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Plant-microbe interactions as a cause of ring formation in *Bouteloua gracilis*

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ABSTRACT

Patterned vegetation growth such as grass rings is found in many arid ecosystems, yet the mechanisms behind their formation are often unknown and have been minimally tested in the field. One explanation is pathogen accumulation in the center of a long-lived plant, which could cause central dieback and the formation of a ring as the plant grows toward pathogen-free soil. We tested this mechanism by comparing the growth of blue grama grass (*Bouteloua gracilis*) in live and sterilized soils from inside or outside naturally occurring grass rings. Field-collected roots from the inner edge of grass rings had higher fungal colonization than roots from the outer edge, suggesting the potential for intensified pathogen interactions on the inside of rings. However, while plants grown in live soils performed worse than those in sterile soils, this pathogenic effect did not differ between soils collected from inside versus outside of grass rings. Further work on the spatial distribution of plant-microbe interactions is needed to confirm their direct role in ring formation. Our findings suggest that soil and root microbes, in addition to known mechanisms such as soil hydrology, potentially promote ring formation of a widespread North American grass species.

1. Introduction

Spatial pattern formation in clonal plants is a well-documented phenomenon, particularly in arid regions (Klausmeier, 1999; HilleRisLambers et al., 2001; Bonanomi et al., 2014; Tarnita et al., 2017). One example is the ring-shaped growth of clonal plants such as perennial bunch grasses (Bonanomi et al., 2014). While ring pattern formation is widespread, the hypothesized mechanisms behind it are equally wide-ranging. Proposed mechanisms include physiological or demographic growth constraints (White, 1989; Danin and Orshan, 1995; Wikberg and Svensson, 2003), external disturbances such as fire or grazing (Lewis et al., 2001), inter-ramet competition for water and nutrients (Sheffer et al., 2007, 2011), interactions between local vegetation dynamics and abiotic factors (such as aeolian deposition, Ravi et al., 2008), and negative plant-soil feedbacks (Bonanomi et al., 2005; Cartenì et al., 2012; Vincenot et al., 2017). However, investigations of ring formation in plants have mostly been limited to theoretical models or observational studies, which often make it difficult to evaluate alternative hypotheses.

One of the few experimentally-tested mechanisms of grass ring formation is that of negative plant-soil feedback (Bonanomi et al., 2005). This is a broad category of mechanisms in which a plant species interacts with and changes its local soil abiotic and microbial environment as it grows in ways that makes the soil less hospitable for its own growth (Bever et al., 1997; Ehrenfeld et al., 2005; van der Putten et al., 2013). In a clonal grass species, these detrimental conditions are hypothesized to be most prevalent in the center of plants, where plant biomass is oldest and most dense, thus causing central decline and favoring growth toward the outer edges. Potential pathways for negative feedbacks to promote ring formation include the depletion of nutrients or water (Sheffer et al., 2011), the buildup of autotoxic compounds (Curtis and Cottam, 1950; Bonanomi et al., 2005), and the buildup of plant species-specific soil and root pathogens. For example, it is well known that as plants grow and mature, they can cultivate species-specific microbial communities around and inside their roots, and these microbes can be detrimental to host plant health (Kulmatiski et al., 2008; van der Putten et al., 2013). Experimental tests of negative plant-soil feedback as a ring-forming mechanism typically grow focal plants in soils collected from the center of the ring versus from the matrix outside of the ring. These tests have shown that experimental plants performed worse in soil collected from the center of rings relative to the outer edges (Bonanomi et al., 2005). However, past studies have not elucidated whether potential pathways underlying the observed pattern were abiotic (competition for abiotic resources) or biotic (accumulation of pathogens). Further, it remains unknown whether plant pathogen load is higher in the ring centers versus the

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outer edges of naturally ring-forming plants, and whether microbially-driven feedback is sufficiently detrimental to cause central dieback in plants.

