

ALLELOPATHY IN THE INVASIVE WARM-SEASON GRASS *BOTHRIOCHLOA*
ISCHAEMUM: INVESTIGATION OF ITS MECHANISM AND EFFECTS ON
COMPETITOR SPECIES OF THE EDWARDS PLATEAU

by

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I. INTRODUCTION

Much research has been devoted to understanding what mechanisms contribute to the success of invasive plant species (Mack et al. 2000; Callaway and Ridenour 2004; Mitchell et al. 2006). One such mechanism is allelopathy, a phenomenon by which one organism produces secondary metabolites that influence the germination, growth, survival, development and/or reproduction of other organisms (Rice 1984; Einhellig 1995; Cheng and Cheng 2015). Allelopathy can include both inhibitory and stimulatory effects on other plant species (Rice 1984; Einhellig 1995), but in the invasion context, the focus is usually on a negative allelopathic effect on one or more resident community members. A link between allelopathy and the success of invasive species was first discussed by Callaway and Ridenour (2004), in a theory they called the “novel weapons hypothesis”. They proposed that many invasive plant species produce harmful secondary metabolites (allelochemicals) which may have been relatively benign to plants in their native communities due to the co-evolution of interspecific defense mechanisms, but might be powerfully inhibitory to plant species in a novel newly-invaded community (Callaway and Ridenour 2004; Callaway et al. 2008). While its definition is relatively straight-forward, the underlying mechanisms of allelopathy are varied and complex.

A distinction can be made between direct plant-plant allelopathic interference (allelopathy in the narrow sense) and indirect allelopathy (Inderjit and Weiner 2001). Direct allelopathy refers to the direct action of an allelochemical produced and released by a donor plant on a receiver plant. Indirect allelopathy includes all the effects of allelochemicals on abiotic and biotic soil processes that affect other plants (Scavo 2019). Allelopathy can also function through a combination of direct and indirect mechanisms

(Scavo 2019). Direct allelopathy can impact receiver plants at any stage of development, including during seed germination, seedling establishment, vegetative growth, or development of reproductive structures (Cai and Mu 2012; Segesso et al. 2019). Direct allelopathic effects act to disrupt plant processes at different levels of organization by altering cellular structure or inhibiting cell division and elongation, creating imbalances in the antioxidant system, affecting the plant growth regulator system, changing cell membrane permeability, affecting photosynthesis, influencing respiration and water and nutrient intake, and finally, by influencing protein and nucleic acid synthesis and metabolism (Cheng and Cheng 2015). Indirect allelopathy also functions in a variety of different ways, including altering soil pH and thus nutrient availability (Li et al. 2007), and/or altering microbial activity and thus nutrient availability (Kraus et al. 2003; Stinson et al. 2006). For example, allelochemicals can delay decomposition and mineralization of soil organic matter, with negative effects on nutrient release to plants (Segesso et al. 2019). According to Zeng (2014) in a commentary on four decades of allelopathy research, indirect microbial mediation characterizes many, if not most, allelopathic interactions. Due to the broad spectrum of their effects, allelochemicals are likely to play a role in the evolution of plant communities everywhere, and by extension, in plant invasions worldwide (Callaway and Ridenour 2004). However, due to the complexity of allelopathic interactions, a unifying theory of allelopathy is still far off. Related to the complexity and unpredictability of allelochemical effects, it has also been difficult to replicate the effects of allelochemicals in experimental settings (Inderjit et al. 2005).

Almost any experimental detail can potentially interfere with the observation of allelochemical effects. For example, the production of allelochemicals depends on several

factors, including the developmental stage of the donor plant (Iannucci et al. 2012), and environmental stress the donor plant might be under (Cipollini and Gruner 2007). The impact of allelochemicals on a recipient plant also depends on many factors. For example, all toxins are dose-dependent; it takes a minimal concentration at the receptor site to impact the receiver plant (Reigosa and Pazos-Malvido 2007). As such, external factors such as soil type, pot volume, and rainfall or irrigation amounts can potentially modify the effects of allelochemicals. For example, Wang et al. (2019) highlighted the importance of soil type: allelochemicals from a pea plant accumulated at higher concentrations and were phytotoxic to wheat in denser sodosols and chromosols, but not in vertisols. The difference in allelopathy between soil types was attributed to poor aeration and compaction in the sodosols and chromosols, and better microbial decomposition of allelochemicals in the vertisols. Barring experimental designs that cover all permutations of relevant factors, any specific experimental design may fail to show allelopathic effects, where effects might be possible in another circumstance.

Experimenters typically seek to control the dose of allelochemicals by preparing extracts from the parts of the donor plant that are known to, or most likely to contain, allelochemical compounds. Some researchers use only the above-ground plant parts; some use below-ground parts, and some use the whole plant (Favaretto et al. 2018), each of which could contain different amounts of allelochemical compounds and ultimately affect experimental outcomes. Collection of only above-ground parts could exclude indirect effects that root exudates may have on soil chemical properties or the soil microbial community. Depending on which developmental stage the plant was in when it was harvested, allelochemical content of plant organs could vary further (Inderjit and

Dakshini 1995, Cheng and Cheng 2015).

A second source of variation influencing results of allelopathy experiments is how the extracts are prepared. The most common type of extract used is an aqueous extract in which plant material is soaked in water, so that any water-soluble compound contained in the plant tissue can leach out into the solution. A standardized concentration and amount of the extract can then be applied to the recipient plants in a controlled study. A raw extract would presumably contain field microbes from plant surfaces, roots, and the surrounding soil, in addition to chemical compounds from the plant tissue. Further, the soil used to grow the recipient or target plants in the study would also contain microbes. Presence of microbes, both in an allelopathic extract or in the soil used in the study, makes it difficult to isolate a direct allelopathic effect from an indirect microbial effect. Therefore, another source of variation between allelopathy studies is how researchers attempt to control microbial effects by sterilizing (or not) the soil, the extract, or both.

Finally, a likely source of variation among allelopathy studies is the species of the recipient or target plant, and the developmental stage of the target plant when the extracts are applied to it. Not all species in a community may be susceptible, and allelopathic effects may only affect some life stages of the target plants; for example, not germination but seedling growth, or vice versa. How allelopathy is quantified is therefore also important and a potential source of variation, whether by germination, dry biomass of roots and shoots, shoot length, or root length (Favaretto et al. 2018).

Given these sources of variation in the outcomes of allelopathy studies, what kinds of studies are needed? Due to the specificity of allelopathy, most studies are local case studies. Therefore, it might be most fruitful to expand studies in which allelopathy

has been previously reported, to learn more about the circumstances under which a specific allelopathic interaction can be observed. In this way researchers can add experimental components that narrow the range of mechanisms such as distinguishing between direct chemical versus microbially mediated effects, or discern which plants in a native community are most susceptible to the allelopathy of an invader.

With this purpose in mind, I set out to expand a study that previously demonstrated a very strong allelopathic effect of a widely distributed exotic grass, *Bothriochloa ischaemum*, on native warm-season grass species of the mixed grass prairie of central Texas. *Bothriochloa ischaemum* is widely invasive in the Southern United States, and has been known to replace diverse prairie communities with near monocultures (Gabbard and Fowler 2007). Because of this nearly complete suppression of native grasses irrespective of their growth strategies, there has been speculation that allelopathy might help to explain its invasion success.

The limited experimental work on potential allelopathy in *B. ischaemum* has produced mixed results. One study, which included both lab and greenhouse experiments using *B. ischaemum* whole-plant aqueous extracts, found strong inhibitory effects on both the germination and vegetative growth of competing native grasses of the Andropogonae tribe (*Schizachyrium scoparium* and *Andropogon gerardii*, Little and Big Bluestem) (Greer et al. 2014). In contrast, in a study of plant-soil feedback, Hawkes et al. (2012) found that the native congener *Bothriochloa laguroides* (Silver Bluestem) actually benefited when grown in soil conditioned by *B. ischaemum*, compared to other treatments. To my knowledge, these are the only studies that explore potential allelopathy in *B. ischaemum*. Together, they were inconclusive and left questions of mechanism

unanswered. For example, Hawkes et al. (2012) did not resolve whether *B. ischaemum* altered soil chemistry or the microbial community, while Greer et al. (2014) did not resolve whether the strong responses observed were due to direct chemical or indirect microbial effects.

