

**HOW GLOBAL CHANGE AFFECTS SEED GERMINATION  
AND EMERGENCE IN LONGLEAF PINE ECOSYSTEMS**

**By**

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**Chapter One**  
**LITTER DEPTH MEDIATES THE EFFECT OF EXTREME PRECIPITATION**  
**ON EMERGENCE OF UNDERSTORY PLANTS**

A manuscript prepared for submission to *Forest Ecology and Management*:

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**Highlights**

- Climate change may alter key ecological processes: seed arrival and emergence.
- Seedling emergence is affected by pulsed precipitation, litter depth, and fungi.
- Fungi may play a mutualistic rather than pathogenic role in seedling emergence.
- The timing of seedling emergence is delayed as litter depth increases.
- Species respond uniquely; seed traits tied to dormancy may predict outcomes.

**Abstract**

Extreme precipitation events (periods of intense rainfall) are becoming more frequent in many regions as the climate changes. The amount and timing of precipitation are key drivers of seed germination and seedling emergence, yet the effects of extreme precipitation are still poorly understood. This knowledge gap is critical for understanding the composition and diversity of understory plant communities because changes to precipitation regimes may shift community composition over time due to alterations in germination and establishment patterns. Our study used a factorial greenhouse experiment to assess how extreme precipitation affected the germination and emergence of seven understory plant species native to longleaf pine (*Pinus*

*palustris*) ecosystems. We evaluated the effects of pulsed precipitation in conjunction with two other important drivers of seedling emergence and seedling survival - the depth of leaf litter and the presence of soil fungal communities. Leaf litter depth is strongly regulated by the fire regime, which historically included frequent, low-intensity fires every 1-5 years. We found that extreme precipitation and fungicide application did not affect seedling emergence or timing. However, increased litter depth strongly reduced seedling emergence. Despite changes in soil moisture due to pulsed precipitation, the herbaceous understory species examined here were still able to emerge. Given the negative impacts of deep leaf litter on seedling emergence, effective ground-cover management may become essential for maintaining suitable microsites for seedling emergence. Additionally, species-specific responses to climate change highlight the need for management strategies tailored to each species' dormancy-breaking requirements. Future research is needed to better understand these species-specific cues.

**Keywords:** Climate change, community assembly, seed, leaf litter, groundcover, restoration

## 1. Introduction

Climate change is a leading threat to ecosystems and the diversity of plant communities (Mukherji et al., 2023; Thomas et al., 2004). Extreme precipitation events are an increasing component of these changes (Mukherji et al., 2023), defined by the 99th and 99.9th percentile precipitation values for a set period, such as a growing season or over a month (Moore et al., 2015). These extreme events are often followed by dry periods or drought conditions (Fay & Schulz, 2009; Jentsch and Beierkuhnlein, 2008; Larson et al., 2020). For plants, the amount, duration, and timing of precipitation strongly limit species membership within communities

(Classen et al., 2010; Kardol et al., 2010). In one experiment, precipitation accounted for 12% of the variation in herbaceous plant community composition (Kardol et al., 2010). Extreme precipitation events can affect seed dormancy-breaking cues, seedling establishment rates, and plant survival and growth, which can alter community assembly and composition (Cochrane et al., 2015; Jentsch et al., 2007; Thomas et al., 2004; Walck et al., 2011).

Seed and seedling survival is strongly tied to the composition and diversity of plant communities (Orrock et al., 2023; Walck et al., 2011). The transition from seed to seedling is one of the most vulnerable stages in plant development (Clark, 2007; Jimenez et al., 2016; Nelson, 2018) and is strongly affected by the amount and timing of soil moisture (Fay and Schultz, 2009; Lloret et al., 2009). Increasingly frequent extreme precipitation events under climate change could cue seed germination and emergence (Fay and Schultz, 2009), but if followed by periods of drought, seed and seedling desiccation may occur, increasing mortality (Cochrane et al., 2015; Walck et al., 2011) and limiting the ability of species to establish and persist in the community. Predicting the impact of extreme precipitation events will also require understanding not only the direct impacts of changes in precipitation on plants, but also indirect interactions with other physical and biological processes (Classen et al., 2010; Kardol et al., 2010).

The availability of suitable microsites is a physical property that may interact with extreme precipitation events to affect seed germination and establishment. In ecosystems like grasslands and savannas maintained by low-intensity, frequent fire, the availability of microsites can play a key role in regulating species establishment and community diversity (Coffey and Kirkman, 2006; Loydi et al., 2013). Fire combusts leaf litter and reduces plant competition for space and nutrients, increasing the likelihood of seed-soil contact, facilitating seed germination (Harms et al., 2017). Yet, the presence of at least some leaf litter may also be beneficial to seed

emergence and seedling survival by minimizing desiccation risk (Coffey et al., 2013). The impact of extreme precipitation events on seed germination and seedling survival may depend on the availability and quality of suitable microsites (Walck et al., 2011).

In addition to changing the physical processes that may lead to seed mortality, such as desiccation, extreme precipitation events can alter biological processes that can impact seed survival, such as increases in soil microbe diversity and abundance, which can grow more quickly with increased humidity generated by greater amounts of precipitation and warmer temperatures (Leishman et al., 2000; Walck et al., 2011). Soil fungal pathogens, for example, promote seed death (Leishman et al., 2000) and alter germination, emergence, and establishment rates (Nelson 2018). Disentangling these direct and indirect effects of extreme precipitation events on plant establishment is critical for predicting plant species persistence in the future and the consequences for plant community diversity. Experiments that can isolate these factors are needed.

The direct and indirect impacts of extreme precipitation events on seed and seedling survival are especially important to understand in grassland and savanna ecosystems because they are globally imperiled due to widespread habitat loss and degradation (Nerlekar and Veldman, 2020) and are projected to be impacted by climate change-induced extreme precipitation events in many regions of the world (Carter et al., 2018; Lyons et al., 2023). These ecosystems are also limited by seed arrival and establishment (Kirkman et al., 2004). Furthermore, soil microbial communities and the availability of open microsites both alter the likelihood of seed survival and establishment in grassland and savanna ecosystems (Blaney and Kotanen, 2001; Classen et al., 2010; Damschen et al., 2008). Therefore, extreme precipitation events may impact seed germination and establishment directly and indirectly through altered



species interactions with soil microbes (Classen et al., 2015) and abiotic conditions such as microsite availability and soil moisture (Fay and Schultz, 2009).

We tackled these challenges by conducting an experimental study that manipulated extreme precipitation, microsite availability (litter presence and depth), and species interactions with fungi for seven different understory plant species associated with the longleaf pine (*Pinus palustris*) ecosystem. Plant communities in the longleaf ecosystem may be especially useful for studying emergence and seedling emergence timing under future precipitation scenarios as this ecosystem is expected to experience increases in extreme precipitation events in the southeastern United States, as predicted in mild to extreme climate scenarios (Carter et al., 2018). Currently, longleaf pine savannas are managed by prescribed fire, typically burning every 1-3 years, which reduces fine fuels on the forest floor, creating microsites needed for seedling emergence and plant growth (Mitchell 2014). Studies have demonstrated that precipitation and litter depth are crucial to seed germination, establishment, and persistence in longleaf pine ecosystems (Orrock et al., 2023). Today, this biodiversity hotspot is considered imperiled as less than 5% of its original extent remains due to anthropogenic land use changes such as logging, agriculture, fire suppression, and urbanization (Brudvig et al., 2013; Frost, 2006; Jose et al., 2006; Mitchell et al., 2014; Orrock et al., 2023). In anticipation of a changing climate, understanding how understory plant communities respond to shifts in episodic precipitation events will be vital for achieving conservation and restoration goals.

In this study, we asked how the interactions between extreme precipitation, microsite availability, and soil fungi affected: 1) seedling emergence and 2) the timing of seedling emergence and survival (“seedling emergence timing”). We use a controlled experiment to manipulate extreme precipitation, available microsites (leaf litter depth), and seed-fungi

interactions (fungicide) to determine how their direct and interacting effects alter the above two processes. We hypothesized that: H1) seedling emergence will decrease with increased extreme precipitation and litter depth and increase with fungicide application, and H2) the timing of seedling emergence will become increasingly delayed as extreme precipitation events increase in severity.

## 2. Materials and Methods

To test our two hypotheses, we conducted a fully factorial greenhouse experiment that manipulated extreme precipitation events, microsite availability (litter depth), and the presence of soil fungi to determine their direct and indirect impacts on the rate of seedling emergence and how they affect the timing of seedling emergence and survival (seedling emergence timing).

### 2.1 Study Design

This study was conducted in a ~13m<sup>2</sup>, climate-controlled greenhouse at the University of Wisconsin-Madison's (UW-Madison) Walnut Street Greenhouse. The greenhouse was temperature and light-controlled to create an environment that mimicked seasonal temperature changes throughout the growing season at Savannah River Site (near Aiken, SC) from April to September (Table S1).