In the southwestern United States, *Bouteloua gracilis* (blue grama grass) is a common rangeland species known to form rings naturally. Past work has suggested that ring formation could be driven by a difference in aeolian deposition rates in the center versus the outside of the plant leading to decreased water infiltration in the center (Ravi et al., 2008). Blue grama is also known to accumulate microbially-driven negative plant-soil feedbacks (Chung and Rudgers, 2016). Here, we provide a new empirical test of plant-microbe interactions in grass ring formation. We hypothesized that negative interactions caused by soil- and root-associated microbes underlie the central dieback found in ring-forming blue grama. We addressed the specific questions: (1) Is fungal colonization of roots higher inside versus outside of blue grama rings in the field? (2) Is fungal colonization of roots higher when plants are experimentally inoculated with soils originating from inside of blue grama rings than outside? (3) Does soil sterilization eliminate plant growth differences caused by soil origin, implicating microbes as causal agents of higher growth outside of rings? (4) Does the intensity of root colonization correlate with the growth of experimental plants, and does this relationship depend on ring location? If root-associated microbes were responsible for ring formation, we should detect a significant soil origin by sterilization interaction, with reduced plant performance in live soils from inside the ring relative to live soils from the ring edge, but no effect of soil origin when soils were sterilized. This study aims to shed light on a significant mechanism that leads to ring formation in perennial grasses in arid ecosystems.

2. Methods

2.1. Study system

The study site was a semiarid grassland located in the McKenzie flats region of the Sevilleta National Wildlife Refuge in New Mexico (106.6917°W, 34.3529°N). Mean annual temperature is 13.2 °C, and mean annual precipitation is approximately 250 mm. The vegetation at the site is co-dominated *Bouteloua eriopoda* (black grama grass) and *Bouteloua gracilis* (blue grama grass).

2.2. Field collections

A prior field study by Ravi et al. (2008) found that blue grama plants of a medium size (40–60 cm diameter) showed a larger edge vs. center difference in soil characteristics, such as infiltration rate and hydraulic conductivity, than did plants of both smaller and larger ring sizes. Thus, we focused on plants in this size range for our study. Forty medium-sized (40–60 cm diameter) blue grama plants were chosen at random along two transects in a semiarid grassland in the Sevilleta NWR. Plants were ≥1 m apart and formed an obvious single ring.

During the week of 10 June 2013, we collected four soil samples (approx. 66 ml each) at each of the 40 target plants: two from the center and two from the outer edge of the ring using a soil corer and hand trowel to collect to depths between 10 and 20 cm. The soil corer and trowel were sterilized between soil collections using a 10% bleach solution. We also collected roots from the inner and outer edges of the ring (10 cm per sample) to quantify natural levels of fungal root colonization. Roots were rinsed with tap water to remove soil particles and then segmented to fit into tissue cassettes (Simport, Quebec, Canada) that were stored in 50% glycerol prior to staining and microscopy.

2.3. Bioassay experiment

To determine whether ring formation in blue grama was driven by soil microbes, we grew blue grama seedlings in the soils collected from inside or outside 40 naturally-occurring blue grama rings in the field. One of the pair of soil samples collected at each location was sterilized by autoclaving (two rounds of 3 h at 121 °C separated by 24 h cooling period). Sterilized and live soils were added to bleached containers (2.5 cm diameter, 16 cm deep, Ray Leach Containers, Stuewe and Sons, Corvalis, OR), which were kept at room temperature for 6 days until seeds were planted.

We sowed five blue grama seeds from a local distributor (Curtis and Curtis, Clovis, NM) into each pot on 18 June 2013. Pots were supplemented with additional seeds on 24 June (after some seeds had been lost due to wind) for a total of 6 seeds per pot. Pots were misted with water twice each day in the greenhouse for germination, and all but one seedling were pruned from each pot to avoid competition. After germination, the pots were moved to a rack outside and given 5 ml of water two times daily and allowed to grow for 4 weeks.

2.4. Response variables

We estimated plant performance by monitoring seedling emergence and survival, and weighing belowground and aboveground biomass. Emergence was determined by counting the total number of seeds that sprouted in each pot and dividing that by the total number of seeds planted. Survival was determined as the mean survival of a germinated seedling at 20 days after seed addition. After 4 weeks of growth, the seedlings were harvested, soil was rinsed from roots, and plants were dried at 60 °C for 3 days to determine above and below-ground dry biomass. A subset of fresh roots (approx. 10 cm) from experimental seedlings were stored in ethanol for further microscopy.

