



KR bluestem (*Bothriochloa ischaemum* var. *songarica*) has some direct allelopathic effects on germination but not on seedling growth in a native/non-native mixed community of warm season grasses in Texas

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Abstract

Purpose Allelopathy is a plant interaction in which a donor species releases chemicals that suppress the development of receptor species. Allelopathy has been suggested as one explanation for catastrophic loss of native biodiversity in some invaded biomes; however, experimental tests of this hypothesis have had inconsistent results. Here, we examine if a previous finding of strong allelopathic effects of the warm-season, invasive C4 grass *Bothriochloa ischaemum* on North American prairie grasses can be reproduced in a different geographic setting.

Methods We examined the effects of sterilized (autoclaved or microfiltered) and unsterilized leachate on germination and the effect of sterilized leachate on seedling growth, including five native species and two exotic warm season grass species. For nine weeks, seedlings were irrigated with water or

autoclaved leachate from *B. ischaemum* or *Schizachyrium scoparium*, a native species.

Results Germination rates were significantly suppressed only in the two invasive species and only when treated with sterilized leachates. Seedling biomass at harvest was largely insensitive to leachate application.

Conclusions The present study did not replicate earlier results, though many details of the experimental designs were similar. However, we used sterilized leachates and soils, whereas the previous study did not, which could indicate mediation by indirect microbial effects in the previous study. In addition, historic differences in the introduction of *B. ischaemum* in Oklahoma and Texas, along with climatic differences, may have affected the evolution of allelopathy post-invasion. Future studies would benefit from comparisons of allelopathic effects across invasive species' ecotypes, using sterilized and unsterilized extracts.

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Introduction

Identifying and understanding mechanisms that determine invasive species success is the key to their management (Albers et al. 2018). Substantial research has been devoted to understanding the factors that contribute to invasive plant success, although the drivers

may be likely as varied as the invading species themselves (Kueffer et al. 2013). Nonetheless, contributing mechanisms generally fall into three categories. 1) Invasive species are often superior in exploiting available resources. For example, many have higher photosynthetic rates with lower nutrient requirements combined with quick nutrient acquisition and high propagule pressure and germination rates (Gioria and Osborne 2014). 2) Invasive species may be ignored by local consumers and pathogens, thereby reducing losses relative to what they experience in their native range or relative to other species in their new range. In these scenarios enemy escape may simultaneously intensify enemy pressure on native species on the invasion frontier (Orrock et al. 2010) and/or alter the environment wholesale, in essence destroying native species' habitat (Reynolds et al. 2017). 3) Invasive species may arrive with 'novel weapons', allelopathic secondary metabolites that lower native species' vital rates (Hierro and Callaway 2003). Here, we examine evidence for the third type of mechanism.

Allelochemicals act through a variety of mechanisms that typically suppress germination and growth with negative consequences for survival, development and reproduction (Einhellig 1995; Rice 1987). How these metabolites operate is rarely known but may include several different proximate mechanisms, including direct phytotoxicity that disrupts enzyme function (Cai and Mu 2012), altering soil pH and thus nutrient availability (Cai and Mu 2012), or altering microbial activity and thus nutrient availability (Kraus et al. 2003; Stinson et al. 2006). Symptoms of suppression include reduced shoot and root growth, changes in morphology or low chlorophyll concentration (Dayan and Duke 2009). Root:shoot ratios are frequently reduced, perhaps because growth-inhibiting phytotoxins enter the plants through roots and may maximize effects in that organ (Elisante et al. 2013).

The toxic effects of certain plant compounds on other plants have been known to agriculturalists for hundreds if not thousands of years (Chou 2006) and were acknowledged as a natural form of plant interaction by Hans Molisch who coined the term 'allelopathy' (Narwal and Jain 1994). The conceptual connection with native-exotic plant interactions dates to the 1990s and was initially proposed as a mechanism whereby native species repel the invasion of exotic species (Hobbs and Atkins

1991), recently coined 'homeland security hypothesis' (Cummings et al. 2012; Yuan et al. 2021), before the alternative 'novel weapons hypothesis' was introduced (Bais et al. 2003). Allelopathy is an attractive explanation for some of the more catastrophic effects of plant invasion on diversity because it seems to explain how one species can suppress so many native species to near-exclusion (Hierro and Callaway 2003). After all, classic niche theory suggests that in a multidimensional resource space with many locally adapted species, newcomers should be the ones most limited in growth (Callaway and Ridenour 2004). The novel weapons hypothesis proposes instead that native species could be systemically disadvantaged because they did not co-evolve with the invasive species' allelochemical weapons arsenal (Kalisz et al. 2021), unlike members of the invasive species' home community (Callaway and Ridenour 2004; Callaway et al. 2008). Though many non-invasive exotic species may be suppressed by the allelochemicals of resident native species, invasive species are evidently composed of species that have successfully evaded homeland weaponry.