Greer et al. (2014) utilized an aqueous extract (“leachate”), made from the shoots and roots of *B. ischaemum*. The leachate was not sterilized before use, and would have contained microbes present on the plants or in particles of field soil clinging to the roots. The results were striking: *B. ischaemum* leachate reduced the germination rates of both *A. gerardii* and *S. scoparium* by over 90% relative to a water control, while treatment of the same species with a leachate made from the native *A. gerardii* yielded no significant difference from the water control. Biomass production was also very significantly reduced in both native target species after application of *B. ischaemum* leachate, when compared with *A. gerardii* leachate and with the water control. In *S. scoparium* and *A. gerardii* plants, both above-ground and below-ground biomass were reduced by nearly 100% compared with the water control.

Greer et al. (2014) also carried out chemical analysis of both *B. ischaemum* and *A. gerardii*, searching for differences between the two leachates that might explain *B. ischaemum*’s strong inhibitory effects. They tested PH, tested for the presence of phenols, and compared the elemental composition of the two leachates. The only difference found was that phenolic content was significantly higher in the native *A. gerardii* leachate. Allelopathy in some *Bothriochloa* species has been attributed to high levels of sesquiterpenes and monoterpenes (Scrivanti 2010), which Greer et al. (2014) did not analyze. The chemicals or compounds responsible for *B. ischaemum*’s allelopathic

effect, and also the mechanism of that effect (direct, or mediated by microbial interactions) therefore remains uncertain. Greer et al. (2014) concluded that “it is unclear if these allelopathic effects directly hinder competitors, or indirectly hinder them through alterations in soil microbial communities, however, reductions in germination of native seeds strongly support direct allelopathic effects.”

The study presented herein was designed to 1) determine whether the effects observed in the Greer et al. (2014) studies were repeatable; 2) further explore whether the allelopathic effects of *B. ischaemum* leachate are due to a direct chemical or microbially mediated effect, and 3) test the effects of *B. ischaemum* leachate on four additional species. Specifically, my experiments tested the effects of *B. ischaemum* leachate on the germination and growth of five native grasses and two non-native grasses which characterize the grasslands of the Edwards Plateau of Central Texas.

I conducted two experiments, one on vegetative growth and one on germination. In the growth experiment, I used both sterilized leachates and sterilized growth medium, to test for direct allelopathic effects and rule out microbially mediated effects. In the germination experiment, I used both sterilized and unsterilized (‘raw’) leachates, to test for and discern between direct chemical allelopathy and microbially mediated effects. Since it is possible that autoclaving as a method of sterilization could denature active chemicals, I used both autoclaving and vacuum filtration as two independent methods of sterilization in my germination experiment. Similar to Greer et al. (2014), I used the leachate of a dominant native species as a reference. Because all plants produce compounds that are phytotoxic to another plant species at some concentration (Dayan and Duke 2009), it was essential in both experiments to compare the effects of *B. ischaemum*

leachate not only to a pure water control, but also to the leachate of a similar native species that is not known to be allelopathic. Greer et al. (2014) used *Andropogon gerardii* for this purpose; I selected *Schizochyrium scoparium* because it is more common in native plant communities on the Edwards Plateau, where my studies took place.

II. HYPOTHESES

First, I expected the germination and the growth of all target species to be significantly suppressed by *B. ischaemum* leachate compared to the leachate of the native reference species or the water control, as Greer et al. (2014) observed in target species *A. gerardii* and *S. scoparium*. I expected the degree of suppression to vary somewhat between target species. Second, I expected *B. ischaemum* leachate to have less of an inhibitory effect on the growth or germination of *B. ischaemum* itself, compared to its effects on *S. scoparium* and *A. gerardii*, because this species frequently grows in near-monocultures (Gabbard and Fowler 2007) and because it was less inhibited by its own leachate than the other two target species were in Greer et al.'s (2014) study. Third, I expected the results of both experiments to indicate direct chemical allelopathic effect as the mechanism of allelopathy in *B. ischaemum*. This would be confirmed if a) there were significant inhibitory effects in the greenhouse growth study, in which the leachates and soil were sterilized, and also b) if the effects of the sterilized and unsterilized leachates were the same in the germination experiment. A contrary result, in which a) no suppression was observed in the growth study and b) the unsterilized *B. ischaemum* leachate used in the germination study was inhibitory but the sterilized leachates were not, would indicate an indirect allelopathic effect mediated by microbial communities. Fourth, I expected that the autoclaved and vacuum filtered *B. ischaemum* leachates used in the germination study would have the same effects as one another, indicating that the allelopathic effect is due to chemical compounds that are heat-stable.

III. METHODS

Study species

The seven species studied included two non-native grasses and five native grasses (Table 1).

Table 1. Seven Poaceae species used as target species in the growth experiment and the germination experiment. All species are warm season C4 grasses that grow on the Edwards Plateau of Central Texas.

Latin name	Common name	Edwards Plateau native or non-native	Used in growth study	Used in germination study
<i>Schizachyrium scoparium</i>	Little Bluestem	Native	x	x
<i>Andropogon gerardii</i>	Big Bluestem	Native	x	x
<i>Bouteloua curtipendula</i>	Sideoats Grama	Native	x	x
<i>Bothriochloa laguroides</i>	Silver Bluestem	Native	x	x
<i>Sorghastrum nutans</i>	Indian Grass	Native	x	
<i>Bothriochloa ischaemum</i>	King Ranch Bluestem	Non-native	x	x
<i>Dicanthium sericeum</i>	Silky Bluestem	Non-native	x	x

Bothriochloa ischaemum (King Ranch Bluestem) is native to parts of Europe and Asia (Gabbard and Fowler 2007) and is widely invasive in the grassland and savannah habitats of the Southern United States. Due to its drought-hardiness and quick establishment, it was originally introduced to stabilize roadsides and railroad right-of-ways, and also cultivated in pastures as cattle forage (White and Dewald 1996; Harmoney and Hickman 2004; Harmoney et al. 2007). *Bothriochloa ischaemum* readily escaped containment, displaced native grasses as it spread throughout the southern US, and can

now be found in 17 Southern states (Sammon and Wilkens 2005).

The five native target species (Table 1) all have large geographic ranges in North America, and overlap on the Edwards Plateau, albeit occupying different habitats.

Bothriochloa ischaemum, like many invasive species, is a generalist that can occupy and will invade all of these native species' habitats (Gabbard and Fowler 2007.)

Schizachyrium scoparium is a dominant upland native grass species on the Edwards Plateau. It is moderately palatable to livestock, and therefore is often absent from heavily grazed pastures; it still occurs in stable upland grassland communities that have not been intensively or recently over-grazed (Tober and Jenson 2013). *Sorghastrum nutans* and *A. gerardii* are both associated with upland drainages and full sun riparian habitat, and are both more palatable and more sensitive to continuous grazing than *S. scoparium* (USDA NRCS 2002; Wennerberg 2004). These species may have been more prevalent on the Edwards Plateau prior to widespread overgrazing and invasion by non-native invasive species, notably *B. ischaemum*. *Schizachyrium scoparium*, *A. gerardii*, and *S. nutans* are three of the “big four” native prairie grasses in North America and are all associated with high-quality native grasslands. *Bouteloua curtipendula*, another of the dominant upland species on the Edwards Plateau, establishes readily from seed, even on dry or otherwise unproductive sites, and tends to increase following drought (Wynia 2017). *Bothriochloa laguroides* is the native study species most associated with disturbance. Like *B. ischaemum*, it thrives in a range of soil types and weather conditions, and in many years produces seed throughout the growing season (Maher and Reilley 2018). *Dicanthium sericeum*, the non-native target species in my studies, is an Australian grass that arrived in Texas sometime in the 1940s, around the same time as *B.*

ischaemum. It is considered “not very widespread to date,” according to TexasInvasives.org, and noted as present only in Texas, Mississippi, Florida, and Hawaii. In recent years, biologists with both the Lady Bird Johnson Wildflower Center in Austin (M. Bertelsen and D. Davis, personal communication, 2016) and the City of Austin’s Water Quality Protection Lands (D. Grobert, personal communication, 2016) have observed that *D. sericeum* is becoming more prevalent on the land that they manage in Travis and Hays counties (though they had not seen it on these sites prior to 2013), and growing in close proximity with *B. ischaemum*. *Dicanthium sericeum*’s new presence on land in and around South Austin seems to represent an increase of its overall range in Texas.