2.5. Root fungal staining and microscopy

Roots were stained using the ink and vinegar method (Vierheilig et al., 1998) and mounted on slides with polyvinyl-lacto-glycerol. We used the grid-line intercept method (McGonigle et al., 1990) to determine levels of fungal colonization with 100 views per slide. Prior, randomly selected slides were observed to create morphological categories for the fungi: Dark Septate Endophyte (DSE), Blue Hyphae (BLUE), and Arbuscular Mycorrhizal Fungi (AMF). DSE were categorized by a dark brown color, septae, and thin hyphae with disordered growth. AMF were characterized by blue color, generally thicker hyphae, and structured growth in-between and into cells, absence of septae, and presence of arbuscules and vesicles. BLUE were categorized by a light blue color, thin hyphae, disordered intracellular growth, absence of septae, and lack of other structures, and are likely a morphotype of AMF. Extra-radical (ER) hyphae, any hyphal strands that surrounded the root tissue but did not penetrate, were also counted for each sample.

2.6. Data analysis

All analyses were conducted using R version 3.3.2 (R Core Team, 2016).

2.6.1. Q1: Fungal colonization of field-collected roots

We predicted higher levels of colonization of roots inside than outside the ring. To test for these differences we used a paired *t*-test, with observations paired by plant identity to examine total and DSE fungal colonization. Colonization by blue, AMF, and extra-radical hyphae were at levels too low for individual statistical analysis (Table 1).

2.6.2. Q2-Q3: Bioassay of root colonization potential and bioassay of blue grama performance

If plant-microbe interactions were responsible for ring formation, we should detect a significant soil origin by sterilization interaction, with reduced plant performance in soils from inside the ring relative to outside the ring, but only for live soils. To test for these effects on seedling survival, plant growth, and root colonization, we used linear

mixed models with field plant identity as a random variable (package nlme Pinheiro et al., 2016). Total fungal colonization and plant biomass were natural log transformed for analysis to meet normality of residuals assumptions. Differential variance among groups was modeled using the varIdent option to correct for heteroscedasticity. *Post hoc* pairwise comparisons of DSE and total fungal colonization were conducted using the lsmeans function with Tukey adjustments for multiple comparisons (package lsmeans, Lenth, 2016).

2.6.3. Q4: Correlation between plant growth and root colonization

We predicted that plant biomass would decrease as total fungal colonization increased, and that this relationship would be stronger for soils originating inside of blue grama rings than for soils outside of rings. Natural-log-transformed biomass of live-inoculated plants was modeled using total fungal colonization, soil origin, and their interaction as predictors.

3. Results

3.1. Q1: Fungal colonization of field-collected roots

Field roots on the inside edge of the ring had 42% higher total fungal colonization than roots on the outside of the ring (Table 1; $t = 4.81$, $df = 39$, $p < 0.001$). This pattern was consistent in each fungal group examined, although we could only test for statistical significance in DSE ($t = 4.97$, $df = 39$, $p < 0.001$). Field roots were predominately colonized by DSE (78% of total colonization) with lower levels of colonization by other fungal morphotypes (Table 1).

3.2. Q2: Bioassay of root colonization potential

Roots from the bioassay experiment were colonized by different fungal morphotypes than roots collected from the field. While the DSE morphotype was still the most common (49% of total fungal colonization), the potted bioassay plants had more AMF and aseptate blue-staining fungi than field plants (Table 1), which may reflect differences in plant age or pot versus field conditions. Soil origin (inside vs. outside of the ring) did not significantly alter total fungal root colonization ($p = 0.38$). However, there was significantly less fungal root colonization in sterilized soil than live soil ($t = -2.90$, $df = 78$, $p = 0.005$; Fig. 1A). There was no significant interaction between soil sterilization and soil origin ($p = 0.53$). DSE colonization followed a similar, although statistically insignificant, pattern to total fungal colonization (soil sterilization $t = -1.76$, $df = 78$, $p = 0.08$).