We selected seeds of seven perennial, herbaceous study species (Table 1) including two C4 grasses (Poaceae; *Aristida beyrichiana*, *Sorghastrum secundum*), one legume (Fabaceae; *Lespedeza hirta*), and four composites (Asteraceae; *Coreopsis major*, *Eupatorium album*, *Silphium compositum*, and *Solidago odora*). These seven species were chosen because they are indicators of remnant longleaf pine understory communities (Brudvig et al., 2014) and because

they differed in seed traits known to drive establishment (Jimenez et al., 2016; Orrock et al., 2023) and resistance to pathogens (Jimenez et al., 2016), including the average number of days seeds take to germinate (i.e., germination days) and seed coat thickness (Table 1). We collected seeds from natural populations at the Department of Energy's Savannah River Site (SRS), a National Environmental Research Park in South Carolina, United States. We used this location as a source for the seeds in this study because it lies in the central portion of the range of the longleaf ecosystem. Previous studies have documented the importance of seed limitation, precipitation, litter depth, and fungal pathogens for plant survival and community diversity (but not their potential interactions) (Kirkman et al., 2004; Orrock and Damschen, 2005; Orrock et al., 2023).

Seeds were hand-collected from three to five populations at SRS in the fall and winter of 2021. Populations of seeds were mixed by species to create a homogenized sample of seeds for each species. A cold stratification treatment was applied by storing seeds in a covered shed outdoors from late fall through early winter (December) until nighttime temperatures approached freezing. Prior to planting, seeds were washed with deionized water, then disinfected in a 0.5% bleach solution for 10 minutes, rinsed, and dried for at least 72 hours (Baskin & Baskin 2014). Once dried, seeds were stored in paper bags in a cool climate-controlled environment until sorted and prepared for this experiment. Twenty seeds of each of the seven species were sown approximately 1 cm under the soil by species in separate rows to ensure seed bags were covered and maximize seed contact with soil microbes. Each species was randomly assigned to one of ten rows in each greenhouse tray.

Soils were collected from SRS in March 2023 from the field, stored in gallon-sized plastic bags, and then frozen at  $-18^{\circ}\text{C}$  until transported to the greenhouse at UW-Madison as

lowering temperature can decrease the deterioration of nucleic acid and preserve fungal taxa while inhibiting continued growth (Guerrieri et al., 2020). Upon arrival, the soil was transferred to a walk-in cooler until the experiment was set up. All field soil was homogenized before being used as an inoculum. Greenhouse trays measuring  $23 \times 30$  cm were filled with  $\sim 1996$  cm<sup>3</sup> of autoclaved growing medium (ProMix LP15) and sand mix, creating a 4:1 ratio (Coffey and Kirkman, 2006). Seedling substrate consisted of this autoclaved mix that was then inoculated with the field soil. The ratio of field soil to growing medium mix, by volume, was 1:6. Total depth of soil in each greenhouse tray was approximately 2.0-2.5 cm.

Leaf litter was collected from SRS in March 2023. Pine needles and oak leaves were collected by hand from at least eight sites across the SRS from previous study sites with a known land-use history of agriculture that had not been burned in the last year. The collected litter was stored in brown paper bags until transported to the greenhouse at UW-Madison. Litter was homogenized to an 80:20 pine-to-broadleaf mixture, which is representative of the litter composition on the forest floor from longleaf pine sites (Chapter 3). After sowing seeds, leaf litter was gently placed on top of the soil (see Experimental Design below for further details).

## *2.2 Experimental Design*

Our fully factorial study design consisted of a precipitation treatment (3 levels), a litter-depth treatment (3 levels), and a fungicide application (2 levels). The eighteen treatment combinations were replicated five times each and applied at the tray level ( $N = 90$ ). Treatments were randomly assigned to greenhouse trays.

The precipitation treatment consisted of three levels to mimic the variation in soil moisture during extreme precipitation events. Two of the levels manipulated extreme

precipitation events (“Pulse I” and “Pulse II”), while the third level served as a comparison treatment level for germination under optimal conditions where water was not limiting (“Ambient”). Greenhouse trays receiving the Ambient treatment level were watered daily until water seeped from the bottom (~500-700 mL). The Pulse I treatment level simulates current precipitation conditions similar to those in the region encompassed by SRS, receiving an average of 2.5 cm of rainfall per week during one to two precipitation events (The South Carolina State Climatology Office; U.S. Climate Data, 2024). For this treatment level, 700 mL of precipitation was added to each greenhouse tray every other day to prevent trays from drying out between watering. The Pulse II treatment level simulates projected future conditions characterized by large pulses of precipitation followed by longer periods with no rainfall, where soil moisture is not retained between precipitation events, leading to periods of drought in between these larger pulsed precipitation events. For this treatment level, we added 700 mL of precipitation to each greenhouse tray for three consecutive days, followed by three days without water, where trays were able to dry out between watering events.

We manipulated litter depth to mimic suitable microsite availability regulated by fire regimes. We applied three litter depth treatment levels to represent differences between low and high-frequency fire regimes: litter was added on top of the soil surface within the greenhouse trays at depths of 0, 2, or 4 cm. Field sites with low-frequency fire typically have 4 cm of leaf litter on average compared to high-frequency sites which have 2 cm (Brudvig et al., 2014). This was compared to a control level with no litter present (0 cm).

To evaluate the role of fungal biotic interactions on seedling emergence in each scenario, an even layer of approximately 1 cm (0.27L) of soil from the field was laid over each greenhouse tray to inoculate the greenhouse trays with the fungal community that is present at our study site

(Savannah River Site, SC) in the Southeast (Crawford et al., 2019). Before adding seeds to the experimental trays, half of the seeds were dusted with Captan soil fungicide (Strid et al., 2018) by adding approximately 0.5 tsp to each seed bag and shaking it to eliminate the fungal community. Captan has successfully been used to eliminate fungi in previous studies of seed survival and germination (Blaney and Kotanen, 2001; Schafer and Kotanen, 2003). This resulted in two treatment levels: seeds treated with fungicide vs. seeds not treated with fungicide.

### *2.3 Plant Sampling*

Seedling emergence and death were monitored daily for sixteen weeks for every individual in all trays. Emerging seedlings (cotyledons emerging from the soil) were marked with colored pins so that each individual could be followed over time and identified to species. Individual seedling survival or death was recorded daily until the end of the experiment. We allowed the experiment to continue until all seedlings in the Pulse II treatment and over 75% in the Pulse I treatment died, and no new seedlings emerged for seven days. The experiment concluded on October 26, 2023.

These data were used to calculate two response variables that were used to test our two hypotheses, respectively: 1) the proportion of seedlings that emerged from seed (hereafter, “seedling emergence”). The denominator in this measure is all of the seeds that were sown, including those that failed to emerge. 2) the number of days it took for each seedling to emerge and whether that seedling survived to the end of the study (hereafter, “seedling emergence timing”).

## *2.4 Statistical Analyses*

### *2.4.1 Seedling Emergence*

Seedling emergence was analyzed using a fully factorial negative binomial generalized linear model (GLM.nb), fitting the main effects of precipitation, litter depth, soil fungicide, species, and all their interactions (Venables and Ripley, 2004). To determine the interaction between abiotic and biotic factors influencing emergence of species-specific responses, individual generalized linear models were run for each species fitting the main effects of precipitation, litter depth, soil fungicide, and all their interactions with a Poisson distribution.

### *2.4.2 Seedling Emergence Timing*

We used a Cox proportional-hazards model (CPH; Cox and Oakes, 1984) to examine the impact of precipitation, litter depth, soil fungicide, and their interaction, in addition to species, on seedling emergence timing. The probability of survival based on the timing of seedling emergence (seedling emergence timing) served as the response variable, defined as the number of days it took for an individual seedling to emerge from the soil following the start of the study (DTE = days to emergence) and whether it survived until the end of the study. A fully factorial CPH analyzed interactions between the study treatments (precipitation, litter depth, fungicide) and species as fixed effects with greenhouse tray as a random effect.

### *2.4.3 Statistical Modeling*

Models were run using the ‘MASS’ package (Venables and Ripley, 2004), ‘coxme’ package (Therneau, 2020), and base code in R version 2024.4.1+402 (RStudio Team, 2020).

### 3. Results

#### 3.1 Seedling Emergence

Seedling emergence was generally low overall, with averages among treatments ranging from 5 to 15% across all species (Figure 1). Species identity accounted for most of the variation in emergence ( $p < 0.001$ , Table 2). In addition, three-way interactions between precipitation, fungicide, and species ( $p = 0.028$ ) and litter depth, fungicide, and species ( $p = 0.020$ ) were found to be driven by species differences (Figure 1). A trend was found between the four-way interaction among precipitation, litter depth, fungicide, and species ( $p = 0.057$ , Table 2), further supporting the role of species-specific responses on emergence. Some species had very high emergence rates, while others had less than 1% emergence (Figure 2). Three species of forbs (*C. major*, *L. hirta*, and *S. compositum*), including the one legume and two of the four composites, exhibited very low levels of seedling emergence compared to the two C4 grasses, which had the highest seedling emergence rates across all of the species (Figure 2, *A. beyrichiana* and *S. secundum*). Three species of forbs (*E. album*, *S. compositum*, and *S. odora*) and one grass (*A. beyrichiana*) exhibited significant species-specific responses to the three-way interaction between precipitation, litter depth, and fungicide (Table S2, Figure S1). All except *S. compositum* had lower rates of emergence when precipitation was pulsed (Pulse I and II), leaf litter was deep, and fungicide was applied compared to Ambient precipitation, no litter, and no fungicide application.



