biotic (e.g. pollinators, seed dispersers), which ultimately shape the composition of plant communities (Violle et al. 2009). These interactions can be significantly altered when off-target herbicide exposures affect plant species' phenotypes, such as their growth or flowering, which can in turn affect plant-insect interactions with pollinators or herbivores via changes in vegetative/floral food resources. In Chapter 1, my co-authors and I found that off-target exposure to dicamba via 'drift' (atmospheric movement; Egan et al. 2012), representative of only ~1% of the field application rate, resulted in high amounts of instantaneous leaf damage and reduced final biomass for about a quarter of the 25 plant species tested, but it affected others less, and even enhanced the size of one species, indicating significant interspecific variation in both initial resistance and longer-term tolerance of dicamba-related damages. Similarly, flowering was delayed and/or shortened for many species, but was accelerated/prolonged for others, resulting in different patterns of floral resource deployment among dicamba-exposed plants. Although the greenhouse setting did not allow us to directly test for plant-plant competition for resources or its impacts on plant-insect interactions, our study contributed necessary knowledge of the breadth of variation in the phenotypic responses to drift-level dicamba among agro-eco plant species, which future field studies could expand upon to gain these insights. For example, Johnson and Baucom (2022) recently found evidence of a negative correlation in velvetleaf between resisting leaf damage caused by dicamba drift and deterring herbivory caused by whiteflies. If this pattern extends to other plant species, then plant-herbivore interactions could be markedly changed by dicamba drift exposure in diverse agro-eco plant communities.

Moreover, it is yet unknown whether species have varied responses to low-level herbicide exposures in many other traits beyond immediate vegetative damage responses, growth, and flowering that could affect plant-plant or plant-insect interactions. For instance, Chapters 2 and 3 revealed that off-target levels of dicamba generally diminished trait values related to the resource mutualism between legumes and rhizobial bacteria, including the amount of symbiotically fixed nitrogen legumes received from rhizobia. From an ecological community perspective, this legume-specific impact of herbicide exposure could overturn competitive dynamics between plant species, wherein legumes would often otherwise receive a competitive advantage over other plants, especially when soil nitrogen is limited (reviewed in Traveset and Richardson 2014). Additionally, some studies in crop systems have indicated that herbicides (e.g. imazethapyr and 2,4-D) at ≤ field rates can reduce or increase the release of allelopathic chemicals that are antagonistic for neighboring plants, depending on the species (e.g. in ryegrass and wheat [Zhang et al. 2024]; sunflower [Dieterman et al. 1964] and tomato [van Bragt et al. 1965]), which could thereby also alter outcomes of plant-plant competition. In regards to plant-insect interactions, it has been speculated that low-level herbicide exposures could likewise result in changes in floral or leaf volatile emissions which could thus affect visitation to plants by pollinators or herbivores, but this has rarely if ever been tested in either wild or cultivated plant species (reviewed in Ramos et al. 2022). Furthermore, limited work on honeybees has indicated that directly exposing these insects to herbicides (i.e. glyphosate, mesotrione, and atrazinecan) can disrupt their learning, locomotor activity, and foraging behavior (Farina et al. 2019; dos Santos Araújo et al. 2023); however these effects are often tested for at high concentrations and related information on other insect species is scarce. In the future, experiments that simulate off-target herbicide exposures directly onto both plant and insect species and also quantify a suite of biologically-relevant traits, e.g. as de Manincor

et al. (2023) did in studying the effects of warming on plant-pollinator interactions, would provide a clearer understanding of not only the ecological costs of herbicide exposures for these species interactions but also the mechanisms which are driving them.

### 5.2 Impacts of Herbicide Exposures on Plant-*Microbe* Interactions

Plant-microbe interactions which make essential contributions to terrestrial ecosystems include resource mutualisms wherein plants exchange carbon for nutrients provided by rhizospheric microbes (e.g. rhizobia and arbuscular mycorrhizal fungi [AMF]) to enhance plant growth (Reynolds et al. 2003). However, these interactions can be significantly altered when off-target herbicide exposures affect the ability of partners to trade resources. In Chapter 2, we found that aboveground application of dicamba drift onto red clover hindered its growth and ability to benefit from rhizobial interactions; however, these effects were modulated by rhizobial genotypes (i.e. strains, N = 2). Meanwhile, although many more were tested, plant genotypes (N = 17) had lesser effects on these mutualism outcomes. Following from this, in Chapter 3, we used many more rhizobial strains (N = 8) while still accounting for clover variation (N = 2 genotypes) and again found that rhizobial variation was the predominant driver of plant-rhizobia interactions, despite the fact that this time dicamba was applied in the belowground rhizospheric environment but at a similar level (~0.5% of the field application rate). Moreover, in both cases, the rhizobial strains which often resulted in the largest declines in plant benefit under dicamba were those that, regardless of herbicide treatment, led to similar nodule numbers (e.g. strain 14479 in Chapter 2;

strain 2063 in Chapter 3) and/or were similarly active in providing fixed nitrogen to plants (e.g. strains 4386, 2063, and 2316; Chapter 3)—indicating that this consequence may be driven by strain-specific increases in symbiotic costs associated with nodulation/nitrogen fixation rather than decreases in nutritional benefits received. On the other hand, results from Chapter 3 also demonstrated that some rhizobia (e.g. strain 2214) can ameliorate the negative effects of herbicides for plant growth despite their lack of change in nodule numbers or nitrogen fixation under herbicidal conditions, thereby raising the question: what other benefits beyond nitrogen do rhizobia provide that could help plants cope with herbicide stress? For example, free-living rhizobia are known to vary in their production of phytohormones and enzymes that can enhance tolerance to abiotic stressors including foreign chemicals (although herbicides have yet to be thoroughly tested; reviewed in Jaiswal et al. 2021), and in rare cases evidence for herbicide-degrading rhizobia have been reported (e.g. Fabra et al. 1997). Ultimately, investigations into rhizobial metabolism in both in and out of symbiosis and with/without herbicide treatments will help illuminate the mechanisms underpinning rhizobial mediation of plant-rhizobia interactions following off-target herbicide exposures. By extension, it is possible that certain rhizobial strains with heightened capabilities to enhance herbicide tolerance could defend against herbicide-related damages to nontarget plants. This would be a particularly exciting future direction to research for applied ecological and agricultural uses.

Additionally, although the greenhouse/growth chamber experiments conducted in Chapters 2 and 3 provided novel insights concerning herbicide-induced changes in plant-microbe interactions and their biotic drivers, future field studies will be needed to determine the extent to which our results are transferrable to present-day agro-eco plant communities. For instance, in natural rhizospheres, various rhizobial strains compete with each other as well as numerous other

microbes, and this competition can affect rhizobial colonization of roots, nitrogen fixation, and plant growth (Mendoza-Suarez et al. 2021; Batstone et al. 2023; Burghardt and DiCenzo 2023; Rahman et al. 2023). Therefore, it would be especially valuable to explore how off-target herbicide exposures affect rhizospheric microbial diversity and in turn how this diversity influences outcomes of plant-rhizobia interactions under herbicide stress. Furthermore, another ecologically important plant-microbe resource mutualism that was not researched here is that between plants and AMF: 80-90% of vascular plant species partake in this symbiosis wherein plants trade carbon for AMF-enhanced access to nutrients and water (Smith et al. 2004). Although there is some evidence that exposures to herbicides via off-target movement onto the foliage of host plants affects AMF root colonization (e.g. studies on glyphosate: Druille et al. 2013; Carvalho et al. 2014), we especially do not know whether they could cause similar augmentations of AMF-associated symbiotic costs similar to what we observed in the plant-rhizobia mutualism. Thus, studies which consider as we did, both above- and belowground exposures to environmentally realistic levels of herbicides on plant-microbe interactions, but expand our work by considering a greater span of potential microbial associates, will allow us to better gauge the potential for herbicides to influence symbioses between plants and rhizospheric microbes and the ecosystem services they provide.

## 5.3 Final Remarks

Altogether, the three Chapters of my dissertation converge on one message: off-target exposures to the contemporary herbicide dicamba, despite constituting only a small percentage of the total amount that is normally applied for agricultural purposes, can have appreciable impacts on the growth of wild plants and crucial species interactions they are involved in at the agro-ecological interface. At the same time however, there exists significant genetic variation, either among plant species or their ecological partners, that mediate the ecological consequences of these exposures and could also influence species evolution in response to them, if assumptions from eco-evolutionary theory hold true. While numerous questions remain, future research can take important steps forward to test the predictions made here on the ecology and evolution of agro-ecosystems in herbicide-exposed contexts.

# Appendix A Supplementary Tables and Figures for Chapter 1

**Appendix A: Table 6. Collection site information for seeds used in this study.**

| Site | Species | Latitude | Longitude | County, State in USA |
|---|---|---|---|---|
| 15 | *Amaranthus palmeri* | 37.1281 | -86.6596 | Butler County, KY |
| 22 | *Abutilon theophrasti, Amaranthus palmeri, Erigeron annuus, Lepidium virginicum* | 36.7271 | -88.1781 | Calloway County, KY |
| 23 | *Rumex crispus* | 36.6906 | -88.189 | Calloway County, KY |
| 24 | *Taraxacum officinale, Daucus carota, Erigeron annuus, Oxalis stricta* | 36.6614 | -88.2168 | Calloway County, KY |
| 25 | *Commelina virginica* | 36.5696 | -88.2472 | Calloway County, KY |
| 24A | *Eupatorium serotinum, Solidago canadensis* | 36.6614 | -88.2168 | Calloway County, KY |
| 20 | *Plantago virginica* | 36.8494 | -87.3016 | Christian County, KY |
| 21 | *Sida spinosa, Abutilon theophrasti* | 36.854 | -87.5514 | Christian County, KY |
| 27 | *Trifolium pratense* | 36.8133 | -87.4098 | Christian County, KY |
| 28 | *Persicaria pennsylvanica* | 36.8993 | -87.5339 | Christian County, KY |
| 30 | *Physalis philadelphica, Plantago virginica* | 36.7929 | -87.3796 | Christian County, KY |
| 29A | *Solanum carolinense, Lepidium virginicum, Plantago lanceolata, Trifolium pratense* | 36.7995 | -87.3839 | Christian County, KY |
| 30A | *Oxalis stricta* | 36.7929 | -87.3796 | Christian County, KY |
| 27A | *Ipomoea hederacea* | 36.8257 | -87.477 | Christian County, KY |
| 13 | *Persicaria pennsylvanica* | 36.7988 | -86.8762 | Logan County, KY |
| 17 | *Asclepias syriaca, Cardiospermum halicacabum* | 36.9771 | -86.7835 | Logan County, KY |
| 31 | *Sida spinosa, Abutilon theophrasti, Cardiospermum halicacabum* | 36.7014 | -87.0415 | Logan County, KY |

| | | | | |
|---|---|---|---|---|
| 32A | *Commelina virginica, Geum canadense, Solanum carolinense, Oxalis stricta* | 36.6409 | -87.1576 | Montgomery County, TN |
| 11 | *Ipomoea lacunosa* | 36.5976 | -86.8511 | Robertson County, TN |
| 33A | *Plantago lanceolata, Trifolium pratense, Daucus carota,* | 36.44 | -86.8106 | Robertson County, TN |
| DG07 | *Amaranthus palmeri, Ipomoea lacunosa* | 36.6233 | -86.8413 | Robertson County, TN |
| DG08 | *Ipomoea hederacea* | 36.6319 | -86.8636 | Robertson County, TN |
| HJ01 | *Desmanthus illinoensis, Senna obtusifolia, Sida spinosa, Taraxacum officinale* | 35.751 | -86.6208 | Rutherford County, TN |
| HJ03 | *Ipomoea 108acunose, Cardiospermum halicacabum* | 35.7333 | -86.5902 | Rutherford County, TN |
| HJ04 | *Solanum carolinense, Desmanthus illinoensis* | 35.7333 | -86.5902 | Rutherford County, TN |
| JB06 | *Physalis philadelphica* | 35.8729 | -86.4745 | Rutherford County, TN |
| 19 | *Taraxacum officinale* | 36.8153 | -87.1768 | Todd County, KY |
| 14 | *Lepidium virginicum, Persicaria pennsylvanica* | 37.0655 | -86.6151 | Warren County, KY |

**Appendix A: Table 7. Contrast analysis to test for significant differences between herbicide treatments in flowering duration across species.**

'Estimate' is the estimated marginal mean of flowering duration in days in the control treatment subtracted by that of the drift treatment. 'Lower CI' shows the lower and 'Upper CI' the upper bound of the asymptotic 95% confidence interval for the estimate. '*z*-ratio' denotes the *z*-statistic calculated to determine the *P*-value of the difference between herbicide treatments. Significant *P*-values are in bold. Degrees of freedom = infinity. Subscripts denote species whose flowering duration was determined by the termination of the experiment rather than flowering in the control[a] or drift[b] treatment or both[c].

| **Species** | **Estimate** | **Lower CI** | **Upper CI** | ***z*-ratio** | ***P*-value** |
|---|---|---|---|---|---|
| *Abutilon theophrasti* | 10.000 | 2.222 | 17.778 | 2.520 | **0.0117** |
| *Amaranthus palmeri*[b] | -11.600 | -18.148 | -5.052 | -3.472 | **0.0005** |
| *Cardiospermum halicacabum*[c] | -2.300 | -11.081 | 6.481 | -0.513 | 0.6077 |
| *Commelina virginica* | 19.167 | 12.914 | 25.420 | 6.008 | **<.0001** |
| *Daucus carota*[a] | 13.833 | 0.0650 | 27.602 | 1.969 | **0.0489** |
| *Desmanthus illinoensis* | -4.500 | -13.392 | 4.392 | -0.992 | 0.3213 |
| *Eupatorium serotinum* | -8.056 | -13.804 | -2.308 | -2.747 | **0.0060** |
| *Ipomoea hederaceae* | 0.500 | -3.224 | 4.224 | 0.263 | 0.7924 |
| *Ipomoea lacunosa* | -10.800 | -15.165 | -6.434 | -4.849 | **<.0001** |
| *Oxalis stricta* | -7.500 | -15.366 | 0.366 | -1.869 | 0.0617 |
| *Persicaria pennsylvanica*[c] | 3.033 | -7.594 | 13.660 | 0.559 | 0.5759 |
| *Physalis philadelphica* | 7.286 | 3.118 | 11.454 | 3.426 | **0.0006** |
| *Plantago lanceolata* | 13.500 | 7.225 | 19.775 | 4.217 | **0.0001** |
| *Senna obtusifolia* | 4.095 | 0.198 | 7.993 | 2.059 | **0.0395** |
| *Sida spinosa* | 1.033 | -2.908 | 4.975 | 0.514 | 0.6074 |

| | | | | | |
|---|---|---|---|---|---|
| *Taraxicum officionale* | 25.57 | 18.828 | 32.315 | 7.432 | **<.0001** |
| *Trifolium pratense* | 47.300 | 40.263 | 54.337 | 13.175 | **<.0001** |

**Appendix A: Table 8. Estimates of goodness of fit for each model in this study based on the Akaike Information Criterion (AIC) with and without phylogenetic correction.**

Phylogenetic correction was applied to models using the 'phylolm' package in R (version 1.2 1335). 'K' indicates the number of model parameters and 'ΔAIC' denotes the model AIC with phylogenetic correction ('Phylogenetic AIC') subtracted by the model AIC without phylogenetic correction ('Non-phylogenetic AIC'). Abbreviation: Sqrt = Square-root.

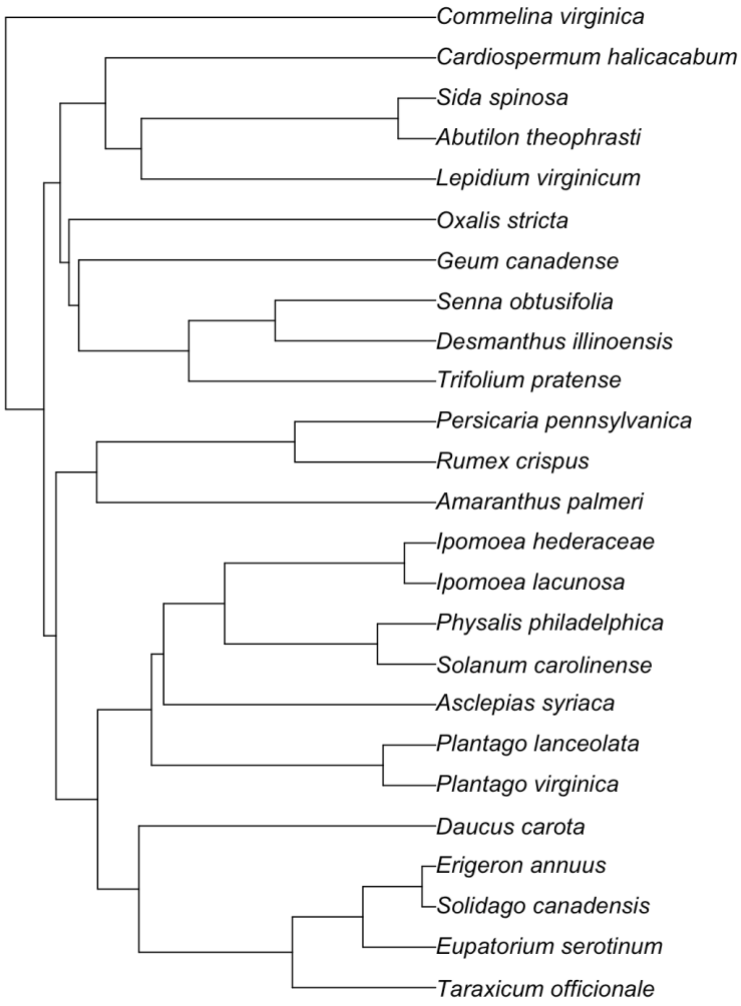
| **Model** | **K** | **Phylogenetic AIC** | **Non-phylogenetic AIC** | **ΔAIC** |
|---|---|---|---|---|
| Proportion of undamaged Leaves ~ Species | 1 | -126.58 | -157.54 | 30.96 |
| Sqrt(size) 21 days post-treatment ~ Treatment x Species + sqrt(Pre-treatment Plant Size) + (1\| transplanted) | 4 | 2996.51 | 2818.83 | 177.67 |
| Sqrt(biomass) 145 days post-treatment ~ Treatment x Species + sqrt(Pre-Treatment Plant Size) | 3 | 284.53 | 282.53 | 2.00 |
| Sqrt(day of first flower) ~ Treatment x Species | 2 | 280.64 | 278.64 | 2.00 |
| Log(biomass per Flower) ~ Treatment x Species | 2 | 127.55 | 125.55 | 2.00 |
| Log(floral display) ~ Treatment x Species | 2 | 531.94 | 529.94 | 2.00 |
| Flowering duration ~ Treatment x Species | 3 | 3667.00 | 3339.73 | 327.27 |

**Appendix A: Table 9. ANOVA type III tests for all measured traits.**

'Response' indicates the response variable (i.e., measured plant trait) tested. 'Fixed Effects' shows all explanatory variables inputted in the ANOVA to predict each response variable. 'Sum Squares', 'df', and '*F*-value' denote the sum of squares, degrees of freedom, and overall *F*-statistic, respectively, associated with each fixed effect. If an ANOVA was performed on a mixed effects linear model, the outputted Wald chi-square statistic is reported in the 'Chisq' column. The fixed effect 'Treatment' includes two levels (control and drift) and 'Species' includes 17-25, depending on the response (see Statistical Analysis in main text). Dashes indicate that a statistic was not calculated for the corresponding fixed effect. Significant *P*-values are in bold. Abbreviations: Sqrt = Square-root.

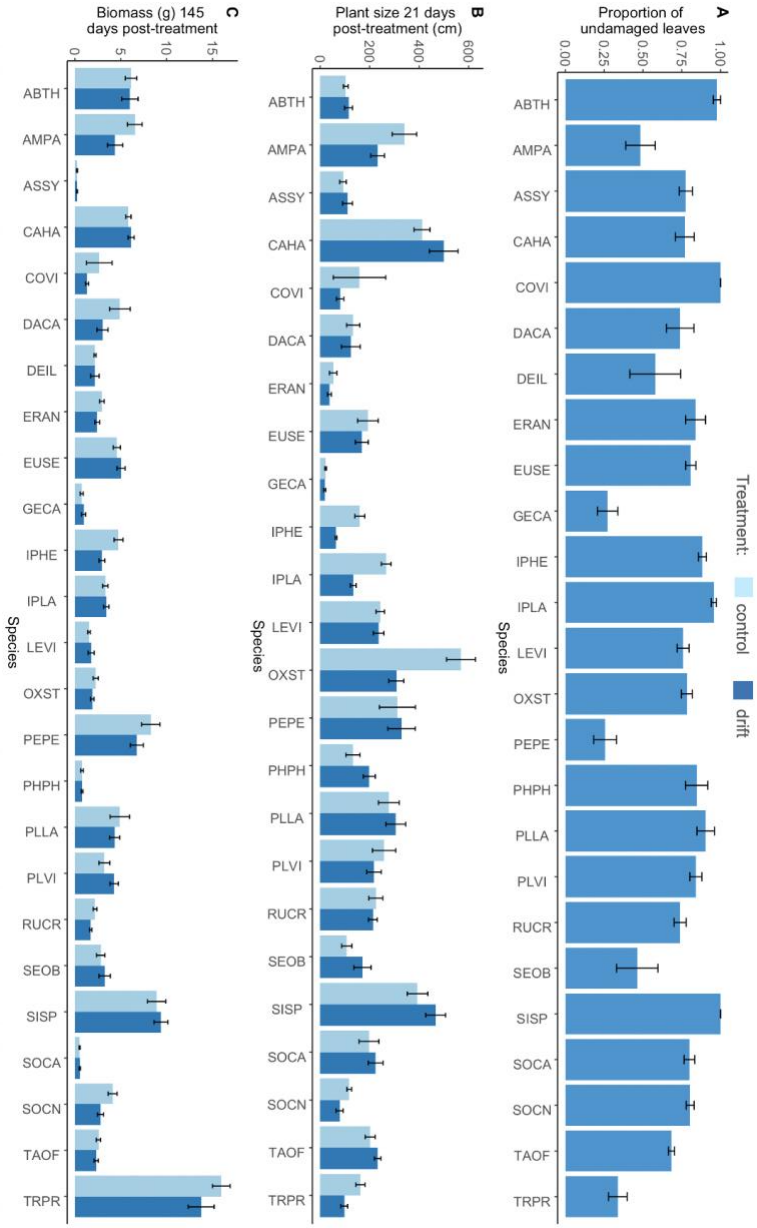
| **Response** | **Fixed Effects** | **Sum Squares** | **df** | ***F*-Value** | **Chisq** | ***P*-value** |
|---|---|---|---|---|---|---|
| Proportion of undamaged Leaves | Species | 8.89 | 24 | 14.94 | - | **<2.2e-16** |
| Sqrt(size) 21 days post-treatment | Treatment | - | 1 | - | 0.11 | 0.700 |
| | Species | - | 23 | - | 370.91 | **<2.2e-16** |
| | Sqrt(Pre-Treatment Plant Size) | - | 1 | - | 188.57 | **<2.2e-16** |
| | Treatment*Species | - | 23 | - | 91.11 | **4.6e-10** |
| | Transplanted | - | 1 | - | 5.84[b] | **0.015** |
| Sqrt(biomass) 145 days post-treatment | Treatment | 0.01 | 1 | 157.45 | - | 0.759 |
| | Species | 131.91 | 24 | 53.18 | - | **<2.2e-16** |
| | Sqrt(Pre-Treatment Plant Size) | 8.63 | 1 | 83.54 | - | **<2.2e-16** |
| | Treatment*Species | 5.77 | 24 | 2.33 | - | **<0.001** |

| | | | | | | |
|---|---|---|---|---|---|---|
| Sqrt(day of first flower) | Treatment | 0.50 | 1 | 3.31 | - | 0.070 |
| | Species | 64.67 | 16 | 26.70 | - | **<2.2e-16** |
| | Treatment*Species | 17.87 | 16 | 7.38 | - | **9.53e-14** |
| Log(biomass per flower) | Treatment | 0.25 | 1 | 2.79 | - | 0.096 |
| | Species | 289.84 | 16 | 204.62 | - | **<2.2e-16** |
| | Treatment*Species | 2.57 | 16 | 1.82 | - | **0.030** |
| Log(floral display) | Treatment | 0.16 | 1 | 0.34 | - | 0.561 |
| | Species | 576.91 | 16 | 75.89 | - | **<2.2e-16** |
| | Treatment*Species | 5.63 | 16 | 0.74 | - | 0.750 |
| Log(flowering duration) | Treatment | - | 1 | - | 6.38 | **0.012** |
| | Species | - | 16 | - | 2152.34 | **<2.2e-16** |
| | Treatment*Species | - | 16 | - | 335.72 | **<2.2e-16** |




**Appednix A: Figure 11. Phylogeny of the 25 agro-eco plant species investigated in this study.**

The tree was constructed using the R package *V.PhyloMaker* (Jin and Qian 2019), wherein we altered the format of species' genus, species, and families to match those used by *V.Phylomaker* and used the options: tree = GBOTB.extended (indicates that phylogenetic information was extracted from a corrected combination of the mega-phylogenetic tree 'GBOTB' for seed plants [Smith and Brown 2018]), nodes = nodes.info1 (genus- and family-level node and branch length information provided by the mega-tree), and scenarios = "S1" (new species tips are binded to genus- or family-level basal nodes).




