Effect of Prior Herbicide Exposure on Rhizobial Response to Glyphosate

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ABSTRACT

The effects of herbicides on rhizobia have been well-documented, but little focus has been given to understanding if rhizobia tolerances to herbicide can be explained by prior exposure history. I aimed to test the effect of prior exposure in the field on in-vitro rhizobia tolerances by comparing bacterial growth in increasing glyphosate concentrations between strains isolated from a prior-exposed environment (UC Berkeley's campus) and a non prior-exposed environment (Ohlone Park, Berkeley, CA). I also isolated rhizobia from four sites within these two larger environments, to further test the role of host strain/site in defining strain tolerances. The strains' life histories were defined by these two nested conditions, but I found that prior exposure history and site of origin did not significantly define strain tolerances and that strains had incredible variation in tolerances as well as in patterns of tolerance. This suggests that the diverse responses of rhizobia to glyphosate found in previous studies is likely encoded on a strain-by-strain basis with little relation to environmental boundaries. These diverses tolerances within a single species of *Rhizobium* could be relevant in selecting optimal strains for the agricultural practice of pre-inoculating legume seeds with their compatible rhizobia.

KEYWORDS

Life history ecology, pesticide exposure, environmental adaptation, rhizobia, glyphosate

INTRODUCTION

Rhizobia are soil bacteria that establish nitrogen-fixing symbiosis in the nodules of legume roots, converting atmospheric nitrogen into a reduced form usable by plants. Host legumes incorporate this reduced form of nitrogen into important biomolecules required for plant growth and development, including photosynthetic pigments, ATP, and proteins, and they in turn provide photosynthetic sugars to the rhizobia to fuel the fixation process (Wagner 2011). Symbiosis with rhizobia is thus critical for the agricultural production of legume crops such as soybeans, which were the second most-valued crop in the United States in 2020, at $46.1 billion (USDA 2021). Given this integral role of rhizobia for agriculture, legume seeds are often pre-inoculated with their compatible species of rhizobia (USDA 2021). Additionally, as commercially important crops often grown in monoculture conditions that make them susceptible to pests and weeds, legume seeds are often pre-treated with herbicide (Mallik and Tesfai 1983), and crops continue receiving herbicide treatment once growing in the field. Therefore, legumes and their symbiotic rhizobia, especially those involved in agriculture, often have a history of exposure to pesticides.

Glyphosate is one of the main pesticides that legumes and their symbiotic partners are exposed to in different stages of crop development. Glyphosate's effectiveness as a herbicide was discovered by Monsanto, and, as a commercial product subsequently sold under the name Roundup, it is now the most widely used herbicide in the United States in terms of treated area (EPA 2019). Although glyphosate is also used in non-agricultural settings such as residential, landscaping, and right-of-way applications, the majority of glyphosate is used in agriculture and specifically on soybeans (Benbrook 2016). Additionally, agricultural usage has increased 300-fold in the four decades following its introduction in 1974 (Benbrook 2016). This ubiquitous glyphosate application will likely continue to increase because of continuing use of Roundup-Ready (glyphosate tolerant) crops, emergence of glyphosate-resistant weeds, and its use as a desiccant for grain and bean harvest (Benbrook 2016, Myers et al. 2016). As such, it is critical to see how rhizobia are affected by long-term herbicide exposure in their role as symbiotic partners to important host plants.

Rhizobia exposure to pesticides including glyphosate has been well-studied. However, most studies evaluating pesticide effects have focused on 1) whether the pesticides have adverse

effects on legumes, rhizobia, or their symbiosis (Castro et al. 1997), and 2) the wide range of strain tolerances to pesticides (Zabaloy and Gómez 2005, Drouin et al. 2010, Hamuda 2020). Less focus has been given to understanding the role that prior exposure history and potential environmental adaptation plays in explaining these diverse responses. Drouin et al. (2010) started exploring this question using arctic (pesticide-free) and agricultural (pesticide-exposed) rhizobial strains; however, notwithstanding pesticide exposure differences, the strains originated from a variety of geographic backgrounds, which could make the question of the role of prior environmental exposure difficult to discern if other environmental differences due to geography were more significant in shaping the rhizobia. As such, I aim to expand on this question of the role of prior environmental exposure to glyphosate in explaining rhizobial response to herbicide by using strains isolated from pesticide and non-pesticide environments both within the city of Berkeley.

In the present study, I investigate the role of prior herbicide exposure in explaining rhizobial response to glyphosate. Specifically, I want to know if there is a difference in rhizobial growth in response to increasing glyphosate concentrations between two groups: rhizobia isolated from host plants on UC Berkeley's campus (prior herbicide exposure) and rhizobial strains isolated from a Berkeley city park (no prior exposure). Strains were isolated from four sites within each of these environments. Through this design, I also ask if there is a difference in rhizobia tolerance to glyphosate between individual sites/hosts.

METHODS

Study site description

To see if there was a difference in rhizobial response to increasing glyphosate concentrations between prior-exposed and non prior-exposed environments, I sampled from multiple sites within University of California, Berkeley and Ohlone Park, also located in Berkeley, California (Figure 1). I used sites on UC Berkeley's campus, which had a known history of herbicide application to control weeds (EL Simms, personal observation), as my prior exposed environment, while Ohlone Park, which was unlikely to have a history of herbicide application (City of Berkeley), served as my non prior-exposed environment.

Sample collection

To gather prior herbicide-exposed rhizobial strains, I sampled five sites (sites 1-5) on UC Berkeley's campus in April 2019 and isolated strains of *Rhizobium leguminosarum bv. trifolii* from the nodules of white clover plants (*Trifolium repens*) (Figure 1). To gather non-prior exposed rhizobial strains, I sampled four sites (sites 6-9) in Ohlone Park in September 2019 and isolated strains of *Rhizobium leguminosarum bv. trifolii* from the nodules of white clover plants (*Trifolium repens*) (Figure 1). All strains within each site originated from one host plant with the exception of site 4, where I sampled from three host clovers. I isolated 73 total strains from host clovers but dropped site 1 because sampling there yielded only one isolated strain. In all, I used 72 strains throughout the experiment.