Evidence that exotic invaders come to dominate native communities through allelopathy has been mixed. According to Zhang et al. (2021), allelopathic research has slowed in recent years, in part because of difficulties reproducing previously published results. In a meta-analysis of 384 studies, aimed at quantifying the average strength of allelopathy and identifying leading causes for variance, Zhang et al. (2021) found that overall allelopathic effects reduced plant performance by 25%, which is less than the average effect size of competition identified in another meta-analysis (Lekberg et al. 2018). They also found statistical evidence of publication bias against no-effect results, suggesting that an unbiased average effect of allelopathy could be even weaker. While the study attributed some of the effect size variation to aspects of experimental design (e.g., natural vs. controlled conditions, effects on growth or germination), 50% of the overall effect size variance in these studies remained unexplained.

A likely reason for this unsatisfactory state of affairs is that mechanisms underlying allelopathy are varied and complex. Allelopathic effects can be direct or indirect (Inderjit and Weiner 2001). If direct, an allelochemical released by a donor

plant comes into contact with a receptor plant and disrupts cellular biochemistry (Cheng and Cheng 2015). Indirect allelopathy includes all effects mediated by biotic and abiotic soil processes, which often vary in time and space related to site conditions (Scavo et al. 2018). According to Zeng, indirect microbial mediation characterizes many, if not most, allelopathic interactions. Due to the potentially broad spectrum of their effects, allelochemicals are likely to play a role in the evolution and succession of plant communities everywhere and by extension, in plant invasions (Callaway and Ridenour 2004), but it is not clear whether the effect is uniform, net-negative, and bound to give invasive species a decisive edge.

An uncommonly strong allelopathic effect was recently reported for an invasive plant species with nearly worldwide distribution. *Bothriochloa ischaemum* var. *songarica* (L.) Keng. (a.k.a. yellow bluestem) is a productive grassland species native to southern China, Myanmar, and Taiwan (Wilson 2021). The species has been widely introduced to North and South America, Australia and some Pacific Islands (Clayton et al. 2006; Gao et al. 2004). Though its original introduction to North America was accidental, seed was commercially produced and distributed in Texas since 1949 under the name King Ranch (KR) bluestem and was later more widely distributed under different names by the USDA Soil Conservation Service as a remedy for soil erosion and low grassland productivity (Clayton et al. 2017; Wied et al. 2020). Today, the variety is present in 16 mostly southern US states and is considered highly invasive (USDA NRCS 2021). A habitat generalist, *B. ischaemum* has invaded many diverse prairie communities and replaced them with near monocultures (White 2021). Once established, the loss of native species diversity proves nearly impossible to reverse (Clayton et al. 2017).

Two studies have linked the invasiveness of *B. ischaemum* to allelopathy or soil conditioning. In a greenhouse study, whole-plant aqueous extract ('leachate') of *B. ischaemum* was applied to seeds and seedlings of two native prairie species, little bluestem (*Schizachyrium scoparium*), and big bluestem (*Andropogon gerardii*), as well as *B. ischaemum* itself (Greer et al. 2014). All native plants treated with *B. ischaemum* leachate died within 12 weeks and the remaining harvestable

biomass was reduced by >98% relative to a water control treatment. Survival in *B. ischaemum* was about 70% but biomass was similarly reduced to >90%, suggesting strong autotoxicity. Seed germination was reduced by >90% in the native species but was not significantly reduced in *B. ischaemum* itself. In the only other published study examining the allelopathy of *B. ischaemum*, Hawkes et al. (2013) found that two native species (*Bothriochloa laguroides* and *Panicum virgatum*) and *B. ischaemum* itself benefited when grown in soil conditioned by *B. ischaemum*.

Given these contradictory prior results, we set out to replicate Greer et al's (2014) study but also modified it to gain further understanding of potential mechanisms. Whereas Greer et al (2014) used raw leachate and unsterilized soil and was thus unable to determine whether *B. ischaemum* acted through direct or indirect microbial effects, we used a sterilized leachate treatment to test for a direct, chemical effect on seedling growth. We also increased the number of warm-season grass species under consideration from three to seven, with five common native species and two invasive species; *B. ischaemum* itself, and an emergent invasive species from Australia, *Dicanthium sericum* or silky bluestem. Like Greer et al (2014), we also used a leachate derived from a native species as a reference treatment. Finally, while Greer et al's (2014) study was conducted in Oklahoma (USA), this experiment was conducted with populations from the Edwards Plateau (central Texas, USA), where *B. ischaemum* has a different introduction and management history (Harmony et al. 2004) and is potentially even more invasive due to warmer, more arid conditions (CABI 2022; Gabbard and Fowler 2007; USDA Forest Service 2018). Overall, our working hypotheses were that 1) Leachate prepared from a local population of *B. ischaemum* applied to local populations of native species (and one recent invader) in central Texas will impose similar allelopathic effects to those observed for Oklahoma populations (novel weapons hypothesis); 2) Leachate prepared from a native species will impose allelopathic effects on the exotic species, but not on native species (homeland security hypothesis); 3) Different leachate preparation methods will have similar allelopathic effects, on the assumption that the allelopathic effect is direct and chemical in nature.