**Appendix A: Figure 12. Vegetative growth responses by species and herbicide treatment.**

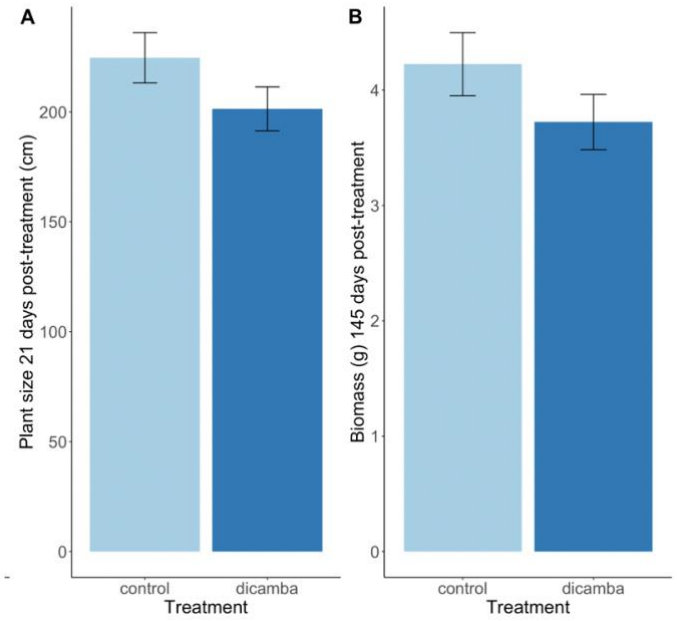
Responses are proportion of undamaged leaves 48 hours post-treatment (A), plant size in cm at 21 days post-treatment (B), and biomass in g at 145 days post-treatment (C). Four-letter codes are used for species (see Table 2). Bars show means ± SE.

**Appendix A: Table 10. *t*-tests to score species for dicamba drift resistance.**

The null hypothesis was that, for each species, the estimated marginal mean ('emmean') of the proportion of undamaged leaves (1 - # leaves damaged / total # leaves) at 48 hours post-herbicide treatment was equal to one. The alternative hypothesis was that the emmean was less than 1. 'Lower CI' shows the lower and 'Upper CI' the upper bound of the 95% confidence interval for the emmean. '*t*-ratio' denotes the *t*-statistic calculated from each test. Species with nonsignificant *P*-values were characterized as resistant. Significant *P*-values are in bold. Degrees of freedom = 188.

| **Species** | **Emmean** | **Lower CI** | **Upper CI** | ***t*-ratio** | ***P*-value** |
|---|---|---|---|---|---|
| *Abutilon theophrasti* | 0.976 | 0.849 | 1.103 | 0.370 | 0.3558 |
| *Amaranthus palmeri* | 0.484 | 0.386 | 0.582 | 10.364 | **<.0001** |
| *Asclepias syriaca* | 0.776 | 0.659 | 0.894 | -3.760 | **0.0001** |
| *Cardiospermum halicacabum* | 0.770 | 0.671 | 0.868 | -4.628 | **<.0001** |
| *Commelina virginica* | 1.000 | 0.890 | 1.110 | 0.000 | 0.5000 |
| *Daucus carota* | 0.739 | 0.612 | 0.866 | -4.054 | **<.0001** |
| *Desmanthus illinoensis* | 0.579 | 0.399 | 0.758 | -4.633 | **<.0001** |
| *Erigeron annuus* | 0.838 | 0.711 | 0.965 | -2.518 | **0.0063** |
| *Eupatorium serotinum* | 0.808 | 0.709 | 0.906 | -3.864 | **0.0001** |
| *Geum canadense* | 0.272 | 0.169 | 0.376 | -13.863 | **<.0001** |
| *Ipomoea hederaceae* | 0.883 | 0.785 | 0.981 | -2.344 | **0.0101** |
| *Ipomoea lacunosa* | 0.956 | 0.858 | 1.054 | -0.882 | 0.1894 |
| *Lepidium virginicum* | 0.759 | 0.661 | 0.857 | -4.839 | **<.0001** |
| *Oxalis stricta* | 0.782 | 0.684 | 0.881 | -4.370 | **<.0001** |
| *Persicaria pennsylvanica* | 0.256 | 0.117 | 0.395 | -10.566 | **<.0001** |
| *Physalis philadelphica* | 0.845 | 0.747 | 0.943 | -3.107 | **0.0011** |

| | | | | | |
|---|---|---|---|---|---|
| *Plantago lanceolata* | 0.904 | 0.806 | 1.002 | -1.924 | **0.0279** |
| *Plantago virginica* | 0.840 | 0.742 | 0.939 | -3.203 | **0.0008** |
| *Rumex crispus* | 0.739 | 0.641 | 0.837 | -5.238 | **<.0001** |
| *Senna obtusifolia* | 0.463 | 0.336 | 0.590 | -8.350 | **<.0001** |
| *Sida spinosa* | 1.000 | 0.902 | 1.098 | 0.000 | 0.5000 |
| *Solanum carolinense* | 0.799 | 0.696 | 0.903 | -3.827 | **0.0001** |
| *Solidago canadensis* | 0.804 | 0.694 | 0.913 | -3.528 | **0.0003** |
| *Taraxicum officionale* | 0.683 | 0.585 | 0.781 | -6.362 | **<.0001** |
| *Trifolium pratense* | 0.338 | 0.240 | 0.436 | -13.292 | **<.0001** |




**Appendix A: Figure 13. Vegetative growth responses by herbicide treatment, averaged over species.**

Responses (mean ± SE) are plant size in cm at 21 days post-treatment (A) and biomass in g at 145 days post-treatment (B).

**Appendix A: Table 11. Contrast analysis to test for significant differences between herbicide treatments in size 21 days post-treatment across species.**

'Estimate' is the estimated marginal mean of plant size in cm at 21 days post-treatment (on the square-root scale) in the control treatment subtracted by that of the drift treatment. 'Lower CI' shows the lower and 'Upper CI' the upper bound of the 95% confidence interval for the estimate. '*t*-ratio' denotes the *t*-statistic calculated to determine the *P*-value of the difference between herbicide treatments. Significant *P*-values are in bold. Degrees of freedom = 370.

| **Species** | **Estimate** | **Lower CI** | **Upper CI** | ***t*-ratio** | ***P*-value** |
|---|---|---|---|---|---|
| *Abutilon theophrasti* | -0.152 | -1.070 | 0.765 | -0.326 | 0.7442 |
| *Amaranthus palmeri* | 1.635 | 0.917 | 2.353 | 4.478 | **<.0001** |
| *Asclepias syriaca* | -0.583 | -1.433 | 0.267 | -1.349 | 0.1783 |
| *Cardiospermum halicacabum* | -0.592 | -1.302 | 0.117 | -1.642 | 0.1015 |
| *Commelina virginica* | 0.059 | -0.764 | 0.882 | 0.140 | 0.8884 |
| *Daucus carota* | 0.221 | -0.696 | 1.138 | 0.473 | 0.6362 |
| *Erigeron annuus* | 0.204 | -0.724 | 1.131 | 0.432 | 0.6660 |
| *Eupatorium serotinum* | 0.082 | -0.627 | 0.792 | 0.228 | 0.8198 |
| *Geum canadense* | 0.254 | -0.476 | 0.983 | 0.684 | 0.4944 |
| *Ipomoea hederaceae* | 1.282 | 0.571 | 1.992 | 3.548 | **0.0004** |
| *Ipomoea lacunosa* | 1.609 | 0.900 | 2.319 | 4.459 | **<.0001** |
| *Lepidium virginicum* | -0.159 | -0.871 | 0.552 | -0.441 | 0.6595 |
| *Oxalis stricta* | 1.658 | 0.947 | 2.369 | 4.586 | **<.0001** |
| *Persicaria pennsylvanica* | 0.024 | -0.943 | 0.990 | 0.048 | 0.9615 |
| *Physalis philadelphica* | -0.823 | -1.535 | -0.111 | -2.274 | **0.0235** |
| *Plantago lanceolata* | -0.046 | -0.759 | 0.667 | -0.128 | 0.8985 |

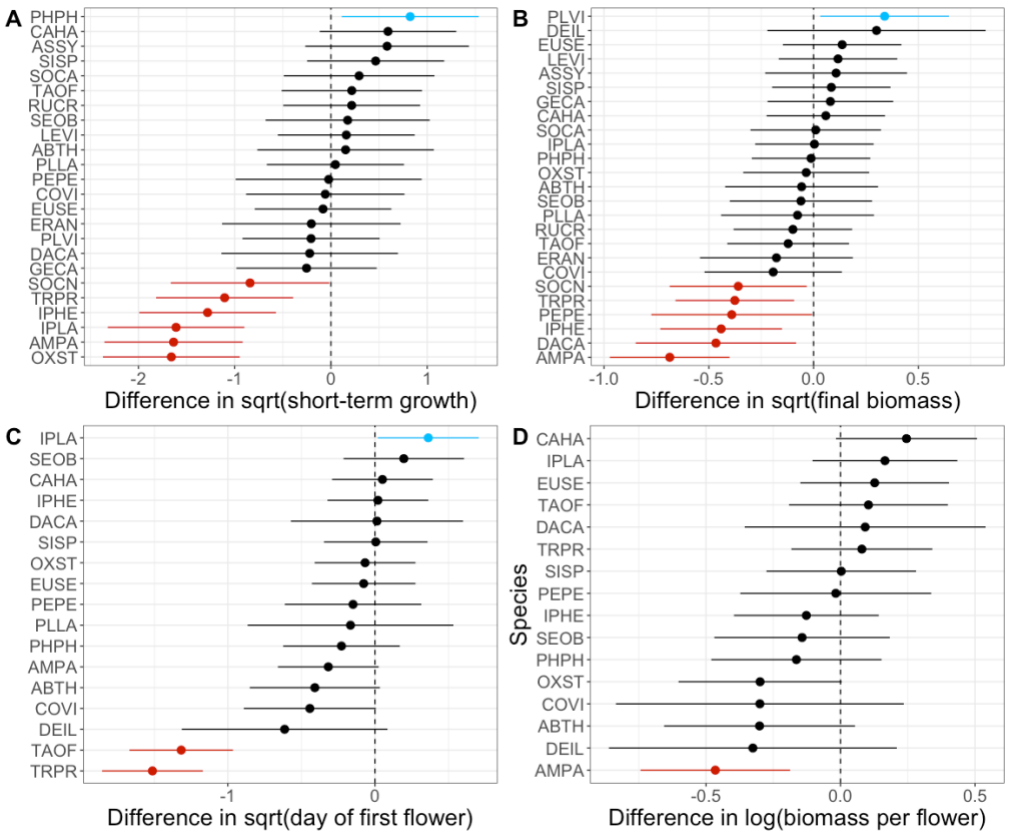
| | | | | | |
|---|---|---|---|---|---|
| *Plantago virginica* | 0.207 | -0.506 | 0.919 | 0.570 | 0.5688 |
| *Rumex crispus* | -0.215 | -0.927 | 0.496 | -0.595 | 0.5521 |
| *Senna obtusifolia* | -0.174 | -1.026 | 0.679 | -0.401 | 0.6890 |
| *Sida spinosa* | -0.464 | -1.177 | 0.248 | -1.282 | 0.2005 |
| *Solanum carolinense* | -0.293 | -1.075 | 0.490 | -0.735 | 0.4646 |
| *Solidago canadensis* | 0.841 | 0.017 | 1.664 | 2.007 | **0.0455** |
| *Taraxicum officionale* | -0.217 | -0.946 | 0.513 | -0.584 | 0.5595 |
| *Trifolium pratense* | 1.105 | 0.393 | 1.817 | 3.050 | **0.0025** |

**Appendix A: Table 12. Contrast analysis to test for significant differences between herbicide treatments in biomass 145 days post-treatment across species.**

'Estimate' is the estimated marginal mean of biomass in g 145 days post-treatment (on the square-root scale) in the control treatment subtracted by that of the drift treatment. 'Lower CI' shows the lower and 'Upper CI' the upper bound of the 95% confidence interval for the estimate. '*t*-ratio' denotes the t-statistic calculated to determine the *P*-value of the difference between herbicide treatments. Significant *P*-values are in bold. Degrees of freedom = 359.

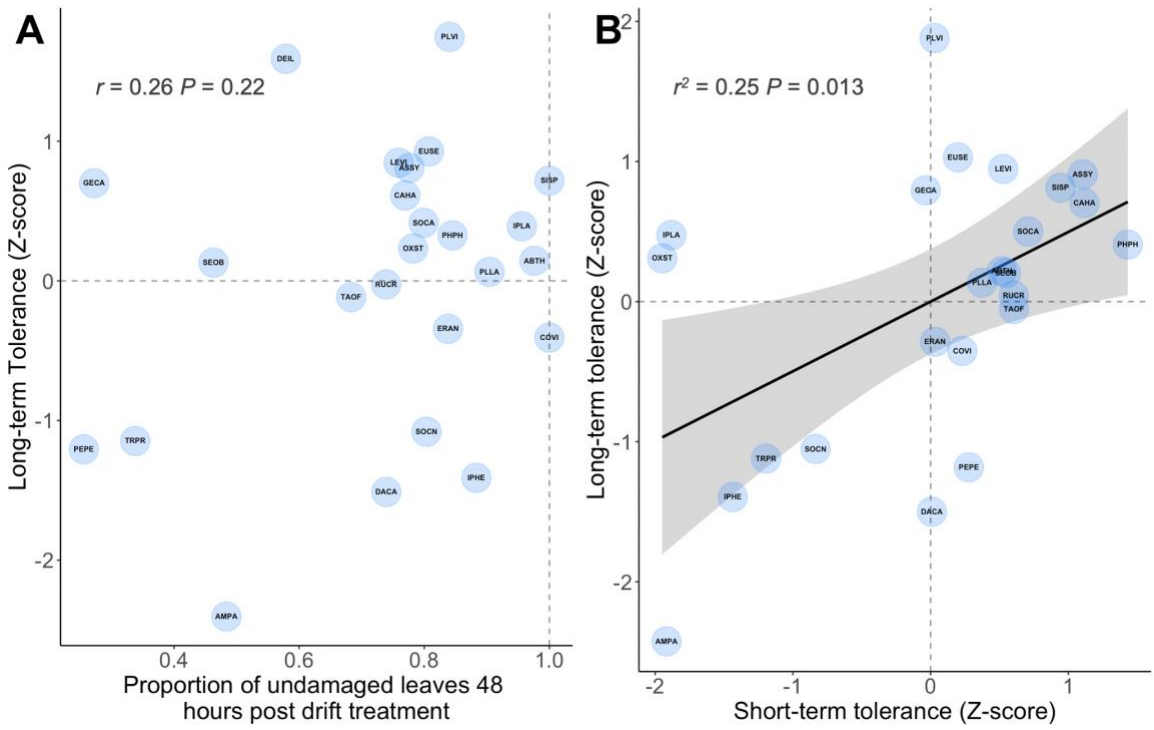
| **Species** | **Estimate** | **Lower CI** | **Upper CI** | ***t*-ratio** | ***P*-value** |
|---|---|---|---|---|---|
| *Abutilon theophrasti* | 0.057 | -0.308 | 0.422 | 0.308 | 0.7586 |
| *Amaranthus palmeri* | 0.687 | 0.401 | 0.972 | 4.724 | **<.0001** |
| *Asclepias syriaca* | -0.108 | -0.446 | 0.231 | -0.625 | 0.5325 |
| *Cardiospermum halicacabum* | -0.059 | -0.341 | 0.224 | -0.407 | 0.6843 |
| *Commelina virginica* | 0.193 | -0.135 | 0.521 | 1.156 | 0.2484 |
| *Daucus carota* | 0.466 | 0.083 | 0.849 | 2.394 | **0.0172** |
| *Desmanthus illinoensis* | -0.300 | -0.821 | 0.220 | -1.134 | 0.2575 |
| *Erigeron annuus* | 0.177 | -0.188 | 0.542 | 0.955 | 0.3401 |
| *Eupatorium serotinum* | -0.136 | -0.419 | 0.146 | -0.949 | 0.3433 |
| *Geum canadense* | -0.080 | -0.380 | 0.220 | -0.527 | 0.5988 |
| *Ipomoea hederaceae* | 0.441 | 0.151 | 0.732 | 2.986 | **0.0030** |
| *Ipomoea lacunosa* | -0.004 | -0.287 | 0.279 | -0.029 | 0.9772 |
| *Lepidium virginicum* | -0.117 | -0.400 | 0.166 | -0.811 | 0.4181 |
| *Oxalis stricta* | 0.035 | -0.265 | 0.336 | 0.231 | 0.8178 |
| *Persicaria pennsylvanica* | 0.391 | 0.007 | 0.774 | 2.001 | **0.0461** |
| *Physalis philadelphica* | 0.012 | -0.271 | 0.295 | 0.083 | 0.9338 |
| *Plantago lanceolata* | 0.077 | -0.288 | 0.442 | 0.412 | 0.6803 |

| | | | | | |
|---|---|---|---|---|---|
| *Plantago virginica* | -0.339 | -0.646 | -0.032 | -2.171 | **0.0306** |
| *Rumex crispus* | 0.099 | -0.184 | 0.382 | 0.686 | 0.4931 |
| *Senna obtusifolia* | 0.060 | -0.279 | 0.400 | 0.349 | 0.7271 |
| *Sida spinosa* | -0.085 | -0.368 | 0.198 | -0.590 | 0.5557 |
| *Solanum carolinense* | -0.010 | -0.322 | 0.301 | -0.065 | 0.9482 |
| *Solidago canadensis* | 0.360 | 0.032 | 0.687 | 2.161 | **0.0313** |
| *Taraxicum officionale* | 0.121 | -0.169 | 0.412 | 0.821 | 0.4120 |
| *Trifolium pratense* | 0.376 | 0.093 | 0.659 | 2.611 | **0.0094** |



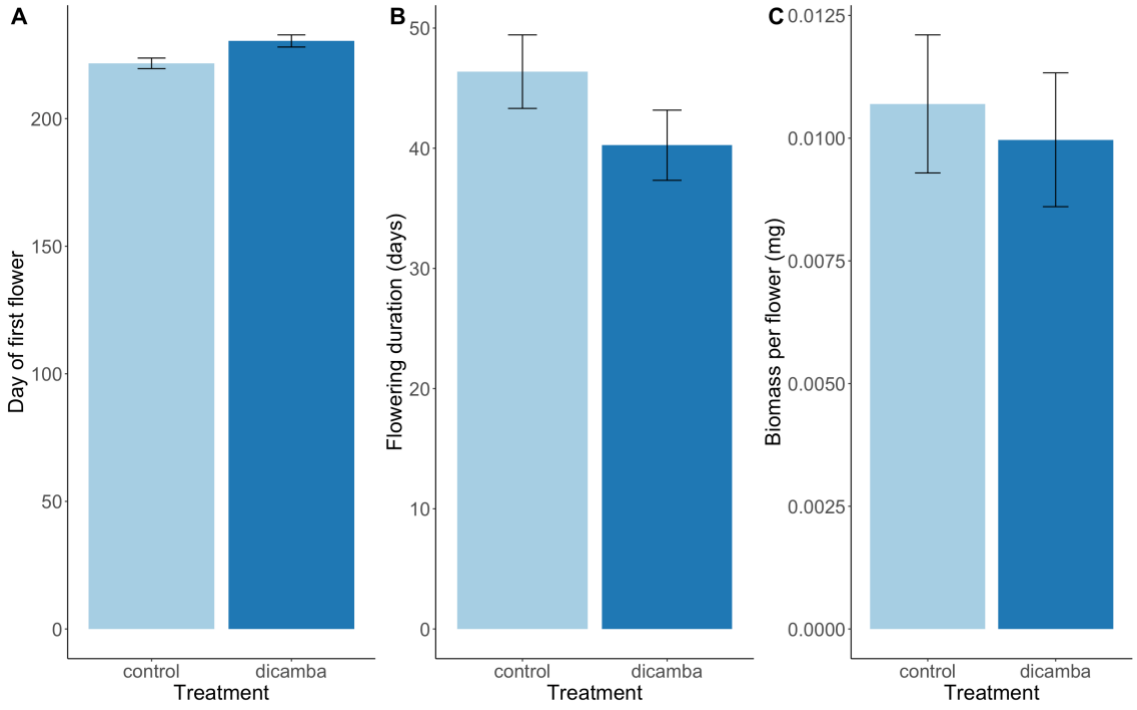

**Appendix A: Figure 14. Plant species vary in tolerance to dicamba drift and in whether drift affects flowering onset and biomass per flower.**

Contrast estimates ± 95% confidence intervals show the difference between dicamba drift-treated plants and control plants, relative to control plants, in short-term tolerance (i.e. short-term growth, plant size at 21 days post-treatment in cm; A) and long-term tolerance (i.e. final biomass in g at 145 days post-treatment; B), day of first flower (C), and biomass per flower in mg (D). Red denotes species that (A-B) were significantly negatively impacted by dicamba drift, (C) dicamba drift delayed the day of first flower, or (D) decreased biomass per flower. Light blue shows significant effects in the opposite direction and black indicates no significant change. Species are designated by four-letter codes as in Table 2. X-axes for A-C are on the sqrt scale and on the log scale for D. The vertical dashed line at 0 is a reference for determining whether species were positively or negatively affected by dicamba drift treatment.




**Appendix A: Figure 15. Resistance and tolerance levels to dicamba drift were uncorrelated, but short-term and long-term tolerance were positively related.**

Species (blue points labeled with four-letter codes; Table 2) estimated marginal means for the proportion of undamaged leaves (A; Figure 2A; Appendix A: Table 10) and standardized z-scores for contrast estimates (i.e., the difference between dicamba drift and control treatments, relative to control) for short-term growth (B; Appendix A: Figure 14A, Table 11) are plotted on the x-axis and standardized contrast estimates for final biomass (Appendix A: Figure 14B, Table 12) on the y-axes. The dashed line at x = 1 (A) references no dicamba-related injury immediately after herbicide treatment, whereas those at x =0 and y = 0 determine whether short-term growth and/or final biomass was positively or negatively affected by dicamba treatment. The shaded area in B reflects the 95% confidence interval for the regression line.



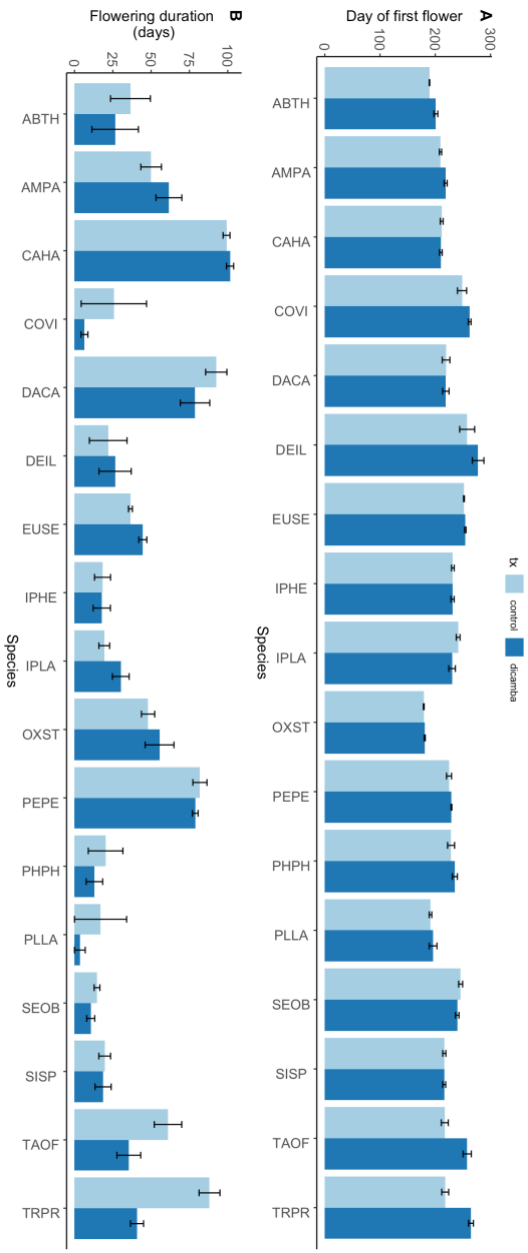

**Appendix A: Figure 16. Floral trait responses by herbicide treatment, averaged over species.**

Responses (mean ± SE) are day of first flower (A), flowering duration in days (B), and biomass per flower in mg (C).