After initial collection in 2019, I stored the strains at -80℃. In December 2021, I made fresh stocks of the 72 strains from the -80℃ freezer and stored them in glycerol at -20℃ for use throughout the experiment.

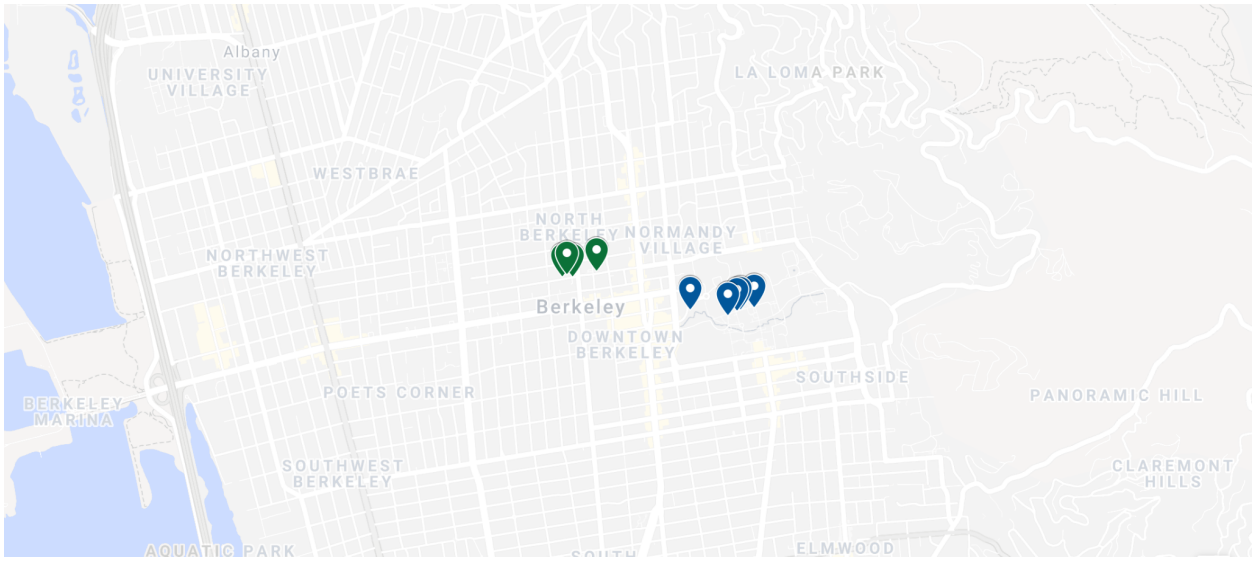


Figure 1. Site locations. Sites 1-5 (Berkeley: prior exposed) are labeled in blue, and sites 6-9 (Ohlone Park: non-prior exposed) are labeled in green.

Herbicide information

To test the role of prior exposure to herbicide on glyphosate tolerance, I prepared four stock solutions of glyphosate in distilled water solvent: 6.5 mg mL^{-1}; 13 mg mL^{-1}; 39 mg mL^{-1}; and 117 mg mL^{-1}. These concentrations are presented in terms of acid equivalent glyphosate and were made as serial dilutions of 53.8% aquatic glyphosate (glyphosate, without surfactants, in its isopropylamine salt form). I sterilized all solutions by passing them through a 0.22 micron syringe filter.

Direct enrichment method for glyphosate exposure

To quantify rhizobia strain tolerance to glyphosate, I used a direct enrichment method (Maldani et al. 2018). I first grew rhizobial strains in tryptone-yeast (TY) broth for 1-2 days and scaled them to a uniform OD_{650} = 0.05. I applied 40 µL of the four stock glyphosate solutions to cells of a 96 deep-well plate (2 mL capacity), each containing 1 mL of the strain at OD_{650} = 0.05, to expose the bacteria to final glyphosate concentrations of 0.25 mg mL^{-1}; 0.5 mg mL^{-1}; 1.5 mg mL^{-1}; and 4.5 mg mL^{-1} of solution. I also inoculated 1 mL of each strain at OD_{650} = 0.05 with 40 µL of sterile distilled water to act as the 0 mg mL^{-1} glyphosate solution and to account for any solvent effects.

In all, there were five cells for each strain, containing 0 mg mL^{-1}; 0.25 mg mL^{-1}; 0.5 mg mL^{-1}; 1.5 mg mL^{-1}; and 4.5 mg mL^{-1} of glyphosate solution. I incubated the 96 deep-well plates containing the bacterial herbicide solutions in a shaker at 30 ℃ and 220 rpm for 45 hours (Singh and Wright 2002). After 45 hours, I measured the optical density at 650 nm (OD_{650}) to quantify bacterial growth and repeated the experiment twice.

Statistical analysis

To determine if prior herbicide exposure could explain rhizobial tolerance to increasing glyphosate concentrations, as well as if there were significant differences in tolerance between hosts/locations, I used multiple regression analysis on the log-transformed growth data to test for significant interactions (1) between glyphosate concentration and environment, and (2) between

glyphosate concentration and site. I also used regression analysis to test for the main effect of glyphosate concentration.

RESULTS

Data collection and analysis

I isolated 72 strains of *Rhizobium leguminosarum bv. trifolii* from locations in Ohlone Park (non prior-exposed) and on UC Berkeley's campus (prior-exposed). Of the 72 strains, 38 came from 4 sites on Berkeley's campus and 34 were isolated from 4 sites in Ohlone Park.