3.3. Q3: Bioassay of blue grama performance

Plants grown in sterile soil had 206% greater aboveground biomass and 269% greater belowground biomass than plants grown in live soil, indicating that soil microbes had a net pathogenic effect on plant growth in the bioassay experiment (Fig. 1B - C; aboveground biomass $t = 8.15$, $df = 83$, $p < 0.0001$, belowground biomass, $t = 7.93$, $df = 83$, $p < 0.0001$). Growth of plants inoculated with soils from the inside or outside of grass rings was not affected by sterilization, contrary to our initial hypothesis (Soil origin x Sterilization, $p = 0.97$ for above- and belowground biomass).

3.4. Q4: Correlation between plant growth and root colonization

Opposite to our prediction, total root colonization was positively, rather than negatively, correlated with belowground plant biomass in the bioassay experiment (slope = 0.02 ± 0.01SE, $t = 2.23$, $df = 57$, $p = 0.03$; Fig. 2). However, the full statistical model explained little of the variation in belowground biomass (adjusted $r^2 = 0.05$, $F_{3,57} = 2.0$, $p = 0.13$). In addition, there was no significant relationship between fungal colonization of roots and aboveground plant biomass (adjusted $r^2 = 0.00$, $F_{3,57} = 0.11$, $p = 0.96$).

4. Discussion

4.1. Fungal colonization of roots was higher inside than outside of rings in the field

In the field, fungal colonization of the roots was greater on the inner edge of blue grama rings than on the outside edge. This pattern is consistent with the hypothesis that negative plant-soil feedback is caused by pathogen accumulation, if observed fungi are net pathogenic. Alternatively, roots from the inside of rings may have higher fungal colonization due to their older age, although fine root turnover in blue grama is ~120 days (Gill et al., 2002). The bioassay experiment showed that seedlings grown in live soils that included the entire microbial community achieved much smaller biomass compared to plant growth in sterilized soils, suggesting overall pathogenicity of the microbial community. However, it is not possible to conclude from microscopy alone that the observed fungi were pathogens. The majority of fungi found in field roots were dark septate endophytes (DSE), consistent with previous work at this site (Porras-Alfaro et al., 2008). While DSE are known to have positive effects on plant growth in some laboratory studies, they can also be pathogenic (reviewed by Newsham, 2011), and for most taxa, their ecological roles are unknown (Mandyam and Jumpponen, 2005). Further study to determine the identity and ecological functions of root-associated fungi in blue grama could shed light on potential candidates that may drive ring formation.

4.2. Live soils were net pathogenic, but pathogenicity did not vary with ring location

Live soils reduced seedling growth by 60–70% and increased root fungal colonization by 43% relative to sterilized soils, but these effects did not differ between live soils collected from inside versus outside of grass rings. Discrepancies between the bioassay results and patterns of fungal colonization in the field could be driven by differences in the

Table 1
Average root colonization (% views) by each fungal morphotype for plants in the field and greenhouse experiment: arbuscular mycorrhizal fungi (AMF), blue-staining aspetate hyphae (BLUE), dark septate endophytes (DSE), extra-radical fungi (ER). Values are mean ± standard error. For fungal groups (DSE and Total) with sufficient data to conduct statistics, superscripts with different letters indicate significant pairwise differences ($p < 0.05$) between treatments.

	AMF	BLUE	DSE	ER	Total
Field roots					
Inside ring	3.30 ± 0.83	8.48 ± 1.75	41.85 ± 1.90^{a}	1.05 ± 0.22	53.63 ± 2.82^{a}
Outside ring	2.08 ± 0.62	5.08 ± 1.15	30.73 ± 1.95^{b}	0.50 ± 0.18	37.88 ± 2.67^{b}
Greenhouse roots					
Live - Inside	5.09 ± 0.93	3.29 ± 0.70	9.74 ± 1.32^{a}	0.21 ± 0.09	18.32 ± 1.89^{a}
Live - Outside	4.85 ± 1.11	3.30 ± 0.83	7.67 ± 1.62^{ab}	0.37 ± 0.19	16.19 ± 2.24^{ab}
Sterile - Inside	0.93 ± 0.23	2.00 ± 0.66	6.00 ± 1.02^{ab}	0.35 ± 0.12	9.28 ± 1.56^{b}
Sterile - Outside	2.90 ± 0.80	5.52 ± 1.65	5.61 ± 1.29^{b}	0.84 ± 0.40	14.87 ± 2.98^{b}