**Appendix A: Table 13. Contrast anlaysis to test for significant differences between herbicide treatments in day of first flower across species.**

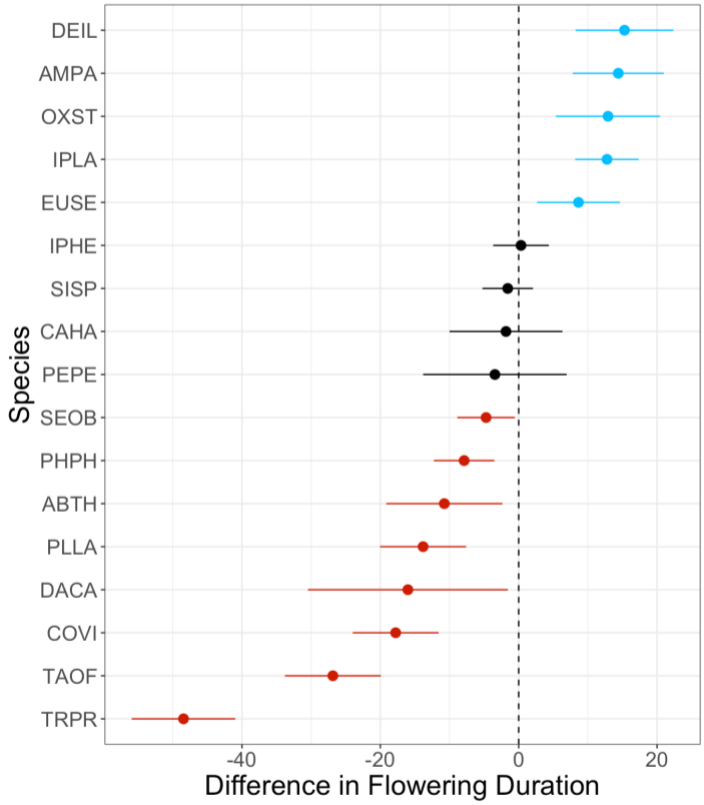
'Estimate' is the estimated marginal mean of day of first flower (on the square-root scale) in the control treatment subtracted by that of the drift treatment. 'Lower CI' shows the lower and 'Upper CI' the upper bound of the 95% confidence interval for the estimate. '*t*-ratio' denotes the *t*-statistic calculated to determine the *P*-value of the difference between herbicide treatments. Significant *P*-values are in bold. Degrees of freedom = 224.

| **Species** | **Estimate** | **Lower CI** | **Upper CI** | ***t*-ratio** | ***P*-value** |
|---|---|---|---|---|---|
| *Abutilon theophrasti* | -0.409 | -0.851 | 0.034 | -1.819 | 0.0703 |
| *Amaranthus palmeri* | -0.317 | -0.660 | 0.026 | -1.821 | 0.0699 |
| *Cardiospermum halicacabum* | 0.051 | -0.291 | 0.394 | 0.295 | 0.7679 |
| *Commelina virginica* | -0.443 | -0.892 | 0.006 | -1.943 | 0.0533 |
| *Daucus carota* | 0.014 | -0.572 | 0.600 | 0.047 | 0.9627 |
| *Desmanthus illinoensis* | -0.614 | -1.314 | 0.086 | -1.729 | 0.0851 |
| *Eupatorium serotinum* | -0.763 | -0.429 | 0.276 | -0.427 | 0.6701 |
| *Ipomoea hederaceae* | 0.021 | -0.322 | 0.363 | 0.118 | 0.6701 |
| *Ipomoea lacunosa* | 0.363 | 0.020 | 0.706 | 2.087 | **0.0381** |
| *Oxalis stricta* | -0.067 | -0.410 | 0.276 | -0.384 | 0.7013 |
| *Persicaria pennsylvanica* | -0.149 | -0.613 | 0.316 | -0.631 | 0.5286 |
| *Physalis philadelphica* | -0.227 | -0.624 | 0.170 | -1.129 | 0.2603 |
| *Plantago lanceolata* | -0.166 | -0.866 | 0.534 | -0.467 | 0.6408 |
| *Senna obtusifolia* | 0.197 | -0.213 | 0.606 | 0.945 | 0.3456 |
| *Sida spinosa* | 0.006 | -0.346 | 0.358 | 0.033 | 0.9736 |
| *Taraxicum officionale* | -1.318 | -1.670 | -0.965 | -7.370 | **<.0001** |
| *Trifolium pratense* | -1.513 | -1.856 | -1.171 | -8.698 | **<.0001** |




**Appendix A: Figure 17. Floral trait responses by species and herbicide treatment.**

Responses (means ± SE) show day of first flower (A) and flowering duration in days (B). Four-letter codes are used for species (see Table 2).



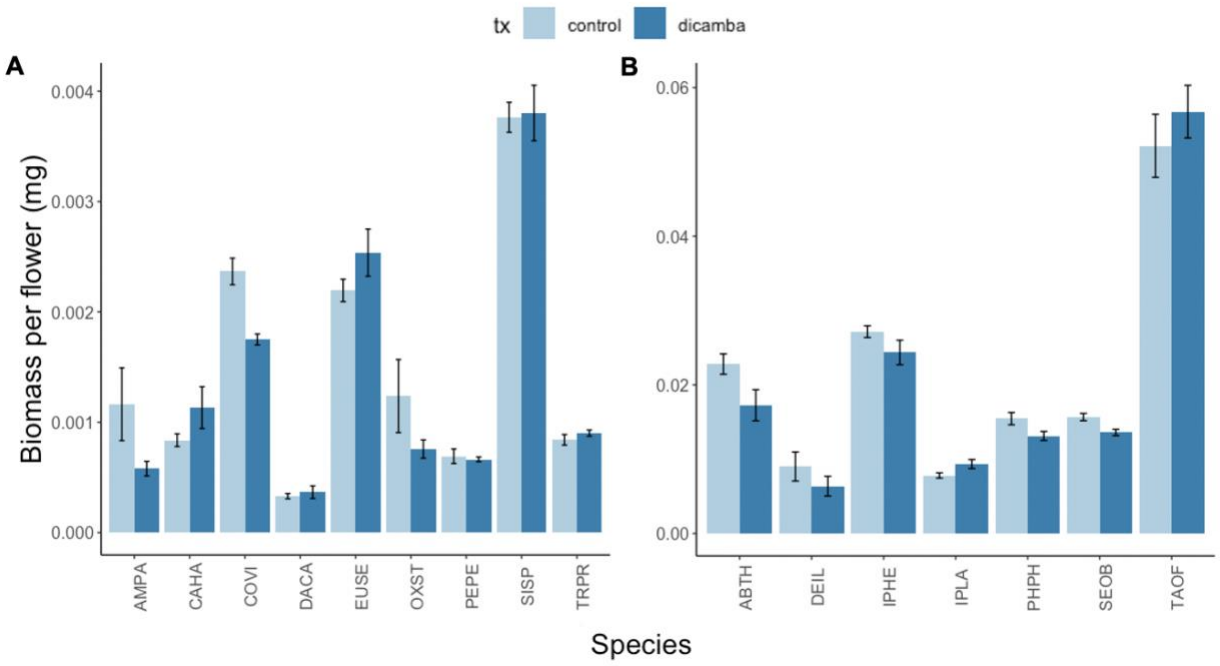

**Appendix A: Figure 18. Dicamba drift has species-specific effects on flowering duration, even after accounting for plant size.**

Contrast estimates ± 95% confidence intervals show the difference between dicamba drift-treated and control plants (i.e., drift – control) in flowering duration. The vertical dashed line at 0 is a reference for determining whether species were positively or negatively affected by dicamba drift treatment compared to the control. Black signifies unaffected species, red denotes species whose flowering duration was shortened by drift, and blue shows species whose flowering duration was lengthened by drift. Results were extracted from a generalized linear model with the equation: Flowering Duration ~ Treatment*Species + Plant Size at 21 Days Post-treatment. Four-letter codes are used for species (see Table 2).

**Appendix A: Table 14. Contrast analysis to test for significant differences between herbicide treatments in biomass per flower across species.**

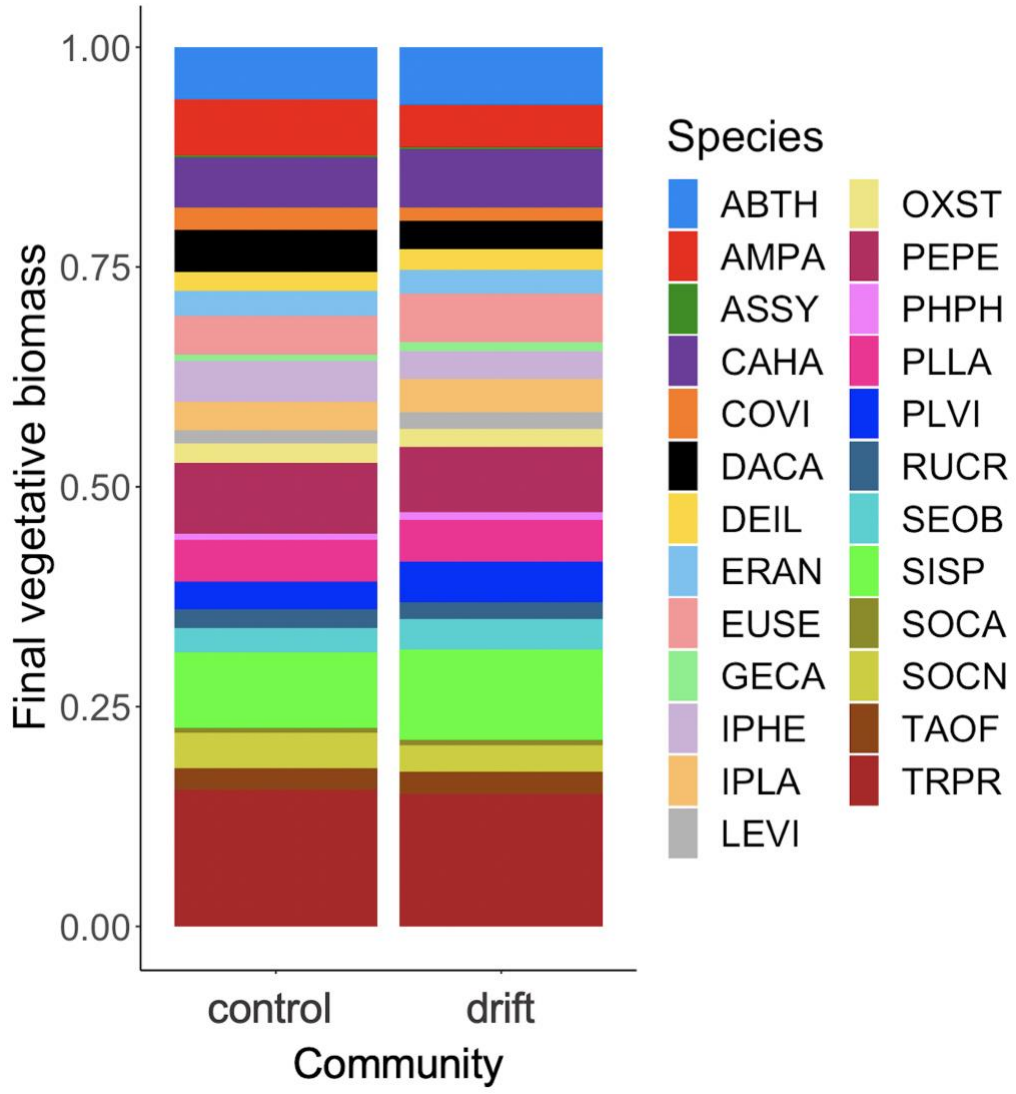
'Estimate' is the estimated marginal mean of log(biomass per flower in mg) in the control treatment subtracted by that of the drift treatment. 'Lower CI' shows the lower and 'Upper CI' the upper bound of the 95% confidence interval for the estimate. '*t*-ratio' denotes the *t*-statistic calculated to determine the *P*-value of the difference between herbicide treatments. Significant *P*-values are in bold. Degrees of freedom = 197.

| **Species** | **Estimate** | **Lower CI** | **Upper CI** | ***t*-ratio** | ***P*-value** |
|---|---|---|---|---|---|
| *Abutilon theophrasti* | 0.30095 | -0.054 | 0.656 | 1.673 | 0.0960 |
| *Amaranthus palmeri* | 0.46523 | 0.187 | 0.743 | 3.301 | **0.0011** |
| *Cardiospermum halicacabum* | -0.2451 | -0.507 | 0.017 | -1.845 | 0.0666 |
| *Commelina virginica* | 0.29975 | -0.235 | 0.835 | 1.105 | 0.2704 |
| *Daucus carota* | -0.0915 | -0.539 | 0.356 | -0.403 | 0.6872 |
| *Desmanthus illinoensis* | 0.32578 | -0.209 | 0.861 | 1.201 | 0.2311 |
| *Eupatorium serotinum* | -0.12692 | -0.403 | 0.149 | -0.906 | 0.3659 |
| *Ipomoea hederaceae* | 0.12706 | -0.142 | 0.396 | 0.931 | 0.3531 |
| *Ipomoea lacunosa* | -0.16513 | -0.434 | 0.104 | -1.21 | 0.2279 |
| *Oxalis stricta* | 0.299 | -0.004 | 0.602 | 1.944 | 0.0533 |
| *Persicaria pennsylvanica* | 0.0177 | -0.337 | 0.372 | 0.098 | 0.9217 |
| *Physalis philadelphica* | 0.16398 | -0.152 | 0.480 | 1.022 | 0.3081 |
| *Senna obtusifolia* | 0.14288 | -0.183 | 0.469 | 0.864 | 0.3884 |
| *Sida spinosa* | -0.00281 | -0.281 | 0.275 | -0.02 | 0.9841 |
| *Taraxicum officionale* | -0.10381 | -0.399 | 0.191 | -0.693 | 0.4889 |
| *Trifolium pratense* | -0.07926 | -0.341 | 0.183 | -0.597 | 0.5515 |



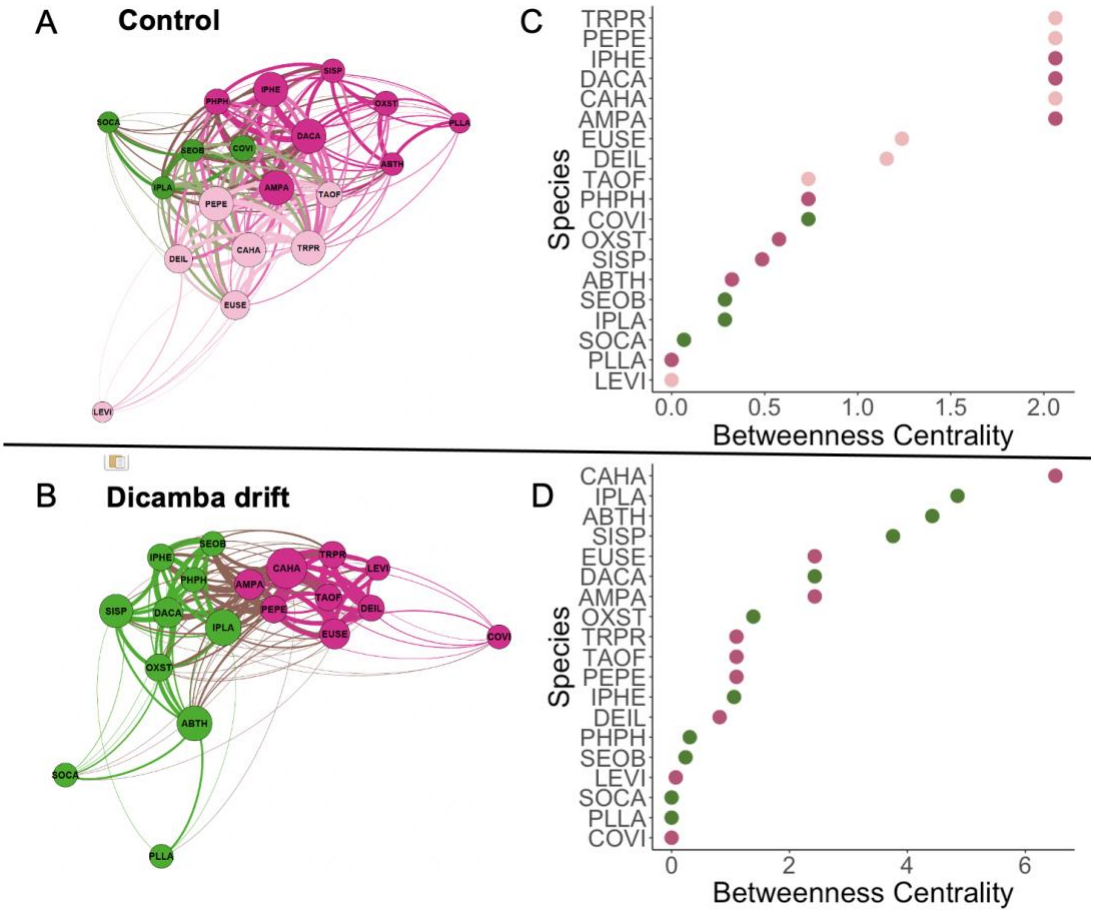

**Appendix A: Figure 19. Biomass per flower responses by species and herbicide treatment.**

Species with smaller (<0.004mg) flowers are shown in A and larger ones (> 0.004mg) in B. Four-letter codes are used for species (see Table 2). Bars show means ± SE.




**Appendix A: Figure 20. Species evenness of synthetic greenhouse community is not significantly impacted by dicamba drift.**

Percent stacked bar charts show the relative proportion of mean final vegetative, shoot biomass occupied by each species (represented using species codes; Table 2) in the control and dicamba drift synthetic communities. See text for statistics.




**Appendix A: Figure 21. Subset networks showing only species (*n* = 19) that flowered in both synthetic plant communities.**

A-B: Each plant species is represented as a circle, and links between them represent coflowering interactions in the control (A) and dicamba drift (B) community. The thickness of the lines reflects the strength of coflowering overlap (duration and intensity), and the size of the circles reflects species betweenness centrality (relative importance of species for network stability). C-D: Betweenness centrality for each species according to the subset networks in rank order for the control (C) and drift (D) community. High values reflect higher relative importance in the network. A-D: Different colors represent different modules (groups of species that coflower more strongly with each other than with other species). See Table 2 for species codes noted in circles (A-B) and on y-axes (C-D).

**Appendix A: Table 15. Coflowering network metrics in the subset control and dicamba drift synthetic communities.**

Analyses included only species ($n$ = 19) wherein at least one plant flowered in both communities. Network metric denotes the network-level property addressed. 'Control' reports the network-level metric values found for the control synthetic community and 'Dicamba Drift' reports those for the dicamba drift community.

| Network Metric | Community | |
|---|---|---|
| | **Control** | **Dicamba Drift** |
| Degree | 16.00 | 14.42 |
| Strength | 0.261 | 0.203 |
| Weighted Degree | 4.698 | 3.647 |
| Connectance | 0.889 | 0.801 |
| Modularity | 0.099 | 0.202 |

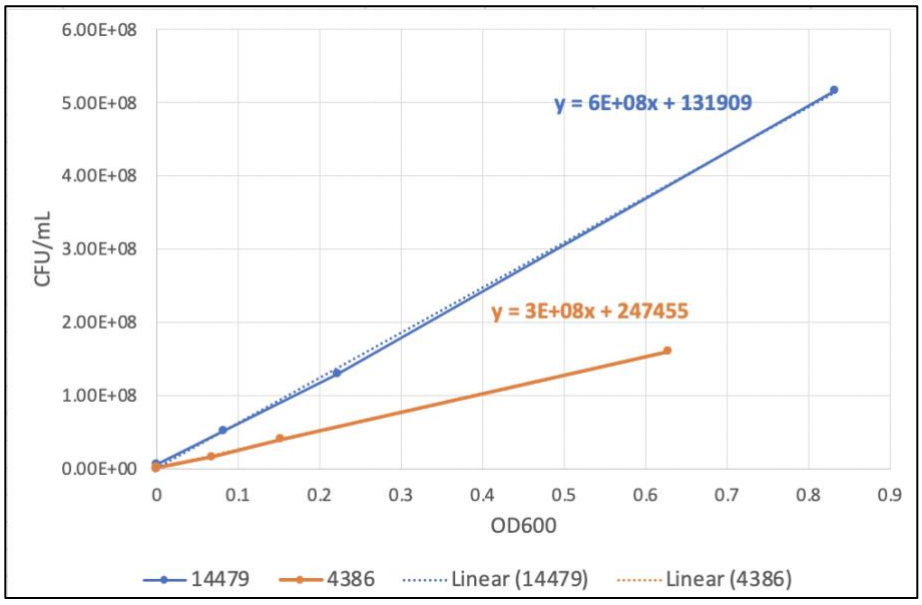
# Appendix B Supplementary Tables and Figures for Chapter 2

**Appendix B: Table 16. Accession information for red clover (*Trifolium pratense*) and *Rhizobium leguminosarum* genotypyes used in this study.**

Seventeen full-sibling families of red clover (*Trifolium pratense*) were created from accessions obtained from the USDA National Genetic Resources Program ('GRIN'; http://www.npgsweb.ars-grin.gov/, accessed 27 January 2020) or collected by the authors and grown in symbiosis with two *Rhizobium leguminosarum* strains obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA) or the Northern Regional Research Lab (NRRL; Peoria, IL, USA) to assess plant-rhizobia interactions in response to herbicide drift. 'Geographic origin' refers to the country where the accession was originally collected from according to government/laboratory records. If from the USA, the state is listed in parentheses. Plant families with sample sizes of ≥ 2 plants/treatment were analyzed in a reduced dataset for some dependent variables to corroborate results (see Statistical Analysis). Variable codes: instantaneous damage (ID), nodule number (NN), nodule size (NS), symbiotically fixed nitrogen (SFN), and shoot biomass (SB) or none of the above (-). Plant accession 'USA18-S33A' was collected from wild populations in Robertson County, TN, USA. Origin of rhizobial strain NRRL B-4386 was estimated from NRRL records indicating the strain was originally obtained from J. Burton at Nitragin Co. in Milwaukee, WI, USA.

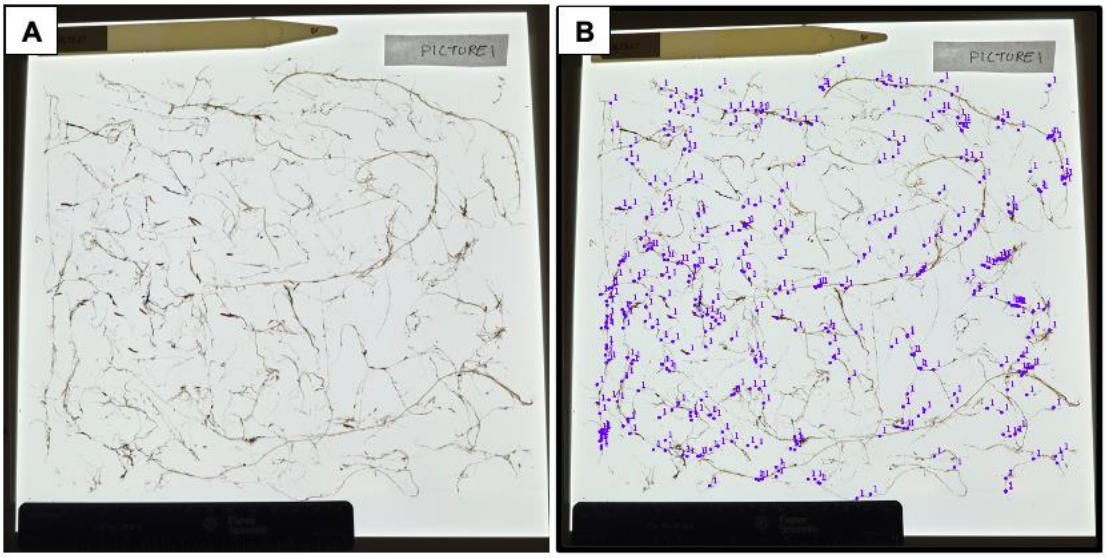
| **Species Partner** | **Family/Strain code** | **Accession #** | **Geographic origin** | **Variables analyzed using reduced dataset** |
|---|---|---|---|---|
| *T. pratense* | AUT57 | PI 236609 | Austria | ID, NN, NS, SFN, SB |
| | AUT64 | PI 294481 | Austria | - |
| | CAN39 | PI 632210 | Canada | ID, SB |
| | CAN66 | PI 315534 | Canada | - |
| | GRC52 | PI 199263 | Greece | ID |

| | | | | |
|---|---|---|---|---|
| | HUN71 | PI 368023 | Hungary | ID, NN, NS, SFN |
| | JPN53 | PI 205446 | Japan | ID, SB, SFN |
| | JPN91 | PI 655650 | Japan | ID |
| | NOR39 | PI 632217 | Norway | ID, NN, NS, SB, SFN |
| | NZL47 | PI 158384 | New Zealand | ID, NN, NS, SB, SFN |
| | NZL77 | PI 376880 | New Zealand | ID, SB |
| | POL73 | PI 384058 | Poland | ID, SB |
| | SRB91 | PI 597514 | Serbia | ID |
| | SWE49 | PI 174775 | Sweden | ID |
| | TJK04 | PI 655928 | Tajikistan | ID |
| | TUR37 | PI 120105 | Turkey | ID |
| | USA18-S33A | NA | USA (TN) | - |
| *R. leguminosarum* | 14479 | ATCC 14479 | USA (VA) | - |
| | 4386 | NRRL B-4386 | USA (WI) | - |




**Appendix B: Figure 22. Relationship between optical density at 600 nm (OD600) and Colony Forming Units (CFU) per mL for rhizobial strains 14479 and 4386.**

Data for strain 14479 is shown in blue and data for strain 4386 is shown in orange. Points connected by solid lines are raw data obtained from liquid rhizobial cultures; dotted lines are best-fit linear trends. Equations describing each linear trend are displayed. OD600 was measured using a Spectronic 200 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and CFU/mL was estimated following lab protocols.




**Appendix B: Figure 23. Example photo of a root sample from a red clover plant before (A) and after (B) nodules were counted using the application Fiji.**

In B, each blue point labeled with a '1' marks where a nodule was identified.