The majority of the seed for my studies was collected by hand during Fall of 2016, from three sites in western Travis County, Texas. A small amount of supplemental *A. gerardii* and *S. nutans* seed was purchased from Native American Seed Company (Junction, TX), to assure sufficient numbers for the studies. Within each species, seed from different sources was mixed, to ensure that seed mixes were distributed evenly across treatment groups.

Leachate preparation

Leachates were prepared from fresh whole *B. ischaemum* and *S. scoparium* plants, to capture both water-soluble leaf and stem compounds as well as root exudates. The plants were harvested from a site in western Travis County (which was also the main seed collection site for my studies). After the roots were manually cleaned of debris, the still-fresh biomass was soaked for 72 hours in distilled water (5mL/gram of plant material) at room temperature (approximately 70°F.) (Roberts and Anderson 2001;

Dorning and Cipollini 2006; Harnden et al. 2011; Greer et al. 2014.) The plant biomass was then removed, and the leachates were filtered through a double layer of sterile cheesecloth to remove any remaining soil or plant debris. For the growth experiment, plants were harvested in early June of 2017, when plants were in the vegetative phase. Both species' leachates were autoclaved and stored at 40°F for the duration of the experiment. For the germination experiment, a separate leachate was prepared from plants collected in late October 2017, when plants were in the flowering stage. One third of the batch was left "raw"; one third was centrifuged, then vacuum filtered through a 0.2 micron filter to remove microbes (Korczynski 1981), and one third was autoclaved. All preparations were stored at 40°F until use.

Growth study: Experimental design and analysis

The growth study employed a complete factorial design in which the seven species were each grown with three treatments (autoclaved *B. ischaemum* leachate, autoclaved *S. scoparium* leachate, and a distilled water control), in soil-filled Cone-tainers (Leach and Bergeron 1975) in a greenhouse for 9 weeks. Each species x treatment combination was replicated 21x, and the arrangement in the greenhouse followed a blocked design with three blocks, within which species and treatments were fully randomized.

Seeds were planted in triplicate, directly into the in Cone-tainers, in a soil mix based on the UC Soil Mix III formulation from University of California Riverside (<http://agops.ucr.edu/soil/>). This mixture consists primarily of sand and peat moss, with small amounts of added macro and micronutrients. Sand and peat moss were autoclaved before being mixed. This mixture was used instead of clay-heavy local field soil so that

the roots could be extracted and cleaned without damage. The sterilization of the soil mixture components was to further ensure any significant leachate effect observed would be due to chemical allelopathy directly inhibiting vegetative growth.

After containers were seeded, any duplicate seedlings per container were carefully removed as soon as they emerged, so there would be only one plant per container. I kept the most central seedling in each container, and removed the others. For the first two weeks, all seedlings were watered with captured rainwater. Two weeks after seeding, plant height from the soil to the tip of the longest blade was recorded (to be used in later analysis as a covariate), and treatment applications began. Twenty-seven mL of undiluted leachate or water, enough to saturate the soil in each Cone-tainer, was applied once a week, for seven weeks. Supplemental water sufficient to saturate the soil but not drain from the Cone-tainers was given to all plants once weekly between each treatment application, to keep the seedlings well-watered. All seedlings survived the seven-week treatment period. A final plant height was recorded before plants were uprooted and washed free of most soil, separated into root and shoot portions, and dried at 70°C. The removal of remaining peat moss was continued on dried root material. Roots and shoots were then weighed separately.

The four response variables measured in this experiment were above-ground height achieved (i.e., the difference between pre-treatment height and final height), below-ground biomass, above-ground biomass, and total biomass. Log-transformed root, shoot and total biomass, as well as the root/shoot ratio and the root/total biomass ratio, were analyzed using ANCOVA, with species and treatment as factors and pre-treatment plant height as a covariate. Above ground height achieved was analyzed using two-way

ANOVA, with species and treatment as factors. I tested for significant block effects but since there were none, I removed block as a factor from the final analysis. Target species and treatment differences were examined by post-hoc analysis. Height and biomass produced under each treatment were compared within each grass species, to determine whether a species was inhibited by *B. ischaemum* leachate, compared with the effects of *S. scoparium* leachate and water. An inhibitory effect would be indicated by significantly reduced height or biomass measurements in the plants that received *B. ischaemum* leachate, compared with plants of the same species that received *S. scoparium* leachate or water.

Germination study: Experimental design and analysis

This study also followed a factorial design, with six target species and seven treatments (three different preparations of *B. ischaemum* leachate, three different preparations of *S. scoparium* leachate, and distilled water as a control). Each species-treatment combination was replicated three times, and each sample consisted of 50 seeds in a petri dish. Dishes were arranged in the lab in a blocked design with three blocks, within which species and treatment were fully randomized.

Each petri dish was lined with filter paper, and fifty seeds of a single species were placed on top of the filter paper. Dishes were placed on a lab table and maintained at room temperature (approximately 70°F) during the experiment. The filter papers were soaked to saturation with 2.5mL of leachate or distilled water and the petri dishes were covered with lids. Since the filter paper remained damp for the duration of the experiment, treatments were not repeated.

The response variable for this experiment was the number of seeds germinated in

each dish. Germinated seeds were counted on day 6 and again on day 8. On day 5, germination was still extremely low, around 1%. On day 6, germination averaged over 15%, so I began data collection. A seed was considered to have germinated when the radical had emerged at least 2 mm from the seed coat (Beligni and Lamattina 2000; Greer et al. 2014). Each germinated seedling was removed after counting (Harnden et al., 2011; Greer et al., 2014). I planned to count germinated seeds every other day (Greer et al. 2014), so I counted again on day 8. By day 10 of the study, mold had grown in some of the dishes and obscured germinated seeds, so the experiment was terminated. Since amount of fungal growth varied among dishes, I scored the amount of fungal growth in each dish on a scale of 0 to 3, where 0 indicated no visible fungal growth and 3 indicated the largest amount of visible fungal growth.

Counts of germinated seeds were first converted to proportions of total seeds per dish, then logit transformed. Two of the seed species (*B. laguroides* and *S. scoparium*) exhibited extremely low germination across all treatments and thus were omitted from the analyses. Four target species were analyzed: *A. gerardii*, *B. curtispindula*, *D. sericeum*, and *B. ischaemum*.

Since the design was somewhat asymmetric (e.g., only one water control, but three preparations of each leachate), the analysis was broken into several steps to address my hypotheses. In summary, I first compared effects of the two leachate species and all three leachate preparations, while omitting the water control. I next compared effects of the two leachate species, one processing method at a time, with the water control. All of the following analyses were conducted twice, first on the counts from day 6, and again on the counts from day 8.