### 3.2 Seedling Emergence Timing

Overall, the probability that an emerging seedling survived decreased as seeds emerged later in the growing season (Figure 3). Pulsed precipitation, which caused seeds to emerge earlier, led to a sharper decline in survival probability than ambient precipitation, with 50% of seeds dying at 20 and 32 days, respectively (Figure S2). Seedling emergence timing was significantly impacted by the main effects of precipitation, litter depth, and species identity (Table 3). The interaction between precipitation and litter depth strongly impacted seedling emergence timing ( $p < 0.001$ ; Table 3), such that seedling emergence was delayed when precipitation was pulsed (Pulse I and Pulse II) and litter was deep, corresponding with lower rates of survival (Figure 3). Seedling emergence timing increased when precipitation was pulsed (Pulse I and Pulse II) and litter depth increased, with 4 cm of litter having the greatest delay, yet survival increased as litter depth increased (Figure 3). Post-hoc tests showed that *S. odora* accounted for most of the difference between species. The probability of survival declined more with later emergence for *S. odora* over time compared to the other six species (Figure 4).

## 4. Discussion

Extreme precipitation events may have consequential effects on plant communities by altering seedling emergence and the timing of seedling emergence. Our findings demonstrate that seeds of understory plant species are affected by extreme precipitation events, but these impacts act in concert with other ecological processes, are species-specific, and differ in magnitude depending on the stage of seed development. We also found that while species often respond uniquely, seed traits can predict successful emergence in understory plant communities. Species with thinner seed coats were more likely to emerge earlier and persist longer (Figure 4). Our

results demonstrate that higher soil moisture variability, induced by pulsed precipitation (Pulse II), negatively impacted seedling emergence, supporting our hypothesis. The extended intervals between precipitation events may have led to seed desiccation, which is known to increase seed mortality (Fay and Schulz, 2009).

In contrast, consistent soil moisture (Ambient) increased seedling emergence, but only under conditions where the native fungal community was intact and there was a thin layer of leaf litter. This unexpected result suggests a mutualistic role of fungi in seed germination and emergence for at least some of the species native to the longleaf ecosystem. The presence of leaf litter also positively affected seedling emergence, affirming previous research that underscores litter's role in forming favorable microsite conditions for seedling emergence (Facelli et al., 1999; Zhang et al., 2017). The interaction between consistent soil moisture and a thin layer of leaf litter likely creates a humid environment optimal for seedling emergence. This is further supported by higher levels of seedling emergence observed in control trays when water was not limiting (Ambient), indicating that seedling emergence in these understory species may be constrained by water availability in the absence of litter.

Seedling emergence was significantly influenced by species identity, primarily driven by the high emergence rates of the grasses *A. beyrichiana* and *S. secundum*, compared to the very low emergence rates of two of the forbs, *C. major* and *L. hirta* (Figure 2). These grasses, characterized by their thinner seed coats and transient seed banking ability, typically do not persist in the soil for more than 1-2 years and germinate quickly (Kaesler and Kirkman, 2012). In contrast, the species with lower seedling emergence rates had the thickest seed coats (Table 1). Thick seed coats may make it more difficult for the radicle to break the seed coat without scarification, which increases seed coat permeability (Kobziar et al., 2015). These findings

suggest that seed traits related to dormancy breaking may be especially useful for predicting whether plant species will germinate and establish in community assembly. Since the ability to regenerate from seed can play a key role in determining plant community composition and associated ecosystem functions (Larson and Funk, 2016), trait-based predictions such as these might be very important for anticipating future community change. Such traits may also enable restoration practitioners to identify species that may require additional management actions (e.g., scarification) when trying to increase the emergence and establishment of plant species from seed (Barak et al., 2018).

Species-specific responses to the interaction between precipitation, litter depth, and soil fungicide application were more pronounced in non-leguminous forbs compared to grasses or leguminous forbs (Figure S1). Three forbs, *E. album*, *S. compositum*, and *S. odora*, exhibited a significant response to this three-way interaction, with lower emergence rates observed under pulsed precipitation conditions (Pulse I and Pulse II) when fungicide was applied. These species showed reduced emergence in the presence of both altered soil moisture and disturbance to the native fungal community, suggesting that seed survival and growth in some longleaf pine understory species may be closely linked to soil fungi. This finding is consistent with previous research, which indicated that the removal of fungal communities can benefit species in wetland ecosystems where soil moisture is elevated, but may not have the same effect in upland habitats with fluctuating moisture levels (Blaney and Kotanen, 2001).

As predicted, seeds sown beneath a deeper leaf litter layer took longer to emerge; however, the probability of survival increased with delayed emergence (Figure 3). Delayed emergence under deeper leaf litter suggests that species may be triggered to germinate and emerge later in the growing season when temperatures and the risk of desiccation are higher,

potentially hindering their establishment within the plant community (Walck et al., 2011). However, a protective layer of leaf litter can mediate this effect (Figure 3), though it does not guarantee seedling persistence (Facelli et al., 1999; Zhang et al., 2017). The significant interaction between extreme precipitation and litter depth might be explained by independent cues regulating germination and establishment; dormancy is primarily broken by soil temperature (which can be regulated by leaf litter), while germination and seedling recruitment are determined by the amount and timing of precipitation (Baskin and Baskin, 2014; Fenner and Thompson, 2005; Ooi, 2012). Extreme rainfall events can induce a pulse of seed germination and emergence, but seedlings may perish without a protective layer of litter to retain soil moisture in periods of drought between extreme rainfall events. Failing to persist could have cascading effects on the assembly of the understory plant community, as conditions suitable for emergence and establishment are not necessarily the same as in later stages of plant growth (Fay and Schultz, 2009).

Our research revealed that seeds of understory herbaceous species can emerge despite changes in pulsed versus consistent precipitation regimes. The direct effects of extreme precipitation events were never significant on their own. Rather, the pulsed precipitation treatments interacted with litter depth and the presence of fungi, to affect two critical ecological processes (seedling emergence and seedling emergence timing). Once seeds emerged, pulsed precipitation treatments alone had no effect on seedling survival. This may be because plant species in the longleaf pine ecosystem must tolerate well-drained, sandy soils and seasonal growing season precipitation events, including hurricanes, floods, and periods of drought (Carter et al., 2018). Established individuals, therefore, may have adaptations that allow individuals to survive periods of drought. What is not known is whether future climate changes will lead to

such extreme periods of drought between extreme precipitation that at least some individuals or species will be unable to survive.

Against our expectations, removing the fungal community alone by applying soil fungicide was detrimental or did not affect seedling emergence (Table 2). Mutualistic fungal interactions with plant seeds and seedlings may outweigh the risk of fungal pathogens within these early plant-soil interactions. Similar results were found in a study comparing buried seeds of various species in wetland versus upland habitats that were treated with the same type of fungicide used in our study (Captan, Blaney and Kotanen, 2001). Their study was conducted in different habitat types and for different species than the ones in our study, but found that the benefit of fungal community removal was limited to mostly wetland conditions (Blaney and Kotanen, 2001). Other studies found fungicides to be beneficial for only some of their study species, concluding that results are highly species-specific (Connolly, 2017; Leishman et al., 2000). These mixed results open the door to asking additional questions about the role of soil fungi in seedling emergence and establishment. Using fungicides may not be helpful as a broad application technique for augmenting seedling emergence in all restoration management contexts. Evidence thus far suggests that using fungicide to reduce fungal pathogen attack on seeds will be most beneficial in wetter habitats (Blaney and Kotanen, 2001), but this will require further examination.

Interestingly, it is also possible that naturally occurring soil microbes play an essential mutualistic role in breaking dormancy, leading to germination and emergence (Van Der Heijden et al., 2008). All seeds used in this study were disinfected by soaking them in a 0.5% bleach solution to remove surface microbes without causing damage to the seed's internal organisms (Sauer, 1986). Removing surface microbes may have reduced or eliminated naturally occurring

microbes on the seed coat surface that serve a mutualistic role. Additionally, our findings could be consistent with the idea that fungicides could reduce pathogenic and beneficial soil fungi, supporting the idea that seedling emergence could have been negatively impacted by removing the seed's microbial community (Connolly et al., 2017). Only one type of fungicide (non-systemic broad spectrum) was tested in our study design. Without comparison to other kinds of fungicides, we cannot definitively conclude that fungicide application is not beneficial for increasing the seedling emergence probability of understory plant species in a longleaf savanna. Additional testing of both systemic and non-systemic fungicides needs to be conducted.

These results imply that predicted shifts in precipitation variation may impact how and when understory plants can regenerate from seed, creating novel plant communities better suited for withstanding extreme precipitation events. Understanding the species-specific responses to precipitation and litter depth can help restoration and conservation practitioners manage available site conditions to promote seedling emergence and establishment of understory plant communities.