**Appendix B: Table 17. Summary of all analyses.**

Rows correspond to different statistical models shown in 'Model', which are grouped according to the dependent variable ('Response') being analyzed. 'Data' refers to whether the full or reduced dataset was used (see Statistical Analysis in main text for details). 'K' shows the number of parameters in the model. 'AICc' and 'ΔAICc' show the results of Akaike Information Criterion scoring for each model, adjusted for small sample sizes.

| **Response** | **Data** | **Model** | **K** | **AICc** | **ΔAICc** |
|---|---|---|---|---|---|
| Instantaneous Leaf Damage | Full | family*rhizobia_trt | 49 | 1180.98 | 0.00 |
| | | family + rhizobia_trt | 20 | 1200.38 | 19.41 |
| | Reduced | family*rhizobia_trt | 43 | 1069.45 | 0.00 |
| | | family + rhizobia_trt | 17 | 1088.94 | 19.50 |
| Nodule Number | Full | family + rhizobia_trt*dicamba_trt | 23 | 355.80 | 0.00 |
| | | family*rhizobia_trt + dicamba_trt*rhizobia_trt | 22 | 361.42 | 5.62 |
| | | family + rhizobia_trt + dicamba_trt | 35 | 374.32 | 18.52 |
| | | dicamba_trt*rhizobia_trt + dicamba_trt*family | 35 | 375.37 | 19.57 |
| | | family*rhizobia_trt + dicamba_trt | 34 | 376.38 | 20.58 |
| | | family*rhizobia_trt + dicamba_trt*rhizobia_trt + family*dicamba_trt | 34 | 380.61 | 24.81 |
| | | family*dicamba_trt*rhizobia_trt | 47 | 398.43 | 42.63 |
| | | family*dicamba_trt + rhizobia_trt | 46 | 398.71 | 42.91 |
| | | family*rhizobia_trt + dicamba_trt*family | 53 | 401.43 | 45.63 |
| | Reduced | family + rhizobia_trt*dicamba_trt | 10 | 184.16 | 0.00 |
| | | family*rhizobia_trt + dicamba_trt*rhizobia_trt | 13 | 186.03 | 1.86 |
| | | family + rhizobia_trt + dicamba_trt | 9 | 188.94 | 4.78 |

| | | | | | |
|---|---|---|---|---|---|
| | | dicamba_trt*rhizobia_trt + dicamba_trt*family | 13 | 189.78 | 5.62 |
| | | family*rhizobia_trt + dicamba_trt | 12 | 189.95 | 5.79 |
| | | family*rhizobia_trt + dicamba_trt*rhizobia_trt + family*dicamba_trt | 16 | 192.15 | 7.98 |
| | | family*dicamba_trt*rhizobia_trt | 19 | 193.77 | 9.61 |
| | | family*dicamba_trt + rhizobia_trt | 12 | 195.35 | 11.18 |
| | | family*rhizobia_trt + dicamba_trt*family | 15 | 196.81 | 12.65 |
| Nodule biomass | Full | family + rhizobia_trt*dicamba_trt | 23 | 285.95 | 0.00 |
| | | family + rhizobia_trt + dicamba_trt | 22 | 293.47 | 7.53 |
| | | family*rhizobia_trt + dicamba_trt*rhizobia_trt | 35 | 305.10 | 19.15 |
| | | dicamba_trt*rhizobia_trt + dicamba_trt*family | 35 | 314.86 | 28.91 |
| | | family*rhizobia_trt + dicamba_trt | 34 | 316.34 | 30.39 |
| | | family*dicamba_trt + rhizobia_trt | 34 | 321.27 | 35.33 |
| | | family*rhizobia_trt + dicamba_trt*rhizobia_trt + family*dicamba_trt | 47 | 346.92 | 60.97 |
| | | family*rhizobia_trt + dicamba_trt*family | 46 | 357.10 | 71.16 |
| | | family*dicamba_trt*rhizobia_trt | 54 | 363.02 | 77.08 |
| | Reduced | family + rhizobia_trt*dicamba_trt | 10 | 145.48 | 0.00 |
| | | family*rhizobia_trt + dicamba_trt*rhizobia_trt | 13 | 150.94 | 5.45 |
| | | dicamba_trt*rhizobia_trt + dicamba_trt*family | 13 | 152.48 | 6.99 |
| | | family + rhizobia_trt + dicamba_trt | 9 | 155.65 | 10.17 |
| | | family*rhizobia_trt + dicamba_trt*rhizobia_trt + family*dicamba_trt | 16 | 158.63 | 13.15 |
| | | family*dicamba_trt + rhizobia_trt | 19 | 161.53 | 16.05 |
| | | family*dicamba_trt*rhizobia_trt | 12 | 161.86 | 16.38 |

| | | | | | |
|---|---|---|---|---|---|
| | | family*dicamba_trt + rhizobia_trt | 12 | 162.24 | 16.76 |
| | | family*rhizobia_trt + dicamba_trt*family | 15 | 169.11 | 23.62 |
| Fixed N received | Full | family + rhizobia_trt + dicamba_trt | 22 | 94.39 | 0.00 |
| | | family + rhizobia_trt*dicamba_trt | 23 | 97.33 | 2.94 |
| | | family*dicamba_trt + rhizobia_trt | 34 | 115.06 | 20.67 |
| | | family*rhizobia_trt + dicamba_trt | 34 | 117.78 | 23.39 |
| | | dicamba_trt*rhizobia_trt + dicamba_trt*family | 35 | 118.79 | 24.40 |
| | | family*rhizobia_trt + dicamba_trt*rhizobia_trt | 35 | 121.51 | 27.12 |
| | | family*rhizobia_trt + dicamba_trt*family | 46 | 149.98 | 55.59 |
| | | family*rhizobia_trt + dicamba_trt*rhizobia_trt + family*dicamba_trt | 47 | 154.84 | 60.46 |
| | | family*rhizobia_trt*dicamba_trt | 52 | 169.04 | 74.65 |
| | Reduced | family + rhizobia_trt + dicamba_trt | 9 | 62.77 | 0.00 |
| | | family + rhizobia_trt*dicamba_trt | 10 | 65.48 | 2.71 |
| | | family*dicamba_trt + rhizobia_trt | 12 | 67.52 | 4.75 |
| | | family*rhizobia_trt + dicamba_trt | 12 | 68.46 | 5.69 |
| | | dicamba_trt*rhizobia_trt + dicamba_trt*family | 13 | 70.55 | 7.78 |
| | | family*rhizobia_trt + dicamba_trt*rhizobia_trt | 13 | 71.50 | 8.73 |
| | | family*rhizobia_trt + dicamba_trt*family | 15 | 73.92 | 11.15 |
| | | family*rhizobia_trt + dicamba_trt*rhizobia_trt + family*dicamba_trt | 16 | 77.28 | 14.51 |
| | | family*rhizobia_trt*dicamba_trt | 19 | 81.24 | 18.47 |
| Shoot biomass | Full | family + rhizobia_trt*dicamba_trt | 25 | 608.54 | 0.00 |
| | | family + rhizobia_trt + dicamba_trt | 23 | 612.60 | 4.06 |
| | | dicamba_trt*rhizobia_trt + dicamba_trt*family | 41 | 631.36 | 22.82 |

| | | | | | |
|---|---|---|---|---|---|
| | | family*dicamba_trt + rhizobia_trt | 39 | 634.52 | 25.98 |
| | | family*rhizobia_trt + dicamba_trt*rhizobia_trt | 54 | 648.94 | 40.40 |
| | | family*rhizobia_trt + dicamba_trt | 52 | 649.74 | 41.20 |
| | | family*rhizobia_trt + dicamba_trt*family | 68 | 672.04 | 63.50 |
| | | family*rhizobia_trt + dicamba_trt*rhizobia_trt + family*dicamba_trt | 70 | 673.03 | 64.50 |
| | | family*dicamba_trt*rhizobia_trt | 95 | 749.77 | 141.24 |
| | Reduced | family + rhizobia_trt + dicamba_trt | 13 | 375.09 | 0.00 |
| | | family + rhizobia_trt*dicamba_trt | 15 | 376.67 | 1.57 |
| | | family*dicamba_trt + rhizobia_trt | 19 | 379.30 | 4.21 |
| | | dicamba_trt*rhizobia_trt + dicamba_trt*family | 21 | 381.57 | 6.48 |
| | | family*rhizobia_trt + dicamba_trt | 25 | 386.24 | 11.14 |
| | | family*rhizobia_trt + dicamba_trt*rhizobia_trt | 27 | 389.61 | 14.51 |
| | | family*rhizobia_trt + dicamba_trt*family | 31 | 391.61 | 16.52 |
| | | family*rhizobia_trt + dicamba_trt*rhizobia_trt + family*dicamba_trt | 33 | 395.97 | 20.88 |
| | | family*dicamba_trt*rhizobia_trt | 45 | 422.70 | 47.61 |

**Appendix B: Table 18. ANOVA results using reduced datasets.**

Effects of inoculation treatment (IT) or rhizobial strain (RS), plant family (F), herbicide treatment (HT), their interactions, and covariates (pre-HT size, root biomass, nodule number) on red clover traits related to herbicide injury (a), the plant-rhizobia symbiosis (b), and plant fitness (c). Results are based on a reduced dataset of plant families (Table S1). All trait data was log-transformed prior to analysis except for instantaneous leaf damage. Covariates (Pre-HT size, root biomass, nodule number) were also log-transformed. See Methods in main text for how traits were measured. Fixed effects were determined based on the best-fitting statistical model (Appendix B: Table 17).

| | **Trait** | **Fixed Effects** | $\chi^2$ | **df** | ***P*** |
|---|---|---|---|---|---|
| a. Plant Herbicide Injury | Instantaneous Leaf Damage | **Inoculation treatment** | 27.03 | 2 | **<.0001** |
| | | **Family** | 44.99 | 13 | **<.0001** |
| | | **F x IT** | 87.46 | 26 | **<.0001** |
| b. Symbiosis | Nodule No. | **Rhizobial Strain** | 7.37 | 1 | **0.0066** |
| | | **Family** | 13.42 | 3 | **0.0038** |
| | | Herbicide Treatment | 0.23 | 1 | 0.63 |
| | | **Root Biomass** | 134.79 | 1 | **<.0001** |
| | | **RS x HT** | 7.93 | 1 | **0.0049** |
| | Nodule Size | Rhizobial Strain | 3.04 | 1 | 0.082 |
| | | Family | 1.83 | 3 | 0.61 |
| | | **Herbicide treatment** | 8.10 | 1 | **0.0044** |
| | | **Root Biomass** | 3.06 | 1 | **0.080** |
| | | **RS x HT** | 14.09 | 1 | **0.00020** |
| | Symbiotically Fixed N | **Rhizobial Strain** | 6.62 | 1 | **0.01** |
| | | Family | 0.45 | 3 | 0.93 |

| | | | | | |
|---|---|---|---|---|---|
| | | **Herbicide treatment** | 6.44 | 1 | **0.011** |
| | | Nodule No. | 1.15 | 1 | 0.28 |
| c. Plant Fitness | Shoot Biomass | **Inoculation Treatment** | 56.77 | 2 | **<.0001** |
| | | **Family** | 22.01 | 6 | **0.0012** |
| | | **Herbicide treatment** | 23.16 | 1 | **<.0001** |
| | | **Pre-HT Size** | 712.43 | 1 | **<.0001** |
| | | IT x HT | 3.20 | 2 | 0.203 |

**Appendix B: Table 19. Contrast analysis results for immediate leaf damage.**

'Estimate' reflects the difference in the estimated marginal means for the number of damaged leaves 48 hours post dicamba drift treatment between inoculation treatments ('Contrast') according to plant family ('Family'). '*z*-ratio' denotes the *z*-statistic calculated to determine the *p*-value of the contrast. Degrees of freedom = infinity. Significant *p* values are bolded. 'NA' signifies that the statistic could not be calculated because replication was lacking (due to limited seed supply) for some families.

| **Family** | **Contrast** | **Estimate** | **SE** | ***z*-ratio** | ***p*** |
|---|---|---|---|---|---|
| AUT57 | strain_14479 – strain_4386 | 29.49 | 6.82 | 4.328 | **<.0001** |
| | strain_14479 – uninoculated | 30.35 | 6.10 | 4.974 | **<.0001** |
| | strain_4386 – uninoculated | 0.86 | 6.78 | 0.126 | 0.9913 |
| AUT64 | strain_14479 – strain_4386 | *NA* | *NA* | *NA* | *NA* |
| | strain_14479 – uninoculated | *NA* | *NA* | *NA* | *NA* |
| | strain_4386 – uninoculated | -22.72 | 14.55 | -1.561 | 0.2625 |
| CAN39 | strain_14479 – strain_4386 | 8.01 | 8.09 | 0.991 | 0.5828 |
| | strain_14479 – uninoculated | -1.58 | 8.41 | -0.188 | 0.9807 |
| | strain_4386 – uninoculated | -9.59 | 8.59 | -1.117 | 0.5034 |
| CAN66 | strain_14479 – strain_4386 | *NA* | *NA* | *NA* | *NA* |
| | strain_14479 – uninoculated | 41.33 | 9.47 | 4.364 | **<.0001** |
| | strain_4386 – uninoculated | *NA* | *NA* | *NA* | *NA* |
| GRC52 | strain_14479 – strain_4386 | 9.05 | 17.67 | 0.512 | 0.8654 |
| | strain_14479 – uninoculated | -26.86 | 11.35 | -2.366 | 0.0472 |
| | strain_4386 – uninoculated | -35.91 | 18.00 | -1.995 | 0.1134 |
| HUN71 | strain_14479 – strain_4386 | 8.62 | 8.71 | 0.989 | 0.5837 |
| | strain_14479 – uninoculated | 22.70 | 9.17 | 2.477 | **0.0354** |

| | | | | | |
|---|---|---|---|---|---|
| | strain_4386 – uninoculated | 14.08 | 10.49 | 1.342 | 0.3718 |
| JPN53 | strain_14479 – strain_4386 | 26.14 | 16.88 | 1.548 | 0.2686 |
| | strain_14479 – uninoculated | -6.14 | 13.26 | -0.463 | 0.8886 |
| | strain_4386 – uninoculated | -32.28 | 17.11 | -1.886 | 0.1426 |
| JPN91 | strain_14479 – strain_4386 | -17.33 | 10.04 | -1.726 | 0.1954 |
| | strain_14479 – uninoculated | -19.87 | 8.55 | -2.324 | 0.0525 |
| | strain_4386 – uninoculated | -2.55 | 9.97 | -0.255 | 0.9647 |
| NOR39 | strain_14479 – strain_4386 | 4.18 | 8.74 | 0.479 | 0.8814 |
| | strain_14479 – uninoculated | 12.03 | 7.77 | 1.549 | 0.2681 |
| | strain_4386 – uninoculated | 7.84 | 8.63 | 0.909 | 0.6344 |
| NZL47 | strain_14479 – strain_4386 | 33.37 | 8.00 | 4.173 | **0.0001** |
| | strain_14479 – uninoculated | 24.67 | 7.27 | 3.394 | **0.0020** |
| | strain_4386 – uninoculated | -8.70 | 7.79 | -1.117 | 0.5035 |
| NZL77 | strain_14479 – strain_4386 | -27.54 | 13.49 | -2.041 | 0.1024 |
| | strain_14479 – uninoculated | -36.76 | 11.48 | -3.201 | **0.0039** |
| | strain_4386 – uninoculated | -9.22 | 13.79 | -0.669 | 0.7817 |
| POL73 | strain_14479 – strain_4386 | 31.41 | 14.83 | 2.119 | 0.0861 |
| | strain_14479 – uninoculated | 22.47 | 14.00 | 1.605 | 0.2436 |
| | strain_4386 – uninoculated | -8.94 | 13.11 | -0.682 | 0.7739 |
| SRB91 | strain_14479 – strain_4386 | -18.60 | 12.04 | -1.545 | 0.2698 |
| | strain_14479 – uninoculated | 35.29 | 10.60 | 3.329 | **0.0025** |
| | strain_4386 – uninoculated | 53.89 | 11.49 | 4.689 | **<.0001** |
| SWE49 | strain_14479 – strain_4386 | 28.43 | 14.40 | 1.974 | 0.1186 |
| | strain_14479 – uninoculated | 43.90 | 14.03 | 3.129 | **0.0050** |
| | strain_4386 – uninoculated | 15.47 | 15.92 | 0.972 | 0.5945 |
| TJK04 | strain_14479 – strain_4386 | -3.24 | 15.00 | -0.216 | 0.9746 |

| | | | | | |
|---|---|---|---|---|---|
| | strain_14479 – uninoculated | -12.45 | 13.78 | -0.903 | 0.6383 |
| | strain_4386 – uninoculated | -9.21 | 12.72 | -0.724 | 0.7493 |
| TUR37 | strain_14479 – strain_4386 | 44.67 | 11.67 | 3.826 | **0.0004** |
| | strain_14479 – uninoculated | 42.91 | 13.94 | 3.078 | **0.0059** |
| | strain_4386 – uninoculated | -1.76 | 11.42 | -0.154 | 0.9870 |
| USA18-S33A | strain_14479 – strain_4386 | *NA* | *NA* | *NA* | *NA* |
| | strain_14479 – uninoculated | *NA* | *NA* | *NA* | *NA* |
| | strain_4386 – uninoculated | 9.01 | 17.86 | 0.505 | 0.8691 |

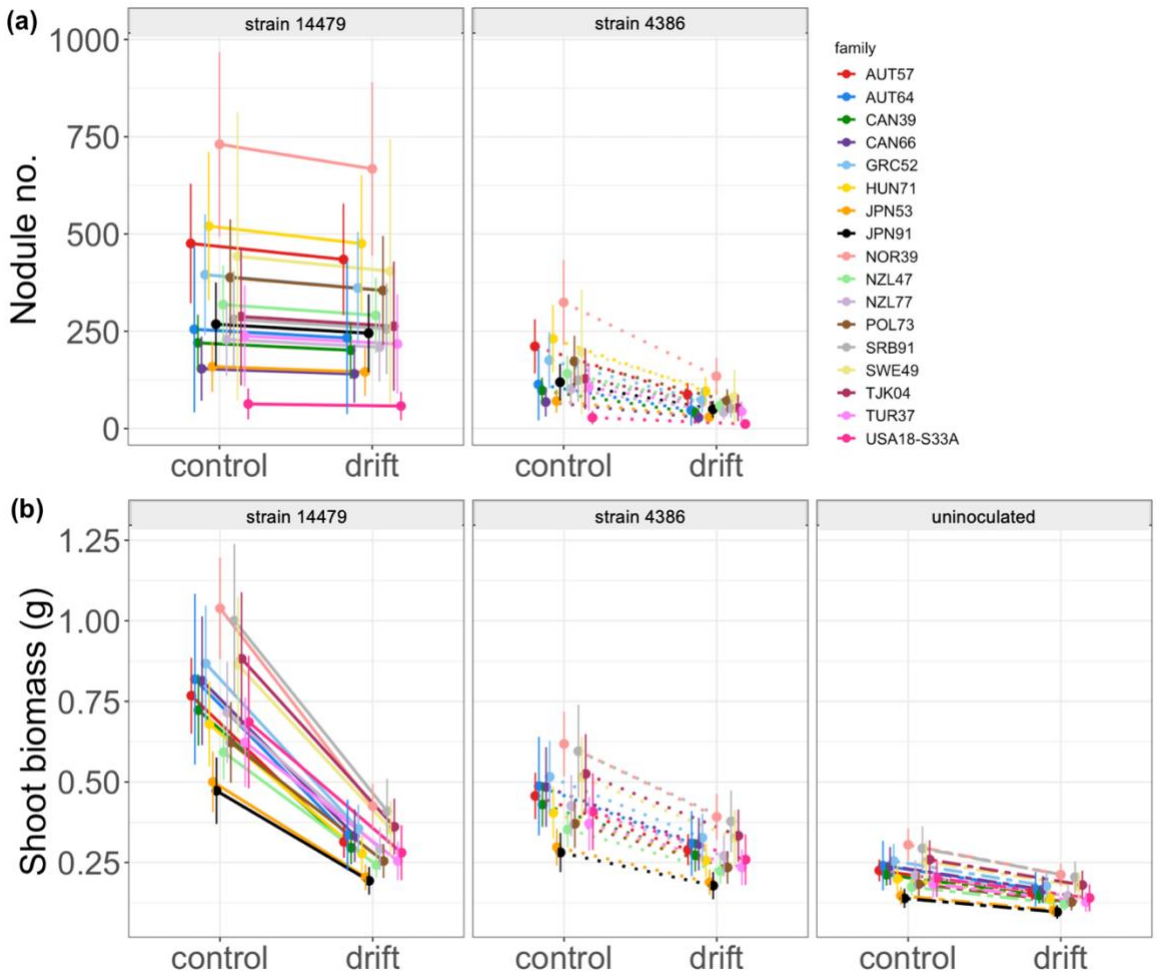
**Appendix B: Table 20. Estimated marginal means (EMM) for immediate leaf damage (percentage of leaves that show symptoms of herbicide-related injury 48 hours post-treatment with dicamba drift) according to plant family and inoculation treatment.**

'Lower CL' and `Upper CL' reflect the lower and upper limits respectively of the asymptotic 95% confidence interval of the EMM. 'NA' signifies that the statistic could not be calculated because replication was lacking (due to limited seed supply) for some families.

| Family | Inoculation Treatment | EMM % leaf damage | Lower CL | Upper CL |
|---|---|---|---|---|
| AUT57 | strain_14479 | 62.7 | 53.5 | 71.1 |
| AUT57 | strain_4386 | 33.2 | 23.6 | 44.5 |
| AUT57 | uninoculated | 32.4 | 24.3 | 41.7 |
| AUT64 | strain_14479 | *NA* | *NA* | *NA* |
| AUT64 | uninoculated | 58.6 | 41.9 | 73.5 |
| AUT64 | strain_4386 | 35.9 | 16.7 | 61.0 |
| CAN39 | uninoculated | 61.4 | 48.3 | 73.0 |
| CAN39 | strain_14479 | 59.8 | 48.2 | 70.4 |
| CAN39 | strain_4386 | 51.8 | 40.2 | 63.1 |
| CAN66 | strain_4386 | *NA* | *NA* | *NA* |
| CAN66 | strain_14479 | 62.8 | 46.8 | 76.4 |
| CAN66 | uninoculated | 21.5 | 12.5 | 34.4 |
| GRC52 | uninoculated | 70.0 | 51.4 | 83.7 |
| GRC52 | strain_14479 | 43.1 | 28.9 | 58.6 |
| GRC52 | strain_4386 | 34.1 | 11.4 | 67.6 |
| HUN71 | strain_14479 | 66.0 | 55.3 | 75.4 |
| HUN71 | strain_4386 | 57.4 | 42.8 | 70.8 |

| | | | | |
|---|---|---|---|---|
| HUN71 | uninoculated | 43.3 | 29.1 | 58.7 |
| JPN53 | uninoculated | 55.1 | 36.2 | 72.6 |
| JPN53 | strain_14479 | 48.9 | 31.6 | 66.5 |
| JPN53 | strain_4386 | 22.8 | 5.7 | 58.9 |
| JPN91 | uninoculated | 30.8 | 20.2 | 44.0 |
| JPN91 | strain_4386 | 28.3 | 15.3 | 46.3 |
| JPN91 | strain_14479 | 11.0 | 3.5 | 29.2 |
| NOR39 | strain_14479 | 61.3 | 49.4 | 72.0 |
| NOR39 | strain_4386 | 57.1 | 43.3 | 70.0 |
| NOR39 | uninoculated | 49.3 | 38.4 | 60.2 |
| NZL47 | strain_14479 | 67.2 | 55.8 | 76.9 |
| NZL47 | uninoculated | 42.5 | 32.7 | 53.0 |
| NZL47 | strain_4386 | 33.8 | 23.0 | 46.7 |
| NZL77 | uninoculated | 70.0 | 51.4 | 83.7 |
| NZL77 | strain_4386 | 60.8 | 38.5 | 79.3 |
| NZL77 | strain_14479 | 33.2 | 19.8 | 50.1 |
| POL73 | strain_14479 | 67.7 | 43.8 | 85.0 |
| POL73 | uninoculated | 45.3 | 29.2 | 62.3 |
| POL73 | strain_4386 | 36.3 | 19.7 | 56.9 |
| SRB91 | strain_4386 | 66.9 | 47.2 | 82.0 |
| SRB91 | strain_14479 | 48.3 | 33.1 | 63.8 |
| SRB91 | uninoculated | 13.0 | 4.2 | 33.6 |
| SWE49 | strain_14479 | 75.9 | 55.3 | 88.9 |
| SWE49 | strain_4386 | 47.5 | 26.6 | 69.2 |
| SWE49 | uninoculated | 32.0 | 14.9 | 55.9 |
| TJK04 | uninoculated | 46.3 | 31.3 | 62.1 |

| | | | | |
|---|---|---|---|---|
| TJK04 | strain_4386 | 37.1 | 20.3 | 57.7 |
| TJK04 | strain_14479 | 33.9 | 16.0 | 57.9 |
| TUR37 | strain_14479 | 70.7 | 48.2 | 86.3 |
| TUR37 | uninoculated | 27.8 | 12.9 | 50.1 |
| TUR37 | strain_4386 | 26.1 | 16.2 | 39.2 |
| USA18-S33A | strain_14479 | *NA* | *NA* | *NA* |
| USA18-S33A | strain_4386 | 48.3 | 22.9 | 74.6 |
| USA18-S33A | uninoculated | 39.3 | 21.8 | 60.1 |




**Appendix B: Figure 24. Plant family additively influenced nodule number and plant fitness, while rhizobial inoculation and herbicide treatment interactively influenced them.**

Points are estimated marginal means (back-transformed to the response scale) ± SE for nodule number (a) and shoot biomass for red clover plant families (color-coded) across herbicide treatment conditions (control and drift; x-axes) and inoculated with rhizobial strain 14479 (first column; solid lines), rhizobial strain 4386 (second column; dotted lines), or uninoculated (third column; dashed lines; only shown in b since uninoculated plants did not interact with rhizobia).