To test for the overall differences in the effects of *B. ischaemum* and *S. scoparium* leachates, as well differences in effects among target species, and leachate preparations (autoclaved, vacuum filtered, or unsterilized) on inhibiting seed germination, I first implemented a three-factor ANOVA with leachate species, leachate sterilization, and seed species as the three factors, omitting the water control treatment. With a replication number of 3, I did not test for 3-way interactive effects. A post-hoc Tukey's test at $\alpha = 0.05$ was used in pairwise comparisons between treatments and species (Freund and Wilson 2003). Since leachate sterilization interacted with seed species, separate ANOVAs were then carried out to compare the effects of sterilization within each seed species.

To examine the strength of the allelopathic effect relative to water as a control, I then conducted a separate two-way ANOVA for each leachate sterilization method (for example, comparing raw *B. ischaemum*, raw *S. scoparium*, and the water control), with leachate species and seed species as factors. A post-hoc Tukey's test at $\alpha = 0.05$ was again used to test for pair-wise differences between treatments and species.

To examine the potential effect of leachate treatment and seed species on the amount of mold that developed, I used an ordinal regression analysis (Harrell 2015). To examine if the amount of mold affected seed germination, I conducted an ANOVA to examine the mold score as a potentially significant factor in seed germination.

IV. RESULTS

Leachate effects on growth

ANCOVA showed that across all seven grass species, there were no significant effects of autoclaved leachate treatment on root biomass, shoot biomass, total biomass, the root/shoot ratio, or the root/total biomass ratio (Table 2). However, there were significant species effects on all of those variables (Table 2, Figure 1) as well as significant species x treatment interactions affecting root biomass and total biomass (Table 2).

Table 2. Results of ANCOVA showing effects of factors grass species and autoclaved leachate treatment on log transformed dry biomass (root, shoot, and total; the root/shoot biomass ratio, and the root/total biomass ratio) of seven target species at the end of seven weeks of treatments. Initial height of seedlings at the time treatment began was used as a covariate.

Variable	Factor	df	F	Sig.
Root Biomass	Height Covariate	1	206.358	<.001
	Grass Species	6	50.263	<.001
	Leachate	2	.209	.812
	Grass Species x Leachate	12	2.231	.010
Shoot Biomass	Height Covariate	1	187.978	<.001
	Grass Species	6	54.838	<.001
	Leachate	2	1.745	.176
	Grass Species x Leachate	12	1.766	.052
Total Biomass	Height Covariate	1	205.196	<.001
	Grass Species	6	35.815	<.001
	Leachate	2	.754	.471
	Grass Species x Leachate	12	1.964	.026
Root / Shoot Biomass Ratio	Height Covariate	1	.013	.909
	Grass Species	6	231.433	<.001
	Leachate	2	1.948	.144
	Grass Species x Leachate	12	1.489	.126

Root / Total Biomass Ratio

Height Covariate	1	.068	.794
Grass Species	6	169.104	<.001
Leachate	2	1.234	.292
Grass Species x Leachate	12	1.319	.205

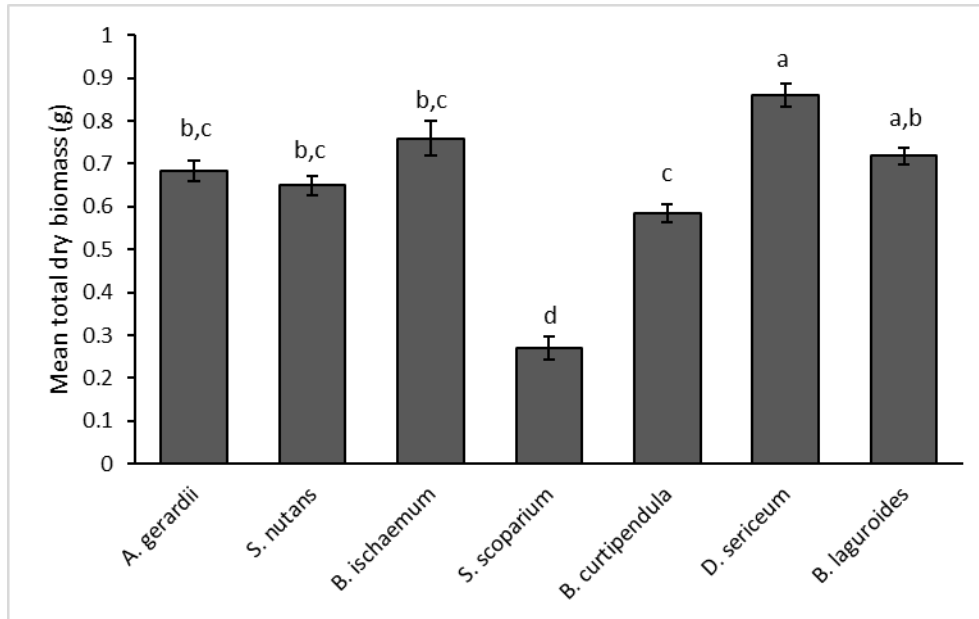


Figure 1. Mean total biomass by species, following seven weeks of treatments in the growth study. Significant differences (alpha = 0.05) are indicated by different lower-case letters. Errors bars represent ± 1 SE.

Single-species ANOVAs determined that two of the target species, *B. laguroides* and *D. sericeum*, responded to treatments and were the source of the significant species x treatment interactions (Figure 2). In *D. sericeum*, *B. ischaemum* leachate had a slight, but significant, negative effect on root biomass, which showed up in the root/shoot and root/total ratios as well. This effect was just strong enough to be significantly different than the water control, but not significantly different than the *S. scoparium* leachate. For target species *B. laguroides*, *B. ischaemum* had the same slight but significant negative

effect on root biomass, this time significantly different than the *S. scoparium* leachate, but not the water control. This effect also showed up as a significant effect on total biomass in this species, and affected the root/shoot and root/total ratios as well (Figure 2).

