Land use history influences recruitment of soilborne microbes to seeds, and seed germination, of *Prosopis velutina* (velvet mesquite)

By

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Abstract

A better understanding of interactions between native plants and endophytic microbes can help improve regional strategies for revegetation of damaged lands. One native plant of interest for revegetation in the Sonoran Desert is *Prosopis velutina* (velvet mesquite). I used a field experiment to evaluate how communities of microbes that recruit to seeds in soil might differ between more natural, riparian soils and degraded, post-agricultural soils. I exposed surface-sterilized *P. velutina* seeds to soils in Tucson, Arizona; isolated microbes associated with seed interiors; and evaluated germination frequency *in vitro*. Regardless of soil type, infection frequency increased after exposure to soil. This increase was due to an increase in fungal infections, not bacterial infections. Germination frequency was negatively associated with fungal isolation frequency, but not bacteria, and germination was particularly poor in seeds exposed to degraded soils. The most abundant fungal morphotypes differed between the soil types (*Fusarium brachygibbosum* in riparian soil; *Aspergillus tubingensis* in degraded soil). Together these findings provide a first perspective on soilborne microbes that infect seeds of an economically and ecologically important plant and set the stage for experiments to measure the effects of focal microbes on seed germination and establishment under field conditions.

Introduction

Plant microbiomes are increasingly appreciated for their important roles in defining plant health and productivity (Vandenkoornhuyse et al., 2015). Such impacts are important not only in agriculture, where they can enhance crop yield and stress tolerance, but also as part of revegetation efforts designed to mitigate land disturbance (Barea & Jeffries, 1995). Maximizing the factors that influence the ability of native plants to establish in devegetated or degraded sites can be especially important for re-establishing ecosystem function and related ecosystem services (Isaacs et al., 2009). For many native plants, the role of plant microbiomes in defining their establishment in native and degraded sites has not yet been explored.

Endophytes are microbes that live within healthy plant tissue without causing symptoms of disease (Wilson, 1995). These microbes are associated with every major land plant lineage and are found in all biomes (Rodriguez et al., 2009). Endophytes can confer important functional traits to their host, such as disease resistance, enhanced nutrient acquisition, and tolerance to abiotic stresses (Rodriguez et al., 2004). Many plants appear to acquire endophytes via infection of seeds by soilborne microbes (Truyens et al., 2015). Endophytes that infect seeds from soil can have host-specific effects on germination and seed viability (Sarmiento et. al., 2017; Hamzazai et. al., in press). Seed endophytes also can play a number of other roles, such as serving as biological control agents against pathogens and improving chances for seedling establishment (Truyens et al., 2015). In some cases, revegetation is desired in post-agricultural lands (Wade et al., 2007). Use of land for agricultural purposes can impact composition of microbial communities in soil over the long term (Cai et al., 2018). This change in the overall soil microbial community may alter community composition of soil-borne endophytes (Hamzazai et al., in press). However, how such changes impact seed colonization and seed germination is not known for important restoration species.

Prosopis velutina (velvet mesquite) is a native tree to the Sonoran Desert. This species has been used in revegetation projects in ecologically disturbed areas (Abella et al., 2015). A better understanding of interactions between *P. velutina* and its seed endophytes has the potential to enhance restoration and revegetation efforts in the Sonoran Desert region by informing strategic plantings that take microbes into account.

To better understand the effects of land use on soil-borne endophytes and their potential relevance for revegetation, this study investigates microbes that associate with the seeds of *P. velutina* in degraded (post-agricultural) and riparian (more native) soils. I exposed surface-sterilized seeds of mesquite to soils of these types in Tucson, Arizona, USA, then retrieved the seeds; surface sterilized them; isolated microbes associated with seeds; and evaluated seed germination frequency *in vitro*. Here I use this experiment to address the following questions: Does the frequency of microbial infection increase in seeds exposed to soils relative to controls (not exposed to soils)? Does that infection frequency differ for seeds exposed to degraded vs. riparian soil? How do seeds exposed to such soils differ in germination frequency? Are bacterial and fungal infections generally associated positively with germination, or negatively? Finally, what are the most common microbial associates of seeds in these soils? Together these data can help establish a framework for future experiments evaluating the potential costs and benefits of focal microbes for the establishment of mesquite in native and degraded soils, relevant for revegetation programs in Arizona and beyond.