**Appendix B: Table 21. Estimated marginal means (EMM) for nodule number, back-transformed to the response scale, according to herbicide treatment and rhizobial strain inoculation.**

'Lower CL' and `Upper CL' reflect the lower and upper limits respectively of the exact 95% confidence interval of the EMM.

| Herbicide Treatment | Rhizobial Strain | EMM nodule no. | Lower CL | Upper CL |
|---|---|---|---|---|
| control | 14479 | 280.1 | 104.7 | 749.2 |
| | 4386 | 124.3 | 46.4 | 332.7 |
| drift | 14479 | 255.9 | 96.5 | 678.4 |
| | 4386 | 51.5 | 19.0 | 139.2 |

**Appendix B: Table 22. Estimated marginal means (EMM) for nodule size, back-transformed to the response scale (mg), according to rhizobial strain inoculation and herbicide treatment.**

'Lower CL' and `Upper CL' reflect the lower and upper limits respectively of the exact 95% confidence interval of the EMM.

| Herbicide Treatment | Rhizobia Strain | EMM nodule size (mg) | Lower CL | Upper CL |
|---|---|---|---|---|
| control | 14479 | 0.135 | 0.090 | 0.201 |
| | 4386 | 0.128 | 0.085 | 0.195 |
| drift | strain_14479 | 0.089 | 0.057 | 0.139 |
| | strain_4386 | 0.163 | 0.098 | 0.270 |

**Appendix B: Table 23. Estimated marginal means (EMM) for symbiotic N fixation (estimated by foliar δ15N quantity), back-transformed to the response scale, according to herbicide treatment and rhizobial strain inoculation.**

'Lower CL' and `Upper CL' reflect the lower and upper limits respectively of the exact 95% confidence interval of the EMM.

| Herbicide Treatment | Rhizobial Strain | EMM foliar $\delta^{15}$N | Lower CL | Upper CL |
|---|---|---|---|---|
| control | 14479 | -0.640 | -1.57 | 1.08 |
| | 4386 | -0.215 | -1.32 | 1.80 |
| drift | 14479 | 0.009 | -1.19 | 2.16 |
| | 4386 | 0.570 | -0.85 | 3.10 |

**Appendix B: Table 24. Estimated marginal means (EMM) for shoot biomass, back-transformed to the response scale (mg), according to herbicide treatment and rhizobial inoculation.**

'Lower CL' and `Upper CL' reflect the lower and upper limits respectively of the exact 95% confidence interval of the EMM.

| Herbicide Treatment | Inoculation Treatment | EMM shoot biomass (g) | Lower CL | Upper CL |
|---|---|---|---|---|
| control | strain 14479 | 0.729 | 0.541 | 0.982 |
| | strain 4386 | 0.434 | 0.318 | 0.592 |
| | uninoculated | 0.214 | 0.158 | 0.289 |
| drift | strain 14479 | 0.298 | 0.214 | 0.416 |
| | strain 4386 | 0.275 | 0.188 | 0.402 |
| | uninoculated | 0.149 | 0.107 | 0.208 |

# Appendix C Supplementary Methods for Chapter 3

### Jensen's medium agar plates protocol

Adapted from Jones et al. (2013)

1. Add ingredients in table below into a flask and mix with a magnetic stir bar.

| Final concentration per L | Quantity of stock solution | Stock concentration |
|---|---|---|
| 1 g $CaHPO_4$ | NA | NA |
| 0.2 g $MgSO_4 \cdot 7H_20$ | 1 ml | 0.2 g/ml |
| 0.2 g $KH_2P0_4$ | 1 ml | 0.2 g/ml |
| 0.2 g NaCl | 1 ml | 0.2 g/ml |
| 0.1 g $FeCl_3 \cdot 6H20$ | 1 ml | 0.1 g/ml |
| DI $H_20$ to 1 L | - | - |

2. Add 15 g of agar per L and autoclave on liquid cycle for 30 minutes. Note: $CaHPO_4$ and $FeCl_3$ will precipitate out during autoclaving. Stir to get them back into suspension; media will still remain cloudy.
3. Cool with stirring until it is not painful to pick up the flask with your bare hands.
4. Add 1 ml trace minerals per L (see below for recipe) while stirring. Note: Be sure to resuspend the precipitate in the trace minerals before dispensing.
5. Add 0.25 ml 4 N NaOH (filter-sterilized) per L, while stirring.

6. Check pH by transferring a small amount of media into a sterile beaker and reading its pH with pH meter. pH should be between 6.5-7. If pH is not correct, there may have been a preparation error.
7. Pour plates (100mm petri dishes) to ~50% capacity, remixing flask by returning to the magnetic stir plate as needed.

**Trace minerals stock 1 L (sterilize by autoclaving)**

1 g $H_3BO_3$

1 g $ZnSO_4 \cdot 7H_20$

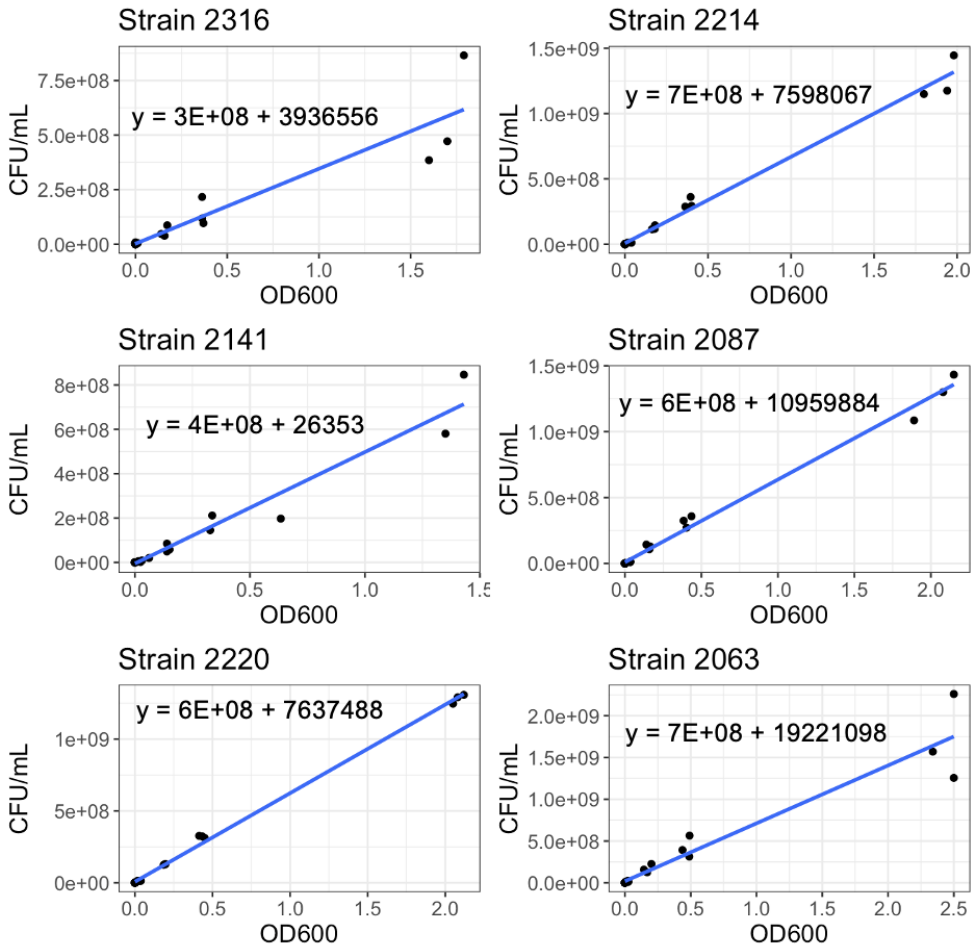
0.5 g $CuSO_4 \cdot 5H_20$

0.5 g $MnCl_2 \cdot 4H_20$

1 g $NaMoO_4 \cdot 2H_20$

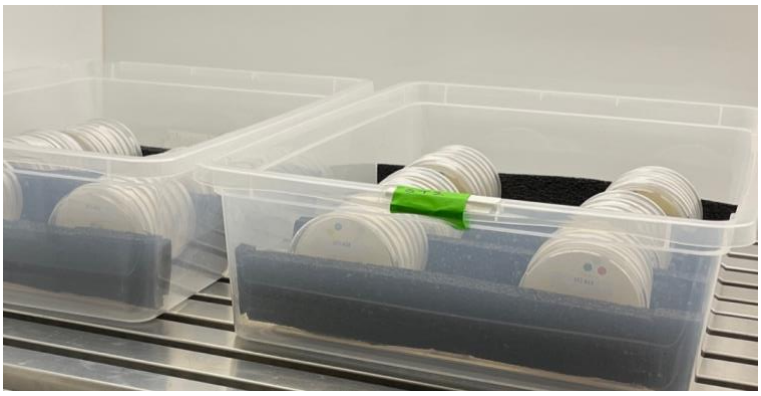
DI $H_20$ to 1 L

# Appendix D Supplementary Tables and Figures for Chapter 3




**Appendix D: Figure 25. Relationship between optical density at 600 nm (OD600) and Colony Forming Units (CFU) per mL for rhizobial strains 2316, 2214, 2141, 2087, 2220, and 2063.**

Points are raw data obtained from liquid rhizobial cultures; solid lines are best-fit linear trends. Equations describing each linear trend are displayed. OD600 was measured using a Spectronic 200 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and CFU/mL was estimated following lab protocols.



**Appendix D: Figure 26. Photograph of experimental replicate trays.**

**Appendix D: Table 25. Foliar δ15N abundance (raw means) ± SE and sample size (N) by rhizobial inoculation treatment.**

| Inoculation Treatment | $\delta^{15}$N | SE | N |
|---|---|---|---|
| Strain 2214 | 1.967 | 0.41 | 20 |
| Strain 2316 | 1.817 | 0.32 | 20 |
| uninoculated | 1.655 | 0.36 | 4 |
| Strain 2141 | 1.353 | 0.22 | 20 |
| Strain 4386 | 1.199 | 0.25 | 20 |
| Strain 14479 | 1.163 | 0.27 | 20 |
| Strain 2063 | 1.007 | 0.30 | 20 |
| Strain 2087 | 0.882 | 0.23 | 20 |
| Strain 2220 | 0.580 | 0.30 | 20 |

**Appendix D: Table 26. Model selection results.**

Rows correspond to different statistical models, grouped according to the response variable analyzed. 'K' is the number of parameters in the model. 'AICc' was the results of Akaike Information Criterion scoring for each model, adjusted for small sample sizes. 'ΔAICc' was the change in AICc relative to the model that best fit the data (i.e. had the lowest AICc). The fixed effect explanatory variables considered were: herbicide treatment (Herbicide), rhizobial strain (Strain), plant genotype (Plant), temporal block (Block), and Minor Fungal Infection (MFI). Every model included the variable Tray as a random effect. Nodulation time was analyzed using a mixed effects cox proportional hazards model. All other response variables were analyzed using linear mixed effects models.

| **Response** | **Model** | **K** | **AICc** | **ΔAICc** |
|---|---|---|---|---|
| Nodulation Time | Herbicide x Strain + Herbicide x Plant + (1\|Tray) + Block | 19 | 2704.6 | 0.0 |
| | Herbicide x Strain + Herbicide x Plant + Strain x Plant + (1\|Tray) + Block | 26 | 2713.2 | 8.6 |
| | Herbicide x Strain x Plant + (1\|Tray) + Block | 33 | 2721.1 | 16.5 |
| | Herbicide x Strain x Plant + (1\|Tray) + Block + MFI | 34 | 2723.0 | 18.4 |
| Nodule Number | Herbicide x Strain + Herbicide x Plant + (1\|Tray) + Block + MFI | 22 | 550.7 | 0.0 |
| | Herbicide x Strain + Herbicide x Plant + Strain x Plant + (1\|Tray) + Block + MFI | 29 | 551.6 | 0.9 |
| | Herbicide x Strain x Plant + (1\|Tray) + Block + MFI | 35 | 558.8 | 8.0 |
| | Herbicide x Strain x Plant + (1\|Tray) + Block | 34 | 566.0 | 15.2 |
| BNF($\delta^{15}$N) | Herbicide x Strain + Herbicide x Plant + (1\|Tray) + MFI | 23 | 570.6 | 0.0 |
| | Herbicide x Strain + Herbicide x Plant + Strain x Plant + (1\|Tray) + MFI | 31 | 588.8 | 18.2 |
| | Herbicide x Strain x Plant + (1\|Tray) + MFI | 39 | 606.5 | 35.9 |

| | | | | |
|---|---|---|---|---|
| | Herbicide x Strain x Plant + (1\|Tray) | 38 | 608.9 | 38.3 |
| Week 4 Plant Size | Herbicide x Strain + Herbicide x Plant + (1\|Tray) + Block + MFI | 22 | 1244.3 | 0.0 |
| | Herbicide x Strain + Herbicide x Plant + Strain x Plant + (1\|Tray) + Block + MFI | 29 | 1258.3 | 14.0 |
| | Herbicide x Strain x Plant + (1\|Tray) + Block + MFI | 35 | 1258.3 | 14.0 |
| | Herbicide x Strain x Plant + (1\|Tray) + Block | 36 | 1261.1 | 16.8 |
| Week 6 Plant Size | Herbicide x Strain + Herbicide x Plant + (1\|Tray) | 20 | 231.3 | 0.0 |
| | Herbicide x Strain + Herbicide x Plant + Strain x Plant + (1\|Tray) | 27 | 242.3 | 11.1 |
| | Herbicide x Strain x Plant + (1\|Tray) | 34 | 247.3 | 16.0 |
| | Herbicide x Strain x Plant + (1\|Tray) + MFI | 36 | 252.4 | 21.2 |

**Appendix D: Table 27. Means and standard errors (SE) of time until nodulation data in *T. pratense* according to Herbicide Treatment (A) and Rhizobial Strain Inoculation (B).**

Means were determined by calculating the area under the curve of the probability that nodulation would occur over time until Week 4 of the study using Kaplan-Meier methods (see Statistical Analysis in main text).

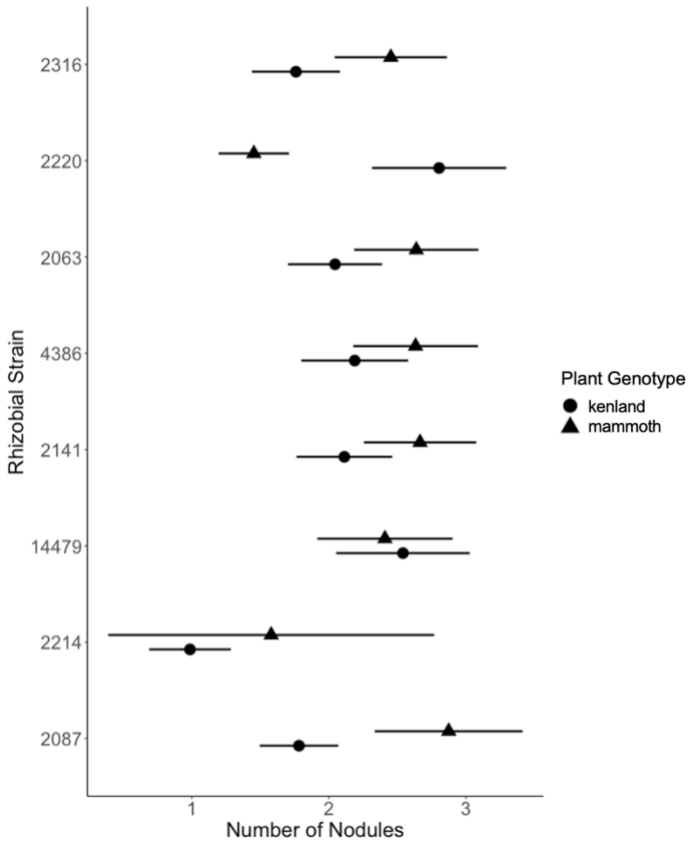
| | | Mean (weeks) | SE |
|---|---|---|---|
| **A. Herbicide Treatment** | Dicamba | 2.55 | 0.0741 |
| | No Dicamba | 3.29 | 0.0700 |
| **B. Rhizobial Strain** | 14479 | 2.92 | 0.1613 |
| | 2063 | 2.70 | 0.1581 |
| | 2087 | 2.69 | 0.1453 |
| | 2141 | 2.56 | 0.1450 |
| | 2214 | 3.85 | 0.0788 |
| | 2220 | 2.85 | 0.1474 |
| | 2316 | 2.82 | 0.1511 |
| | 4386 | 2.96 | 0.1501 |

**Appendix D: Table 28. *Post hoc* pairwise comparisons of herbicide treatment × rhizobial strain interactions for nodule number (A), BNF as estimated by Foliar δ15N abundance (B), Week 4 plant size (C) and Week 6 plant size (D).**

'EMM No Dicamba – EMM Dicamba' shows the difference in the estimated marginal means (EMMs; extracted from the highest performing statistical models) between herbicide treatments according to rhizobial inoculum (Rhizobial Strain) for each response variable (A-C). 'SE' is the standard error of this difference. *T*-statistics (*t*-stat), degrees of freedom (df), and *P*-values (*P*; adjusted via Dunnett's test) were calculated to test for significant differences between herbicide treatments EMMs by strain.

| Response | Rhizobial Strain | EMM No Dicamba – EMM Dicamba | SE | df | *t*-stat | *P* |
|---|---|---|---|---|---|---|
| A. Nodule Number | 14479 | 0.789 | 0.23 | 279 | -0.801 | 0.91 |
| | 2063 | 1.200 | 0.28 | 275 | 0.772 | 0.92 |
| | 2087 | 1.997 | 0.48 | 273 | 2.903 | **0.027** |
| | 2141 | 1.546 | 0.35 | 275 | 1.942 | 0.27 |
| | 2214 | 0.679 | 0.41 | 280 | -0.648 | 0.95 |
| | 2220 | 2.130 | 0.52 | 278 | 3.125 | **0.014** |
| | 2316 | 1.908 | 0.47 | 275 | 2.611 | 0.061 |
| | 4386 | 2.987 | 0.74 | 273 | 4.445 | **0.0001** |
| B. BNF ($\delta^{15}$N) | 14479 | 0.777 | 0.56 | 176 | 1.387 | 0.61 |
| | 2063 | 0.006 | 0.56 | 175 | 0.011 | 1.0 |
| | 2087 | -0.404 | 0.57 | 175 | -0.715 | 0.94 |
| | 2141 | 0.468 | 0.56 | 175 | 0.837 | 0.9 |
| | 2214 | -0.707 | 0.56 | 176 | -1.258 | 0.67 |
| | 2220 | -0.942 | 0.56 | 174 | -1.686 | 0.41 |

| | | | | | | |
|---|---|---|---|---|---|---|
| | 2316 | -1.222 | 0.56 | 173 | -2.195 | 0.17 |
| | 4386 | 0.974 | 0.56 | 176 | 1.733 | 0.39 |
| C. Week 4 Plant Size | 14479 | 2.129 | 0.29 | 376 | 7.327 | **<.0001** |
| | 2063 | 1.967 | 0.29 | 377 | 6.695 | **<.0001** |
| | 2087 | 1.704 | 0.29 | 376 | 5.930 | **<.0001** |
| | 2141 | 1.930 | 0.29 | 376 | 6.722 | **<.0001** |
| | 2214 | 1.005 | 0.29 | 376 | 3.499 | **0.0039** |
| | 2220 | 1.444 | 0.29 | 376 | 4.937 | **<.0001** |
| | 2316 | 2.308 | 0.29 | 376 | 7.943 | **<.0001** |
| | 4386 | 2.298 | 0.30 | 377 | 7.672 | **<.0001** |
| D. Week 6 Plant Size | 14479 | 0.431 | 0.13 | 261 | 3.315 | **0.0076** |
| | 2063 | 0.453 | 0.13 | 261 | 3.418 | **0.0054** |
| | 2087 | 0.257 | 0.13 | 260 | 2.009 | 0.24 |
| | 2141 | 0.437 | 0.13 | 260 | 3.415 | **0.0054** |
| | 2214 | 0.055 | 0.13 | 260 | 0.433 | 0.99 |
| | 2220 | 0.491 | 0.13 | 260 | 3.836 | **0.0012** |
| | 2316 | 0.643 | 0.13 | 260 | 5.030 | **<.0001** |
| | 4386 | 0.733 | 0.14 | 261 | 5.415 | **<.0001** |




**Appendix D: Figure 27. Plant genotype and rhizobial genotype interactions influenced nodule number.**

Points show EMMs (± SE) for the number of root nodules on plants (x-axis) according to rhizobial genetic strain (y-axis) and host *T. pratense* genotype, Kenland (circles) or Mammoth (triangles).

**Appendix D: Table 29. *Post hoc* pairwise comparisons of plant genotype × rhizobial strain interactions for nodule number.**

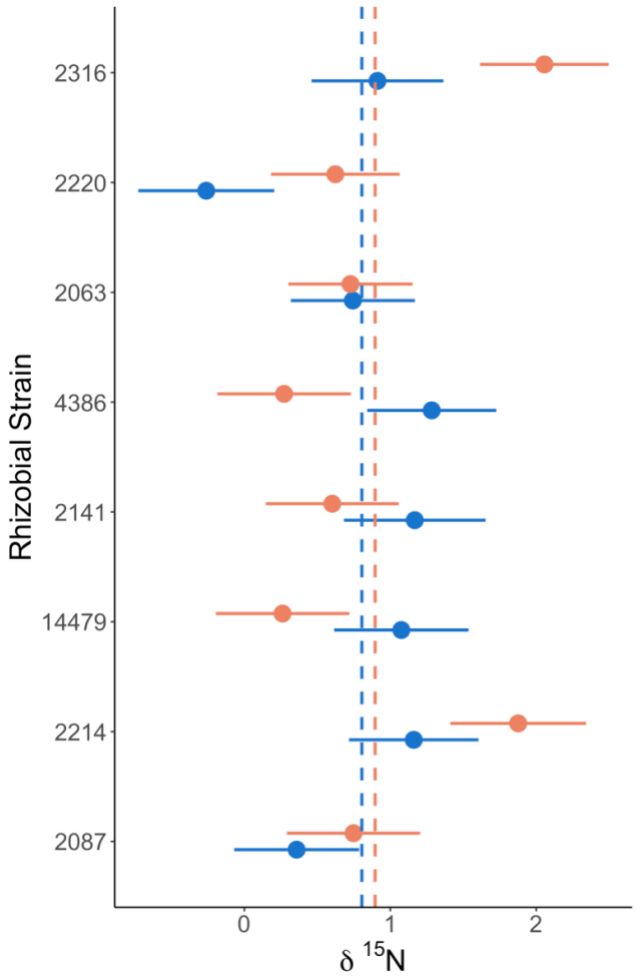
'EMM Kenland – EMM Mammoth' shows the difference in the estimated marginal means (EMMs; extracted from the highest performing statistical model) between *T. pratense* genotypes according to rhizobial inoculum (Rhizobial Strain) for nodule number. 'SE' is the standard error of this difference. *T*-statistics (*t*-stat), degrees of freedom (df), and *P*-values (*P*; adjusted via Dunnett's test) were calculated to test for significant differences between plant genotype EMMs by strain.

| Rhizobial Strain | Kenland EMM – Mammoth EMM | SE | df | *t*-stat | *P* |
|---|---|---|---|---|---|
| 14479 | 1.055 | 0.27 | 272 | 0.210 | 0.99 |
| 2063 | 0.775 | 0.18 | 277 | -1.089 | 0.78 |
| 2087 | 0.620 | 0.15 | 272 | -2.041 | 0.22 |
| 2141 | 0.793 | 0.17 | 272 | -1.066 | 0.80 |
| 2214 | 0.624 | 0.53 | 280 | -0.558 | 0.97 |
| 2220 | 1.930 | 0.46 | 275 | 2.775 | **0.039** |
| 2316 | 0.718 | 0.17 | 273 | -1.424 | 0.58 |
| 4386 | 0.831 | 0.20 | 279 | -0.759 | 0.93 |