## **5. Conclusions**

Species-specific responses to climate change factors underscore the importance of unique management techniques to ensure a diverse community of plants can emerge. Restoration efforts, such as commonly used seed additions, can help combat biodiversity loss by adding seeds of desirable species known to occur in the target habitat (Clark et al., 2007; Orrock et al., 2023). Studies have seen an increase in species richness of 25-50% following a seed addition treatment (Myers and Harms, 2011; Turley et al., 2017). Seeds with thicker seed coats may require additional treatment before sowing to prevent early germinating species from competitively

excluding them. Our results support the demand for litter management in fire-maintained grasslands and savannas to create the necessary microsites for successful emergence and establishment. Litter can be removed and managed through prescribed fire or raking techniques (Kelly and Wentworth, 2009). Routine prescribed fire reduces the depth of leaf litter, and studies on the restoration of plant communities found that decreasing understory litter through fire management increases seedling establishment and species richness over the long term (Orrock et al., 2023; Veldman et al., 2014). Fire and raking, however, can be detrimental to young seedlings and alter community composition based on the timing and frequency of either management tool (Kelly and Wentworth, 2009). Further research is needed to inform decisions on when litter removal is most appropriate and which management technique to use to minimize harmful effects on seedlings. By incorporating these management techniques into restoration and conservation practices, managers can improve the fate of grassland and savanna ecosystems under novel conditions generated by a changing climate.

**Author roles:** Both authors designed the study. EID led and AB contributed to experimental design. AB led and EID contributed to data collection, analysis, and writing.

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## Tables

**Table 1.** *Species and traits* - The seven study species used in the experiment and their germination ability traits. The days to germination and seed coat thickness are taken from Orrock et al. 2023.

<b>Species Code</b>	<b>Family</b>	<b>Genus</b>	<b>Species</b>	<b>Days to germination</b>	<b>Seed coat thickness (mm)</b>
CORMAJ	Asteraceae	<i>Coreopsis</i>	<i>major</i>	27	0.55
EUPALB	Asteraceae	<i>Eupatorium</i>	<i>album</i>	18	0.35
SILCOM	Asteraceae	<i>Silphium</i>	<i>compositum</i>	27	0.55
SOLODO	Asteraceae	<i>Solidago</i>	<i>odora</i>	5	0.31
LESHIR	Fabaceae	<i>Lespedeza</i>	<i>hirta</i>	27	0.60
ARIBEY	Poaceae	<i>Aristida</i>	<i>beyrichiana</i>	9	0.40
SORSEC	Poaceae	<i>Sorghastrum</i>	<i>secundum</i>	16	0.40

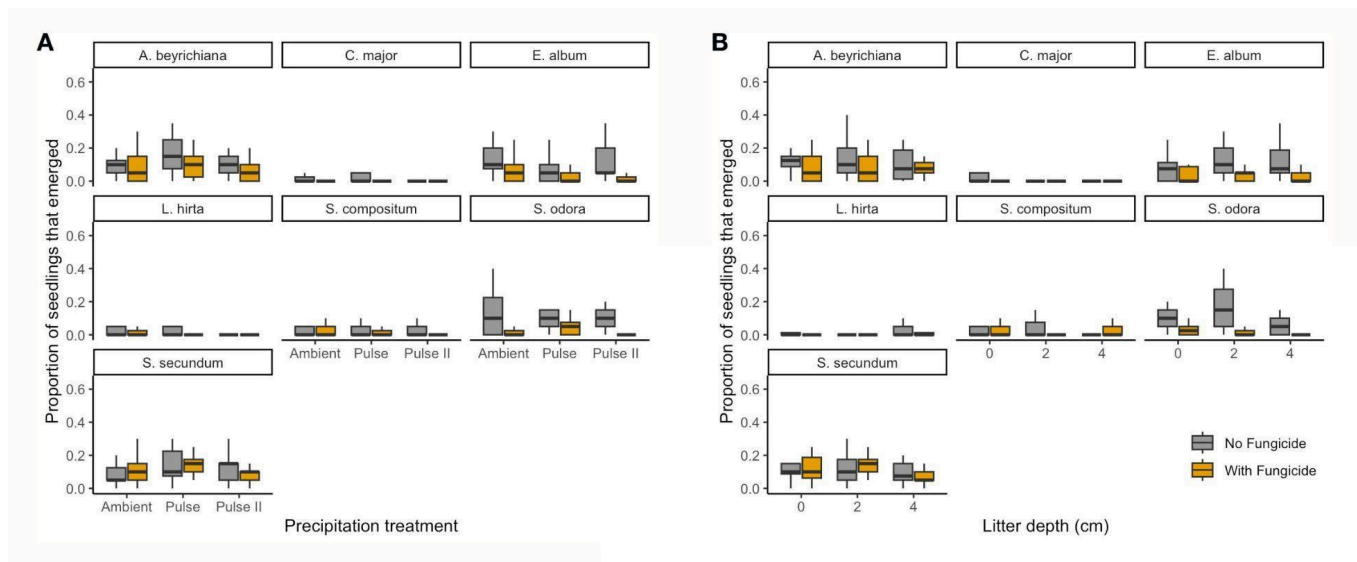
**Table 2.** *Seedling emergence* - Results of the negative binomial generalized linear regression model of the effects of precipitation, litter depth, fungicide, species, and their interactions (indicated by a colon), on seedling emergence. Bolded values indicate significance at the  $p < 0.05$  level.

<b>Treatment</b>	<b>Chisq</b>	<b>Df</b>	<b>Pr(&gt;Chisq)</b>
Precipitation	3.181	2	0.204
Litter Depth	2.857	2	0.240
Fungicide	0.045	1	0.831
Species	27.265	6	<b>&lt;0.0001</b>
Precipitation: Litter Depth	3.004	4	0.557
Precipitation: Fungicide	1.168	2	0.557
Litter Depth: Fungicide	7.776	2	<b>0.020</b>
Precipitation: Species	14.296	12	0.282
Litter Depth: Species	11.906	12	0.453
Fungicide: Species	10.690	6	0.098
Precipitation: Litter Depth: Fungicide	8.676	4	0.070
Precipitation: Litter Depth: Species	19.910	24	0.702
Precipitation: Fungicide: Species	22.938	12	<b>0.028</b>
Litter Depth: Fungicide: Species	23.960	12	<b>0.020</b>
Precipitation: Litter Depth: Fungicide: Species	35.807	24	0.057

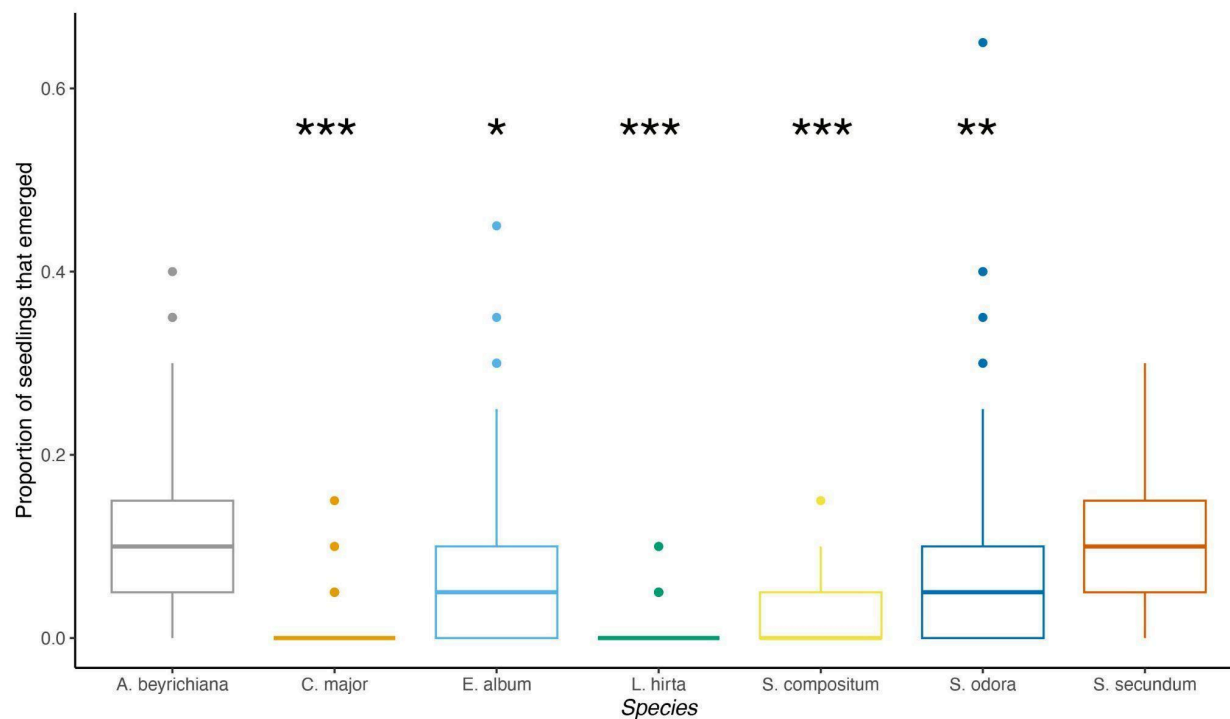
**Table 3.** *Seedling emergence timing* - Results of a Cox proportional-hazards analysis of the effects of precipitation, litter depth, fungicide, and species and their interactions (indicated by a colon) on the probability of survival based on seedling emergence timing. Bolded values indicate significance at the  $p < 0.05$  level.