Materials and methods

Study species

The experiment included five native and two exotic warm-season grasses, all co-occurring on the Edwards Plateau of Central Texas. The native species were: *Schizachyrium scoparium* (Michx.) Nash (little bluestem), *Andropogon gerardii* Vitman (big bluestem), *Bouteloua curtipendula* (Michx.) Torr. (sideoats grama), *Bothriochloa laguroides* (DC.) Herter (silver bluestem) and *Sorghastrum nutans* (L.) Nash (indiangrass). The non-natives were *Bothriochloa ischaemum* (L.) Keng (KR (King Ranch) or yellow bluestem) and *Dicanthium sericum* (R. Br.) A. Camus (silky bluestem). The latter was included to represent an emergent invasive species from a third biogeographic region (Australia), which is closely related to *B. ischaemum* (Sumadijaya 2015) and occupies a similar ecological niche in central Texas. The native species all have broad geographic ranges in North America and overlap on the Edwards Plateau, albeit in different habitats (White 2021). While *B. ischaemum* is very common and widely distributed on the Edwards Plateau (Gabbard and Fowler 2007), *Dicanthium sericum* is currently more limited in its distribution with spotty occurrences in Texas, Mississippi, Florida and Hawaii (Barkworth 2021).

We collected seeds in 2016 from three sites, all within five km of each other, in western Travis County, Texas. Collections of *A. gerardii* and *S. nutans* seeds were supplemented from the Native American Seed Company (Junction, TX), to assure sufficient seed numbers. Seeds from different sources were well mixed.

Leachate preparation

Plant materials for leachate preparation were collected from the same site as the seeds used in this study, in western Travis county. Leachates were prepared only once for each experiment. Whole *B. ischaemum* and *S. scoparium* plants were collected in June 2017 for the seedling growth experiment and in October 2017 for the germination experiment. Following procedures of Greer et al. (2014), roots were manually cleaned and whole plants were soaked for 72 h in distilled water (0.2 g/ml of plant material) at room temperature (21°C). The bulk of the plant

mass was then removed and the leachate was filtered through a double layer of sterile cheesecloth to remove coarse debris.

In the germination experiment, we compared the potency of raw (unsterilized) and sterilized leachate and we used two methods of leachate sterilization: autoclaving, in which leachates are briefly exposed to high temperature and pressure to destroy cells, and filtering through a microporous material (0.2 μ), which retains particles as small as bacterial spores (Korczinky 1981). The first method is conveniently used on large quantities but has the drawback of potentially modifying chemically active compounds, which could weaken allelochemical potency (John et al. 2006). However, phytotoxins such as phenols and catechol remain stable under short-term exposure of up to 150° C (Chen et al. 2014), suggesting that while autoclaved leachate maybe somewhat less potent than raw leachate, it may not neutralize allelopathic activity completely. In addition, autoclaving breaks up cells and releases their contents, including nutrients and potentially endotoxins, which could affect plants either net-positively or net-negatively. Filtering leachates avoids problems of heat instability and released cell content but is cumbersome for large quantities of material and cannot be applied to sterilize soil. Thus, for the seedling growth experiment, which required larger volumes of leachate, and considering the strong effects of similarly prepared leachate in the previous study, we used only autoclaved leachate.

For the germination experiment, one 2 L batch of strained leachate was split three-ways and either left raw, centrifuged and then vacuum filtered through a 0.2 micron filter or autoclaved at 121 °C for 60 min. For the seedling experiment, a total of ca. 30 L of strained leachate was autoclaved in batches of 2 L. Leachate preparations were stored at 4 °C until use.

Germination experiment design

This study followed a complete factorial design, with six target species (not including *S. nutans*, for which we did not have enough seed) and seven treatments: three preparations of *B. ischaemum* leachate, three preparations of *S. scoparium* leachate and a water control. Each species-treatment combination was replicated three times at the scale of a Petri-dish and each Petri-dish had exactly 50 seeds. Seeds were

germinated on top of filter papers soaked with either 2.5 ml of leachate or water. Petri dishes were lidded and maintained at 21 °C on a lab table. The filter paper remained damp for the duration of the experiment. Germinated seeds were counted on Day 6 and Day 8. A seed was considered germinated when the radical had emerged at least 2 mm from the seed coat (Greer et al. 2014). Germinated seedlings were removed after counting (Greer et al. 2014). By Day 6, mold had begun to grow in some of the Petri-dishes, which forced us to terminate the experiment by Day 8. Since we observed differences in the amount of mold by treatment, we decided to score the amount of fungal growth on a scale of 0 to 3 for potential analysis. We later simplified the scoring for analysis to 0 (no visible hyphae) and 1 (hyphae present).