Methods

Seed collection and preparation. Wildlands Restoration provided seed pods of *Prosopis velutina* var. *juliflora* in fall 2018. I extracted ca. 1,200 seeds from these pods. For scarification and surface sterilization, I adapted a protocol from Vilea and Ravatta (2001). I soaked seeds in 1N H_2SO_4 for 15 minutes. I then rinsed seeds with sterile water 3 times for 2 minutes each, soaked them in sterile water for 15 minutes, and left them to surface-dry under sterile conditions.

Seed bag preparation. Seeds were deployed into the field in mesh bags that excluded seed predators but allowed soil microbes to come into contact with seeds (Hamzazai et al., in press). To prepare bags, I cut 10 cm by 10 cm squares of 50 um mesh cloth. I folded these squares in half to form a rectangle and sewed them shut on the long edge and one of the short edges. I made a total of 120 seed bags in this way. I sterilized the seed bags by autoclaving at 121° C for 30 minutes. Under sterile conditions, I placed 10 mesquite seeds into each seed bag. I sealed each seed bag with two rows of staples, offset such that the bar of the staples in one row aligned with the gaps between the staples in the other. This ensured that the bags stayed closed and impervious to seed predators.

Deployment and retrieval. The field experiment was conducted at the University of Arizona Campus Agricultural Center in Tucson, Arizona. At the study site, I placed three plots (each 1 X 2 m) in each of two areas. The first area (32°16'47.3" N, 110°56’14.9” W) was used previously as an agricultural field. It contains few native plants and relatively little plant cover. The second (32°16'47.2" N, 110°56’14.4” W) is located on the edge of a riparian strip with native plants. Common plants in the area include *Parkinsonia sp.* (palo verde), *Datura* sp., and *Baccharis sarothroides* (desert broom). The degraded agricultural plots were typically about 5 m from the nearest riparian plot. Plots within each soil type were typically about 5 m from one another.

I deployed 18 bags into each of the six plots. I buried bags in ca. 5 cm of soil with 10-20 cm spacing between bags. I retained two bags as controls for each plot: one never left the lab (lab control) and one was transported to the field and handled like the deployed seed bags, but it did not come into contact with soil (field control). After 9 days, I retrieved seed bags and returned them to the lab for processing, as described below.

Plant community assessment. I collected data regarding plant communities for each plot by standing at each corner of each plot, facing away from the plot, and recording the following information for the area within 2 m: stem density, stem richness, the slope of the plot (if any), tree cover (if any), the diameter of the largest stem at breast height (if any), and the canopy height (if any). For information on plants in the area surrounding each plot, I ran 10 m transect lines from two random corners of each plot. At 2 m intervals along the transect, I recorded the types of plants immediately surrounding the point, the number of plants, their height, and the diameter of the largest stem at breast height (if any).

Processing. After returning seed bags to the lab, I cut each seed bag open and emptied seeds into a Petri dish. I then surface sterilized seeds in batches by seed bag by rinsing with 95% ethanol for 10 seconds, 0.5% NaOCl for 2 minutes, and 70% ethanol for 2 minutes (Arnold et al., 2007). I allowed seeds to surface-dry under sterile conditions. Under sterile conditions I placed seeds from each bag into 2% malt extract agar (MEA) (Fröhlich & Hyde, 1999) in a 100 mm Petri dish. I placed these plates in the dark for 5 days to induce germination. I then

counted germinated seeds and seeds that were associated with one or more microbes. I also recorded whether or not the seed associated with each microbe germinated and whether each microbe was bacterial or fungal.

Microbe isolation. I isolated emergent microbes with sterile toothpicks under sterile conditions. I re-cultured all microbes onto 60 mm 2% MEA plates and allowed them to grow. I further re-cultured fungi showing signs of bacterial contamination onto 2% MEA containing four antibiotics: kanamycin (50 ul/ml media), ciprofloxacin (40 ul/ml media), ampicillin (100 ul/ml media), and tetracycline (15 ul/ml media) (Shaffer et al., 2017).