I tested strain response to glyphosate at five glyphosate concentrations: 0 mg mL^{-1}; 0.25 mg mL^{-1}; 0.5 mg mL^{-1}; 1.5 mg mL^{-1}; and 4.5 mg mL^{-1} to see if there were significant differences in how strains responded to increasing glyphosate concentrations based on site and prior exposure characteristics. At the 4.5 mg mL^{-1} glyphosate concentration, I found some unusually high optical density values, and subsequent serial dilution analysis showed inconsistencies between strains in whether these values were measuring live or dead cells. As such, I removed this data point in the analyses. I conducted the regression analyses on the log-transformed optical density (growth) values for the remaining four glyphosate concentrations and used a significance level of α=.05.

Comparing prior and non-prior exposed strain tolerances

At each of the four glyphosate concentrations, I found a significant difference in growth between campus rhizobia strains and strains isolated from Ohlone Park ($t(70)$, $p < .05$). At each of the concentrations, I found higher growth in Ohlone Park strains compared to Berkeley strains (Figure 2). This significant difference was largely driven by site 6 (Figure 3): when I removed site 6 from the t-test analysis, growth for Ohlone Park strains was only significantly greater than that of Berkeley strains at the 0 mg mL^{-1} concentration ($t(63)=-2.370$, $p=.021$).

However, I found no significant difference in patterns of strain growth in response to increasing glyphosate concentrations between Berkeley and Ohlone Park (p=.592) (Figure 2).

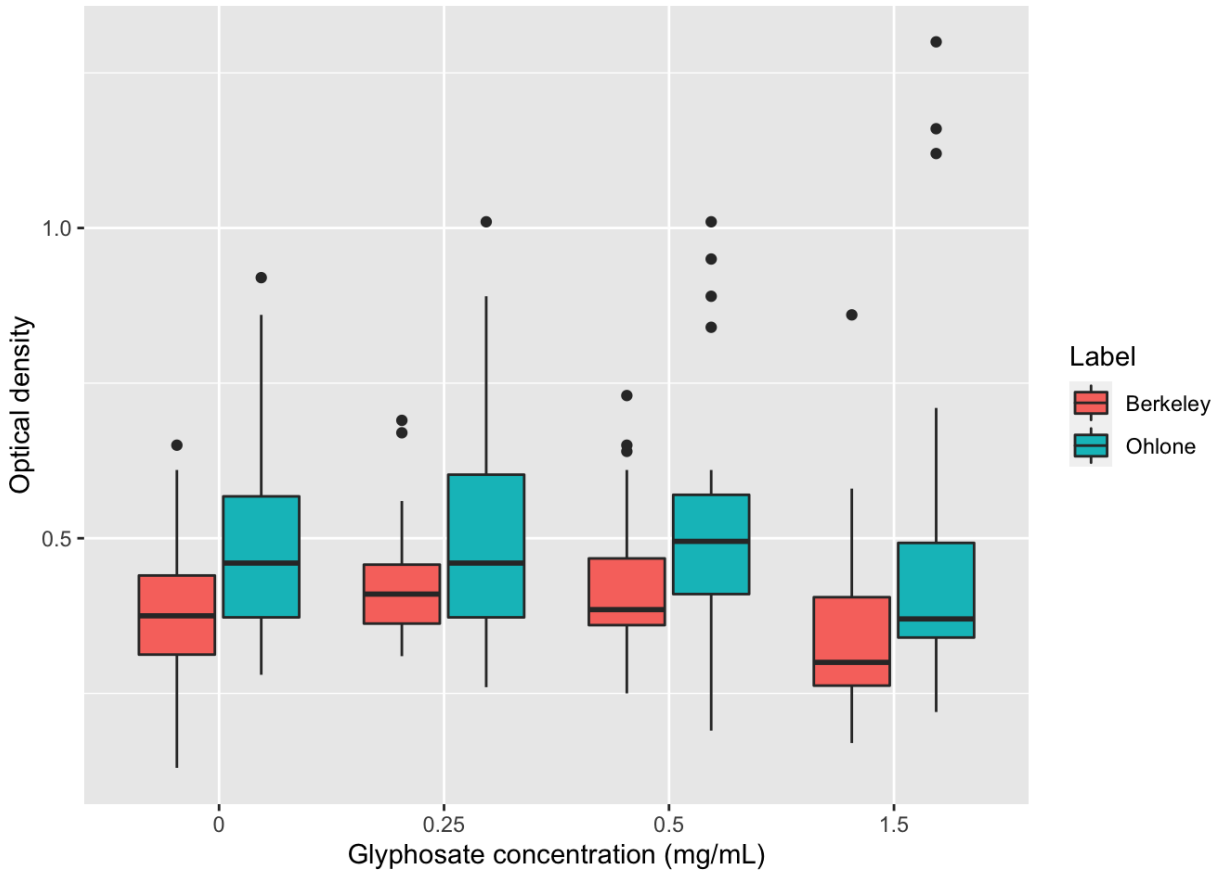


Figure 2. Berkeley vs. Ohlone Park strain growth. Comparing the growth of Berkeley and Ohlone Park strains at four glyphosate concentrations: 0 mg mL^{-1}; 0.25 mg mL^{-1}; 0.5 mg mL^{-1}; 1.5 mg mL^{-1}. Growth is measured as the optical density at 650 nm.