Growth experiment design

The experiment followed a complete-factorial, randomized block design with seven species and three treatments (*B. ischaemum* leachate, *S. scoparium* leachate, and a water control) across three blocks (i.e., greenhouse tables). It was conducted in a greenhouse at Texas State University, San Marcos, TX. In each block, all species x treatment combinations were replicated 7× for a total of 21 replicates per species and treatment and a total planned sample size of 441. Plants were grown for nine weeks in freely draining containers.

Three seeds per container were initially planted into 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 sterile, chemical-grade macro and micronutrients. Sand and peat moss were autoclaved before mixing and sealed in covered containers until use. When all seedlings had emerged, they were thinned (by gently pulling them up with sterilized tweezers) to one per container, with preference given to the seedling in the most central position. For the first two weeks, seedlings were watered with captured rainwater to ensure establishment. For the next seven weeks, 27 ml of undiluted leachate was applied once a week, enough to saturate the soil. To keep seedlings well-watered between applications and to avoid running out of leachate before the experiment was completed, pure water was applied once between treatments, but not so much as to induce drainage.

This protocol of intermittent leachate application also followed Greer et al. (2014), who applied leachate only once every two weeks. Though not all containers had seedling emergence, all seedlings that did emerge, survived the seven-week treatment period.

Plant heights from the soil to the tip of the longest blade were recorded twice, two weeks after seeding (Height 1) just before the first application of leachate, and again on the day of harvest after seven weeks of treatments (Height 2). Plants were harvested whole and washed to remove as much of the soil as possible. Root and shoot were dried separately at 70 °C for three days. Root samples still contained a considerable amount of peat moss after washing, which was carefully removed after drying. Once dried and cleaned, shoots and roots were weighed.

Statistical methods

For the germination experiment, germinant counts per Petri-dish on days 6 and 8 were logit transformed and examined by ANOVA. Two species (*B. laguroides* and *S. scoparium*) had extremely low germination rates across all treatments and were omitted from the analyses. To determine whether leachate affected the germination rates of native and exotic species differently, and whether leachate species or preparation methods made a difference, we omitted the water treatment and conducted a factorial analysis of germination rates by species Status (native versus exotic), Species within Status, Leachate species (*B. ischaemum* or *S. scoparium*) and Preparation method (raw, filtered or autoclaved). To determine the magnitude and direction of the effects, we separately compared each preparation method against the water control.

In the growth experiment, some species established better than others; in total 394 seedlings established, 47 fewer than per design. The lowest seedling numbers were obtained for *S. scoparium* (38 in total). For each species, available seedlings were evenly and randomly distributed among blocks and treatments. At final harvest, three root or shoot samples were accidentally lost (one of each *S. scoparium*, *B. curtipendula*, and *B. laguroides*) and the entire sample was removed from analysis. In addition, we removed two more samples from analysis that were extreme outliers, one very small *B. curtipendula* plant that was 4 standard deviations removed from the mean

total biomass and one *B. laguroides* plant with exceptionally low shoot biomass compared to root biomass.

For the analysis, block effects were omitted since they were non-significant. We used MANOVA using height growth (H2-H1), log shoot dry mass and log

Table 1 Analysis of germination ratios (logit-transformed) on Day 6 after imbibition

Factor	df	MS	F	p
Species (Status)	2	2.404	36.257	<0.001
Status	1	1.04	15.689	<0.001
Status * Leachate preparation	2	0.461	6.952	0.002
Leachate species	1	0.389	5.867	0.019
Leachate preparation	2	0.268	4.035	0.023
Status * Leachate species	1	0.222	3.352	0.072
Leachate species * preparation	2	0.09	1.357	0.266
Status * Leachate species*preparation	2	0.049	0.74	0.482
Error	58	0.066		

The water control was omitted, as were the seed species *S. scoparium* and *B. laguroides* because of very low germination ratios. Status refers to the species' exotic versus native status. Significant effects are highlighted in bold. Treatment effects on Day 9 were not significant

root dry mass as response variables and Status (native or exotic), Species within Status and Leachate treatment as factors. In the first analysis, we contrasted the treatment effects of *B. ischaemum* and *S. scoparium* leachate, omitting the Water control. Upon finding no significant treatment effect, we then pooled across Leachate species to contrast Leachate versus Water.

All analyses were conducted in SPSS Version 26. Throughout, we used the $p=0.05$ threshold to evaluate statistically significant differences.

Results

Leachates had significant effects on seed germination ratios only on Day 6 (Table 1). Overall, *B. ischaemum* leachate reduced germination ratios more than *S. scoparium* (Leachate species main effect; Table 1, Fig. 1). The difference between the effects of the two leachate species was marginally more pronounced in the native species (Status*Leachate species effect). This amounts to weak support for Hypothesis 1 (novel weapons hypothesis) and somewhat stronger support for Hypothesis 2 (homeland security hypothesis).