DNA extraction and vouchering. I extracted DNA from fungal cultures via the Sigma Extract-n-Amp plant PCR kit (Sigma-Aldrich, St. Louis, Missouri, USA) according to the manufacturer's instructions. I added a small sample of mycelium to 100 ul of Sigma Extract-n-Amp extraction buffer under sterile conditions. I then used a sterile pestle to grind the tissue, centrifuged each sample briefly, and heated each sample to 95° C for 10 minutes. After heating, I centrifuged samples briefly and added 100 ul of Sigma Extract-n-Amp dilution buffer to each. I then stored these samples at -20° C. To create vouchers, I transferred a strip of media and fungal tissue approximately 0.5 cm by 2 cm to a 2 ml cryotube and filled the remainder of the tube with sterile molecular water. Each fungal strain was vouchered in sterile water (Shaffer et al., 2017) for future use.

I also extracted DNA from each bacterial culture. Under sterile conditions, I added a toothpick-tip worth of cells to 20 ul of Y-PER extraction buffer (Packeiser et al., 2013). I vortexed each sample for 5 seconds to dissolve cells into the buffer and heated them to 98° C for 5 minutes. I centrifuged samples at 14,000 RPM to pellet cells. I added 80 ul of sterile molecular water and stored these samples at -20° C. To create vouchers, I suspended a toothpick-tip worth of cells in a solution of 50% water and 50% glycerol and stored vouchers at -20° C.

I amplified the diagnostic barcode locus for fungi (nuclear ribosomal internal transcribed spacer and partial large subunit), and the diagnostic barcode region for bacteria (16s rRNA), via the polymerase chain reaction following Shaffer et al. (2017). I evaluated the quality of the reactions via gel electrophoresis, cleaned successful products with ExoSap-IT per the manufacturer's instructions, and submitted each positive product for bidirectional

sequencing. Sequence reads were assembled, edited, and compared against the GenBank database via BLAST following Shaffer et al. (2017).

Results

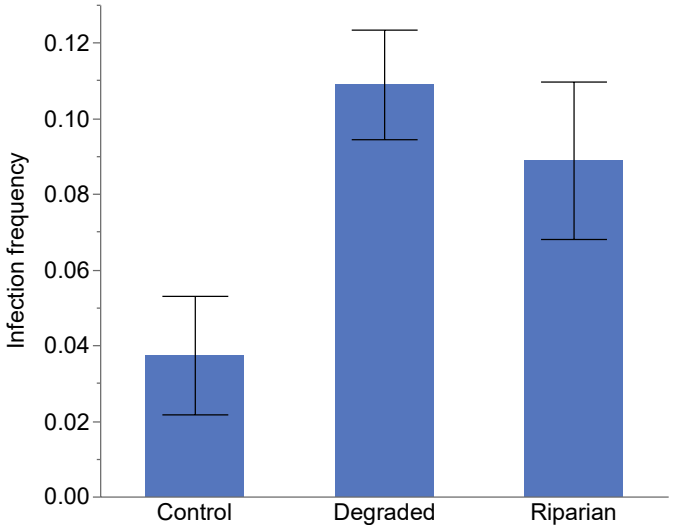
Plot characterization. Characteristics of the study plots are shown in Table 1. Relative to riparian plots, degraded plots had somewhat higher stem density (chi-square = 3.46, DF = 1, P = 0.0630) and higher stem richness (chi-square = 4.57, DF = 1, P = 0.0326) (Table 1). Degraded and riparian plots had similar canopy cover (chi-square = 0.008, DF = 1, P = 0.9292), similar values for the largest diameter at breast height (chi-square = 0.0316, DF = 1, P = 0.8588), and similar canopy height (chi-square = 0.06, DF = 1, P = 0.8072). All plots were flat (no slope or aspect).

Land use	Plot	Stem density	Stem richness	Canopy cover (%)	Largest diameter at breast height (cm)	Canopy height (m)
Degraded	A	2.3 ± 2.1	1.5 ± 1.3	0	NA	0.9 ± 1.7
	B	10.8 ± 1.7	4.8 ± 0.5	0.1 ± 0.1	4.5 ± 5.2	1.2 ± 1.3
	C	7.5 ± 1.3	3.5 ± 0.6	0	NA	0
Riparian	D	4.8 ± 1.0	2.3 ± 0.5	0	NA	0
	E	2.3 ± 1.5	1.5 ± 0.6	0.1 ± 0.1	6.3 ± 12.5	1.1 ± 2.3
	F	5.0 ± 3.6	2.8 ± 0.5	0.1 ± 0.2	4.8 ± 9.5	1.7 ± 3.5

Table 1. Site characteristics for each plot. Values represent the average for observations at four corners of each plot, +/- standard deviations. The degraded plots featured species such as *Datura* sp., *Portulaca oleracea,* and *Caesalpinia* sp. The riparian plots featured species such as *Parkinsonia spp.*, *Datura* spp., *Baccharis sarothroides*, and *Portulaca oleracea*.