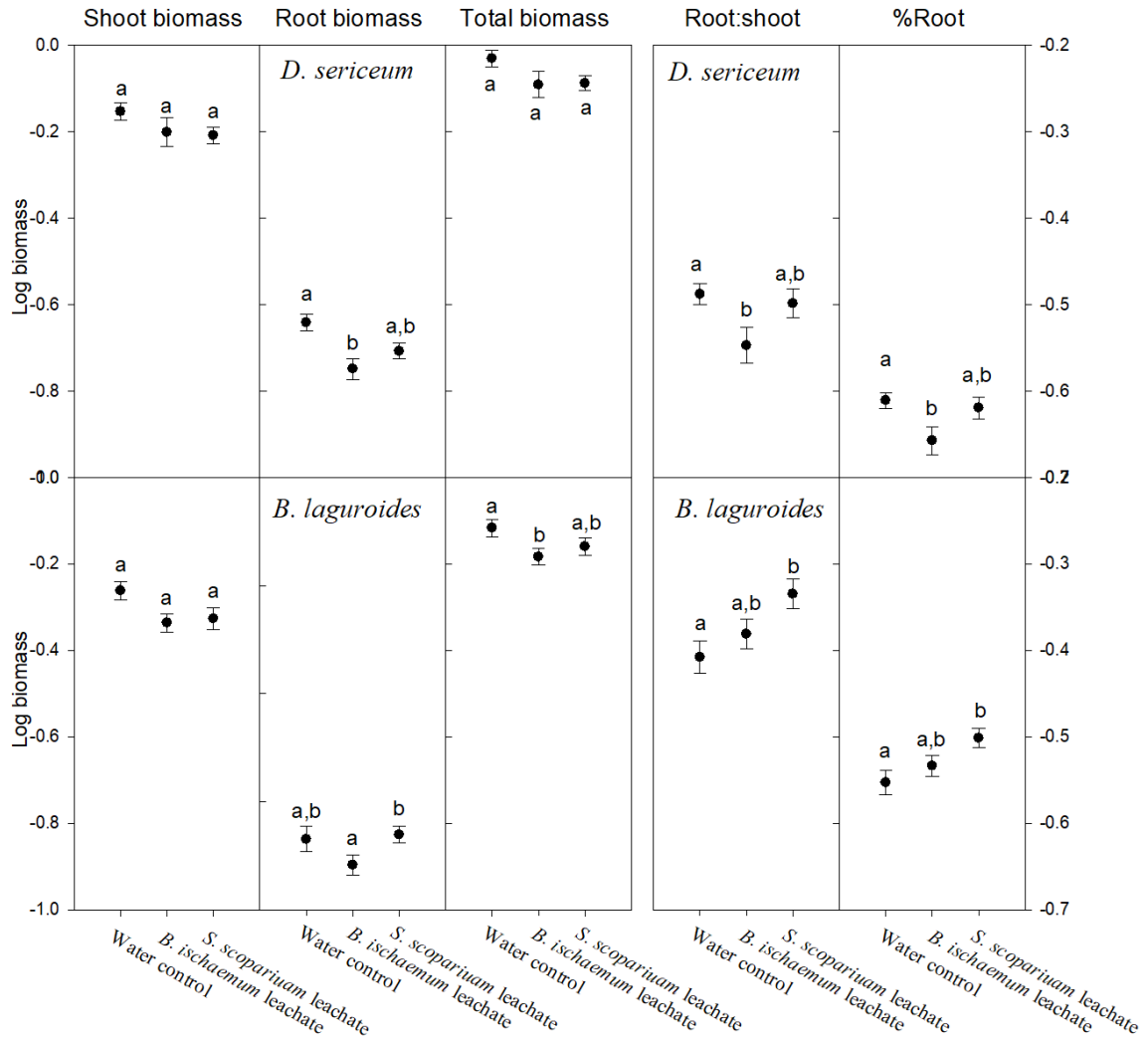


Figure 2. Treatment effects on shoot biomass, root biomass, total biomass, root/shoot ratio, and root/total biomass ratio of target species *D. sericeum* and *B. laguroides*. Lower case letters indicate significant differences (alpha = 0.05). Error bars represent ± 1 SE.

ANOVA results on plant height (difference between pre-treatment height and final height) showed that, similar to the results of ANCOVAs on biomass variables, there were no significant treatment effects on plant height (Table 3), but there were significant

differences in height between grass species (Table 3, Figure 3). The two non-native, invasive species (*B. ischaemum* and *D. sericeum*) and also the native *B. laguroides* grew fastest, reflected in greatest mean heights at the study's end.

Table 3. ANOVA results showing effects of factors grass species and autoclaved leachate treatment on aboveground height achieved (difference between initial pre-treatment height and final height) during treatment period during the growth study.

Factor	df	F	Sig.
Grass Species	6	53.85	<.001
Leachate	2	2.158	.290
Grass Species x Leachate	12	1.434	.136

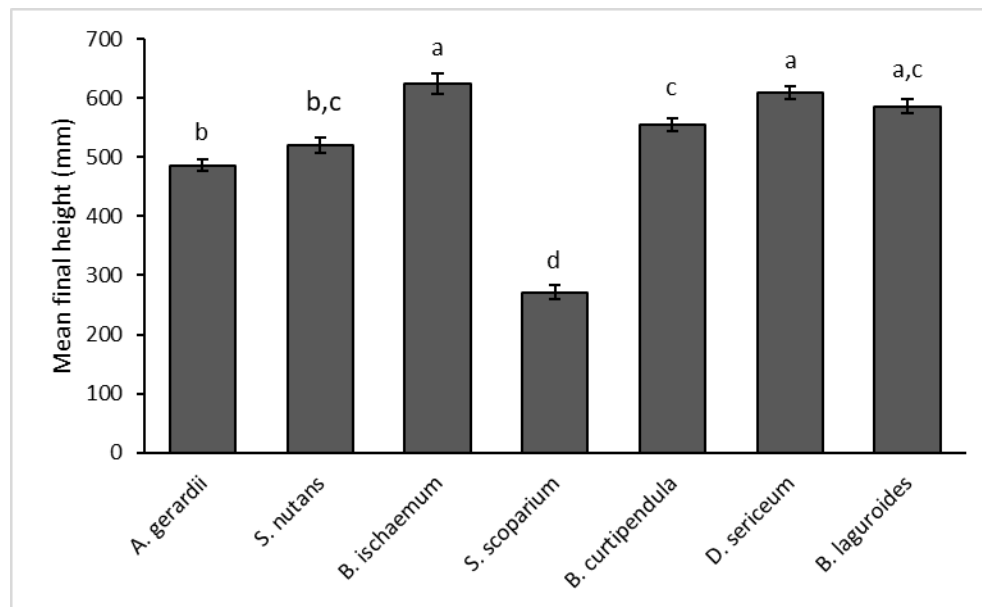


Figure 3. Mean final height by species, following seven weeks of treatments in the growth study. Significant differences ($\alpha = 0.05$) are indicated by different lower-case letters. Errors bars represent ± 1 SE.

Leachate effects on germination

In the germination study, across all treatments, two seed species (*S. scoparium* and *B. laguroides*) exhibited such low germination rates that they were excluded from the analysis (Figure 4).

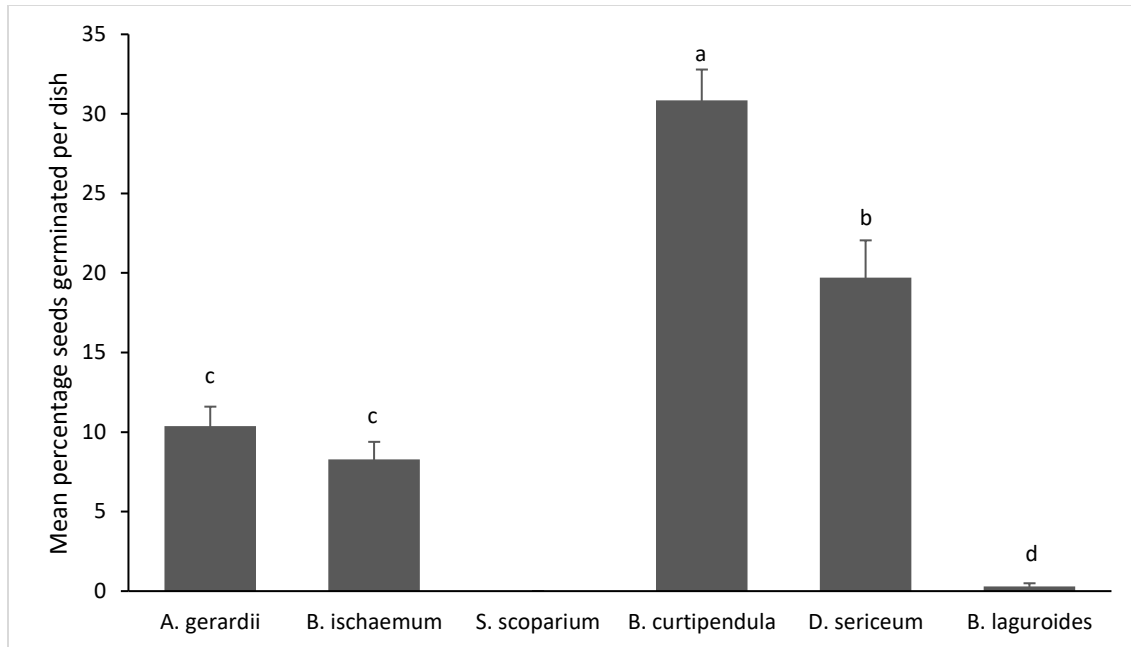


Figure 4. Mean percent germination by species on day 6 of the germination study. Significant differences ($\alpha = 0.05$) are indicated by different lower-case letters. Error bars represent ± 1 SE.