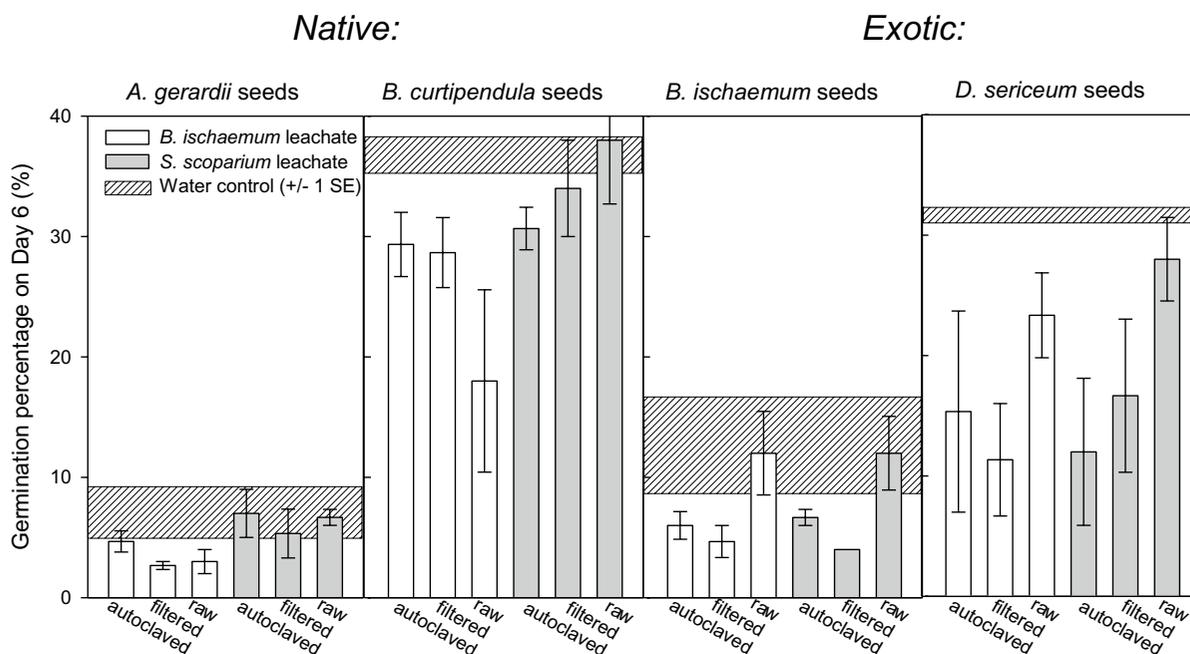


Fig. 1 The effect of leachate species and preparation method on Day-6 germination rates. Error bars represent ± 1 SE. The striped bars indicate average seed germination rates in the water control treatment ± 1 SE

In the exotic species, sterilized leachate reduced the germination ratios more than raw leachate (Status*Leachate preparation effect), with no difference between autoclaved and filtered leachates. This refutes Hypothesis 3 that the effect of leachate does not change with preparation method, but lends support for the contention that autoclaving does not fundamentally alter the allelochemical potency of leachate.

Mold had begun to develop on Day 6. Analysis of the distribution of mold across treatments (described in the Appendix) determined that significantly more Petri dishes developed mold if treated with *B. ischaemum* leachate than with *S. scoparium* leachate, particularly when the *B. ischaemum* leachate was sterilized. There was however no statistical evidence that mold formation affected germination ratios directly (Appendix A).

In the seedling growth experiment, there were no significant differences in the effects of *B. ischaemum* and *S. scoparium* leachate on multivariate growth indicators across species (Table 2), in contravention of Hypothesis 1. Overall, the leachate treatment was statistically not different from the water treatment (Treatment effect and Status*Treatment effect, Table 2), except for idiosyncratic responses in some species. For example, leachate increased or decreased the height growth of two native species (Fig. 2A) and increased or decreased the root dry weight of the two exotic species (Fig. 2C). Overall, there was no

evidence of direct and systematic allelopathic effects of sterilized leachate on seedling growth.

Discussion

In this experiment, we set out to confirm and generalize the allelopathic effects of *B. ischaemum* on native prairie species found previously by Greer et al. (2014). Instead, we found different results, with much weaker allelopathic effects than previously reported, and indications that the strong allelopathic effects on the growth and survival of seedlings in the previous study could have been mediated by microbial associates of *B. ischaemum*, rather than by leachate chemical toxicity.