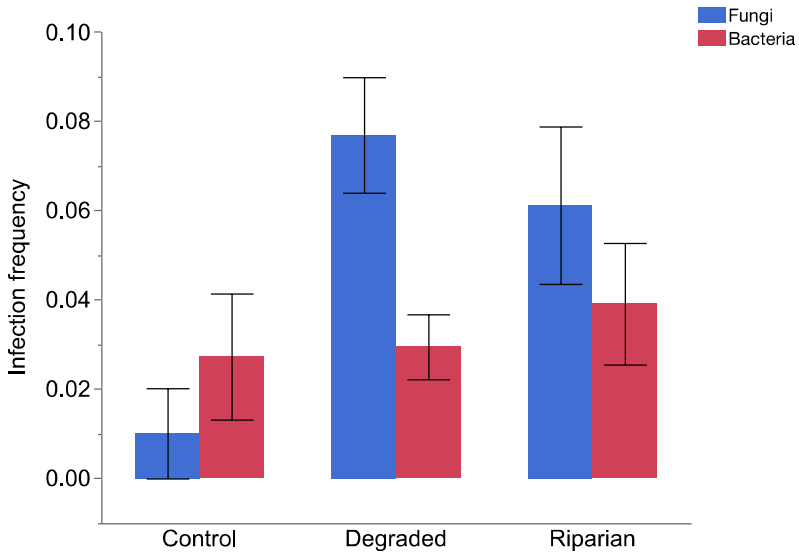
Infection frequency. Infection frequency increased after exposure to soil, regardless of degraded or riparian status (Fig. 1) (chi-square = 6.12, df = 2, P = 0.0469). Infection frequency was typically twofold higher in seeds placed in soil vs. control seeds (combined lab and field controls; one sample removed because infection frequency was > 2 standard deviations from the mean). The trend was for seeds in degraded soils to be infected more frequently than seeds in the riparian soils, but this difference was not significant.

Fig. 1. *Infection frequency increased after exposure to soil, regardless of degraded or riparian status. On average I isolated microbes from ca. 3.9% of control seeds (combined, field and lab controls). Bars indicate standard error.*



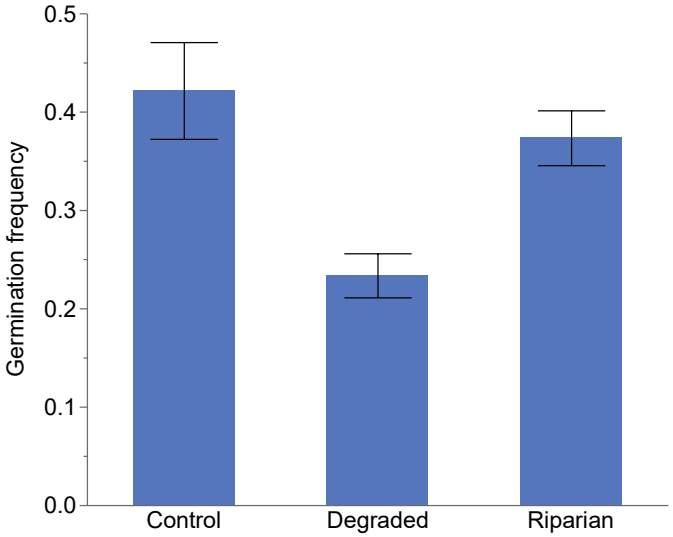
The increase in infection frequency after soil exposure reflected an increase in fungal infections, not bacterial infections (Fig. 2). Infections by fungi were six- to eightfold more common in seeds exposed to soil vs. controls. Overall variation in fungal infection was significant (chi-square = 8.37, DF = 2, P = 0.0152), but there was no meaningful difference in the incidence of fungal infection in seeds after exposure to degraded and riparian soils. Overall there was no increase in the frequency of infections by bacteria after exposure to soil (chi-square = 0.06, DF = 2, P= 0.9697).