**Appendix D: Table 30. Type III sums of squares ANOVAs for foliar δ15N abundance, controlling for nodule number.**

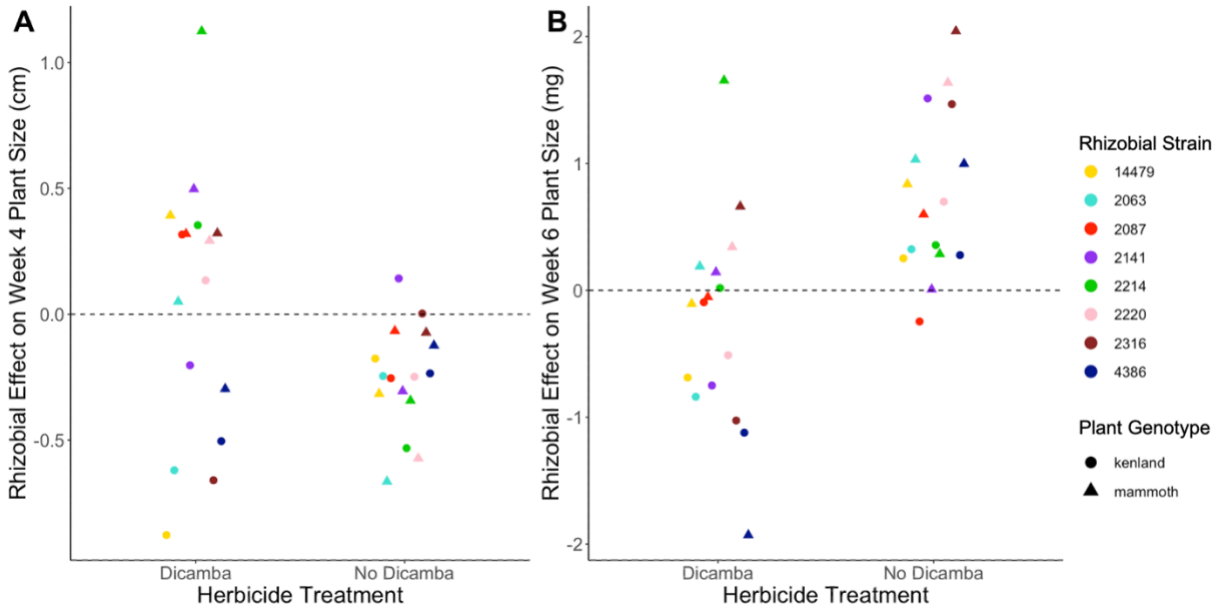
Rows correspond to fixed effect factors ('fixed effect') and their degrees of freedom ('df'), χ2, and *P*-values extracted from a linear mixed effects model. Significant *P* values are bolded.

| | | $\delta^{15}$N | |
|---|---|---|---|
| **Fixed Effect** | **df** | $\chi^2$ | ***P*** |
| Herbicide Treatment € | 1 | 2.62 | 0.10 |
| Rhizobial Strain ($G_R$) | 7 | 13.94 | 0.052 |
| Plant Genotype ($G_P$) | 1 | 0.37 | 0.54 |
| Week 6 nodule number | 1 | 0.41 | 0.52 |
| Minor Fungal Infection | 1 | 5.51 | **0.019** |
| $G_R \times E$ | 7 | 17.11 | **0.017** |
| $G_P \times E$ | 1 | 0.23 | 0.63 |

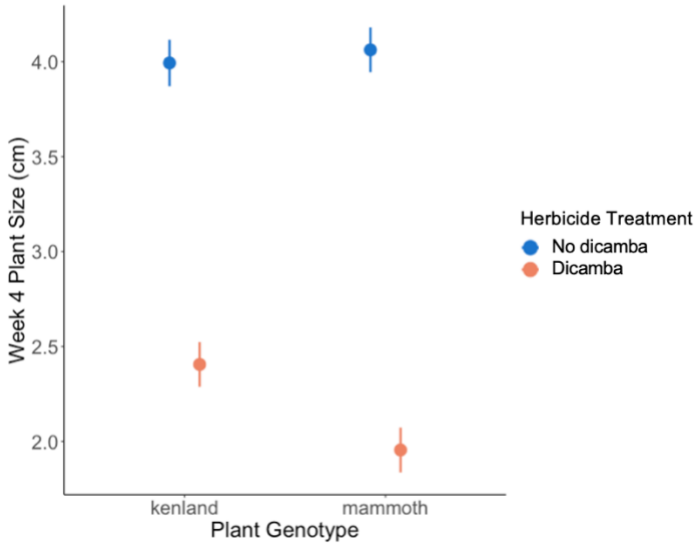



**Appendix D: Figure 28. Herbicide exposure and rhizobial variation interacted to determine foliar δ15N abundance, after controlling for nodule number.**

Points show EMMs (± SE), extracted from a linear mixed effects model incorporating Week 6 nodule number as a covariate (see Methods), according to whether plants were grown in the presence (orange) or absence (blue) of rhizospheric dicamba within microcosms and with which rhizobial strain (y-axis) they were inoculated with. Dashed lines represent herbicide treatment means across inocula.




**Appendix D: Figure 29. Effects of herbicide exposure on plant growth response to rhizobial inoculation.** Points show the mean rhizobial effect (difference in size with vs. without rhizobia) according to herbicide treatment (x-axis), rhizobial strain inoculum (color), and plant genotype (shape). At Week 4 (A), size was estimated from photographs using leaflet number and length; at Week 6 (B) it was measured using shoot biomass. The dashed line at 0 represents the baseline size for plants without rhizobia (uninoculated).




**Appendix D: Figure 30. Herbicide exposure and plant genotypic variation interactively affected early plant growth.**

Points show EMMs (± SE) for Week 4 plant size in cm (y-axis) according to whether *T. pratense* genotypes (x-axis) were grown in the presence (orange) or absence (blue) of rhizospheric dicamba.

**Appendix D: Table 31. *Post hoc* pairwise comparisons of plant genotype × herbicide treatment interactions for Week 4 plant size.**

'EMM Kenland – EMM Mammoth' shows the difference in the estimated marginal means (EMMs; extracted from the highest performing statistical model) between *T. pratense* genotypes according to herbicide treatment for Week 4 plant size (cm). 'SE' is the standard error of this difference. *T*-statistics (*t*-stat), degrees of freedom (df), and *P*-values (*P*; adjusted via Dunnett's test) were calculated to test for significant differences between plant genotype EMMs by herbicide treatment.

| **Herbicide Treatment** | **Kenland EMM – Mammoth EMM** | **SE** | **df** | ***t*-stat** | ***P*** |
|---|---|---|---|---|---|
| No Dicamba | -0.069 | 0.15 | 376 | -0.477 | 0.84 |
| Dicamba | 0.451 | 0.15 | 377 | 3.076 | **0.0044** |

## Bibliography


Abdi, H., and J. Williams. 2010. Contrast analysis. Pages 244-251 *in* N. Salkind, editor. Encyclopedia of Research Design. First edition. Sage, Thousand Oaks, California, USA.

Agrawal, A. A. 2000. Overcompensation of plants in response to herbivory and the by-product benefits of mutualism. Trends in Plant Science 5: 309–313.

Aguilar, A., Y. Mora, A. Dávalos, L. Girard, J. Mora, and H. Peralta. 2018. Analysis of genome sequence and symbiotic ability of rhizobial strains isolated from seeds of common bean (*Phaseolus vulgaris*). BMC Genomics 19: 796-815.

Agustí, M., S. Zaragoza, D.J. Iglesias, V. Almela, E. Primo-Millo, and M. Talón. 2002. The synthetic auxin 3,5,6-TPA stimulates carbohydrate accumulation and growth in citrus fruit. Plant Growth Regulation 36: 141–147.

Ahemad, M., and M. S. Khan. 2010. Growth Promotion and Protection of Lentil (*Lens esculenta*) against herbicide stress by *Rhizobium* species. Annals of Microbiology 60: 735–45.

Ahemad, M., and M. S. Khan. 2012. Ecological assessment of biotoxicity of pesticides towards plant growth promoting activities of pea (*Pisum sativum*)-specific *Rhizobium* sp. strain MRP1. Emirates Journal of Food and Agriculture 24: 334–43.

Akaike, H. 1973. Maximum likelihood identification of Gaussian autoregressive moving average models. Biometrika 60: 255–265.

Albaugh, LLC. 2018. Dicamba DMA Salt Specimen Label. Ankeny, Iowa, USA.

Albor, C., J. G. García-Franco, V. Parra-Tabla, C. Díaz-Castelazo, and G. Arceo-Gómez. 2019. Taxonomic and functional diversity of the co-flowering community differentially affect *Cakile edentula* pollination at different spatial scales. Journal of Ecology 107: 2167–2181.

Alcocer-ruthling, M., D. C. Thill, and B. Shafii. 1992. Differential competitiveness of sulfonylurea resistant and susceptible prickly lettuce (*Lactuca serriola*). Weed Science 6: 303–309.

Allender, W. J., G. C. Cresswell, J. Kaldor, and I. R. Kennedy. 1997. Effect of lithium and lanthanum on herbicide induced hormesis in hydroponically-grown cotton and corn. Journal of Plant Nutrition 20: 81-95.

Altieri, M. A. 1999. The ecological role of biodiversity in agroecosystems. Invertebrate Biodiversity as Bioindicators of Sustainable Landscapes 74: 19–31.

Arceo-Gómez, G., and T.-L. Ashman. 2011. Heterospecific pollen deposition: Does diversity alter the consequences? New Phytologist 192: 738–746.

Arceo-Gómez, G., R. L. Kaczorowski, and T.-L. Ashman. 2018. A network approach to understanding patterns of coflowering in diverse communities. International Journal of Plant Sciences 179: 569–582.

Arceo-Gómez, G. 2021. Spatial variation in the intensity of interactions via heterospecific pollen transfer may contribute to local and global patterns of plant diversity. Annals of Botany 128: 383–394.

Ashman, T.-L., and G. Arceo-Gómez. 2013. Toward a predictive understanding of the fitness costs of heterospecific pollen receipt and its importance in co-flowering communities. American Journal of Botany 100: 1061–1070.

Ashman, T.-L., T. M. Knight, J. A. Steets, P. Amarasekare, M. Burd, D. R. Campbell, M. R. Dudash, M. O. Johnston, S. J. Mazer, R. J. Mitchell, M. T. Morgan, and W. G. Wilson. 2004. Pollen limitation of plant reproduction: Ecological and evolutionary causes and consequences. Ecology 85: 2408–21.

Ashraf, M., and A. Iram. 2005. Drought stress induced changes in some organic substances in nodules and other plant parts of two potential legumes differing in salt tolerance. Flora - Morphology, Distribution, Functional Ecology of Plants 200: 535–46.

Athar, M., and D. A. Johnson. 1996. Nodulation, biomass production, and nitrogen fixation in alfalfa under drought. Journal of Plant Nutrition 19:185–199.

Bader, D. A., S. Kintali, K. Madduri, and M. Mihail. 2007. Approximating betweenness centrality. Pages 124-137 *in* A. Bonato, and F. R. K. Chung, editors. Algorithms and Models for the Web-Graph. First edition. Springer, Berlin, Germany.

Barber, N. A., and N. L. S. Gorden. 2015. How do belowground organisms influence plant–pollinator interactions? Journal of Plant Ecology 8: 1–11.

Barker, D. G., T. Pfaff, D. Moreau, E. Groves, S. Ruffel, M. Lepetit, S. Whitehand, F. Maillet, R. M Nair, and E.-P. Journet. 2007. Growing *M. Truncatula*: Choice of substrates and growth conditions. Pages 1-26 in S. Roberts, editor. The *Medicago truncatula* handbook. Nobel Foundation, Ardmore, Oklahoma, USA.

Barquero, M., J. Poveda, A. M. Laureano-Marín, N. Ortiz-Liébana, J. Brañas, and F. González-Andrés. 2022. Mechanisms involved in drought stress tolerance triggered by rhizobia strains in wheat. Frontiers in Plant Science 13:1036973.

Barrett, L. G., J. D. Bever, A. Bissett, and P. H. Thrall. 2015. Partner diversity and identity impacts on plant productivity in *Acacia*–rhizobial interactions. Journal of Ecology, 103: 130–142.

Barrett, L. G., L. M. Broadhurst, and P. H. Thrall. 2012. Geographic adaptation in plant–soil mutualisms: Tests using *Acacia* spp. and rhizobial bacteria. Functional Ecology 26: 457–68.

Bascompte, J., and P. Jordano. 2007. Plant-animal mutualistic networks: The architecture of biodiversity. Annual Review of Ecology, Evolution, and Systematics 38: 567–593.

Bastian, M., S. Heymann, and M. Jacomy. 2009. Gephi: an open source software for exploring and manipulating networks. Third International AAAI Conference on Weblogs and Social Media. San Jose, California, USA.

Bates, D., M. Maechler, B. Bolker, and S. Walker. 2015. Fitting linear mixed-effects models using lme4. Journal of Statistical Software 67: 1-48.

Batstone, R. T., A. Ibrahim, and L. T. MacLean. 2023. Microbiomes: Getting to the root of the rhizobial competition problem in agriculture. Current Biology 33: R777–80.

Batstone, R. T., E. M. Dutton, D. Wang, M. Yang, and M. E. Frederickson. 2017. The evolution of symbiont preference traits in the model legume Medicago truncatula. New Phytologist 213: 1850–61.

Batstone, R. T., A. Ibrahim, and L. T. MacLean. 2023. Microbiomes: Getting to the root of the rhizobial competition problem in agriculture. Current Biology 33: R777–R780.

Batstone, R. T., L. T. Burghardt, and K. D. Heath. 2022. Phenotypic and genomic signatures of interspecies cooperation and conflict in naturally occurring isolates of a model plant symbiont. Proceedings of the Royal Society B 289: 20220477.

Batstone, R. T., M. A. E. Peters, A. K. Simonsen, J. R. Stinchcombe, and M. E. Frederickson. 2020. Environmental variation impacts trait expression and selection in the legume–rhizobium symbiosis. American Journal of Botany 107: 195–208.

Baucom, R. S., and R. Busi. 2019. Evolutionary epidemiology in the field: A proactive approach for identifying herbicide resistance in problematic crop weeds. New Phytologist 223: 1056-1058.

Baucom, R. S., and R. Mauricio. 2004. Fitness costs and benefits of novel herbicide tolerance in a noxious weed. Proceedings of the National Academy of Sciences 101: 13386–13390.

Baucom, R. S., and R. Mauricio. 2008. Constraints on the evolution of tolerance to herbicide in the common morning glory: Resistance and tolerance are mutually exclusive. Evolution 62: 2842–2854.

Baucom, R. S. 2019. Evolutionary and ecological insights from herbicide-resistant weeds: What have we learned about plant adaptation, and what is left to uncover? New Phytologist 223: 68–82.

Bell, G. 2017. Evolutionary Rescue. Annual Review of Ecology, Evolution, and Systematics 48:605-27.

Belz, R. G., and S. O. Duke. 2014. Herbicides and plant hormesis. Pest Management Science 70: 698–707.

Benner, B. L., and F. A. Bazzaz. 1987. Effects of timing of nutrient addition on competition within and between two annual plant species. The Journal of Ecology 75: 229.

Bernardo, P., T. Charles-Dominique, M. Barakat, P. Ortet, . . . , and P. Roumagnac. 2018. Geometagenomics illuminates the impact of agriculture on the distribution and prevalence of plant viruses at the ecosystem scale. ISME Journal 12: 173–184.

Bhat, S. V., S. C. Booth, S. G. K. Mcgrath, and T. E. S. Dahms. 2015. *Rhizobium leguminosarum* bv. viciae 3841 adapts to 2,4-dichlorophenoxyacetic acid with 'auxin-like' morphological changes, cell envelope remodeling and upregulation of central metabolic pathways. PLoS ONE 10: e0123813.

Bianucci, E., A. Furlan, M. del Carmen Tordable, L. E. Hernández, R. O. Carpena-Ruiz, and S. Castro. 2017. Antioxidant responses of peanut roots exposed to realistic groundwater doses of arsenate: Identification of glutathione S-transferase as a suitable biomarker for metalloid toxicity. Chemosphere 181: 551–561.

Biesmeijer, J. C., S. P. M. Roberts, M. Reemer, R. Ohlemüller, M. Edwards, T. Peeters, A. P. Shaffers, S. G. Potts, R. Kleukers, C. D. Thomas, J. Settele, and W. E. Kunin. 2006. Parallel declines in pollinators and insect-pollinated plants in Britain and the Netherlands. Science 313: 351–354.

Bingham, R., P. King, and A. G. Keck. 2017. An investigation of tradeoffs in herbicide resistant *Brassica rapa*: Effects on pollen and ovule production, biomass and development. American Journal of Environmental Sciences 13: 22–29.

Blackshaw, R. E., L. J. Molnar, and H. H. Janzen. 2004. Nitrogen fertilizer timing and application method affect weed growth and competition with spring wheat. Weed Science 52: 614–622.

Blondel, V. D., J. L. Guillaume, R. Lambiotte, E. Lefebvre. 2008. Fast unfolding of communities in large networks. Journal of Statistical Mechanics: Theory and Experiment: P10008.

Bohnenblust, E. W., A. D. Vaudo, J. F. Egan, D. A. Mortensen, J. F. Tooker. 2016. Effects of the herbicide dicamba on nontarget plants and pollinator visitation. Environmental Toxicology and Chemistry 35: 144–151.

Boutin, C., K. L. Aya, D. Carpenter, P. J. Thomas, O. and Rowland. 2012. Phytotoxicity testing for herbicide regulation: Shortcomings in relation to biodiversity and ecosystem services in agrarian systems. Science of The Total Environment 415: 79–92.

Boutin, C., B. Strandberg, D. Carpenter, S. K. Mathiassen, P. J. Thomas. 2014. Herbicide impact on non-target plant reproduction: What are the toxicological and ecological implications? Environmental Pollution 185: 295–306.

Brandes, U., D. Delling, M. Gaertler, and R. Gorke. 2008. On modularity clustering. IEEE Transactions on Knowledge and Data Engineering 20: 172–188.

Bretagnolle, V., and S. Gaba. 2015. Weeds for bees? A review. Agronomy for Sustainable Development 35: 891–909.

Brockwell, J., P. J. Bottomley, and J. E. Thies. 1995. Manipulation of rhizobia microflora for improving legume productivity and soil fertility: A critical assessment. Plant and Soil 174: 143–180.

Bronstein, J. L. 2001. The Exploitation of Mutualisms. Ecology Letters 4: 277–87.

Brooker, R. W. 2006. Plant–plant interactions and environmental change. New Phytologist 171: 271–284.

Brown, B. E. 1997. Coral bleaching: causes and consequences. Coral Reefs 16: S129–38.

Bruessow, F., and H. Brüssow. 2020. Our extended genotype—An argument for the study of domesticated microbes. Environmental Microbiology 22: 1669–1674.

Brühl, C. A., and J. G. Zaller. 2021. Indirect herbicide effects on biodiversity, ecosystem functions, and interactions with global changes. Pages 231-272 *in* R. Mesnage and J. G. Zaller, editors. Herbicides: Chemistry, efficacy, toxicology, and environmental impacts. First edition. Elsevier, Amsterdam, Netherlands.

Burdon, J. J., and P. H. Thrall. 2008. Pathogen evolution across the agro-ecological interface: Implications for disease management. Evolutionary Applications 1: 57–65.

Burghardt, L. T., and G. C. diCenzo. 2023. The evolutionary ecology of rhizobia: Multiple facets of competition before, during, and after symbiosis with legumes. Current Opinion in Microbiology 72: 102281.

Burghardt, L. T., B. Epstein, J. Guhlin, M. S. Nelson, M. R. Taylor, N. D. Young, M. J. Sadowsky, and P. Tiffin. 2018. Select and resequence reveals relative fitness of bacteria in symbiotic and free-living environments. Proceedings of the National Academy of Sciences of the United States of America 115: 2425–30.

Burghardt, L. T., B. Epstein, M. Hoge, D. I. Trujillo, and P. Tiffin. 2022. Host-associated rhizobial fitness: Dependence on nitrogen, density, community complexity, and legume genotype. Applied and Environmental Microbiology 88: e00526-22.

Burul, F., K. Barić, J. Lakić, and A. Milanović-Litre. 2022. Herbicides effects on symbiotic nitrogen-fixing bacteria. Journal of Central European Agriculture 23: 89–102.

Busi, R., D. E. Goggin, I. M. Heap, M. J. Horak, M. Jugulam, R. A. Masters, R. M. Napier, D. S. Riar, N. M. Satchivi, J. Torra, P. Wetsra, and T. R. Wright. 2018. Weed resistance to synthetic auxin herbicides. Pest Management Science 74: 2265–76.

Cafaro La Menza, N., J. P. Monzon, J. L. Lindquist, T. J. Arkebauer, J. M. H. Knops, M. Unkovich, J. E. Specht, and P. Grassini. 2020. Insufficient nitrogen supply from symbiotic fixation reduces seasonal crop growth and nitrogen mobilization to seed in highly productive soybean crops. Plant, Cell & Environment 43: 1958–1972.

Carbonari, C. A., R. N. Costa, B. F. Giovanelli, N. C. Bevilaqua, M. Palhano, H. Barbosa, R. F. Lopez Ovejero, and E. D. Velini. 2022. Volatilization of dicamba diglycolamine salt in combination with glyphosate formulations and volatility reducers in Brazil. Agronomy 12: 1001.

Carlsen, S. C. K., N. H. Spliid, and B. Svensmark. 2006. Drift of 10 herbicides after tractor spray application. Chemosphere 64: 778–786.

Carlson, S. M., C. J. Cunningham, and P. A. H. Westley. 2014. Evolutionary rescue in a changing world. Trends in Ecology & Evolution 29: 521–530.

Carpenter, D., and C. Boutin. 2010. Sublethal effects of the herbicide glufosinate ammonium on crops and wild plants: Short-term effects compared to vegetative recovery and plant reproduction. Ecotoxicology 19: 1322–1336.

Carson, R. L. 1962. Silent Spring. Mariner Books, Boston, Massachusetts, USA.

Carthey, A. J. R., M. R. Gillings, and D. T. Blumstein. 2018. The extended genotype: Microbially mediated olfactory communication. Trends in Ecology & Evolution 33: 885–894.

Carvalho, F. P., B. P. Souza, A. C. França, E. A. Ferreira, M. H. R. Franco, M. C. M. Kasuya, and F. A. Ferreira. 2014. Glyphosate drift affects arbuscular mycorrhizal association in coffee. Planta Daninha 32: 783–789.

Chamberlain, S. A., J. L. Bronstein, and J. A. Rudgers. 2014. How context dependent are species interactions? Ecology Letters 17: 881–90.

Chaulagain, D., and J. Frugoli. 2021. The regulation of nodule number in legumes is a balance of three signal transduction pathways. International Journal of Molecular Sciences 22: 1–14.

Checcucci, A., and M. Marchetti. 2020. The rhizosphere talk show: The rhizobia on stage. Frontiers in Agronomy 2: 591494.

Chittka, L., and S. Schürkens. 2001. Successful invasion of a floral market. Nature 411: 653–653.

Christian, C. E. 2001. Consequences of a biological invasion reveal the importance of mutualism for plant communities. Nature 413: 635–39.

Collins Johnson, N., G. W.T. Wilson, M. A. Bowker, J. A. Wilson, and R. M. Miller. 2010. Resource limitation is a driver of local adaptation in mycorrhizal symbioses. Proceedings of the National Academy of Sciences of the United States of America 107: 2093–98.

Cousens, R.D., and A. Fournier-Level. 2018. Herbicide resistance costs: What are we actually measuring and why? Pest Management Science 74: 1539–1546.

Cox, D. R. 1972. Regression models and life-tables. Journal of the Royal Statistical Society Series B (Methodological) 34: 187-220.

Craine, J. M., E. N. J. Brookshire, M. D. Cramer, N. J. Hasselquist, K. Koba, E. Marin-Spiotta, and L. Wang. 2015. Ecological interpretations of nitrogen isotope ratios of terrestrial plants and soils. Plant and Soil 396: 1–26.

Crews, T. E. 1999. The presence of nitrogen fixing legumes in terrestrial communities: Evolutionary vs ecological considerations. New Perspectives on Nitrogen Cycling in the Temperate and Tropical Americas 46: 233–46.

Damgaard, C., B. Strandberg, S. K. Mathiassen, and P. Kudsk. 2014. The effect of glyphosate on the growth and competitive effect of perennial grass species in semi-natural grasslands. Journal of Environmental Science and Health 49: 897–908.

David, T. I., J. Storkey, and Stevens, C. J. 2019. Understanding how changing soil nitrogen affects plant–pollinator interactions. Arthropod-Plant Interactions 2019 13: 671–684.

Dawkins, R. 1982. The extended phenotype: The long reach of the gene. Oxford University Press, Oxford, England.

De Cauwer, B., D. Reheul, K. D'hooghe, I. Nijs, and A. Milbau. 2005. Evolution of the vegetation of mown field margins over their first 3 years. Agriculture, Ecosystems and Environment 109: 87–96.

de Manincor, N., A. Fisogni, and N. E. Rafferty. 2023. Warming of experimental plant–pollinator communities advances phenologies, alters traits, reduces interactions and depresses reproduction. Ecology Letters 26: 323–334.

Debban, C. L., S. Okum, K. E. Pieper, A. Wilson, and R. S. Baucom. 2015. An examination of fitness costs of glyphosate resistance in the common morning glory, *Ipomoea purpurea*. Ecology and Evolution 5: 5284-5294.

Denison, R. F. 2021. Legume-imposed selection for more-efficient symbiotic rhizobia. Proceedings of the National Academy of Sciences of the United States of America 118: e2107033118.

Denison, R. F., and E. T. Kiers. 2004. Lifestyle alternatives for rhizobia: Mutualism, parasitism, and forgoing symbiosis. FEMS Microbiology Letters 237: 187–93.