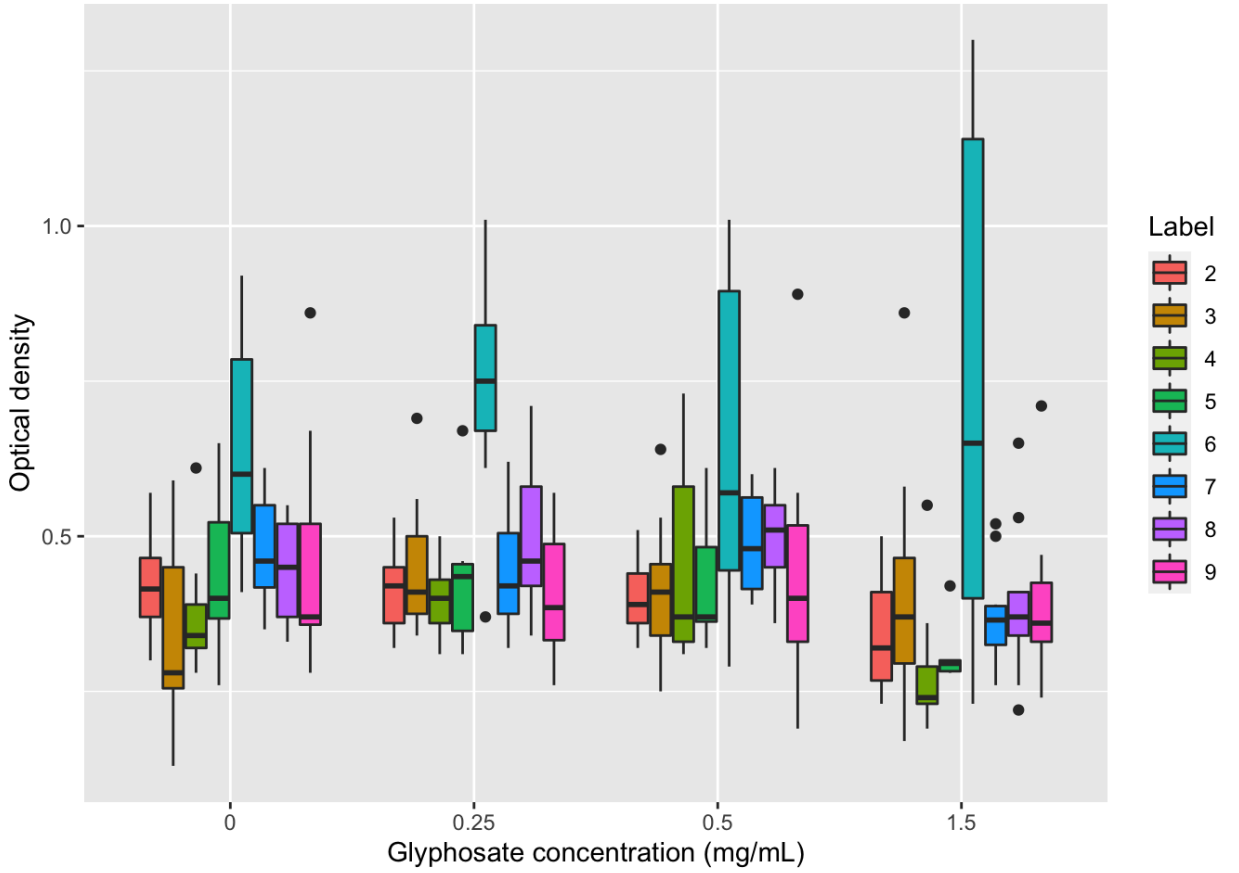


Figure 3. Site specific strain growth. Comparing the growth between the eight sites at four glyphosate concentrations: 0 mg mL^{-1}; 0.25 mg mL^{-1}; 0.5 mg mL^{-1}; 1.5 mg mL^{-1}. Sites 2-5 are located on UC Berkeley's campus and sites 6-9 represent sites in Ohlone Park. Growth is measured as the optical density at 650 nm.

Comparing site-specific tolerances

I found no significant difference in patterns of strain growth in response to increasing glyphosate concentration between any of the sites (p>.05), except for between sites 3 and 4 (p=.025) (Figure 3).

Effect of glyphosate concentration on strain tolerance

I found a significant negative relationship between glyphosate concentration and growth of strain ignoring the effect of strain background (p<.001) (Figure 4, 5). However, only 5 of the 72 strains showed declines in optical density at each increasing glyphosate concentration (Figure 6a). Many strains showed a peak growth (higher than their growth without glyphosate) at 0.25

mg mL^{-1} (Figure 6b); others showed a negative quadratic relationship with peak growth at 0.5 mg mL^{-1} (Figure 6c).