Germination rates

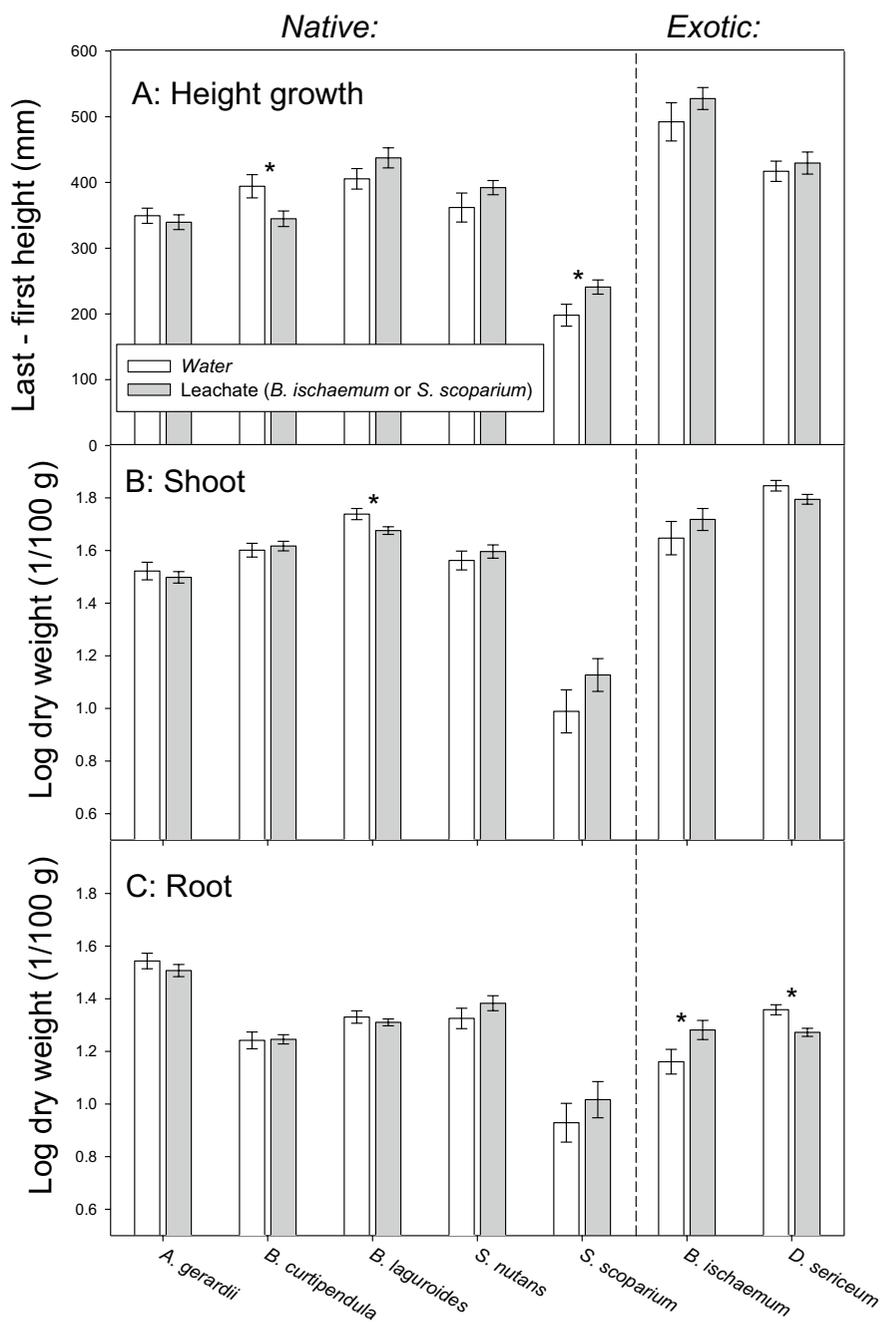
Using raw leachate, Greer et al. (2014) observed a >90% suppression of *A. gerardii* and *S. scoparium* germination rates relative to a water control when treated with *B. ischaemum* leachate but no effect on *B. ischaemum* itself, and no effect of *A. gerardii* leachate on any species, in support of the novel weapons hypothesis. We certainly did not find effects of this magnitude, but we also detected a slightly stronger effect of *B. ischaemum* compared to *S. scoparium* leachate (Table 1). In our experiment, raw leachate also did not reduce *B. ischaemum* germination but

Table 2 Results of MANOVA on seedling height growth, log shoot and log root biomass at final harvest

Factor	Wilk's I F	Hypothesis df	Error df	p
A: Treatment: <i>B. ischaemum</i> versus <i>S. scoparium</i> leachate				
Status	188.8	3	238	< 0.001
Species(Status)	42.6	15	657.4	< 0.001
Treatment	2.0	3	238.0	0.117
Status x Treatment	0.6	3	238.0	0.642
Species(Status) *Treatment	0.9	15	657.4	0.523
B: Treatment: Leachate (<i>B. ischaemum</i> or <i>S. scoparium</i>) versus Water				
Status	244.3	3	373	< 0.001
Species(Status)	62.9	15	1030.1	< 0.001
Treatment	1.1	3	373.0	0.344
Status x Treatment	0.37	3	373.0	0.774
Species(Status) *Treatment	2.5	15	1030.1	0.001