<b>Treatment</b>	<b>Chisq</b>	<b>Df</b>	<b>Pr(&gt;Chisq)</b>
Precipitation	72.850	2	<b>&lt; 2.2e-16</b>
Litter Depth	121.328	2	<b>&lt; 2.2e-16</b>
Fungicide	0.556	1	0.452
Species	41.966	6	<b>1.867e-07</b>
1 tray	0.000	0	<b>1.000</b>
Precipitation: Litter Depth	24.237	4	<b>7.160e-05</b>
Precipitation: Fungicide	0.259	2	0.879
Litter Depth: Fungicide	1.834	2	0.399
Precipitation: Litter Depth: Fungicide	1.039	4	0.904

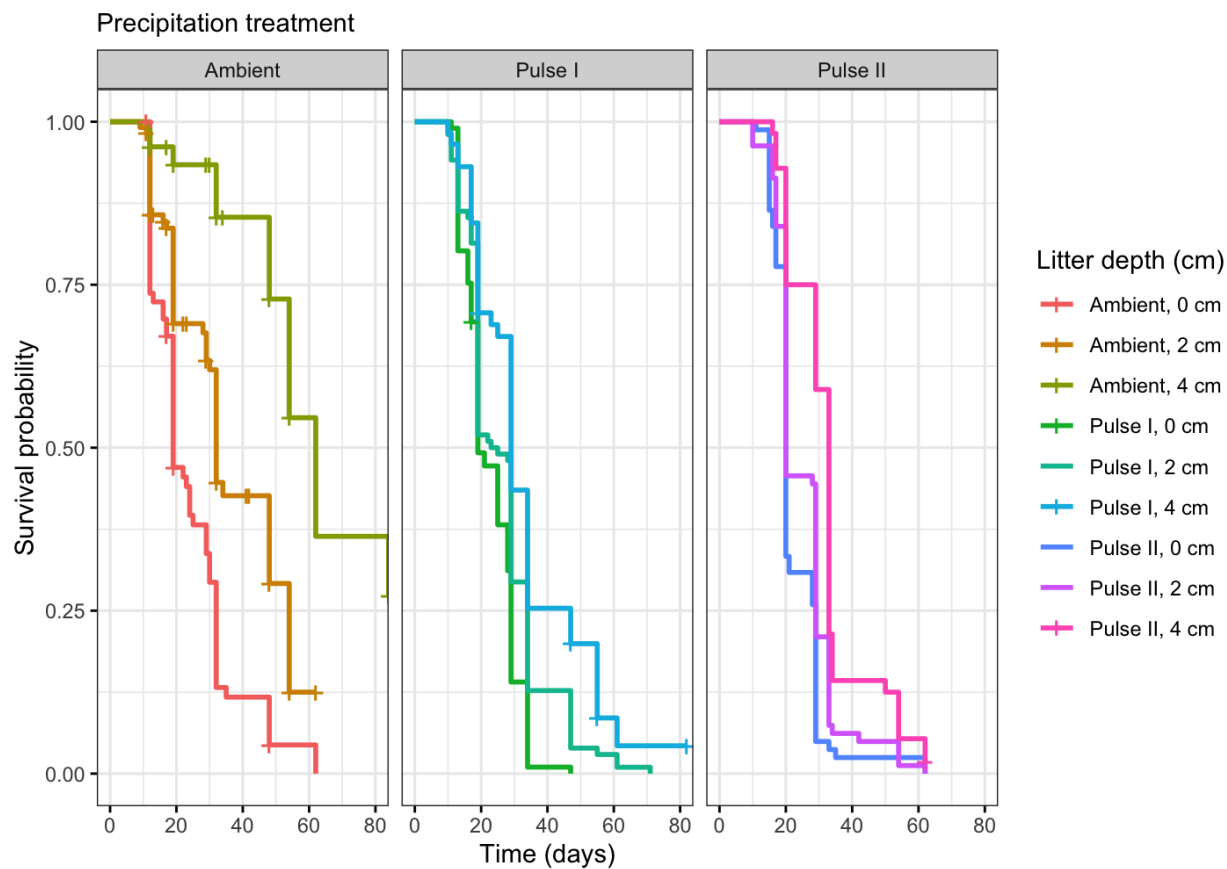
## Figures



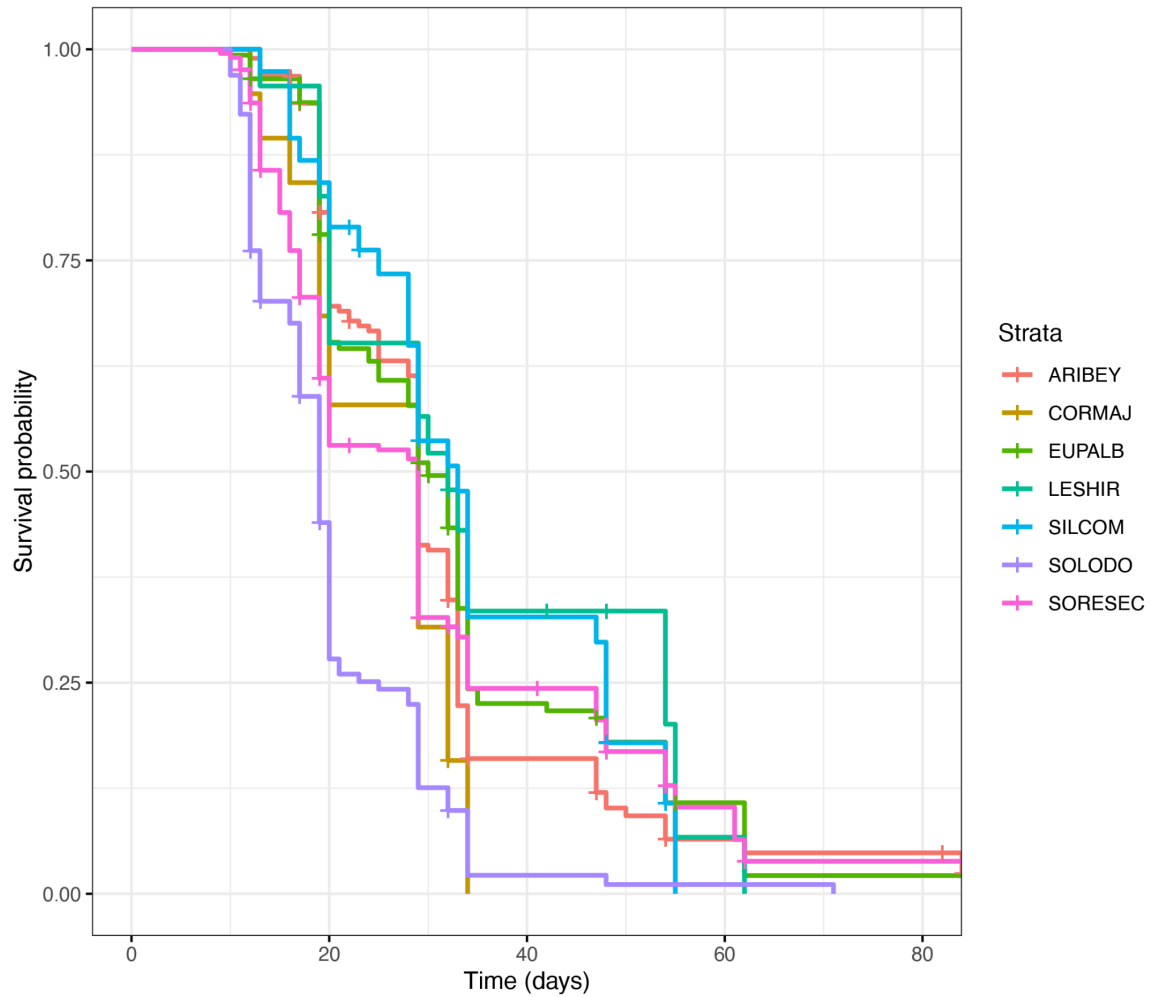
**Figure 1.** Mean seedling emergence is affected by an interaction between (A) precipitation and fungicide treatments and species identity ( $p = 0.03$ ) and (B) litter depth and fungicide treatment and species identity ( $p = 0.02$ ). The bars represent the mean proportion of seeds that emerged and are grouped by precipitation treatment. Error bars represent the first (lower bar) and third (upper bar) quartiles.



**Figure 2.** Mean seedling emergence is significantly different between species. Error bars represent the first (lower bar) and third (upper bar) quartiles. Asterisks indicate significance between species identity at  $p < 0.05$ . Asterisks indicate p-values as follows: ‘\*\*\*’ =  $<0.001$ , ‘\*\*’ =  $<0.01$ , ‘\*’ =  $<0.05$ , and correspond to a decrease in seedling emergence when comparing species variation to *A. beyrichiana*, an important fire-dependent grass species found in understory plant communities of longleaf pine ecosystems.