Six days after inducing germination, leachate species, leachate sterilization method, and seed species all significantly affected seed germination (Table 4). *Bouteloua curtipendula* had significantly higher germination rates than any other species, followed by *D. sericeum*, which had significantly higher germination rates than *A. gerardii* and *B. ischaemum* (Figure 4). Across seed species, significantly fewer seeds germinated when treated with *B. ischaemum* leachate compared to *S. scoparium* leachate and water (Figure 5), and significantly fewer seeds germinated when treated with sterilized leachates compared to raw leachates or water (Figure 6). There was also a significant interaction between seed species and leachate sterilization method on day 6 (Table 4). Two seed species (*B. ischaemum* and *D. sericeum*) responded to leachate sterilization (Figure 7), together giving rise to the significant interaction. *Bothriochloa ischaemum* seed germination was higher under raw leachates than under sterilized leachates (Figure 7). The same pattern of higher germination under the raw leachates was present for *D.*

sericeum seed (Figure 7), but due to the low number of replications in my study, the effect was only marginally significant for this species. Eight days after inducing germination, effect of leachate species and leachate sterilization method were no longer significant, no significant interactions were present and only the overall species effects on germination rates remained significant. (Table 4).

Table 4. Three-way ANOVA on logit transformed data, showing effects of factors seed species, leachate species, and leachate sterilization on seed germination on days 6 and 8 of the germination study. The three-way interaction was omitted because of low sample size.

Factor	df	F	Sig.
<i>Day 6</i>			
Seed Species	3	28.139	<.001
Leachate Species	1	5.615	.021
Leachate Sterilization	2	3.862	.027
Seed Species x Leachate Species	3	1.108	.354
Seed Species x Leachate Sterilization	6	2.687	.024
Leachate Species x Leachate Sterilization	2	1.299	.281
<i>Day 8</i>			
Seed Species	3	53.289	<.001
Leachate Species	1	.015	.572
Leachate Sterilization	2	3.586	.165
Seed Species x Leachate Species	3	.713	.182
Seed Species x Leachate Sterilization	6	1.150	.298
Leachate Species x Leachate Sterilization	2	.505	.404

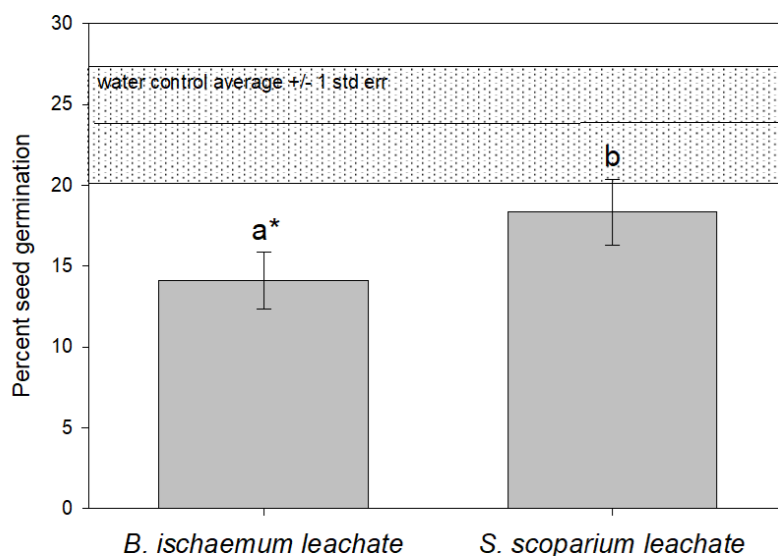


Figure 5. Significant treatment effect of leachate species on mean seed germination, across all seed species and leachate sterilization methods, on day 6 of the germination study. Significant differences ($\alpha = 0.05$) between treatments are indicated by different lower-case letters. * indicates a significant difference with the water control. Error bars represent ± 1 SE.

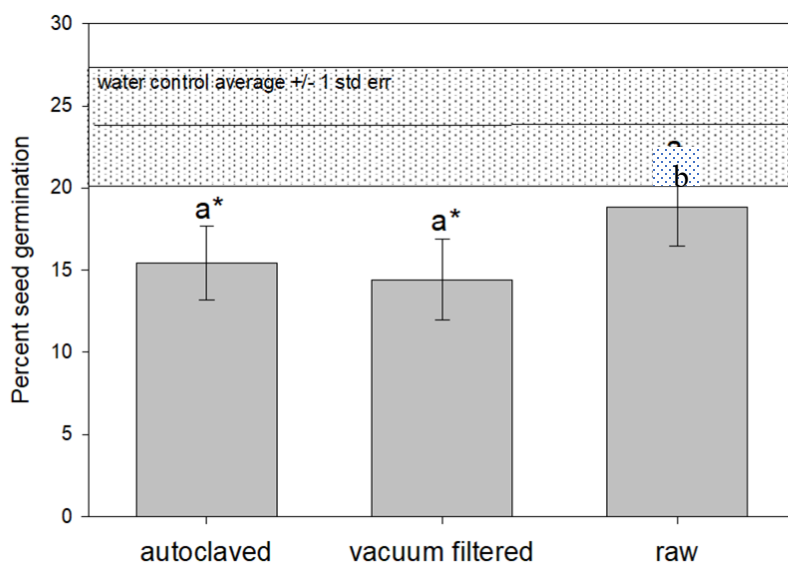


Figure 6. Significant treatment effect of leachate sterilization on mean seed germination, across all seed species and leachate species, on day 6 of the germination study. Significant differences ($\alpha = 0.05$) between treatments are indicated by different lower-case letters. * indicates a significant difference with the water control. Error bars represent ± 1 SE.

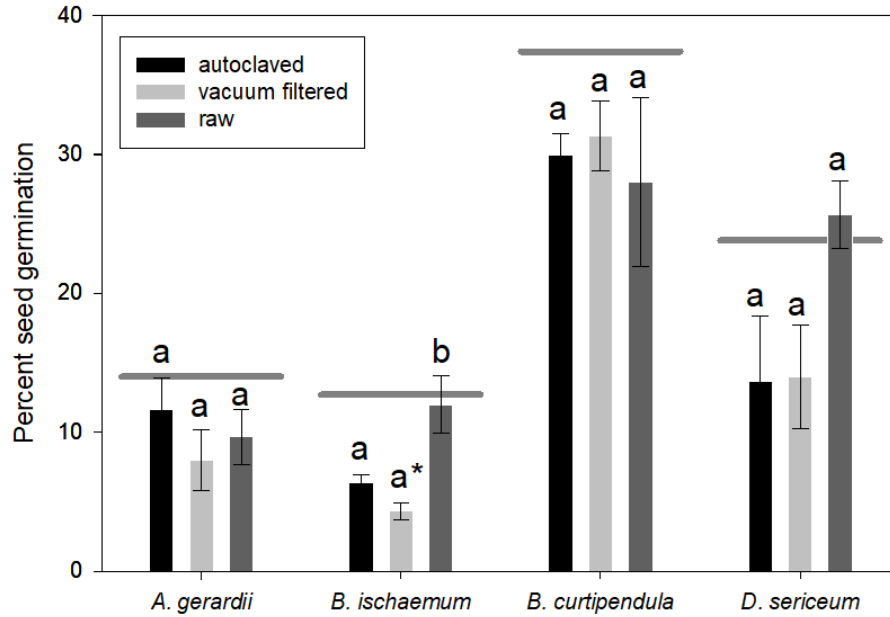


Figure 7. Interaction between seed species and leachate sterilization method on day 6 of the germination study. Germination rates are shown for each target species 6 days after initiating germination. Horizontal bars indicate germination rates under the water control. Lower case letters indicate significant differences ($\alpha = 0.05$) within groups, and * indicates significant difference from the water control.

While the germination experiment was conducted, mold began to grow in the petri dishes. Visual inspection indicated that the amount of fungal growth was related to which treatment the dish had received (Figures 8 & 9). The ordinal regression confirmed that by far the largest amount of fungal growth occurred in dishes that were given sterilized *B. ischaemum* leachates (Table 5). Both autoclaved and vacuum filtered *B. ischaemum* leachate caused this effect, across all seed species. Further, the raw *B. ischaemum* leachate, and all three preparations of *S. scoparium* leachate, had significantly lower amounts of mold, and dishes that received the water control had the least mold of all (Figure 10). An ANOVA including mold score as a factor showed that mold had a significant effect on seed germination (Table 6).