Fig. 2. *Infection frequency by fungi increased after exposure to soil, but infection frequency by bacteria did not. Bars indicate standard error.*



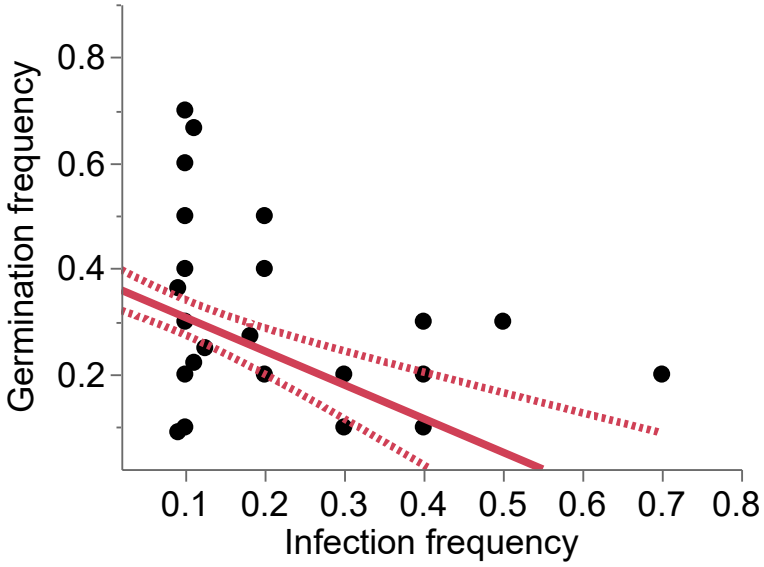
Germination frequency. Germination frequency decreased after exposure to degraded soils relative both to control seeds and seeds exposed to riparian soils (Fig. 3). Overall, germination varied among the three categories ($F = 9.86$, $DF = 2, 116$, $P < 0.0001$). This reflects a nearly twofold decrease in germination frequency in seeds exposed to degraded soil vs. control seeds. Seeds exposed to riparian soil did not differ significantly in germination frequency relative to controls (post-hoc test, $P > 0.05$).

Fig. 3. *Seed germination decreased following exposure to degraded soils, relative to both control seeds and seeds placed in the riparian soil. Bars indicate standard error.*



Relationship of germination frequency to microbial infection. Overall, germination frequency was negatively associated with isolation frequency, such that bags with more infected seeds generally had lower germination ($F = 22.82$, $DF = 1, 115$, $P < 0.0001$) (Fig. 4).

Fig. 4. *Seed germination was lower in bags that had a higher infection frequency. Red line is best fit (R-squared = 0.17); dotted lines indicate 95% confidence intervals.*



The negative relationship between germination and infection frequence reflected a relationship with fungal infections, but not with bacterial infections (Fig. 5 and Fig. 6). Germination frequency was negatively associated with isolation frequency of fungi ($F = 18.87$, $DF = 1, 117$, $P < 0.0001$) but not bacteria ($F = 1.12$, $DF = 1, 117$, $P = 0.2923$).

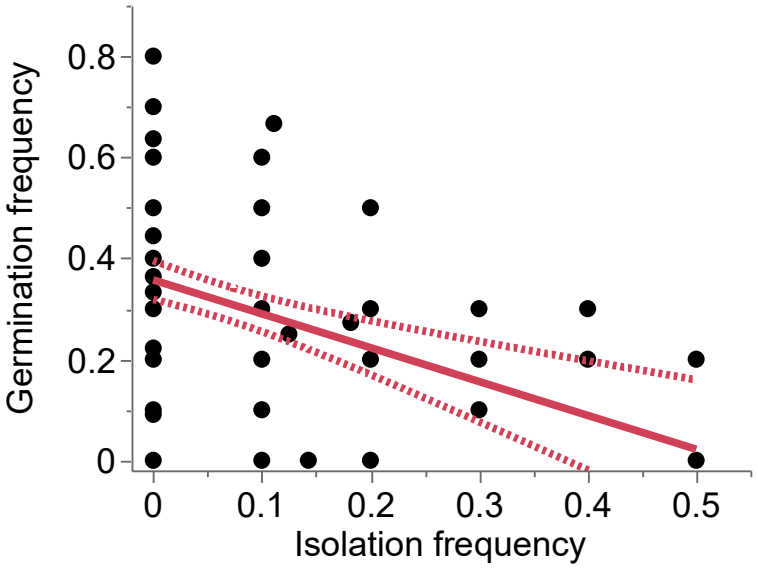


Fig. 5. *Seed germination was lower in bags that had a higher frequency of infection by fungi. Red line is best fit (R-squared = 0.17); dotted lines indicate 95% confidence intervals.*