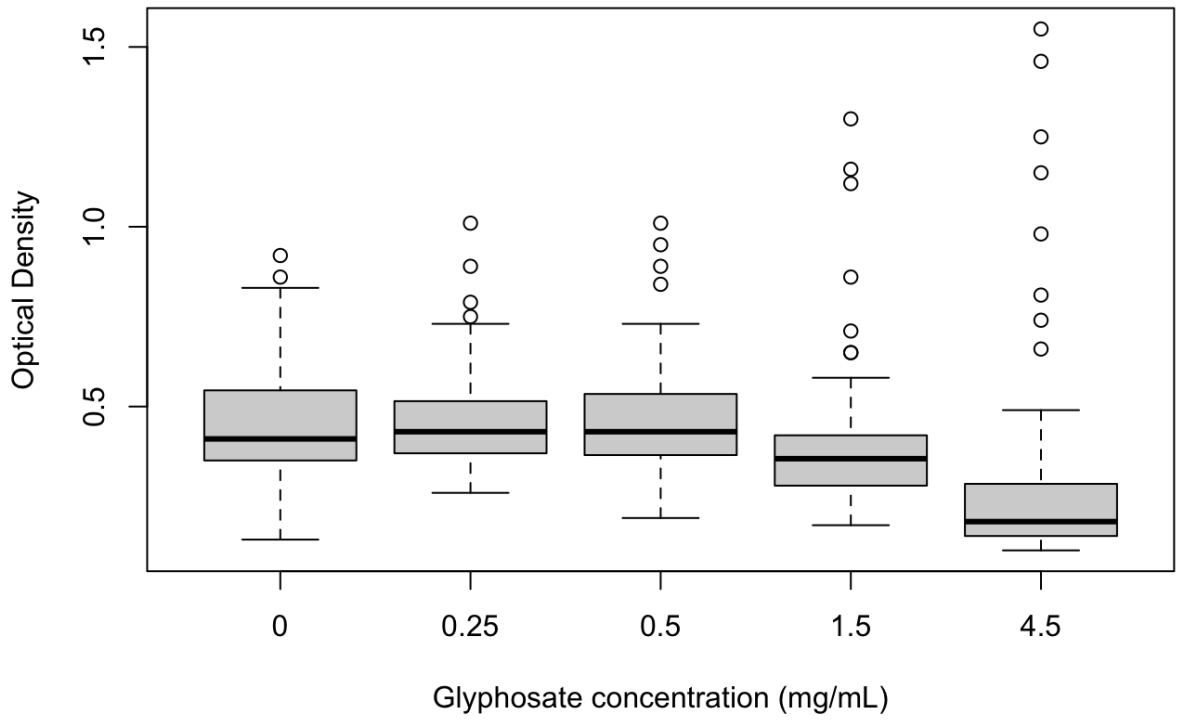


Figure 4. Strain growth vs glyphosate concentration. The relationship between rhizobial strain growth and glyphosate concentration in mg mL^{-1}, without consideration of the site of isolation or exposure environment. The 4.5 mg mL^{-1} data is illustrated here but was not included in the regression analysis. Growth is measured as the optical density at 650 nm.