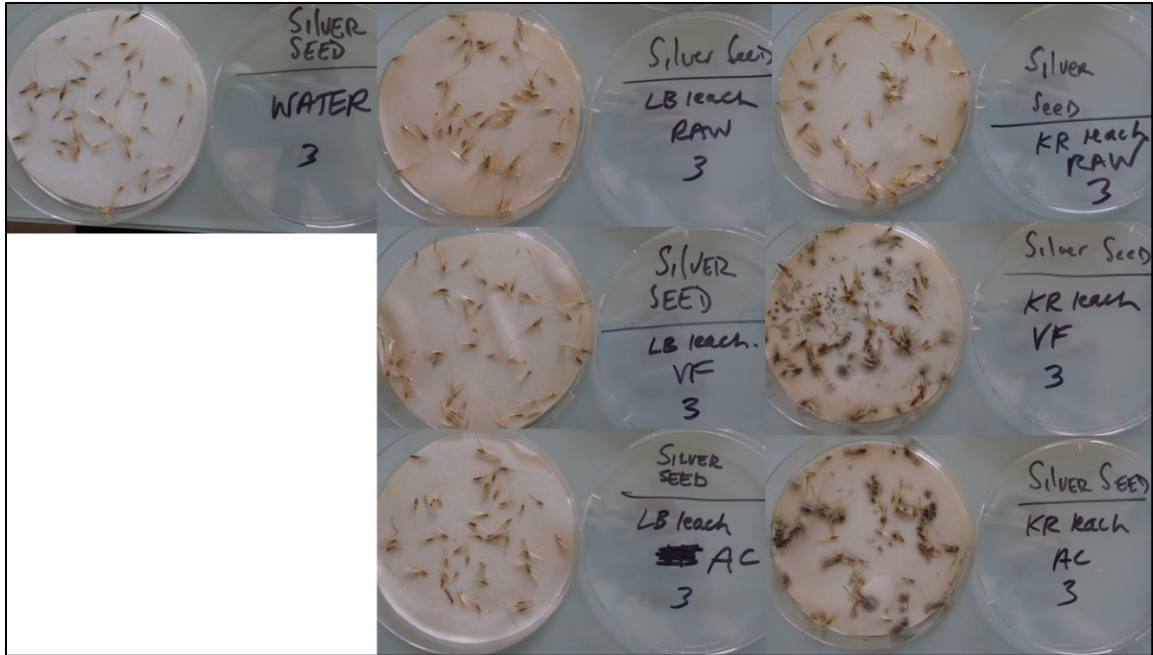


Figure 8. Photographic comparison of fungal growth in dishes containing *B. laguroides* seeds (abbreviated “Silver” for common name). Since different notation was used to label treatments in the experiment vs in this manuscript, treatments are identified as follows. In the first column, the dish received the distilled water control. In the second column, top to bottom, dishes received unsterilized *S. scoparium* leachate, *S. scoparium* leachate sterilized by vacuum filtration, and *S. scoparium* leachate sterilized by autoclave. In the third column, top to bottom, dishes received unsterilized *B. ischaemum* leachate, *B. ischaemum* leachate sterilized by vacuum filtration, and *B. ischaemum* leachate sterilized by autoclave.

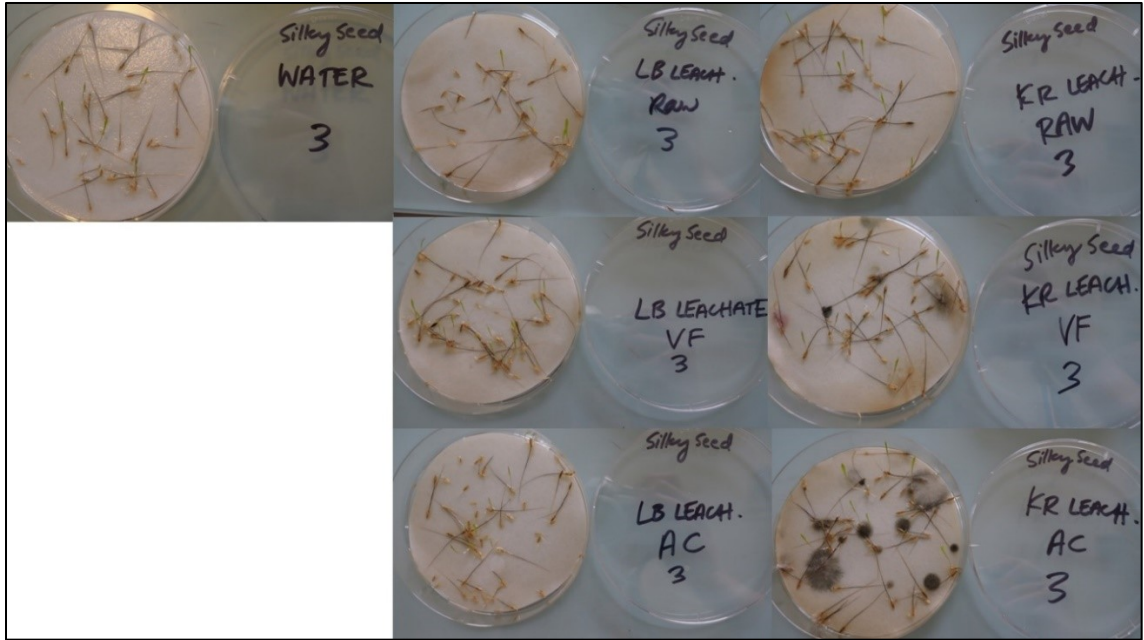


Figure 9. Photographic comparison of fungal growth in dishes containing *D. sericeum* seeds (abbreviated “Silky” for common name). Treatments are organized for comparison in the same way as treatments in Figure 11: distilled water; *S. scoparium* leachates unsterilized, vacuum filtered, and autoclaved; and *B. ischaemum* leachates unsterilized, vacuum filtered, and autoclaved.

Table 5. Results of ordinal regression examining the effects of factors leachate species and leachate sterilization method on amount of mold present in dishes on day 6 of the germination study. * indicates the level used as the reference.

Factor	Observation	Std. Error	df	Sig.
Leachate Species	<i>B. ischaemum</i> leachate	1.009	1	<.001
	<i>S. scoparium</i> leachate*		0	
Leachate Sterilization Method	Autoclaved	.590	1	.917
	Vacuum Filtered	.902	1	<.001
	Raw*		0	