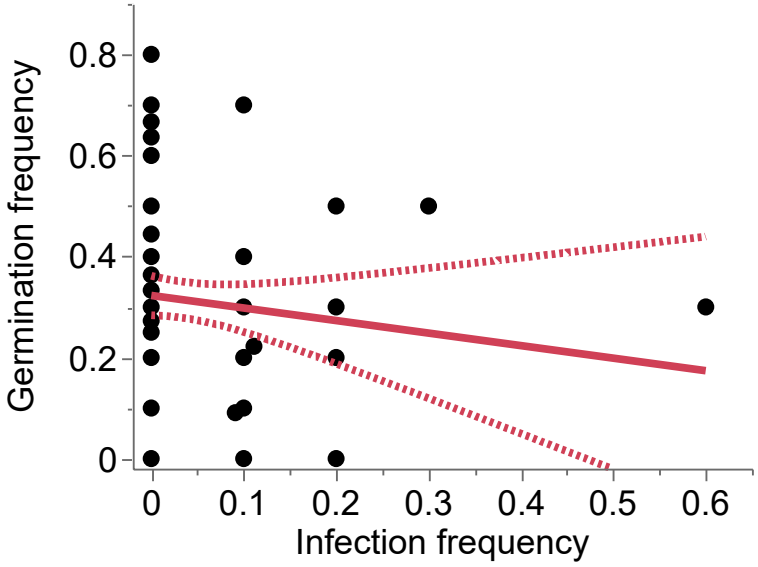


Fig. 6. *Seed germination was not strongly associated with the incidence of bacterial infections. Red line is best fit (R-squared = 0.17); dotted lines indicate 95% confidence intervals*

Microbial communities. A total of 123 cultures were isolated from seeds. Of these, eight were excluded due to lack of growth or contamination, for a total of 115 isolates. Lab controls yielded one bacterial culture and one fungal

culture. Field controls yielded two bacterial cultures. Of the remaining 111 isolates, 74 were fungal (66.7%). Of these, 41 originated from the degraded area and 33 from the riparian strip. I estimated that fungi from the degraded soils included 10 morphotypes, and fungi from the riparian soils included 13 morphotypes. The remaining 37 isolates were bacteria, with 16 originating from the degraded area (3 morphotypes) and 21 from the riparian strip (5 morphotypes).

The most abundant fungal morphotypes were identified tentatively as *Aspergillus tubingensis* in the degraded soil and *Fusarium brachygibbosum* in the riparian soil. Other fungi in the degraded soil included *Dothideomycetes sp.* and *Fusarium sp.*, whereas the riparian soil included *Aspergillus japonicus* and *Kwoniella heveanensis*.

The most abundant bacterial morphotype was not identified for seeds exposed to soil in the degraded area. Other bacteria in the degraded area included *Brevibacterium* sp. and *Bacillus aryabhattai*, whereas the riparian area included *Bacillus safensis* and *Bacillus megaterium*. Overall, *Bacillus subtilis* was the most common bacterium from seeds placed in the riparian soil. One bacterial culture originating from a field control was identified as *Paenibacillus jamilae.* Putative identities for the most common fungal and bacterial morphotypes by soil type are seen in Table 2.

Microbe type	Soil type	Putative ID:	Isolates	Number of plots
Fungal	Degraded	*Aspergillus tubingensis*	11	3
Fungal	Riparian	*Fusarium brachygibbosum*	10	1
Bacterial	Degraded	Not determined	7	2
Bacterial	Riparian	*Bacillus subtilis*	11	3

Table 2. Putative identities for the largest fungal and bacterial morphotypes by soil type. Isolates refers to the number of individual cultures sorted into a given morphotype. Number of plots refers to the number of plots (3 per soil type) from which members of the morphotype were recovered. Even though *F. brachygibbosum* was very common in seeds from the riparian plots overall, it was found only in one of those plots.