**Figure 3.** Seedling emergence timing shown with survival curves. The probability of seed or seedling survival is plotted against the number of days since the start of the experiment (time) for each precipitation treatment level and litter depth (cm) treatment level combination.



**Figure 4.** Seedling emergence timing for each of the seven study species shown above using survival curves. One species, *S. odora* (SOLODO), had significantly lower survival earlier in the time series compared to the remaining six species. ( $p < 0.001$ ).

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## Supplemental Information

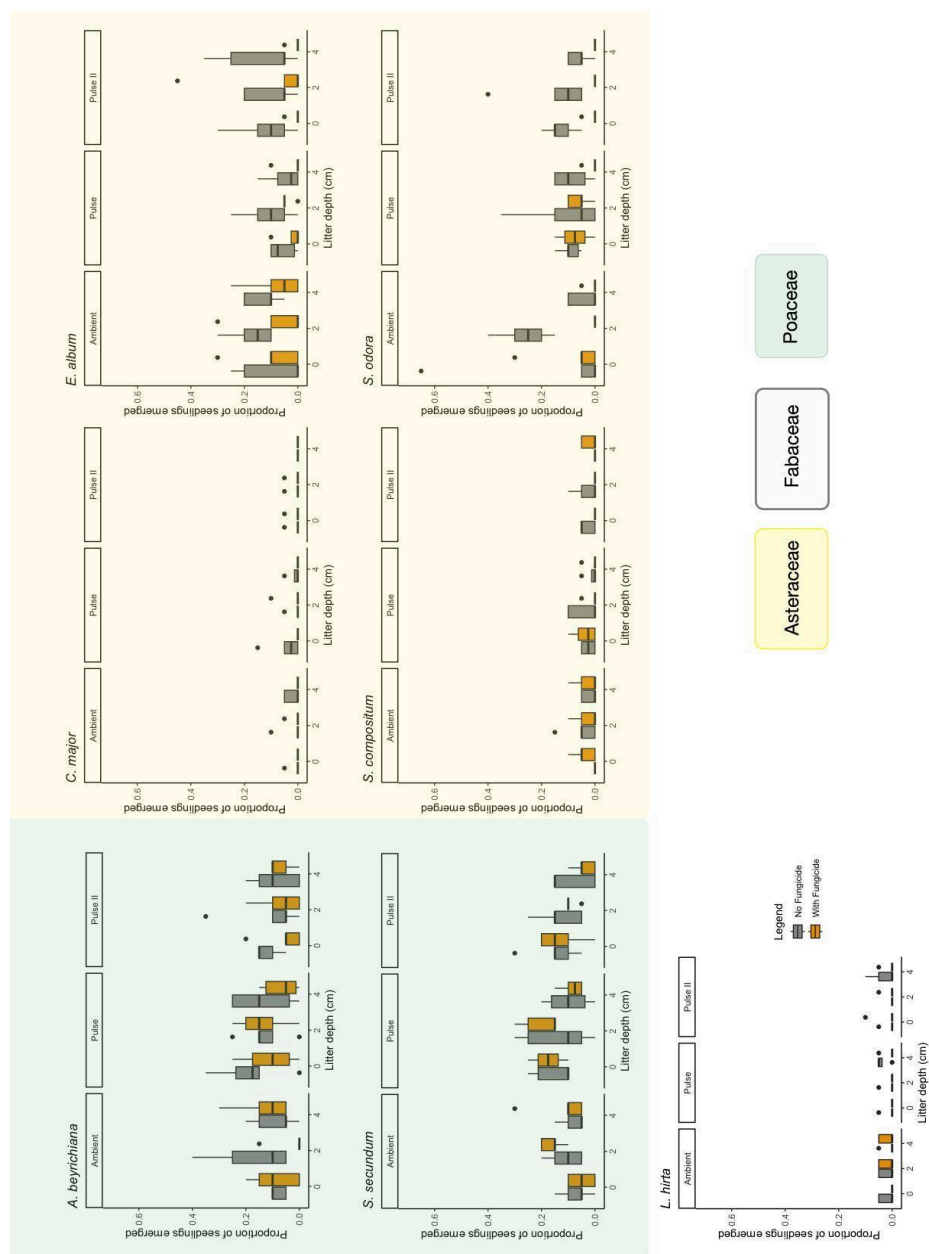
**Table S1.** Greenhouse temperature and light conditions across the duration of the study. Temperature and light values were taken from Orrock et al., 2015 and mimic local field conditions at the Savannah River Site, SC.

Week	Daytime (C°)	Nighttime (C°)	Light on	Light off	Mimics (Averages)	Sunrise	Sunset	Max (C°)	Min (C°)	Avg (C°)
0	24	20	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
1-3	25	10	07:12	17:46	April	07:12	19:46	25	10	18
4-6	29	15	06:35	18:10	May	06:35	20:10	29	15	22
7-9	32	19	06:14	18:32	June	06:14	20:32	32	19	25
10-12	33	22	06:16	18:42	July	06:16	20:42	33	22	28
13-15	33	21	06:35	18:27	August	06:35	20:27	33	21	26
16-end	30	18	06:58	19:51	September	06:58	19:51	30	18	24

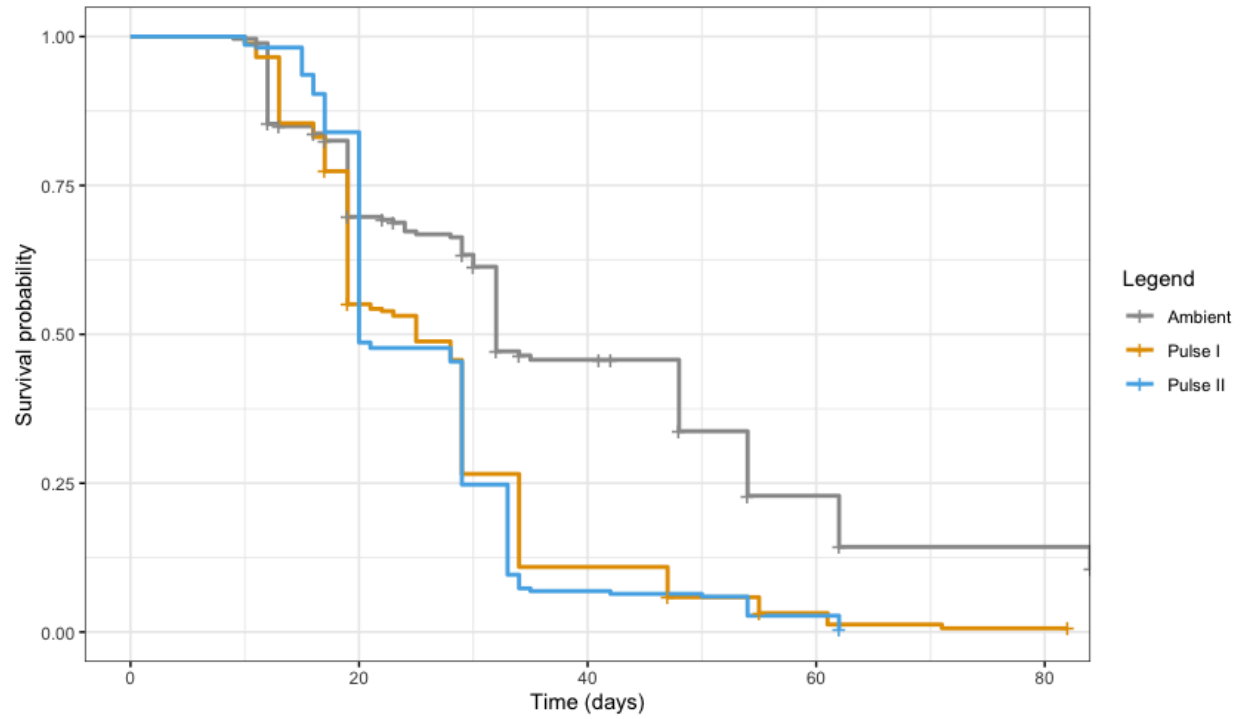


**Table S2.** Results of individual species generalized linear regression models of the effects of precipitation, litter depth, fungicide, and species and their interactions (indicated by a colon) on seedling emergence probability. Bolded values indicate significance at the  $p < 0.05$  level.