Status is with respect of native or exotic species origin. Species was nested within status. Significant effects are highlighted in bold

Fig. 2 The effect of water versus leachate seedling growth. Error bars represent ± 1 SE. Asterisks mark treatment contrasts that are significantly different at the $p=0.05$ level



sterilized leachates significantly reduced the germination rates of both exotic species (Fig. 1). The effects of autoclaved and microfiltered leachates were similar across seed species and leachate species, which makes it unlikely that artefacts associated with cell lysis or heat-instability enhanced leachate toxicity. It is rather more likely that microorganisms associated

with unsterilized *B. ischaemum* either neutralized chemical germination suppressants or compensated for their negative effects. Notable in this context is that unsterilized leachate of *B. ischaemum* also appeared to inhibit mold growth, though model selection analysis suggested that the leachate effects on germination and mold growth were independent.

Also different from the results of Greer et al. (2014), sterilized leachate prepared from a native species had negative effects on the germination rates of both exotic species, in support of the homeland security hypothesis. Overall, the allelopathic effect of *B. ischaemum* on germination rates was broadly reproduced, but unlike Greer et al. (2014), we also found evidence for the reciprocal effect of native species leachate on exotic species germination, as well as evidence of autotoxicity in *B. ischaemum* using sterilized leachate. The production of autotoxic compounds is common in allelopathic plants (producing the phenomenon of ‘soil sickness’), though allelopathic species have also evolved varied mechanisms to evade autotoxicity (Singh et al. 1999). Since unsterilized leachates did not reduce germination in this experiment, perhaps *B. ischaemum*'s avoidance mechanism is linked to microbial associates.

Seedling growth

In the Oklahoma experiment, raw *B. ischaemum* leachate stopped seedling growth soon after the first leachate application and eventually killed all seedlings, including *B. ischaemum* itself. Raw leachate made from a native species also tended to reduce growth, through significantly so only in *B. ischaemum*. We have no direct comparison for the effects of raw leachate in this experiment, but with sterilized leachate, we found only small and idiosyncratic effects of leachates on seedling growth (Fig. 2) and importantly, no seedling died. Shoot biomass was 13% and 17% reduced relative to the water control in *B. laguroides* and *D. sericeum*, respectively. In *S. scoparium*, leachate had a positive effect on growth. Thus overall, we reject the hypothesis that *B. ischaemum* leachate had unique and highly disabling direct (chemical) allelopathic effects on the seedlings of native or exotic species.

Explaining the differences between the two experiments

Much of the experimental approaches between the Oklahoma and Texas experiments were similar. Donor plants were harvested while still actively growing. The leachate was prepared from whole plants using the same biomass to water ratio and soak times. In both experiments, leachate was applied

intermittently (once every 2 weeks in the Oklahoma experiment, once a week in the Texas experiments), and in both experiments, pots were watered with pure water in between as needed. Our greenhouse experiment lasted 9 weeks compared to Greer et al.'s (2014) 12 weeks, but total seedling dry mass of the water control groups was roughly the same, between 0.2 and 0.7 g.

The main difference of experimental design was the use of unsterilized field soil and unsterilized leachate by Greer et al. (2014) compared to the sterilized potting soil and sterilized leachate used here. Comparisons of allelopathic effects in natural versus autoclaved soils consistently suggests that soil microbial activity lessens the concentrations of allelochemicals and their effects on plants (Inderjit and Dakshini 1995), as microbes can utilize these compounds as sources of energy. In addition, soil fine particles can adsorb organic compounds, reducing their concentration in soil water (Ito et al. 1998; Tongma et al. 1998). However, Greer et al. (2014) argued that native soils may not have efficient decomposers for novel chemicals (Inderjit and van der Putten 2010). All things considered, there is no indication that differences in irrigation regime or potting soil should have systematically weakened the interactions between allelochemicals and plant roots in this experiment.

However, it is possible that the leachate produced from Texas plant materials simply contained lower concentrations of allelochemicals. For example, *B. ischaemum* leachate reduced the germination rate by ca. 40% on average in this experiment compared to 90% in Greer et al.'s (2014) experiment. Higher concentrations may have been required to cross response thresholds for allelopathic effects on seedling growth. In the germination assay, we found that autoclaving did not significantly weaken the allelopathic potency of leachates, with the one caveat that different compounds may be responsible for disrupting seedling germination and growth.