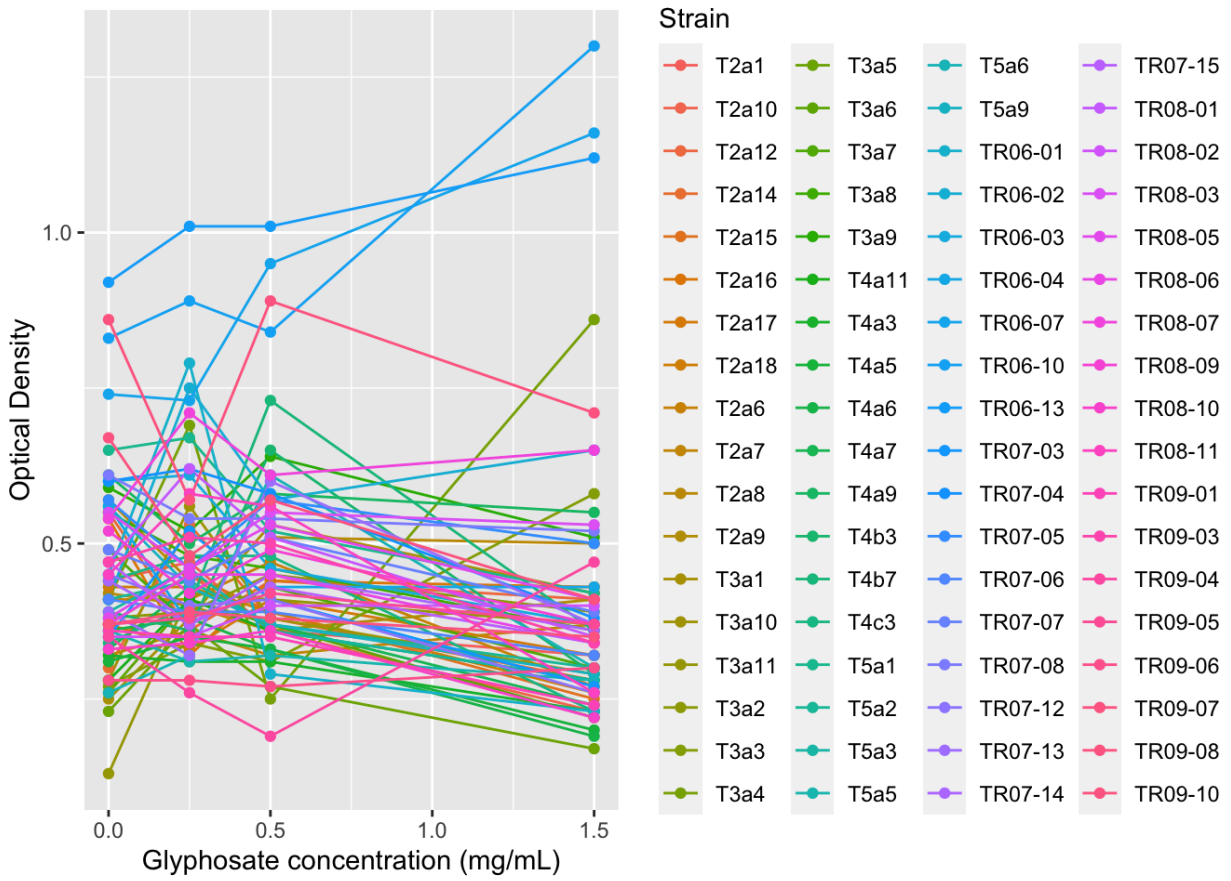
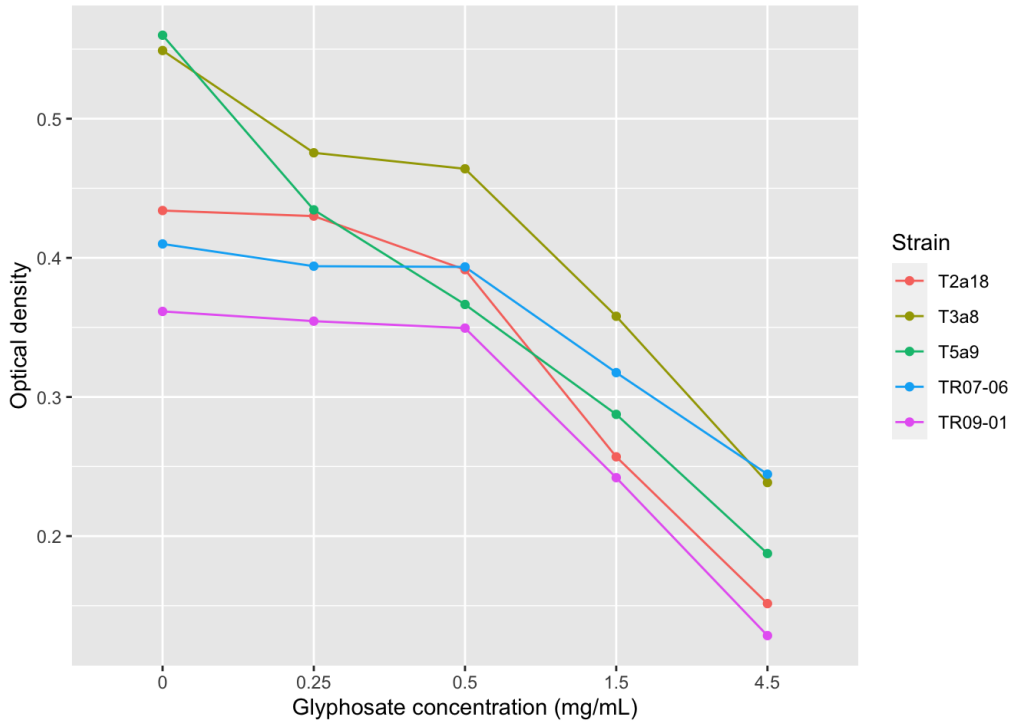
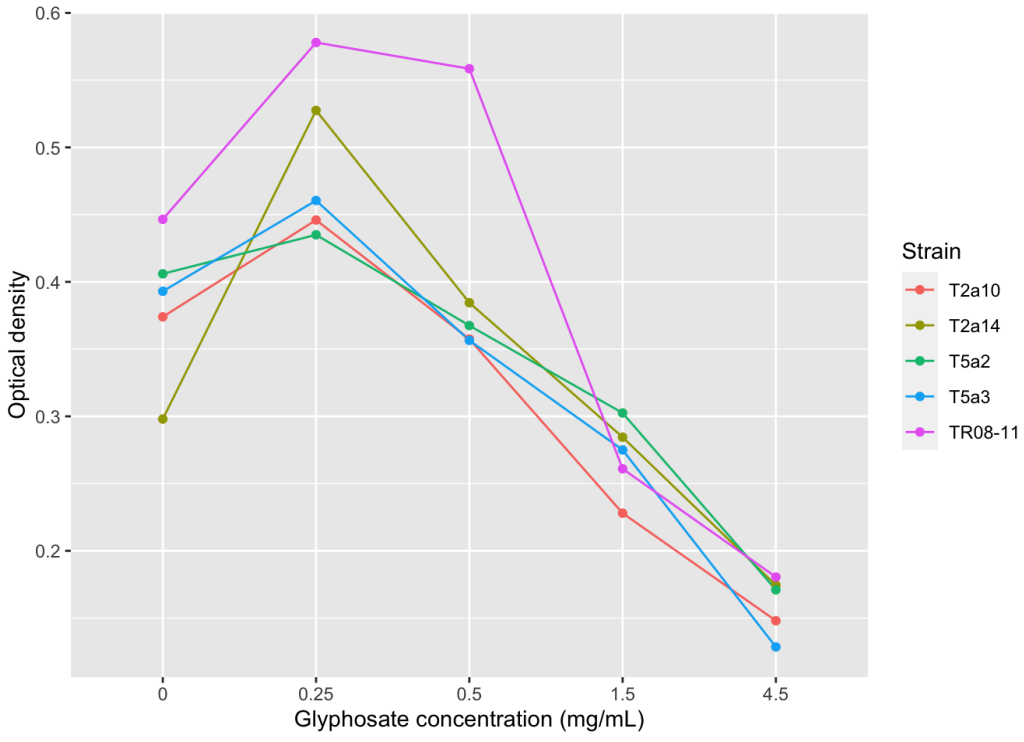


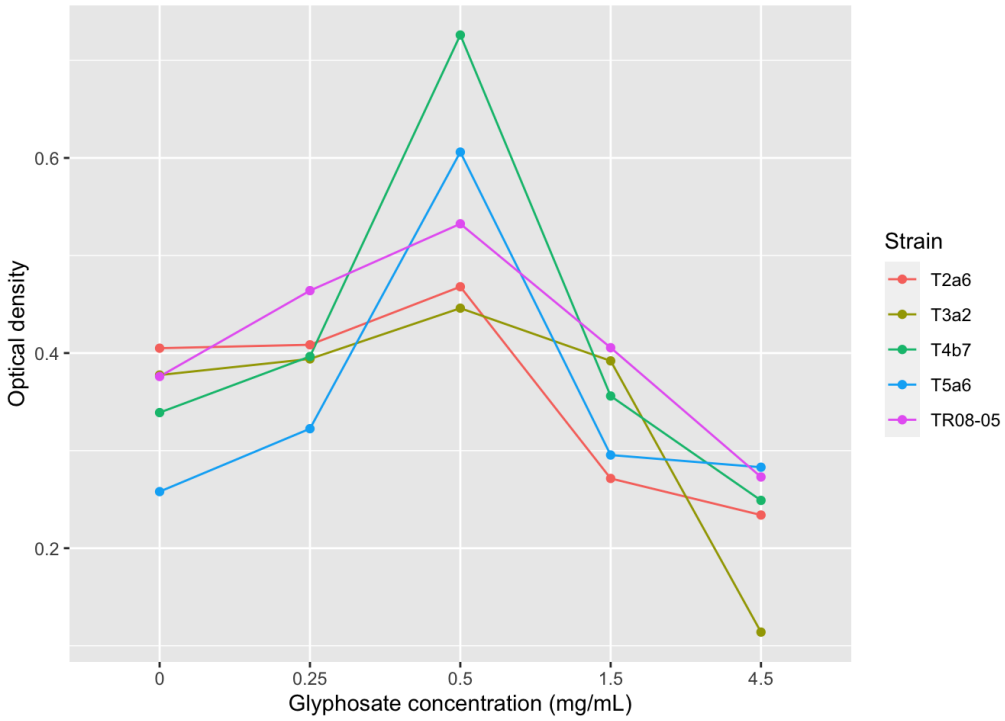
Figure 5. Individual strain trends. The relationship between growth and glyphosate concentration in mg mL^{-1} for each of the 72 strains. Growth is measured as the optical density at 650 nm. The number immediately following the "T" or "TR" represents the site of strain isolation.