Devine, M. D. 2005, Why are there not more herbicide-tolerant crops? Pesticide Management Science 61: 312-317.

Dias, P. M. B., J. Barreto, B. Julier, J.-P. Sampoux, P. Barre, and M. Dall'Agnol. 2008. Genetic diversity in red clover (*Trifolium pratense L.*) revealed by morphological and microsatellite (SSR) markers. Euphytica 160: 189–205.

Dieterman, L. J., C. Y. Lin, L. M. Rohrbaugh, and S. H. Wender. 1964. Accumulation of ayapin and scopolin in sunflower plants treated with 2,4-dichlorophenoxyacetic acid. Archives of Biochemistry and Biophysics 106: 275–279.

Doré, M., C. Fontaine, and E. Thébault. 2021. Relative effects of anthropogenic pressures, climate, and sampling design on the structure of pollination networks at the global scale. Global Change Biology 27: 1266–1280.

Dos Santos Araújo, R., T. A. Viana, L. L. Botina, D. S. S. Bastos, B. C. da Silva Alves, M. Machado-Neves, B. Cupertino Bernardes, R., and G. F. Martins. 2023. Investigating the effects of mesotrione/atrazine-based herbicide on honey bee foragers. Science of The Total Environment 898: 165526.

Druille, M., M. Omacini, R. A. Golluscio, and M. N. Cabello. 2013. Arbuscular mycorrhizal fungi are directly and indirectly affected by glyphosate application. Applied Soil Ecology 72: 143–149.

Dunne, J.A., R. J. Williams, and N. D. Martinez. 2002. Food-web structure and network theory: the role of connectance and size. Proceedings of the National Academy of Sciences 99: 12917–12922.

Egan, J.F., E. Bohnenblust, S. Goslee, D. A. Mortensen, and J. Tooker. 2014. Herbicide drift can affect plant and arthropod communities. Agriculture, Ecosystems and Environment 185: 77–87.

Egan, J.F., and D. A. Mortensen. 2012. Quantifying vapor drift of dicamba herbicides applied to soybean. Environmental Toxicology and Chemistry 31: 1023–1031.

Esfahani, M. N., and A. Mostajeran. 2011. Rhizobial strain involvement in symbiosis efficiency of chickpea-rhizobia under drought stress: Plant growth, nitrogen fixation and antioxidant enzyme activities. Acta Physiologiae Plantarum 33: 1075–1083.

Espeby, L., Å, H. Fogelfors, and P. Milberg. 2011. Susceptibility variation to new and established herbicides: Examples of inter-population sensitivity of grass weeds. Crop Protection 30: 429–35.

Evers, G. 2011. Descriptive information on cool-season forage legumes. Texas AgriLife Research and Extension Center, Overton, Texas, USA.

Fabra, A., R. Duffard, and A. Evangelista de Duffard. 1997. Toxicity of 2,4-dichlorophenoxyacetic acid to *Rhizobium* sp in pure culture. Bulletin of Environmental Contamination and Toxicology 59: 645–652.

Fang, Q., and S. Q. Huang. 2013. A directed network analysis of heterospecific pollen transfer in a biodiverse community. Ecology 94: 1176–1185.

FAO. 2022. Pesticides use, pesticides trade and pesticides indicators global, regional and country trends, 1990-2020. FAOSTAT Analytical Brief 46. Food and Agriculture Organization of the United Nations, Rome, Italy.

Farina, W. M., M. S. Balbuena, L. T. Herbert, C. M. Goñalons, and D. E. Vázquez. 2019. Effects of the herbicide glyphosate on honey bee sensory and cognitive abilities: Individual impairments with implications for the hive. Insects 10: 354.

Felipe-Lucia, M. R., S. Soliveres, C. Penone, M. Fischer, . . . , and E. Allan. 2020. Land-use intensity alters networks between biodiversity, ecosystem functions, and services. Proceedings of the National Academy of Sciences of the United States of America 117: 28140–28149.

Felsot, A. S., J. B. Unsworth, J. B. H. J. Linders, G. Roberts, D. Rautman, C. Harris, and E. Carazo. 2010. Agrochemical spray drift; assessment and mitigation—a review. Journal of Environmental Science and Health, Part B, Pesticides, Food Contaminants, and Agricultural Wastes 46: 1–23.

Felsenstein J. 1985. Phylogenies and the comparative method. The American Naturalist. 125: 1-15.

Ferguson, B. J., M. H. Lin, and P. M. Gresshoff. 2013. Regulation of legume nodulation by acidic growth conditions. Plant Signaling & Behavior 8: e23426

Fineblum, W.L., and M. D. Rausher. 1995. Tradeoff between resistance and tolerance to herbivore damage in a morning glory. Nature 377: 517–520.

Forrest, J., D. W. Inouye, and J. D. Thomson. 2010. Flowering phenology in subalpine meadows: Does climate variation influence community co-flowering patterns? Ecology 91: 431-440.

Foster, M. R., and J. L. Griffin. 2018. Injury criteria associated with soybean exposure to dicamba. Weed Technology 32: 608–617.

Fox, J., and S. Weisberg. 2019. An R companion to applied regression. Third edition. Sage, Thousand Oaks, California, USA.

Fox, J. E., J. Gulledge, E. Engelhaupt, M. E. Burow, and J. A. McLachlan. 2007. Pesticides reduce symbiotic efficiency of nitrogen-fixing rhizobia and host plants. Proceedings of the National Academy of Sciences of the United States of America 104: 10282–10287.

Fox, J. E., M. Starcevic, P. E. Jones, M. E. Burow, and J. A. McLachlan. 2004. Phytoestrogen signaling and symbiotic gene activation are disrupted by endocrine-disrupting chemicals. Environmental Health Perspectives 112: 672–677.

Fox, J. E., M. Starcevic, K. Y. Kow, M. E. Burow, and J. A. McLachlan. 2001. Nitrogen fixation. Endocrine disrupters and flavonoid signaling. Nature 413: 128–129.

France, W., J. Norsworthy, T. Roberts, J. Ross, T. Barber, and E. Gbur. 2022. Effect of cultivar and planting date on soybean response to dicamba. International Journal of Agronomy 2022: 9479650

Friesen, M. L. 2012. Widespread fitness alignment in the legume–rhizobium symbiosis. New Phytologist 194: 1096–1111.

Garcia L. C., and M. D. Eubanks. 2019. Overcompensation for insect herbivory: A review and meta-analysis of the evidence. Ecology 100: e02585.

Gassmann, A. J., and D. J. Futuyma. 2005. Consequence of herbivory for the fitness cost of herbicide resistance: Photosynthetic variation in the context of plant-herbivore interactions. Journal of Evolutionary Biology 18: 447–454.

Ghazoul, J. 2006. Floral diversity and the facilitation of pollination. Journal of Ecology 94: 295–304.

Gianfagna, T. 1995. Natural and synthetic growth regulators and their use in horticultural and agronomic crops. Pages 751-773 *in* P. J. Davies, editor. Plant Hormones. Second edition, volume one. Springer, Berlin, Germany.

Gleason, C., R. C. Foley, and K. B. Singh. 2011. Mutant analysis in *Arabidopsis* provides insight into the molecular mode of action of the auxinic herbicide dicamba. PLoS ONE 6: 17245.

Goggin D. E., G. R. Cawthray, and S. B. Powles. 2016. 2,4-D resistance in wild radish: Reduced herbicide translocation via inhibition of cellular transport. Journal of Experimental Botany 67: 3223–3235.

Goggin D. E., P. Kaur, M. J. Owen, and S. B. Powles. 2018. 2,4-D and dicamba resistance mechanisms in wild radish: subtle, complex and population specific? Annals of Botany 122: 627-640.

Gomes, G. L. B., and K. C. Scortecci. 2021. Auxin and its role in plant development: Structure, signalling, regulation and response mechanisms. Plant Biology 23: 894–904.

Gomulkiewicz, R., and M. Kirkpatrick. 1992. Quantitative genetics and the evolution of reaction norms. Evolution; International Journal of Organic Evolution 46: 390–411.

Gopalakrishnan, S., A. Sathya, R. Vijayabharathi, R. K. Varshney, C. L. Laxmipathi Gowda, and L. Krishnamurthy. 2015. Plant growth promoting rhizobia: Challenges and opportunities. 3 Biotech 5: 355–77.

Gou, X., P. B. Reich, L. Qiu, M. Shao, G. Wei, J. Wang, and X. Wei. 2023. Leguminous plants significantly increase soil nitrogen cycling across global climates and ecosystem types. Global Change Biology 29: 4028–4043.

Goyal, R. K., A. S. Maria, and M. F. Hynes. 2021. Molecular biology in the improvement of biological nitrogen fixation by rhizobia and extending the scope to cereals. Microorganisms 9: 1–24.

Granada Agudelo, M., B. Ruiz, D. Capela, and P. Remigi. 2023. The role of microbial interactions on rhizobial fitness. Frontiers in Plant Science 14: 1277262.

Gray, C., D. J. Baird, S. Baumgartner, U. Jacob, G. B. Jenkins, E. J. O'Gorman, X. Lu, A. Ma, M. J. O. Pococl, N. Schuwirth, M. Thompson, and G. Woodard. 2014. FORUM: Ecological networks: The missing links in biomonitoring science. Journal of Applied Ecology 51: 1444–1449.

Griffin, J. L., M. J. Bauerle, D. O. Stephenson, D. K. Miller, J. M. Boudreaux. 2013. Soybean response to dicamba applied at vegetative and reproductive growth stages. Weed Technology 27: 696– 703.

Grossmann, K. 2010. Auxin herbicides: Current status of mechanism and mode of action. Pest Management Science 66: 113–120.

Grover, R., J. Maybank, and K. Yoshida. 1972. Droplet and vapor drift from butyl ester and dimethylamine salt of 2,4-D. Weed Science 20: 320–324.

Guardiola, J. L., and A. García-Luis. 2000. Increasing fruit size in citrus. Thinning and stimulation of fruit growth. Plant Growth Regulation 31: 121–132.

Hald, A. B. 1999. Weed vegetation (wild flora) of long established organic versus conventional cereal fields in Denmark. Annals of Applied Biology 134: 307–314.

Hall, J. K., and R. O. Mumma. 1994. Dicamba mobility in conventionally tilled and non-tilled soil. Soil and Tillage Research 30: 3–17.

Han, K., and I. Jung. 2022. Restricted mean survival time for survival analysis: A quick guide for clinical researchers. Korean Journal of Radiology 23: 495.

Hansen, A. P., T. Yoneyama, H. Kouchi, and K. Hiraoka. 1993. Respiration and nitrogen fixation of hydroponically cultured *Phaseolus vulgaris* L. cv. OAC Rico and a supernodulating mutant. Planta 189: 546–56.

Hartig, F. 2022. DHARMa: Residual diagnostics for hierarchical (multi-level/mixed) regression models. R package version 0.4.6. http://florianhartig.github.io/DHARMa/.

Heath, K. D., and P. Tiffin, P. 2007. Context dependence in the coevolution of plant and rhizobial mutualists. Proceedings of the Royal Society B: Biological Sciences 274: 1905–1912.

Heath, K. D., A. J. Stock, and J. R. Stinchcombe. 2010. Mutualism variation in the nodulation response to nitrate. Journal of Evolutionary Biology 23: 2494–2500.

Heath, K. D., J. C. Podowski, S. Heniff, C. R. Klinger, P. V. Burke, D. J. Weese, W. H. Yang, and J. A. Lau. 2020. Light availability and rhizobium variation interactively mediate the outcomes of legume–rhizobium symbiosis. American Journal of Botany 107: 229–238.

Hilty, J. 2019. Illinois wildflowers. https://illinoiswildflowers.info

Ho, L. S. T., and C. Ane. 2014. A linear-time algorithm for Gaussian and non-Gaussian trait evolution models. Systematic Biology 63: 397-408.

Hoeksema, J. D., and S. E. Forde. 2008. A meta-analysis of factors affecting local adaptation between interacting species. American Naturalist 171: 275–90.

Hoering, T. 1955. Variations of nitrogen-15 abundance in naturally occurring substances. Science 122: 1233–34.

Holzschuh, A., I. Steffan-Dewenter, and T. Tscharntke. 2008. Agricultural landscapes with organic crops support higher pollinator diversity. Oikos 117: 354–361.

Huang, Y., L. Yuan, K. N. Reddy, and J. Zhang. 2016. In-situ plant gyperspectral sensing for early detection of soybean injury from dicamba. Biosystems Engineering 149: 51–59.

Iriart, V., R. S. Baucom, T.-L. Ashman. 2021. Herbicides as anthropogenic drivers of eco-evo feedbacks in plant communities at the agro-ecological interface. Molecular Ecology 30: 5406–5421.

Iriart, V., R. S. Baucom, and T.-L. Ashman. 2022. Interspecific variation in resistance and tolerance to herbicide drift reveals potential consequences for plant community co-flowering interactions and structure at the agro-eco interface. Annals of Botany 130: 1015–1028.

Iriart, V., E. M. Rarick, and T.-L. Ashman. *In review*. Rhizobial variation, more than plant variation, mediates plant symbiotic and fitness responses to herbicide stress.

Ives, A., R. Dinnage, L. A. Nell, M. Helmus, and D. Li. 2020. phyr: Model based phylogenetic analysis. R package version 1.1.0. https://CRAN.R-project.org/package=phyr.

Jacobsen, D.J., R. A. Raguso. 2018. Lingering effects of herbivory and plant defenses on pollinators. Current Biology 28: R1164–R1169.

Jaiswal, S. K., M. Mohammed, F. Y. I. Ibny, and F. D. Dakora. 2021. Rhizobia as a source of plant growth-promoting molecules: Potential applications and possible operational mechanisms. Frontiers in Sustainable Food Systems 4: 619676.

Janzen, D. H. 1985. The Natural History of Mutualisms. Pages 41-99 *in* D. H. Boucher, editor. The biology of mutualism: Ecology and evolution. Oxford University Press, Oxford, United Kingdom.

Jin, Y., H. Qian. 2019. V.PhyloMaker: an R package that can generate very large phylogenies for vascular plants. Ecography 42: 1353–1359.

Jin, Z., J. Li, and Y. Li. 2015. Interactive effects of arbuscular mycorrhizal fungi and copper stress on flowering phenology and reproduction of *Elsholtzia splendens*. PLoS ONE 10: e0145793.

Johnson, N. C., J. H. Graham, and F. A. Smith. 1997. Functioning of mycorrhizal associations along the mutualism–parasitism continuum. The New Phytologist 135: 575–585.

Johnson, N. M., and R. S. Baucom. 2022. Dicamba drift alters plant-herbivore interactions at the agro-ecological interface. Ecosphere 13: e4272.

Johnson, N. M., G. Zhang, A. Soble, S. Johnson, and R. S. Baucom. 2023. The consequences of synthetic auxin herbicide on plant-herbivore interactions. Trends in Plant Science 28: 765–775.

Jones, K. M., H. C. Mendis, and C. Queiroux. 2013. Single-plant, sterile microcosms for nodulation and growth of the legume plant *Medicago truncatula* with the rhizobial symbiont *Sinorhizobium meliloti*. Journal of Visualized Experiments 80: 50916.

Karamaouna, F., V. Kati, N. Volakakis, K. Varikou, N. Garantonakis, L. Economou, A. Birouraki, E. Markellou, S. Liberopoulou, and M. Edwards. 2019. Ground cover management with mixtures of flowering plants to enhance insect pollinators and natural enemies of pests in olive groves. Agriculture, Ecosystems & Environment 274: 76–89.

Karmakar, K., A. Rana, A. Rajwar, M. Sahgal, and B. N. Johri. 2015. Legume-rhizobia symbiosis under stress. Pages 241-258 *in* N. Arora, editor. Plant microbe symbiosis: Applied facets. First edition. Springer India, New Dehli, India.

Kassambara, A. 2019. ggpubr: 'ggplot2' based publication ready plots. R package version 0.2.2. https://CRAN.R-project.org/package=ggpubr.

Kati, V., F. Karamaouna, L. Economou, P. V. Mylona, M. Samara, M.-D. Mitroiu, M. Barda, M. Edwards, and S. Liberopoulou. 2021. Sown wildflowers enhance habitats of pollinators and beneficial arthropods in a tomato field margin. Plants 10: 1003.

Katti, S. K., A. V. Rao. 1968. Handbook of the Poisson distribution. Technometrics 10: 412.

Kelley, K. B., D. E. Riechers. 2007. Recent developments in auxin biology and new opportunities. Pesticide Biochemistry and Physiology 89: 1-11

Kent, M., P. Coker. 1992. Vegetation description and analysis: A practical approach. Belhaven press, London, United Kingdom.

Kersten, S., J. Chang, C. D. Huber, Y. Voichek, . . ., and F. A. Rabanal. 2023. Standing genetic variation fuels rapid evolution of herbicide resistance in blackgrass. Proceedings of the National Academy of Sciences of the United States of America 120: e2206808120.

Kiers, E. T., R. A. Rousseau, S. A. West, and R. F. Denlson. 2003. Host Sanctions and the Legume–Rhizobium Mutualism. Nature 425: 78–81.

Kiers, E. T., T. M. Palmer, A. R. Ives, J. F. Bruno, and J. L. Bronstein. 2010. Mutualisms in a Changing World: An Evolutionary Perspective. Ecology Letters 13: 1459-1474.

Klein, A. M., B. E. Vaissière, J. H. Cane, I. Steffan-Dewenter, S. A. Cunningham, C. Kremen, and T. Tscharntke. 2007. Importance of pollinators in changing landscapes for world crops. Proceedings of the Royal Society B: Biological Sciences 274: 303–313.

Kleinert, A., R. Thuynsma, A. Magadlela, V. A. Benedito, and A. J. Valentine. 2017. Metabolism and transport of carbon in legume nodules under phosphorus deficiency. Pages 77-95 *in* S. Sulieman, and L. S. Tran, editors. Legume nitrogen fixation in soils with low phosphorus availability. Springer International Publishing, Cham, Switzerland.

Kluser, S., and P. Peduzzi. 2007. Global pollinator decline: A literature review. UNEP/GRID-Europe, Geneva, Switzerland.

Knezevic, S. Z., O. A. Osipitan, and J. E. Scott. 2018. Sensitivity of grape and tomato to micro-rates of dicamba-based herbicides. Journal of Horticulture 5: 1–5.

Kudo, G., T. Y. Ida. 2013. Early onset of spring increases the phenological mismatch between plants and pollinators. Ecology 94: 2311–2320.

Larson, D.L., P. A. Rabie, S. Droege, J. L. Larson, M. Haar. 2016. Exotic plant infestation is associated with decreased modularity and increased numbers of connectors in mixed-grass prairie pollination networks. PLoS ONE 11: e0155068.

Lebrazi, S., M. Fadil, M. Chraibi, and K. Fikri-Benbrahim. 2020. Screening and optimization of indole-3-acetic acid production by Rhizobium sp. strain using response surface methodology. Journal of Genetic Engineering and Biotechnology 18: 21.

Leite, D. C. A., J. F. Salles, E. N. Calderon, C. B. Castro, A. Bianchini, J. A. Marques, J. D. van Elsas, and R. S. Peixoto. 2018. Coral bacterial-core abundance and network complexity as proxies for anthropogenic pollution. Frontiers in Microbiology 0: 833.

Lenth, R., H. Singmann, J. Love, P. Buerkner, and M. Herve. 2018. Package 'emmeans', R package version 4.0-3. http://cran.r-project.org/package=emmeans.

Liao, K., R. W. Gituru, Y.-H. Guo, and Q.-F. Wang. 2011. The presence of co-flowering species facilitates reproductive success of *Pedicularis monbeigiana* (Orobanchaceae) through variation in bumble-bee foraging behaviour. Annals of Botany 108: 877–884.

Lindström, K., and S. A. Mousavi. 2020. Effectiveness of nitrogen fixation in rhizobia. Microbial Biotechnology 13: 1314–35.

Liscum, E., S. K. Askinosie, D. L. Leuchtman, J. Morrow, K. T. Willenburg, and D. R. Coats. 2014. Phototropism: growing towards an understanding of plant movement. The Plant Cell 26: 38–55.

Ma, Q. L., A. E. Smith, J. E. Hook, R. E. Smith, and D. C. Bridges. 1999. Water runoff and pesticide transport from a golf course fairway: Observations vs. Opus model simulations. Journal of Environmental Quality 28: 1463–1473.

Maggi, F., F. H. M. Tang, and F. N. Tubiello. 2023. Agricultural pesticide land budget and river discharge to oceans. Nature 620: 1013–1017.

Malinda Sameera Thilakarathna, R. M. 2013. Genotypic variability among diverse red clover cultivars for nitrogen fixation and transfer. Dissertation. Dalhousi University, Halifax, Canada.

Marshall, E. J. P., V. K. Brown, N. D. Boatman, P. J. W. Lutman, G. R. Squire, and L. K. Ward. 2003. The role of weeds in supporting biological diversity within crop fields. Weed Research 43: 77–89.

Mårtensson, A. M. 1992. Effects of agrochemicals and heavy metals on fast-growing rhizobia and their symbiosis with small-seeded legumes. Soil Biology and Biochemistry 24: 435–445.

Martín González, A. M., B. Dalsgaard, and J. M. Olesen. 2010. Centrality measures and the importance of generalist species in pollination networks. Ecological Complexity 7: 36–43.

Maslennikova, D., K. Nasyrova, O. Chubukova, E. Akimova, A. Baymiev, D. Blagova, A. Ibragimov, and O. Lastochkina. 2022. Effects of *Rhizobium leguminosarum* Thy2 on the growth and tolerance to cadmium stress of wheat plants. Life 12: 1675.

Mayerová M., J. Mikulka, J. Soukup. 2018. Effects of selective herbicide treatment on weed community in cereal crop rotation. Plant, Soil and Environment 64: 413–420.

Mazerolle, M. J. 2023. AICcmodavg: Model selection and multimodel inference based on (Q)AIC(c) R package version 2.3.3. https://cran.r-project.org/package=AICcmodavg.

McCown, M. 2018. Dicamba effects on soybean (*Glycine max*) growth, yield, and offspring. Thesis. University of Arkansas, Arkansas, USA.

Mcguire, K. L. 2007. Common ectomycorrhizal networks may maintain monodominance in a tropical rain forest. Ecology 88: 567–74.

Mehlferber, E. C., M. J. Song, J. N. Pelaez, J. Jaenisch, J. E. Coate, B. Koskella, and C. J. Rothfels. 2022. Polyploidy and microbiome associations mediate similar responses to pathogens in Arabidopsis. Current Biology 32: 2719-2729.e5.

Mendoza-Suárez, M. A., B. A. Geddes, C. Sánchez-Cañizares, R. H. Ramírez-González, C. Kirchhelle, B. Jorrin, and P. S. Poole. 2020. Optimizing rhizobium-legume symbioses by simultaneous measurement of rhizobial competitiveness and $N_2$ fixation in nodules. Proceedings of the National Academy of Sciences of the United States of America 117: 9822–31.

Mendoza-Suárez, M., S. U. Andersen, P. S. Poole, and C. Sánchez-Cañizares. 2021. Competition, nodule occupancy and persistence of inoculant strains: Key factors in the rhizobium-legume symbioses. Frontiers in Plant Science 12: 690567.

Mhadhbi, H., M. Jebara, A. Zitoun, F. Limam, and M. E. Aouani. 2008. Symbiotic effectiveness and response to mannitol-mediated osmotic stress of various chickpea-rhizobia associations. World Journal of Microbiology and Biotechnology 24: 1027–35.

Mikaberidze, A., and B. A. McDonald. 2020. A tradeoff between tolerance and resistance to a major fungal pathogen in elite wheat cultivars. New Phytologist 226: 879–890.

Millar, N., J. Piovia-Scott, and S. S. Porter. 2023. Impacts of domestication on the rhizobial mutualism of five legumes across a gradient of nitrogen-fertilisation. Plant and Soil 491: 479–499.

Miller, J. E. D., A. R. Ives, S. P. Harrison, and E. I. Damschen. 2018. Early- and late-flowering guilds respond differently to landscape spatial structure. Journal of Ecology 106: 1033–1045.

Miller, J. L., T. S. Schmidt, P. C. van Metre, B. J. Mahler, M. W. Sandstrom, L. H. Nowell, D. M. Carlisle, and P. W. Moran. 2020. Common insecticide disrupts aquatic communities: A mesocosm-to-field ecological risk assessment of fipronil and its degradates in U.S. streams. Science Advances 6: eabc1299.

Minchin, F. R., and J. F. Witty. 2005. Respiratory/carbon costs of symbiotic nitrogen fixation in legumes. Pages 195-205 *in* H. Lambers, and M. Ribas-Carbo, editors. Plant Respiration. Advances in Photosynthesis and Respiration. First edition, volume eight. Springer, Dordrecht, Netherlands.

Mitchell R. J., Flanagan R. J., Brown BJ, Waser NM, Karron JD. 2009. New frontiers in competition for pollination. Annals of Botany 103: 1403–1413.

Mithila J, Hall JC, Johnson WG, Kelley KB, Riechers DE. 2011. Evolution of resistance to auxinic herbicides: Historical perspectives, mechanisms of resistance, and implications for broadleaf weed management in agronomic crops. Weed Science 59: 445–457.

Mng'ong'o, M. E., F. Ojija, and B. N. Aloo. 2023. The role of rhizobia toward food production, food and soil security through microbial agro-input utilization in developing countries. Case Studies in Chemical and Environmental Engineering 8: 100404.