Unfortunately, it is impossible to attribute differences in allelochemical concentrations between leachate preparations to specific causes. The expression of secondary metabolites can be induced and may vary with site conditions, herbivory and climate (Metlen et al. 2009). Also, there could be genetic differences between Oklahoma and Texas populations. Ecotypes of the same species can differ in the production of allelochemicals (Kashkooli

and Saharkhiz 2014; Kayanifard and Mohsenzadeh 2017; Mousavi et al. 2021) and there are known ecotypic differences between populations of *B. ischaemum* across the United States (Clayton et al. 2017). Secondary metabolism is just another suite of traits undergoing rapid evolution in invading species (Oduor et al. 2016) and selection pressures in Oklahoma and Texas grasslands may have been different. Conversely (or additionally), Texas ecotypes of native species could have evolutionarily responded to the introduction of *B. ischaemum* by becoming less sensitive. *Bouthriochloa ischaemum* is potentially better adapted to the drier and winter-warmer climate of Austin, Texas compared to Stillwater, Oklahoma (USDA Forest Service 2018) and may gain less from investment in ‘chemical warfare’. Given that the production of allelochemicals is costly, Texas populations may have reduced the production of allelochemicals to increase growth potential, except for autotoxic, germination-suppressing compounds that are generally favored in intraspecific competition (Canals et al. 2005).

A third explanation for the difference in this and the previous study is that the Oklahoma study may have involved inhibitory effects other than direct allelopathy. In Greer et al.’s (2014) study, chemical analysis of leachates showed that *A. gerardii* leachate had more than twice the concentration of phenols than *B. ischaemum* leachate. Of course, other chemical compounds not analyzed may have produced *B. ischaemum*’ allelopathic effects. But it is also possible that the allelopathic effect was not chemical, and that pathogens were transmitted with *B. ischaemum* leachate or litter that harmed both native species and *B. ischaemum* itself in the vulnerable seedling stage. For example, seedling damping-off disease is consistent with the severe effect on seedlings in the Oklahoma experiment, where seedling biomass was reduced to ca. 1% in *A. gerardii* and *S. scoparium* relative to the control, and to 8% in *B. ischaemum*.

Clearly, this study raised more questions than it could answer, but questions of phenotypic plasticity and trait divergence in the allelopathy of invasive species are timely and relevant (van der Putten et al. 2013). It would take more extensive experimental designs—including repeated sampling over several years and reciprocal transplanting of geographically separate populations—to develop a better

understanding for why allelopathic effects can differ so widely between experiments.

Leachate effects on mold growth

A link between fungal growth and *B. ischaemum* leachate was suggested by our germination experiment (Table A1). Petri-dishes were significantly more infected when treated with *B. ischaemum* than *S. scoparium* leachate, which may suggest the presence of compounds stimulating fungal growth or fungal spores in *B. ischaemum* leachate that were missing in *S. scoparium* leachate. However, raw leachate of *B. ischaemum* was significantly less susceptible to fungal growth than sterilized leachate, suggesting that *B. ischaemum* leachate also contained microbes antagonistic to mold.

The Petri dishes containing sterilized *B. ischaemum* leachate were not only most contaminated by mold, they also had consistently lower germination rates across species (Fig. 2). This raised the question whether fungal growth suppressed germination rather than allelochemicals directly. Though we cannot rule out this explanation categorically, model selection analysis showed that neither adding a mold infestation index as an explanatory variable nor substituting a treatment factor with a mold index improved the prediction of germination rates (Table A2). Thus, we considered leachate effects on germination independent from leachate effects on mold growth. For example, sterilized leachates of *B. ischaemum* and *S. scoparium* had very similar effects on *B. ischaemum* seeds (Fig. 1), although 3 out of 6 Petri dishes had mold when treated with sterilized *B. ischaemum* leachate versus none when treated with sterilized *S. scoparium* leachate. In *B. curtipendula*, germination was most suppressed when treated with raw *B. ischaemum* leachate, though Petri dishes had no visible mold. This shows that germination ratios could be suppressed in the presence or absence of mold.

Conclusion

To date, only three published studies examined the allelopathic potential of *B. ischaemum*, a noxious invasive grass from Southern US to 40° N latitude (USDA Forest Service 2018). Together, they highlight important knowledge gaps not just for this

species, but broadly for the investigation of allelopathy in invasive grasses. First, the widely used application of unsterilized leachate or litter in allelopathy research does not resolve mechanism of allelopathy. Without sterilized controls, direct allelopathy cannot be distinguished from synergistic pathogen-mediated effects, which is presumably more contingent on ecological context than on plant genetics. Second, phenotypic plasticity and ecotypic differences in allelopathic competitive strategies are rarely examined but would undoubtedly shine light on the complexity of co-evolutionary dynamics that follows the introduction of new species (Meiners et al. 2012) (Lankau and Strauss 2011).

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Data availability The datasets generated during the current study will be made available on Dryad (dryad.org).

Declarations

Competing interests The authors have no relevant financial or non-financial interests to disclose.

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