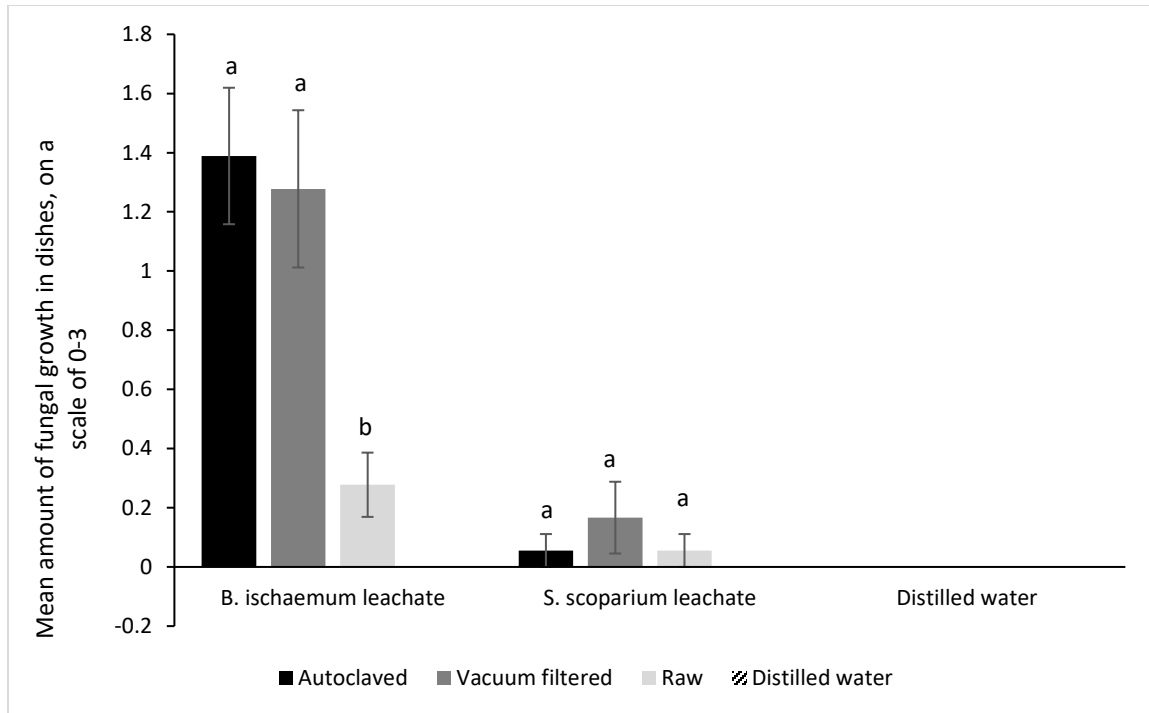


Figure 10. Significant effects of leachate species and leachate sterilization method on amount of fungal growth in dishes, averaged across all seed species. Significant differences ($\alpha=0.05$) within groups are indicated by different lower-case letters. Errors bars represent ± 1 SE. Fungal growth under the distilled water control is shown as a reference.

Table 6. ANOVA results showing significant effect of mold on seed germination.

Factor	df	F	Sig.
Seed species	3	8.586	<.001
Mold score	1	4.352	.046
Seed species x mold score	3	.753	.530

V. DISCUSSION

I hypothesized that an allelopathic effect of *B. ischaemum* leachate would significantly suppress the growth and germination of the target species in my studies, relative to leachate made from the native *S. scoparium* and relative to a water control. I also expected that my experiments would yield evidence of a direct chemical allelopathic effect, rather than a microbially mediated effect. Thus, I expected the sterilized *B. ischaemum* leachate used in the greenhouse study to suppress seedling growth, and for all leachate preparations of *B. ischaemum* to have the same effect on seed germination. Both hypotheses were rejected: while there was evidence of a weak effect of leachate on root growth in two species in the greenhouse study (Figure 2), in one case, growth was equally suppressed by *B. ischaemum* and *S. scoparium* leachate relative to the water control, and, in the other case, *B. ischaemum* leachate suppressed growth relative to *S. scoparium* leachate, but not relative to the water control. Thus, overall, there was a weak, inconsistent, negative effect of *B. ischaemum* leachate on root growth, which was not significantly more negative than that of both water and *S. scoparium* leachate, as would be expected if the effect were allelopathic.

In my germination study, I only observed significant treatment effects on day 6 after inducing germination, and not on day 8. Thus, at most, leachates had a delaying effect on seed germination. Still, in a competitive context, delays can result in significant competitive suppression (Gioria et al. 2016, Gioria and Pyšek 2017). The effect on day 6 was significantly stronger for *B. ischaemum* leachate than for *S. scoparium* leachate and the water control (Figure 5), indicative of a negative effect specific to *B. ischaemum*, rather than a general effect of leachates. However, germination was suppressed the most

by sterilized leachates (Figure 6), which neither supports the hypothesis of a direct chemical effect, which would not have been affected by sterilization, nor the hypothesis of a negative microbial effect, which would have been eliminated by sterilization.

This somewhat confusing result may be explained at least in part by my ancillary observations on the mold that established in the petri dishes during the seed incubation period. Mold growth was much stronger in dishes that received *B. ischaemum* leachates, relative to *S. scoparium* leachates (Figure 10). Furthermore, there was significantly more mold growth in the dishes that received sterilized *B. ischaemum* leachates (both the autoclaved and vacuum filtered) than in raw *B. ischaemum* leachate, in a pattern opposite to seed germination (Figure 6). This seems to indicate that *B. ischaemum* leachate contained chemical compounds that stimulated mold growth, but also microbes that can suppress mold growth. Overall, the patterns are consistent with mold affecting germination success (Table 6), while *B. ischaemum* leachate controlled mold.

These results do suggest an effect consistent with the definition of allelopathy, involving both direct chemical and indirect microbial effects, in which *B. ischaemum* leachate supports the growth of generalist microbial enemies, while at the same time having defenses against such enemies in the form of anti-fungal microbes. However, it is not clear if and how such an effect would manifest in a natural grassland setting.

Several genera of fungi, including in the genus *Colletotrichum*, which is a mold, are strongly associated with *B. ischaemum* (Davidson 2018). Many *Colletotrichum* species are known to be pathogenic endophytes associating with some grasses but not others (Smiley et al. 1992; Browning et al. 1999), and it has been hypothesized that association with *Colletotrichum* fungi might be, at least in part, responsible for *B.*

ischaemum's allelopathy (Davidson, 2018).

Nevertheless, the overall results of my experiments are in stark contrast with the results published by Greer et al. (2014). Most significantly, Greer et al. (2014) documented nearly complete suppression of seed germination and seedling growth by unsterilized extracts of *B. ischaemum*. How can these differences be explained?

First, in the seedling growth study, I used sterilized leachates and growth medium, whereas Greer et al. (2014) used raw leachates and unsterilized field soil. This difference alone could imply that the effect observed by Greer et al. (2014) was microbially mediated and did not involve a direct chemical allelopathic effect. However, in the germination study where our methodologies were similar, our results were still very different. Using raw *B. ischaemum* leachate, Greer et al. (2014) observed nearly complete suppression of germination, whereas I observed only a brief delay in germination. This stark difference casts doubt on leachate sterilization being the decisive factor in the different outcomes of our studies. There may have been other factors at play.

As discussed in the introduction, several confounding factors can interfere with allelochemical effects. The production of allelochemicals can depend on the developmental stage of the donor plant (Iannucci et al. 2012), and environmental stress the donor plant might be under (Cipollini and Gruner 2007). Greer et al. (2014) harvested plants that were “actively growing” for leachate production. I did the same for my seedling growth study, harvesting plants that were in the vegetative stage. However, for my germination study, I harvested grasses that were in the reproductive stage and were flowering. The *B. ischaemum* plants harvested for leachate production in the Greer et al. (2014) studies and the ones I harvested for my studies were collected in different

geographic locations (Kansas and Central Texas), with different soils, different associated microbial communities, different climates and plant genotypes; any of these factors could have impacted allelochemical production.

The effect of allelochemicals on target plants can also be modified by external factors such as soil type (Wang et al. 2019), so another potentially important difference was the choice of growth medium in the seedling study. Greer et al. (2014) used a clay-heavy field soil, whereas I used a well-draining mixture of sand and peat moss. It is possible that in the Greer et al. (2014) study, allelochemicals accumulated at higher concentrations in the clay soil, and in my study, they were flushed away from the roots of the target plants to some extent during supplemental watering, due to the high percentage of sand in my mixture. However, growth medium would not have impacted the results of our germination studies.

In allelopathy studies, since it is not possible for one experimental design to include all combinations of potentially relevant factors, any specific study may fail to show allelopathic effects, even if effects were possible in another circumstance. For this reason, case studies that demonstrate allelopathy seem to warrant repetition and expansion, so we can learn more about the circumstances under which an allelopathic interaction occurs. Studies that most closely replicate field conditions seem most likely to capture such interactions where they do exist. In conclusion, I would argue that the case for *B. ischaemum* allelopathy remains unresolved, because my study and Greer et al.'s (2014) study came to opposite conclusions, for which there could be multiple potential explanations.

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