Discussion

Land use can alter the composition of microbial communities in the soil, which may have an effect on soil-borne endophytes (Cai et al., 2018; Hamzazai et al., in press). Management that takes into account plant-microbe interactions, such as between soil-borne endophytes and their hosts, can improve the efficacy of restoration efforts in degraded ecosystems (Requena et al., 2001).

The experiment described here showed that seeds were infected more frequently by fungi after exposure to soil (vs. controls). Those fungal infections were negatively correlated with germination frequency. Bacterial infection frequency seemed unchanged after soil exposure relative to controls and was not associated meaningfully with germination frequency. Below I explore these observations in greater detail.

Fungal infections

In this study, infection by fungi was negatively correlated with germination, regardless of soil type. Dalling et al. (2011) presented four mechanisms by which seeds may be protected from lethal fungal infection, including physical barriers (e.g. an impermeable seed coat). Chemical scarification with H_2SO_4 breaks physical dormancy in *P. velutina* seeds (Vilea and Ravatta, 2001). As this scarification treatment degrades the impermeable seed coat much more rapidly than would be expected in the environment, it may leave seeds without defense against pathogens, particularly fungi. One future direction for this work may be to evaluate the recruitment of soilborne microbes to *P. velutina* seeds as their seed coat naturally decomposes. To evaluate the role of fungal symbionts in the *P. velutina* seed microbiome, another research direction may be to test the germination rate of *P. velutina* seeds in sterile soil and soil inoculated with the most common fungi identified in this study (*Aspergillus tubingensis* and *Fusarium brachygibbosum*).

Bacterial infections

Contrary to fungal infections, bacterial infections were not strongly associated with seed germination. Further, bacterial infections were often identified in control seeds, which were not exposed to soil. Some plants (e.g., certain grasses) selectively transmit beneficial fungal endophytes from maternal plant to offspring (i.e., vertical transmission) (Rudgers et al., 2009). Similarly, some plants vertically transmit beneficial or commensal bacterial endophytes (Sinnesael et al., 2018). The presence of bacteria in control seeds, coupled with a lack of change in bacterial isolation frequency after soil exposure, suggest that some bacterial endophytes may be vertically transmitted in *P. velutina*. Therefore, one future direction may be to confirm vertical transmission of bacterial endophytes in this species. Further, one of the bacterial cultures recovered from this study, *Bacillus subtilis*, enhances drought tolerance in *Brachypodium distachyon*, a model plant (Gagné-Bourque et al. 2015). Thus,

another future direction may be to evaluate the germination and seedling survival rates of *P. velutina* seeds in sterile soil and soil inoculated with *B. subtilis* under varying degrees of drought stress.

Limitations of this study

This study has several limitations that could be addressed in future work. First, this study relied on culture-based methods to identify seed-associated microbes. This presents a limitation because not all microbes are culturable on the type of media used here. To address this concern, a variety of media types could be used, or culture-free methods could be applied.

Another limitation of this study is its lack of soil chemistry analysis. A better understanding of the soil chemistry in the two sites may serve to contextualize and quantify the physical differences between them, asthese differences may provide insights into the differences in microbial communities.

Finally, the effect of plant communities in the sites was not evaluated. Although *P. velutina* is indigenous to the Sonoran Desert, it has never been grown intentionally at our study site. The native soil area consists of a riparian strip containing other Sonoran Desert native plants. In future work, the plant communities of each soil type may be considered more carefully for their potential effects on both soil microbe communities and seed germination.

Conclusions

This study's goals were twofold: to investigate the differences in soil-borne seed endophytes of *P. velutina* in degraded and riparian soils and to determine if association with these soil-borne seed endophytes was associated with different rates of germination of *P. velutina* seeds. Regardless of soil type, infection rate of seeds increased after exposure to soil, reflecting a six- to eightfold increase in fungal infections after soil exposure. These differences in infection frequency did not seem to be dependent on soil type. However, germination frequency for seeds exposed to degraded soil was much lower than both seeds exposed to riparian soil and control seeds (no soil exposure). Bacterial infection frequency did not appear to be associated with germination, but fungal infection frequency was correlated negatively with germination. Further work is necessary to establish causality with regard

to the importance of symbionts for seed fate and to better understand the role of bacteria and fungi in the *P. velutina* microbiome.

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