Treatment	<i>A. beyrichiana</i>			<i>C. major</i>			<i>E. album</i>			<i>L. hirta</i>		
	Chisq	Df	Pr(>Chisq)	Chisq	Df	Pr(>Chisq)	Chisq	Df	Pr(>Chisq)	Chisq	Df	Pr(>Chisq)
Precipitation	4.63	2	0.098	3.31	2	0.19	2.41	2	0.3	0.62	2	0.73
Litter Depth	4.06	2	0.131	0.44	2	0.8	2.50	2	0.29	0.44	2	0.80
Fungicide	0.06	1	0.808	1.39	1	0.24	0.05	1	0.81	2.77	1	0.09
Precipitation: Litter Depth	4.41	4	0.353	3.82	4	0.43	3.82	4	0.43	4.90	4	0.30
Precipitation: Fungicide	1.53	2	0.465	2.78	2	0.25	7.90	2	<b>0.02</b>	3.57	2	0.17
Litter Depth: Fungicide	10.23	2	<b>0.006</b>	1.59	2	0.45	1.95	2	0.38	3.00	2	0.22
Precipitation: Litter Depth: Fungicide	11.63	4	<b>0.02</b>	2.28	4	0.68	11.93	4	<b>0.02</b>	5.87	4	0.21
<b>Treatment</b>	<i>S. compositum</i>			<i>S. odora</i>			<i>S. secundum</i>					
Precipitation	4.55	2	0.1	1.27	2	0.53	3.91	2	0.14			
Litter Depth	7.00	2	<b>0.03</b>	18.07	2	<b>0.0001</b>	0.97	2	0.61			
Fungicide	5.54	1	<b>0.02</b>	1.66	1	0.2	0.33	1	0.56			
Precipitation: Litter Depth	7.61	4	0.11	5.96	4	0.2	1.41	4	0.84			
Precipitation: Fungicide	9.57	2	<b>0.01</b>	6.37	2	<b>0.04</b>	0.63	2	0.73			
Litter Depth: Fungicide	5.72	2	0.06	14.45	2	<b>0.001</b>	1.24	2	0.54			
Precipitation: Litter Depth: Fungicide	10.36	4	<b>0.03</b>	9.62	4	<b>0.05</b>	12.35	4	0.67			



**Figure S1.** Mean seedling emergence for each species shown as an interaction between the precipitation, litter depth, and fungicide treatments. The bars represent the mean proportion of seeds that emerged and are grouped by precipitation treatment. Error bars represent the first (lower bar) and third (upper bar) quartiles.



**Figure S2.** Seedling emergence timing shown with survival curves. The probability of seed or seedling survival is plotted against the number of days since the start of the experiment (time) for each precipitation treatment level.

**Chapter Two**

**THE DIRECT AND INDIRECT EFFECTS OF LAND-USE HISTORY  
AND FIRE REGIME ON SEED GERMINATION AND SOIL FUNGI  
IN A LONGLEAF PINE ECOSYSTEM**

Bautista, A., and Damschen, E. I. The Direct and Indirect Effects of Land-use History and Fire Regime on Seed Germination and Soil Fungi in a Longleaf Pine Ecosystem.

**Abstract**

Recruitment limitation is a key factor regulating community assembly and long-term ecosystem functioning, particularly in plants, where the transition from seed to seedling is one of the most vulnerable phases in development. In ecosystems like grasslands and savannas, which are known to be limited by seed arrival and establishment, changes in land use and disturbance regimes—especially fire—can significantly affect seed germination. However, these effects are complex, acting both directly and indirectly. Soil fungal communities can also influence establishment through various direct and indirect pathways. Yet, determining their impacts on establishment is difficult because these processes can operate in multiple ways, influencing each other in often unpredictable ways. We assessed how land-use history and altered fire regimes, along with their indirect effects on leaf litter depth and fungal communities, impact seed germination of four understory plant species native to longleaf pine (*Pinus palustris*) ecosystems. Our experiment differed in land-use history (post-agriculture or remnant) and fire regime (low vs. high fire frequency), manipulating litter depth and fungal presence (via fungicide) to

determine how their direct and indirect effects alter the seed germination probability. Despite their importance in structuring mature plant communities, we found that land-use history, fire regime, and fungicide application did not affect seed germination probability. Greater litter depth, however, increased germination across all species, highlighting the importance of local site conditions in creating optimal environments for seed germination. Soil fungal communities were influenced by land-use history but not fire regime. Since disturbance and the availability of suitable microsites are crucial for plant regeneration and seedling establishment, the next step in applying the implications of this work for managing longleaf pine ecosystems is to determine the ideal amount and type of litter that promotes seed germination and supports seedling growth.

**Keywords:** land-use change, fire regime, community assembly, seed, leaf litter, groundcover, restoration

## **Introduction**

Recruitment limitation is a key process regulating community assembly that has consequences for long-term community composition (Clark, 2007; Jimenez et al., 2016; Nelson, 2018) and ecosystem functioning (Walck et al., 2011). Anthropogenic changes, such as habitat degradation and altered disturbance regimes, can impact the ability of species to recruit following dispersal (Török et al., 2020; Turley et al., 2017). For plants, land use and disturbance regime changes can greatly impact recruitment as the transition from seed to seedling is one of the most vulnerable phases in development (Clark, 2007; Jimenez et al., 2016; Nelson, 2018). Seed germination is a critical, all-or-nothing event highly influenced by environmental factors, such as oxygen availability, temperature, salinity, and soil moisture (Fenner, 1991; Jimenez et al.,

2016; Larson et al., 2020; Walck et al., 2011). One of the reasons this phase is so critical is that ideal conditions for seed germination may not be the same as those ideal for early seedling growth and establishment (Fay and Schultz, 2009; Jimenez et al., 2016). For ecosystems like grasslands and savannas that are known to be limited by seed arrival and establishment and which face growing threats from widespread habitat loss and degradation (Iacona et al., 2010; Nerlekar and Veldman, 2020), the effects of anthropogenic land use and changes to disturbance regimes (especially fire) on seed germination are especially crucial to understand. Yet, determining their impacts on establishment is difficult because of the potential for these processes to act not only directly, but indirectly. Anthropogenic land-use change is known to have direct, long-lasting, negative impacts on plant community diversity (Brudvig et al., 2013; Foster et al., 2003; LeProvost et al., 2020) that can persist hundreds of years after agricultural use has been abandoned (Dambrine et al., 2007). Agricultural practices, such as tilling, physically disturb the soil (Brudvig et al., 2013; Foster et al., 2003; Turley et al., 2020), resulting in lower organic matter, carbon, and soil water-holding capacity (Bizzari et al., 2015; Brudvig et al., 2013; Markewitz et al., 2002), all of which can influence the likelihood of seed germination and establishment (Lambers et al., 2008).

Similarly, the loss of fire can reduce diversity in fire-dependent plant communities (Brudvig et al., 2014; Orrock et al., 2023; Veldman et al., 2014). The likelihood of seed germination and establishment may be affected by altered fire regimes since fires create suitable microsites for establishment by reducing leaf litter, minimizing plant competition for space and nutrients, and clearing space for plant regeneration by seed (Ellair and Platt, 2013; Harms et al., 2017; Nelson et al., 2012). Sites managed with fire have indeed been shown to have higher rates of seed germination and establishment (Orrock et al., 2023).

Determining how past agricultural land use and altered fire regimes impact seed and seedling survival will require not only examining their direct effects but also their indirect effects on environmental conditions and species interactions. Since fire frequency reduces leaf litter, it indirectly alters the environmental conditions for seed germination and establishment (Facelli et al., 1999; Iacona et al., 2009; Rotundo and Aguiar, 2005). On the one hand, the presence of litter maintains soil moisture, reduces the risk of desiccation of seeds, and aids germination and seedling establishment (Zhang et al., 2017). On the other hand, deep litter can negatively affect plant regeneration (Loydi et al., 2013; Rotundo and Aguiar, 2005) by creating a physical barrier to seedling emergence. As such, litter depth impacts on seed germination and establishment can influence plant community diversity and ecosystem dynamics (Loydi et al., 2013; Zhang et al., 2017).

Agricultural land use also may have indirect consequences on seed germination and establishment rates by altering interactions with the soil microbial community (Turley et al., 2020). Soil microbes are crucial in shaping plant communities and species distributions through both antagonistic and mutualistic interactions (Turley et al., 2020). Soil fungal pathogens are known to experience increased growth with increased soil moisture and temperature, decreasing the survival of buried seeds (Leishman et al., 2000). This is one reason that fungicides can be used to reduce seed mortality in areas of high moisture (Gallagher, 2013; Schafer and Kotanen, 2003). Interestingly, the indirect impacts of agricultural land use and altered fire regimes can interact themselves. For example, deeper leaf litter can promote the growth of soil pathogens (Rotundo and Aguiar, 2005).

Here, we assess how seed germination is affected by both the direct effects of agricultural land use and altered fire regimes, and their indirect effects on leaf litter depth and fungal

communities. We ask the following questions: 1) Do land-use history and fire frequency directly affect seed germination on the forest floor? 2) Do the indirect effects of agricultural land use and reduced fire frequency on litter depth affect seed germination? And, 3) do the indirect impacts of agricultural land use and reduced fire frequency on soil fungal community composition affect seed germination? We use a distributed experiment with varying land-use history (post-agriculture or remnant) and fire regime (low vs. high fire frequency). Within each site, we manipulated litter depth and the size of the fungal community (using fungicide) to determine how their direct and indirect effects alter seed germination. We hypothesize that: H1) Agricultural land use and low fire frequency will negatively impact seed germination. H2) Seed germination will decrease as litter depth increases due to lower fire frequency in remnant forests. H3) Fungicide application will reduce fungal pathogens and increase seed germination. The impact of the fungicide treatment may be different in sites that have different land use histories (post-agricultural vs. remnant sites).