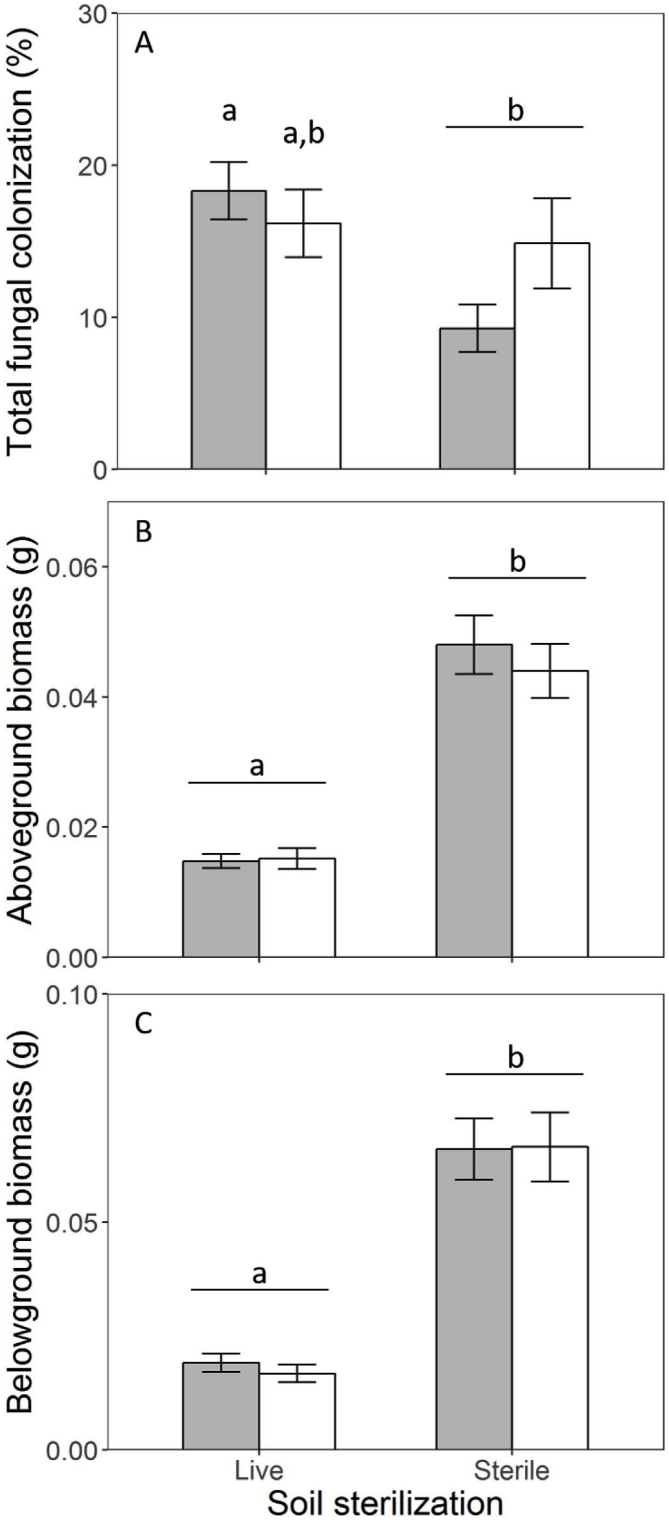

Fig. 1. Effects of sterilization and soil origin from inside (grey) versus outside (white) of grass rings on (A) Total fungal colonization of roots, (B) aboveground biomass, and (C) belowground biomass of plants from the bioassay experiment. Errors indicate SE, and different lowercase letters indicate significant differences between means.

plant growth environment (pot vs. field), soil origins, or life history stage of the focal plant. For example, pot vs. field growth environments could explain the divergence in fungal morphotype composition of field-collected roots versus experimental seedling roots (Sýkorová et al., 2007). In the bioassay, the soil collected from inside the ring was taken from the center of the ring, which was devoid of plant growth. In