Moeller DA. 2004. Facilitative interactions among plants via shared pollinators. Ecology 85: 3289–3301.

Mohd-Radzman, N. A., and C. Drapek. 2023. Compartmentalisation: A strategy for optimising symbiosis and tradeoff management. Plant, Cell & Environment 46: 2998–3011.

Morgan, J. A. W., G. D. Bending, and P. J. White. 2005. Biological costs and benefits to plant–microbe interactions in the rhizosphere. Journal of Experimental Botany 56: 1729–1739.

Mortier, V., M. Holsters, and S. Goormachtig. 2012. Never too many? How legumes control nodule numbers. Plant, Cell & Environment 35: 245–58.

Mothershead, K., R. J. Marquis. 2000. Fitness impacts of herbivory through indirect effects on pollinator interactions in *Oenothera macrocarpa*. Ecology 81: 30–40.

Motta, E. V. S., K. Raymann, and N. A. Moran. 2018. Glyphosate perturbs the gut microbiota of honey bees. Proceedings of the National Academy of Sciences of the United States of America 115: 10305–10310.

Mulligan, G. 1979. The biology of Canadian weeds. First edition, volumes one - thirty-two. The Agricultural Institute of Canada, Ottawa, Canada.

Muschelli, J., J. Betz, and R. Varadhan. 2014. Binomial regression in R. Pages 257-308 *in* C. R. Roa, editor. Handbook of statistics. First edition, volume thirty-two. Elsevier, Amsterdam, Netherlands.

Nascimento, F. X., C. Brígido, B. R. Glick, and M. J. Rossi. 2016. The role of rhizobial ACC deaminase in the nodulation process of leguminous plants. International Journal of Agronomy 4: 1369472.

Nay, M. M., C. Grieder, L. A. Frey, H. Amdahl, J. Radovic, L. Jaluvka, A. Palmé, L. Skøt, T. Ruttink, and R. Kölliker. 2023. Multi-location trials and population-based genotyping reveal high diversity and adaptation to breeding environments in a large collection of red clover. Frontiers in Plant Science 14: 1128823.

Neve, P., and S. Powles. 2005. High survival frequencies at low herbicide use rates in populations of *Lolium rigidum* result in rapid evolution of herbicide resistance. Heredity 95: 485–492.

Nuismer, S. L, J. N. Thompson, and R. Gomulkiewicz. 2000. Coevolutionary Clines Across Selection Mosaics. Evolution 54: 1102–15.

O'Gorman, E. J., J. E. Fitch, and T. P. Crowe. 2012. Multiple anthropogenic stressors and the structural properties of food webs. Ecology 93: 441–448.

Olesen, J. M., J. Bascompte, Y. L. Dupont, P. Jordano. 2007. The modularity of pollination networks. Proceedings of the National Academy of Sciences 104: 19891–19896.

Ollerton, J., R. Winfree, and S. Tarrant. 2011. How many flowering plants are pollinated by animals? Oikos 120: 321–26.

Olszyk, D., T. Pfleeger, E. H. Lee, and M. Plocher. 2015. Glyphosate and dicamba herbicide tank mixture effects on native plant and non-genetically engineered soybean seedlings. Ecotoxicology 24: 1014–1027.

Olszyk, D., T. Pfleeger, T. Shiroyama, M. Blakeley-Smith, E. H. Lee, and M. Plocher. 2017. Plant reproduction is altered by simulated herbicide drift to constructed plant communities. Environmental Toxicology and Chemistry 36: 2799–2813.

Oono, R., C. G. Anderson, and R. F. Denison. 2011. Failure to fix nitrogen by non-reproductive symbiotic rhizobia triggers host sanctions that reduce fitness of their reproductive clonemates. Proceedings of the Royal Society B 278: 2698–2703.

Oseland, E., M. Bish, L. Steckel, and K. Bradley. 2020. Identification of environmental factors that influence the likelihood of off-target movement of dicamba. Pest Management Science, 76: 3282–3291.

Ouvrard, P., A. L. Jacquemart. 2018. Agri-environment schemes targeting farmland bird populations also provide food for pollinating insects. Agricultural and Forest Entomology 20: 558–574.

Parra-Tabla V., and C. F. Vargas. 2004. Phenology and phenotypic natural selection on the flowering time of a deceit-pollinated tropical orchid, *Myrmecophila christinae*. Annals of Botany 94: 243-250.

Patil, V. S., N. R. Shaikh, and S. V. Patil. 2019. Effect of different herbicides on the nodulation property of rhizobial isolates." Universal Journal of Environmental Research and Technology 2: 293-299.

Pelletier, F., and D. W. Coltman. 2018. Will human influences on evolutionary dynamics in the wild pervade the Anthropocene? BMC Biology 16: 1-10.

Peoples, M. B., D. F. Herridge, and J. K. Ladha. 1995. Biological nitrogen fixation: An efficient source of nitrogen for sustainable agricultural production? Management of Biological Nitrogen Fixation for the Development of More Productive and Sustainable Agricultural Systems 174: 3–28.

Petipas, R. H., A. C. Wruck, and M. A. Geber. 2020. Microbe-mediated local adaptation to limestone barrens is context dependent. Ecology 101: e03092.

Pimentel, D. 1995. Amounts of pesticides reaching target pests: Environmental impacts and ethics. Journal of Agricultural and Environmental Ethics 8: 17–29.

Poole, R. W., and B. J. Rathcke. 1979. Regularlity, randomness, and aggregation in flowering phenologies. Science 203: 470-471.

Porter, S. S., and E. L. Simms. 2014. Selection for cheating across disparate environments in the legume-rhizobium mutualism. Ecology Letters 17: 1121–1129.

Porter, S. S., and K. J. Rice. 2013. Trade-offs, spatial heterogeneity, and the maintenance of microbial diversity. Evolution 67: 599–608.

Porter, S. S., R. Bantay, C. A. Friel, A. Garoutte, K. Gdanetz, K. Ibarreta, B. M. Moore, P. Shetty, E. Siler, and M. L. Friesen. 2020. Beneficial microbes ameliorate abiotic and biotic sources of stress on plants. Functional Ecology 34: 2075–86.

Potts, S. G., J. C. Biesmeijer, C. Kremen, P. Neumann, O. Schweiger, and W. E. Kunin. 2010. Global pollinator declines: Trends, impacts and drivers. Trends in Ecology and Evolution 25: 345–353.

Provorov, N. A., and I. A. Tikhonovich. 2003. Genetic resources for improving nitrogen fixation in legume-rhizobia symbiosis. Genetic Resources and Crop Evolution 50: 89–99.

Qi, Y., J. Li, X. Guan, B. Yan, G. Fu, J. He, L. Du, C. Zhao, and D. Zhang. 2020. Effects of herbicides on non-target plant species diversity and the community composition of fallow fields in northern China. Scientific Reports 10: 1–10.

Qin, Y., I. S. Druzhinina, X. Pan, and Z. Yuan. 2016. Microbially mediated plant salt tolerance and microbiome-based solutions for saline agriculture. Biotechnology Advances, 34: 1245–1259.

Quides, K. W., F. Salaheldine, R. Jariwala, and J. L. Sachs. 2021. Dysregulation of host-control causes interspecific conflict over host investment into symbiotic organs. Evolution 75: 1189–1200.

R Core Team. 2019. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna Austria. https://www.R-project.org/.

R Core Team. 2021. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. https://www.R-project.org/.

R Core Team. 2022. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. https://www.R-project.org/.

Rahman, A., M. Manci, C. Nadon, I. A. Perez, W. F. Farsamin, M. T. Lampe, T. H. Le, L. Torres Martínez, A. J. Weisberg, J. H. Chang, and J. L. Sachs. 2023. Competitive interference among rhizobia reduces benefits to hosts. Current Biology 33: 2988-3001.

Rainio, M. J., A. Margus, P. Lehmann, M. Helander, and L. Lindström. 2019. Effects of a glyphosate-based herbicide on survival and oxidative status of a non-target herbivore, the Colorado potato beetle (*Leptinotarsa decemlineata*). Comparative Biochemistry and Physiology. Toxicology & Pharmacology 215: 47–55.

Ramos, S.E., L. D. Rzodkiewicz, M. M. Turcotte, and T.-L. Ashman. 2021. Damage and recovery from drift of synthetic-auxin herbicide dicamba depends on concentration and varies among floral, vegetative, and lifetime traits in rapid cycling *Brassica rapa*. Science of The Total Environment 801: 149732.

Ramos, S. E., M. Bakhtiari, M. Castañeda-Zárate, V. Iriart, and T.-L. Ashman. 2023. Herbicides and their potential to disrupt plant–insect chemical communication. Journal of Systematics and Evolution 61: 550–560.

Rathcke, B. 1988. Flowering phenologies in a shrub community: Competition and constraints. Journal of Ecology 76: 975-994.

Regus, J. U., K. A. Gano, A. C. Hollowell, and J. L. Sachs. 2014. Efficiency of partner choice and sanctions in *Lotus* is not altered by nitrogen fertilization. Proceedings of the Royal Society B 281: 20132587.

Regus, J. U., K. A. Gano, A. C. Hollowell, V. Sofish, and J. L. Sachs. 2015. Lotus hosts delimit the mutualism–parasitism continuum of *Bradyrhizobium*. Journal of Evolutionary Biology 28: 447–456.

Regus, J. U., C. E. Wendlandt, R. M. Bantay, K. A. Gano-Cohen, N. J. Gleason, A. C. Hollowell, M. R. O'Neill, K. K. Shahin, and J. L. Sachs. 2017. Nitrogen deposition decreases the benefits of symbiosis in a native legume. Plant and Soil, 414: 159–170.

Remans, R., L. Ramaekers, S. Schelkens, G. Hernandez, A. Garcia, J. L. Reyes, N. Mendez, V. Toscano, M. Mulling, L. Galvez, and J. Vanderleyden. 2008. Effect of *Rhizobium-Azospirillum* coinoculation on nitrogen fixation and yield of two contrasting *Phaseolus vulgaris L.* genotypes cultivated across different environments in Cuba. Plant and Soil 312: 25–37.

Requier, F., J. F. Odoux, T. Tamic, N. Moreau, M. Henry, A. Decourtye, and V. Bretagnolle. Honey bee diet in intensive farmland habitats reveals an unexpectedly high flower richness and a major role of weeds. Ecological Applications 25: 881–890.

Revell, L. J. 2012. phytools: An R package for phylogenetic comparative biology (and

other things). Methods in Ecology and Evolution 3: 217-223.

Reynolds, H. L., A. Packer, J. D. Bever, and K. Clay. 2003. Grassroots Ecology: Plant-microbe-soil interactions as drivers of plant community structure and dynamics. Ecology, 84: 2281–2291.

Rice, P. J., B. P. Horgan, and J. L. Rittenhouse. 2010. Pesticide transport with runoff from creeping bentgrass turf: Relationship of pesticide properties to mass transport. Environmental Toxicology and Chemistry 29: 1209–1214.

Rich, J. T., J. G. Neely, R. C. Paniello, C. C. J. Voelker, B. Nussenbaum, and E. W. Wang. 2010. A practical guide to understanding Kaplan-Meier curves. Official Journal of American Academy of Otolaryngology-Head and Neck Surgery. 143: 331–336.

Rippy, M. A., A. Deletic, J. Black, R. Aryal, J.-L. Lampard, J. Yat-Man Tang, D. McCarthy, P. Kolotelo, J. Sidhu, and W. Gernjak. 2017. Pesticide occurrence and spatio-temporal variability in urban run-off across Australia. Water Research 115: 245–255.

Ristova, D., M. Giovannetti, K. Metesch, and W. Busch. 2018. Natural genetic variation shapes root system responses to phytohormones in *Arabidopsis*. Plant Journal 96: 468–81.

Riter, L. S., N. Pai, B. C. Vieira, A. Macinnes, R. Reiss, C. J. Hapeman, and G. R. Kruger. 2021. Conversations about the future of dicamba: The science behind off-target movement. Journal of Agricultural and Food Chemistry 69: 14435–14444.

Sachs, J. L., and E. L. Simms. 2006. Pathways to Mutualism Breakdown. Trends in Ecology & Evolution 21: 585–92.

Sachs, J. L., J. E. Russell, Y. E. Lii, K. C. Black, G. Lopez, and A. S. Patil. 2010. Host Control over Infection and Proliferation of a Cheater Symbiont. Journal of Evolutionary Biology 23: 1919–27.

Sachs, J. L., K. W. Quides, and C. E. Wendlandt. 2018. Legumes versus rhizobia: A model for ongoing conflict in symbiosis. New Phytologist 219: 1199–1206.

Saraf, M., N. K. Arora, and V. Kumar. 1999. Effect of 2,4-D on NR, NiR and leghaemoglobin synthesis in root nodules formed by *Bradyrhizobium japonicum*. Microbes and Environments, 14: 219–225.

Sarkar, D., and S. Laha. 2013. Production of phytohormone auxin (IAA) from soil born Rhizobium sp, isolated from different leguminous plant. International Journal of Applied Environmental Sciences 8: 521–28.

Saunders, M. E. 2018. Insect pollinators collect pollen from wind-pollinated plants: Implications for pollination ecology and sustainable agriculture. Insect Conservation and Diversity 11: 13–31.

Schindelin, J., I. Arganda-Carreras, E. Frise, V. Kaynig, M. Longair, T. Pietzsch, . . . , and A. Cardona. 2012. Fiji: An open-source platform for biological-image analysis. Nature 9: 676–82.

Schoener, T. W. 1970. Nonsynchronous spatial overlap of lizards in patchy habitats. Ecology 51: 408-418.

Schütte G., M. Eckerstorfer, V. Rastelli, W. Reichenbecher, S. Restrepo-Vassalli, M. Ruohonen-Lehto, A.-G. Wuest Saucy, and M. Mertens. 2017. Herbicide resistance and biodiversity: Agronomic and environmental aspects of genetically modified herbicide-resistant plants. Environmental Sciences Europe 29: 5.

Schwartz, M. W., and J. D. Hoeksema. 1998. Specialization and resource trade: Biological Markets as a Model of Mutualisms. Ecology 79: 1029–1038.

Searle, S.R., F. M. Speed, and. G. A. Milliken. 1980. Population marginal means in the linear model: An alternative to least squares means. American Statistician 34: 216–221.

Shahid, M., and M. S. Khan. 2018. Glyphosate induced toxicity to chickpea plants and stress alleviation by herbicide tolerant phosphate solubilizing *Burkholderia cepacia* PSBB1 carrying multifarious plant growth promoting activities. 3 Biotech 8: 1–17.

Sharma, A. K. 2005. Text book of correlations and regression. Discovery Publishing House, New Delhi, India.

Smith, S. A., and J. W. Brown. 2018. Constructing a broadly inclusive seed plant phylogeny. American Journal of Botany 105: 302–314.

Smith, S. E., F. A. Smith, and I. Jakobsen. 2004. Functional diversity in arbuscular mycorrhizal (AM) symbioses: the contribution of the mycorrhizal P uptake pathway is not correlated with mycorrhizal responses in growth or total P uptake. New Phytologist 162: 511–524.

Spaepen, S., and J. Vanderleyden. 2011. Auxin and plant-microbe interactions. Cold Spring Harbor Perspectives in Biology 3: a001438.

Sprent, J. I. 2007. Evolving ideas of legume evolution and diversity: A taxonomic perspective on the occurrence of nodulation. The New Phytologist 174: 11–25.

Strandberg, B., P. B. Sørensen, M. Bruus, R. Bossi, Y. L. Dupont, M. Link, and C. F. Damgaard. 2021. Effects of glyphosate spray-drift on plant flowering. Environmental Pollution 280: 116953.

Stuart, A. 1955. A test for homogeneity of the marginal distributions in a two-way classification. Biometrika 42: 412-416.

Tardif, F. J., I. Rajcan, and M. Costea. 2006. A mutation in the herbicide target site acetohydroxyacid synthase produces morphological and structural alterations and reduces fitness in Amaranthus powellii. The New Phytologist 169: 251–264.

Teixido, A. L., L. F. Fuzessy, C. S. Souza, I. N. Gomes, L. A. Kaminski, P. C. Oliveira, and P. K. Maruyama. 2022. Anthropogenic impacts on plant-animal mutualisms: A global synthesis for pollination and seed dispersal. Biological Conservation 266: 109461.

Therneau, T. M., and P. M. Grambsch. 2000. Modeling survival data: Extending the Cox model. Springer, New York City, New York, USA.

Thies, J. E., P. W. Singleton, and B. B. Bohlool. 1995. Phenology, growth, and yield of field-grown soybean and bush bean as a function of varying modes of N nutrition. Soil Biology and Biochemistry 27: 575–583.

Thompson, J. N. 2005 The geographic mosaic of coevolution. University of Chicago Press, Chicago, Illinois, USA.

Thompson, J. N., S. L. Nuismer, and R. Gomulkiewicz. 2002. Coevolution and maladaptation. Integrative and Comparative Biology 42: 381–87.

Thrall, P. H., J. D. Bever, and J. F. Slattery. 2008. Rhizobial mediation of *Acacia* adaptation to soil salinity: Evidence of underlying trade-offs and tests of expected patterns. Journal of Ecology 96: 746–55.

Timberlake, T.P., I. P. Vaughan, J. Memmott. 2019. Phenology of farmland floral resources reveals seasonal gaps in nectar availability for bumblebees. Journal of Applied Ecology 56: 1585–1596.

Tosi, S., G. Burgio, and J. C. Nieh. 2017. A common neonicotinoid pesticide, thiamethoxam, impairs honey bee flight ability. Scientific Reports 7: 1–8.

Traveset, A., and D. M. Richardson. 2014. Mutualistic interactions and biological invasions. Annual Review of Ecology, Evolution, and Systematics 45: 89–113.

Trněný, O., D. Vlk, E. Macková, M. Matoušková, J. Řepková, J. Nedělník, J. Hofbauer, K. Vejražka, H. Jakešová, J. Jansa, L. Piálek, and D. Knotová. 2019. Allelic variants for candidate nitrogen fixation genes revealed by sequencing in red clover (*Trifolium pratense L.*). International Journal of Molecular Sciences 20: 5470.

USDA. 2018. PLANTS database. https://plants.usda.gov/home

U. S. Geological Survey. 2020. Estimated Agricultural Use for dicamba. https://water.usgs.gov/nawqa/pnsp/usage/maps/show_map.php?year=2017&map=DICAMBA&hilo=L&disp=Dicamba.

U. S. Geological Survey. 2021. Estimated agricultural use for dicamba. https://water.usgs.gov/nawqa/pnsp/usage/maps/show_map.php?year=2017&map=DICAMBA&hilo=L&disp=Dicamba

U. S. Geological Survey. 2024. Estimated agricultural use for dicamba. https://water.usgs.gov/nawqa/pnsp/usage/maps/show_map.php?year=2017&map=DICAMBA&hilo=L&disp=Dicamba

US EPA. 2021. Status of over-the-top dicamba: summary of 2021 usage, incidents and consequences of off target movement, and impacts of stakeholder-suggested mitigations (DP # 464173: PC Code 128931). United States Environmental Protection Agency, Washington D. C., USA.

Vaidya, P., and J. R. Stinchcombe. 2020. The potential for genotype-by-environment interactions to maintain genetic variation in a model legume-rhizobia mutualism. Plant Communications 1: 100114.

van Bragt, J., L. M. Rohrbaugh, and S. H. Wender. 1965. The effect of 2,4-dichlorophenoxyacetic acid on the rutin content of tomato plants. Phytochemistry 4: 963–965.

van Cauwenberghe, J., W. Visch, J. Michiels, and O. Honnay. 2016. Selection mosaics differentiate *Rhizobium*–host plant interactions across different nitrogen environments. Oikos 125: 1755–1761.

Vance, G. F., A. J. Krzyszowska, and G. F. Vace. 1994. Monitoring dicamba and picloram movement and fate in the vadose zone for groundwater quality protection in Wyoming. (Report No. WWRC-94-19). The Department of Interior U.S. Geological Survey and Wyoming Water Resources Center, USA.

Vieira, B. C., J. D. Luck, K. L. Amundsen, R. Werle, T. A. Gaines, and G. R. Kruger. 2020. Herbicide drift exposure leads to reduced herbicide sensitivity in *Amaranthus* spp. Scientific Reports 10: 1–11.

Vila-Aiub, M. M., P. Neve, S. B. Powles. 2009. Fitness costs associated with evolved herbicide resistance alleles in plants. New Phytologist 184: 751–767.

Vincent, J. M. 1970. A manual for the practical study of root-nodule bacteria. Blackwell Scientific Publishing, Oxford-Edinburgh, United Kingdom.

Violle, C., E. Garnier, J. Lecoeur, C. Roumet, C. Podeur, A. Blanchard, and M. L. Navas. 2009. Competition, traits and resource depletion in plant communities. Oecologia 160: 747–755.

Vitt, P., K. Havens, C. L. Jolls, and T. M. Knight. 2020. Temporal variation in the roles of exotic and native plant species in plant–pollinator networks. Ecosphere 11: e02981.

Voos, G., P. M. Groffman, and R. M. Groffmann. 1997. Relationships between microbial biomass and dissipation of 2,4-D and dicamba in soil. Biology and Fertility of Soils 24: 106–110.

Wang, Q., J. Liu, and H. Zhu. 2018. Genetic and molecular mechanisms underlying symbiotic specificity in legume-rhizobium interactions. Frontiers in Plant Science 9: 334639.

Wang, Y., M. Wang, Y. Li, A. Wu, and J. Huang. 2018. Effects of arbuscular mycorrhizal fungi on growth and nitrogen uptake of *Chrysanthemum morifolium* under salt stress. PLoS One 13: e0196408.

Wani, P. A., and M. S. Khan. 2013. Nickel detoxification and plant growth promotion by multi metal resistant plant growth promoting rhizobium species RL9. Bulletin of Environmental Contamination and Toxicology 91: 117–124.

Waser, N. M., and L. A. Real LA. 1979. Effective mutualism between sequentially flowering plant species. Nature 281: 670–672.

Watts, S., C. F. Dormann, A. M. Martín González, and J. Ollerton. 2016. The influence of floral traits on specialization and modularity of plant–pollinator networks in a biodiversity hotspot in the Peruvian Andes. Annals of Botany 118: 415–429.

Watts, D. W., and J. K. Hall. 1996. Tillage and application effects on herbicide leaching and runoff. Soil and Tillage Research 39: 241–257.

Weese, D. J., K. D. Heath, B. T. M. Dentinger, and J. A. Lau. 2015. Long-term nitrogen addition causes the evolution of less-cooperative mutualists. Evolution; International Journal of Organic Evolution 69: 631–42.

West, S. A., E. T. Kiers, E. L. Simms, and R. F. Denison. 2002. Sanctions and mutualism stability: Why do rhizobia fix nitrogen? Proceedings of the Royal Society B 269: 685–94.

Westhoek, A., L. J. Clark, M. Culbert, N. Dalchau, . . ., and L. A. Turbull. 2021. Conditional sanctioning in a legume-rhizobium mutualism. Proceedings of the National Academy of Sciences of the United States of America 118: e2025760118.

Wickham, H. 2016. ggplot2: Elegant graphics for data analysis. Springer-Verlag, New York City, New York, USA.

Wilson, S. D., and D. Tilman. 1993. Plant competition and resource availability in response to disturbance and fertilization. Ecology 74: 599–611.

Wood, C. W., and E. D. Brodie. 2015. Environmental effects on the structure of the G-matrix. Evolution; International Journal of Organic Evolution 69: 2927–2940.

Wyatt, G. A. K., E. T. Kiers, A. Gardner, and S. A. West. 2014. A biological market analysis of the plant-mycorrhizal symbiosis. Evolution; International Journal of Organic Evolution 68: 2603–18.

Xiong, Y, and Y. Jiao. 2019. The diverse roles of auxin in regulating leaf development. Plants 8: 243.

Younginger, B. S., D. Sirová, M. B. Cruzan, D. J. Ballhorn. 2017. Is biomass a reliable estimate of plant fitness? Applications in Plant Sciences 5: 1600094.

Zaidi, A., M. S. Khan, and P. Q. Rizvi. 2005. Effect of herbicides on growth, nodulation and nitrogen content of greengram. Agronomy for Sustainable Development 25: 497–504.

Zhang, Q., J. Li, H. Chen, X. Xuan, D. Xu, and Y. Wen. 2024. Mechanisms underlying allelopathic disturbance of herbicide imazethapyr on wheat and its neighboring ryegrass (*Lolium perenne*). Journal of Agricultural and Food Chemistry 72: 3445–3455.

Zhang, Y., J. Xu, R. Li, Y. Ge, Y. Li, and R. Li. 2023. Plants' response to abiotic stress: Mechanisms and strategies. International Journal of Molecular Sciences 24: 10915.

Zheng, Y., Y.-C. Kim, X.-F. Tian, L. Chen, W. Yang, C. Gao, M.-H. Song, X.-L. Xu, and L.-D. Guo. 2014. Differential responses of arbuscular mycorrhizal fungi to nitrogen addition in a near pristine Tibetan alpine meadow. FEMS Microbiology Ecology 89: 594–605.

Zuur, A. F., E. N. Ieno, N. Walker, A. A. Saveliev, and G. M. Smith. 2009. Mixed effects models and extensions in ecology with R. Springer-Verlag, New York City, New York, USA.