Optical density
0.5
0.4
0.3
0.2
0
0.25
0.5
1.5
4.5
Glyphosate concentration (mg/mL)
Strain
T2a18
T3a8
T5a9
TR07-06
TR09-01

(a)


Optical density
0.6
0.5
0.4
0.3
0.2
0
0.25
0.5
1.5
4.5
Glyphosate concentration (mg/mL)
Strain
T2a10
T2a14
T5a2
T5a3
TR08-11

(b)



(c)

Figure 6. Diverse strain responses. The relationship between growth and glyphosate concentration in mg mL^{-1} for a subset of the 72 strains. Growth is measured as the optical density at 650 nm. Figure 6a shows the five strains whose optical density decreased with each increasing glyphosate concentration. Figure 6b shows examples of strains that showed peak growth at 0.25 mg mL^{-1}. Figure 6c shows examples of strains that showed peak growth at 0.5 mg mL^{-1}. The number immediately following the "T" or "TR" represents the site of strain isolation.

DISCUSSION

In my experiment, I isolated strains of *Rhizobium leguminosarum bv. trifolii* from the host clover plant (*Trifolium repens)* from environments both prior-exposed and not prior-exposed to the herbicide glyphosate and tested their tolerances in-vitro. This allowed me to see if strains prior-exposed to herbicide were more tolerant of glyphosate and if diverse responses to glyphosate found in previous studies could be linked to environmental history. Additionally, I isolated strains from four sites within each of these two larger environments to see if sites differed in their glyphosate tolerances, potentially as a result of differences in microenvironment and/or host specificity. Overall I found that the response to increasing glyphosate concentrations was not significantly different between rhizobial strains isolated from Berkeley's campus (prior-exposed) and Ohlone Park (non prior-exposed). As well, most of the sites did not significantly differ from each other in how their growth changed in the presence of glyphosate.

This suggests that prior exposure history or site is not as big a factor in predicting tolerance as individual strain variation, which is also exemplified in the strains' diverse responses to increasing concentrations of glyphosate.

Role of prior exposure on strain tolerances

I found that along the increasing glyphosate concentrations, the Berkeley strains were not significantly more tolerant to glyphosate than the Ohlone Park strains, suggesting that there is little role of prior environmental exposure to herbicide in explaining tolerance in rhizobial strains. This result is in congruence with Drouin et al. 2010, who began exploring this question by comparing the tolerances of arctic and temperate agricultural strains, and found that overall, arctic strains (isolated from presumably pesticide-free environments) were no less tolerant than the agriculturally-isolated strains. However, despite explicitly categorizing strains by pesticide exposure history, the temperature agricultural strains in that study, grouped for their prior exposure to pesticide, included strains originating from many countries around the world. My study used strains isolated from prior-exposed and non prior-exposed environments both located in Berkeley, California, and as such, expanded on Drouin et al's study by also attempting to control for other site-related factors that might confound a role of prior exposure. In the end, I came to a similar conclusion.

This lack of effect of prior exposure is particularly interesting considering the conclusions drawn by Funke et al. (2006) about the molecular characterization of a gene that forms the basis for engineered resistance in Roundup Ready plants. In this study, the authors found that a single active site amino acid mutation was responsible for a glyphosate-insensitive form of the enzyme and that glyphosate's inhibitory effects could be restored by changing this single active site and thus conformation. They concluded with the likely possibility that prolonged glyphosate exposure might select for such favorable mutations, supported by the fact that this tolerant gene was found in an *Agrobacterium sp*. strain isolated from a glyphosate-rich environment. Thus, the study suggests that genetic mutation is a plausible mechanism for connecting tolerance to prior exposure and that long-term herbicide exposure might provide favorable conditions to foster the persistence of such mutations. However, my results suggest

that this relationship between exposure history and development of tolerance for a given rhizobial strain is not guaranteed.

Role of site on strain tolerances

In determining whether there was an effect of herbicide exposure history on strain tolerance, I also sampled multiple sites within these two environments, to see if other aspects of the microenvironment or host specificity could additionally define strain tolerances. Plant hosts exhibit specificity for specific rhizobial genotypes but vary in degrees of this community structuring (Sachs et al. 2005). Abiotic factors can also affect the community of rhizobia in the host nodules (Vuong et al. 2017, Heath et al. 2020). Both observations suggest that to some extent, the isolated rhizobia should cluster more closely with other rhizobia isolated from the same host as compared to rhizobia isolated from different sites.