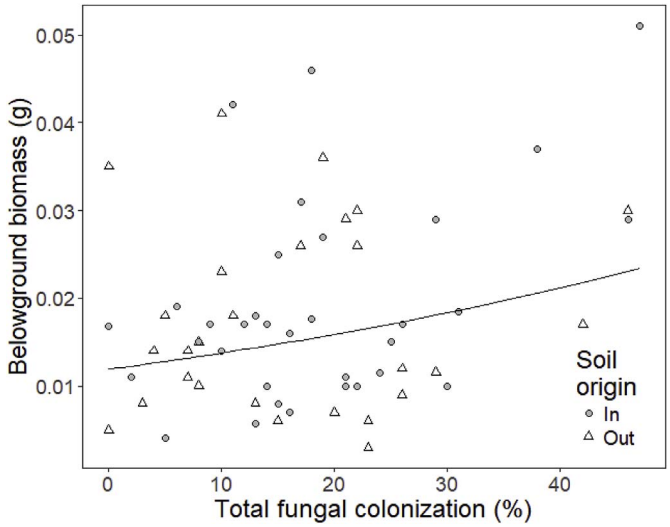

Fig. 2. The relationship between total root fungal colonization and belowground biomass for blue grama seedlings grown in live soil from inside (grey circle) and outside (open triangle) grass rings (overall slope = 0.02 ± 0.01SE, $t = 2.23$, $df = 57$, $p = 0.03$ on ln-transformed data). The interaction between fungal colonization and soil origin was non-significant ($p = 0.25$).

contrast, live roots taken for microscopy were collected near the inside edge of grass rings, because roots were absent from the ring centers. Detailed molecular work in other plant-fungal pathogen systems has shown that pathogen density decreases with increasing distance from the active root surface (Ling et al., 2012). Thus, it is possible that we did not observe an effect of soil origin in the bioassay due to low abundance of host-specific pathogens in soils from the ring center. In addition, plant-microbe associations and their subsequent effects on plant fitness are known to change throughout the life history of the plant host (Rudgers et al., 2010, 2012, Chung et al., 2015). It is possible that the bioassay seedlings did not show similar patterns of root colonization as field plants due to their young age. Future studies might use transects of root and soil collections from the ring center through to the outside ring edge to more finely partition spatial variability in potential microbial interactions.

4.3. Fungal colonization and plant growth

If the dominant root fungi were pathogenic, higher fungal colonization of roots should be negatively correlated with plant growth in live soils. However, there was no significant relationship between fungal colonization and aboveground biomass, and a weakly *positive* relationship for belowground biomass. That the overall effect of live soils compared to sterilized soils was detrimental, yet belowground biomass increased with increasing total fungal colonization seem to be contradicting observations. Most of the fungal morphotypes we could identify via microscopy (AMF and DSE) are typically plant mutualists (Hoeksema et al., 2010; Newsham, 2011). Thus, one explanation is that non-fungal agents, such as bacteria or nematodes, reduced plant growth in live soils, whereas root-associated fungi played a weakly beneficial role. An alternative explanation is that sterilized soils could have increased nutrients levels due to autoclaving (Berns et al., 2008), and thus increased seedling growth, although previous work in this system showed that autoclaving did not significantly alter soil nutrient chemistry (Chung, unpublished data). Future work *in situ* using bioassays in grass rings in the field in combination with manipulations to exclude fungal colonization (e.g. 'microbial cages' in Reed and Martiny, 2007) could help distinguish among these alternative hypotheses.

Our results contribute to the evaluation of alternative hypotheses for how negative plant-soil feedbacks can drive plant ring formation. There were no differences in plant growth between soil origins inside

versus outside of rings when soils were sterilized in the bioassay experiment. This result suggests that blue grama rings are unlikely to be caused by significant depletion of mineral nutrients, the accumulation of autotoxic chemicals, or differences in soil texture (e.g. Bonanomi et al., 2005). However, our design cannot rule out differences in water availability as a potential driver of ring formation (e.g. Sheffer et al., 2007). Notably, neither did our results demonstrate that soil microbes alone were sufficient to drive feedbacks that could cause central dieback and ring formation.

5. Conclusion

Within blue grama rings, roots near the empty center of the ring had higher fungal colonization than roots from the outer ring edges. However, our bioassay using soils from inside versus outside the ring did not detect soil microbial differences that were sufficient to affect early blue grama growth. Our results suggest that this natural pattern is likely caused by a combination of drivers, including plant-microbe interactions as well as abiotic factors such as soil hydrological characteristics.

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