However, as I found in a cross comparison of all the sites that none, aside from sites 3 and 4, differed from each other in how their growth relationships varied with glyphosate concentration, it seems that a clustering of rhizobia due to host specificity and/or microenvironment does not translate to a clustering of their tolerances. Combined with the fact that prior exposure is not a significant driver of rhizobia tolerance, two possibilities emerge: that all the strains had approximately the same tolerances, or that individual strains varied greatly in their tolerances such that there was no pattern by site or environment. My results suggest the latter: that strains have high intrinsic diversity in herbicide tolerance, which dominates over factors that might tend to unite strains isolated from the same conditions.

Diversity in strain tolerances

This diversity in strain tolerances was demonstrated through a diversity in responses to a specific glyphosate concentration as well as in the strains' diverse pattern of tolerance to the increasing glyphosate concentrations. For example, although I found that there was a significant negative relationship between glyphosate concentration and bacterial growth, very few of the strains exhibited a consistent decline in colony density from 0 mg mL^{-1} to 4.5 mg mL^{-1}. Meanwhile, some strains exhibited a peak growth higher than their control at the 0.25 mg mL^{-1}

concentration, while others reached peak growth at the 0.5 mg mL^{-1} and declined in subsequent higher concentrations. dos Santos Malty et al. (2006) found similar results. Some strains exhibited strict decreases in tolerance as concentration increased, while other strains exhibited a negative quadratic growth pattern, with the most rhizobial growth occurring at intermediate glyphosate concentrations. This could be a result of rhizobia metabolizing glyphosate for its benefit as long as the concentration is not high enough to cause significant detriment before.

The findings of other studies also reinforce the diversity in patterns of tolerance to increasing glyphosate exposures as well as other diverse responses in strains. Maldani et al. (2018) tested strains at 0, 0.5, 1, 3, 6, and 12 g/L glyphosate in culture, and the three *Rhizobium* strains had vastly different responses: one steadily decreased growth with increasing concentration; another was not tolerant of any of the concentrations; the last had growth up to the highest glyphosate concentration and even exhibited higher growth at 3 g/L compared to the lower glyphosate concentrations. Finally, dos Santos et al. (2005) found that even within species there was a significant difference between strains: one strain of *Bradyrhizobium elkanii* exhibited a constant colony density through time after inoculation with Roundup, while another grew according to a sigmoidal growth curve.

Limitations

My study tested rhizobia tolerance to glyphosate in-vitro using a direct enrichment/broth culture method, so caution should be taken in applying the conclusions to a field system. For example, Castro et al. (1997) showed that a 50% decrease in strain growth in pure culture did not affect peanut yields under field conditions, while Singh and Singh (2020) found that adverse effects in vitro translated to the field at early stages since pesticide application, but by the time of harvest, the effects of pesticide were not noticeable. These studies point to a role of the soil as a buffer whose mitigating effects are also magnified by an important time scale factor relevant for agriculture.

Additionally, plants in the field receive commercial formulations of glyphosate that include glyphosate as well as surfactant, while my experiment used an aquatic form of glyphosate that lacks surfactant. This is worth mentioning, as there can be greater toxicity to

bacterial growth in some commercial formulations as compared to pure glyphosate, likely due to the effects of additives present in these formulations (dos Santos et al. 2005).

Future directions

Given the limitations of an in-vitro study system, further research might test rhizobial tolerances in greenhouse or field conditions, allowing a role for the soil environment and host-bacteria interactions. Additionally, given that some strains exhibited highest growth at intermediate glyphosate concentrations, the possibility that these strains are benefiting from metabolizing the herbicide is worth further consideration. This could be tested by inoculating strains in minimal media conditions, where nutrient stressors might favor rhizobia capable of glyphosate metabolism. Additionally, a subset of resistant strains could be grown in glyphosate conditions for extended periods of time, allowing an examination of potential adaptation of future generations to glyphosate conditions.

Conclusions and Broader Implications

The long-term exposure effects of glyphosate on soil bacteria and the rhizosphere community at large has been studied. Wicke et al. (2019) found that in the soil bacteria *B. subtilis,* only longer exposure to glyphosate yielded glyphosate resistant mutants. Additionally, long-term glyphosate exposure has been shown to induce shifts in the rhizosphere community at large (Newman et al. 2015), as well as transcriptomic changes (Newman et al. 2016). These studies suggest a role of prior exposure in mediating soil bacterial response to herbicide, in structuring the overall community as well as selecting among individuals within species. However, this role of long-term exposure of glyphosate has not been studied specifically with respect to how it affects rhizobial tolerances to herbicide. Given that rhizobia are important symbiotic partners of crop plants exposed to pesticides such as glyphosate, it is important to understand if strain tolerance can be linked to extended prior exposure to herbicide through the site of origin, or if individual variation rather provides the best predictor of rhizobial tolerance.

I thus studied strains whose tolerances to glyphosate could be linked to two nested identities: their prior exposure history, and site of origin/host plant. Additionally, by selecting

environments that differed in pesticide background but were otherwise very similar, both being located in the city of Berkeley and defined by the same climatic conditions, I could better isolate an effect (or lack of) prior herbicide exposure on strain tolerances. I found that prior exposure history and site of origin did not significantly define strain tolerances and that strains had incredible variation in tolerances as well as in their patterns of tolerance to increasing glyphosate concentrations. These findings suggest that the diverse responses of rhizobia to glyphosate also found in previous studies is likely an intrinsic property of the strains, encoded on a strain-by-strain basis with little relation to environmental boundaries. Drouin et al. (2010) reached a similar conclusion, noting that strain variation within the genera was in accordance with variability in other observed phenotypic traits, such as antibiotic resistance. These diverse tolerances within a single species of *Rhizobium* could be relevant in selecting optimal strains for the agricultural practice of pre-inoculating legume seeds with their compatible rhizobia.

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