## **2. Materials and Methods**

### *2.1 Study system*

We tested our hypotheses in the longleaf pine ecosystem (*Pinus palustris*) of the Southeastern United States, which is an ideal system to assess the direct and indirect impacts of land use and fire regimes on seed germination. The longleaf pine ecosystem once extended from Texas to southern Virginia (Frost, 2006), and historically was maintained by frequent, low-intensity fires that created an open canopy of pines with a diverse herbaceous understory by reducing the abundance of hardwood trees and leaf litter (Kilgo et al., 2005; Kirkman et al., 2001). The



original extent of the ecosystem has been reduced to less than 3% of its historical range due to human impacts including agriculture and the loss of fire (Brudvig et al., 2013; Frost, 2006; Jose et al., 2006), which have altered plant community structure and composition, and may influence seed survival (Brudvig and Damschen, 2011; Brudvig et al., 2013; Mattingly and Orrock, 2013). The longleaf pine ecosystem is a biodiversity hotspot, supporting up to 50 plant species per square meter (Kirkman et al., 2001; Veldman et al., 2014). Understanding the impacts of agricultural land use and fire regimes on seed germination and survival is critical for conserving and restoring this ecosystem (Brudvig et al., 2013; Frost, 2006; Mitchell et al., 2014).

Our study was conducted at the Department of Energy's Savannah River Site (SRS), a National Environmental Research Park near Aiken, SC (Kilgo et al., 2005). Savannah River Site is located in the upper Atlantic coastal plain and represents the central portion of longleaf pine ecosystem range. Soils are typically sandy and well-drained (Brudvig et al., 2013) and the site contains habitat that ranges in its land-use history and fire frequency. Previous research at SRS has demonstrated the role of these factors on plant community composition (Brudvig et al., 2013).

Study species: Four study species were chosen for this study (*Aristida beyrichiana*, *Pityopsis* sp., *Solidago odora*, and *Sorghastrum secundum*) because they are indicators for remnant longleaf pine savanna (Brudvig et al., 2013) and vary in their seed traits (*i.e.*, days to germination and seed coat thickness, Table 1), which are known to influence establishment (Jimenez et al., 2016; Orrock et al., 2023) and resistance to pathogens (Jimenez et al., 2016). Due to difficulty in identifying closely related species within the genus *Pityopsis*, *Pityopsis* spp. includes both *P. aspera* and *P. nervosa*. We hand-collected seeds at SRS in November-December 2021. All seeds collected from the field were homogenized across collection locations by

species, and unfilled (aborted) seeds were removed (Coffey and Kirkman, 2006). Using standardized methods (Baskin and Baskin, 2014), all seeds were disinfected in a 0.5% bleach solution for 10 minutes, thoroughly rinsed, and air-dried for at least 72 hours. We did not test the viability of seeds before placing seeds into the field.

*Experimental design:* We tested our hypotheses using a distributed experiment that manipulated litter depth and the size of the soil fungal community in established sites with varying land-use history (post-agricultural, remnant) and fire frequency (1-12 fires since 1991) (Figure 1, Orrock et al. 2023). We placed one plot in each of the 34 sites previously utilized by Brudvig et al. (2014). To examine land use, half of the sites had a history of agricultural land use, and half were remnant forests (Brudvig et al., 2014). To examine fire frequency, sixteen of the 34 sites had “low” fire frequency (0-7 fires since 1991), and eighteen had “high” fire frequency (7-12 fires since 1991) (Brudvig et al., 2014).

Seeds were buried at each site from March to June to capture the early spring growing season when seeds typically germinate. Ten seeds of a single species were placed in an empty woven cotton blend bag (2×3cm) to prevent seed loss during burial while allowing contact with soil and water. In total, 136 seed bags were created for each of the four species, totaling 544 seed bags and 5,440 seeds. At each site, in March 2022, seed bags (one per species) were buried in 23 x 30 cm plastic trays with drainage holes along the edges of a 3 × 3 m plot (Figure 1). Each tray contained one bag of seeds for each species (four bags per tray), with the order of species placement randomized within each tray, and full factorial treatments (litter present vs. absent, fungicide vs. no fungicide) were randomly applied across trays (Figure 1). The presence or absence of litter acted as a proxy for the indirect effect of fire on site conditions with no litter indicating a high-fire frequency and litter presence acting as a low-fire frequency.

To apply treatments, each tray was placed in position by carefully lifting the existing leaf litter, duff, and ~1 cm of mineral soil from a 23 × 30 cm area (same size as the tray) using a flat shovel, placing the tray beneath the lifted material and gently returning it on top of the tray again. Soil was returned to allow fungal inoculation. For trays assigned the no-litter treatment level, all visible leaf litter was removed from above the ~1 cm of soil that was returned to each tray. For the litter-present treatment level, leaf litter and duff were left on top of the tray. For the fungi treatment level, seeds were dusted with a non-systemic heterocyclic nitrogen fungicide, Captan (Strid et al., 2018), before being placed into seed bags. Captan fungicide is effective against various Ascomycetes and Basidiomycetes, and especially against seed rotting (Schafer and Kotanen, 2003). Seed bags treated with fungicide were kept separate from those that did not. Litter depth was measured for each treatment at the time of burial and remeasured when seed bags were exhumed.

## *2.2 Seed Germination*

Seed bags were exhumed in June 2022 and placed in a paper bag by treatment type. Within 24 hours after being exhumed, each seed bag was carefully cut open and inspected for seed germinants. A seed was marked as having germinated while in the field if a live or desiccated seedling was present or the seed coat was empty but not moldy or otherwise damaged.

## *2.3 Soil Fungal Community Sampling*

Soil from each Control (no fungicide with litter) treatment tray was collected during exhumation in June 2022. Approximately 0.1 kg of soil from the top 2 cm were placed in a sterile Whirl-pak plastic bag and frozen at -40°C until samples were analyzed. Samples were

processed through the Microbial Community Sequencing Service Center at the University of Colorado to obtain fungal ITS sequencing data (Suleiman et al., 2019).

#### *2.4 Statistical Analyses*

To test our hypotheses regarding seed germination, we used generalized linear mixed-effects models (GLMMs) using the ‘glmer’ function in the lme4 package, version 1.3-35.5 (Bates et al., 2015). We used the probability that a seed germinated under study treatments (hereafter, “germination probability”) as a response variable in these models. Germination probability was evaluated at the species level to determine a species’ ability to germinate under treatment conditions. We fit our models with a binomial distribution because germination is a binary event, either the seed breaks dormancy and germinates or it does not. To test whether agricultural land use and low fire frequency negatively impact seed germination (H1), we used land-use history (post-ag, remnant), fire frequency regime (high, low), and species as fixed effects and site as a random effect. To evaluate whether litter depth indirectly affects seed germination probability (H2), we first determined if litter depth is affected by agricultural land use and fire frequency regime. We used a linear mixed-effects model (LME) with litter depth as a response variable, land use and fire frequency as fixed main effects along with their interaction, and site as a random effect. Litter depth was calculated for each tray by averaging the litter depth (cm) above the seed tray at both the time of burial and exhumation and was log-transformed to fit a Gaussian distribution. Next, we conducted a second GLMM model, again using germination probability as the response variable, but using litter depth (continuous), site (random), species (fixed), and their interactions as predictors using a binomial distribution.

To test whether fungicide reduces fungal pathogens and increases seed germination and whether this is dependent on land-use history (H3), we again used germination probability as our response variable in a GLMM and tested the main effects of land-use history, fungicide with varying presence of litter, and site as a random effect. We followed this up with two additional analyses to test whether the fungal community composition is affected by land use and fire frequency. We evaluated the dissimilarity of fungal taxa among land-use history and fire regime by site using PERMANOVA with the ‘adonis2’ function in the vegan package, version 2.6-6.1 (Anderson, 2001), and the Bray-Curtis dissimilarity metric. To evaluate alpha diversity, we calculated the Shannon Diversity metric (Morris et al., 2014). We used it as a response variable in an LME with land-use history and fire regime as fixed effects and site as a random effect.

All analyses were conducted in R version 4.4.1 (R Core Team, 2024).

### 3. Results

Germination ranged from 60% to 80% overall, although no environmental factor significantly impacted germination probability (Figure 3). Our first hypothesis was not supported as land-use history and fire regime showed no significant impact on whether our test species germinated in the field (Table 2). Species identity was the only significant main effect ( $p = 0.029$ , Table 2), with rates ranging from 50% to 88% (Figure 4). Most of the variation was driven by the lower germination rates of *S. odora*.

Neither land-use history, nor fire regime, nor their interaction, were significantly associated with litter depth ( $p > 0.05$ ), showing that these environmental factors may not be driving changes in the amount of litter accumulating on the forest floor (Table 3). However, litter depth indirectly affected seed germination, as we predicted (H2). Overall, germination increased

as litter depth increased ( $p = 0.01$ ). Species identity strongly affected germination ( $p = 0.04$ , Table 4), showing that some longleaf pine species respond differently under varying site conditions.

Applying fungicide did not impact seed germination probability as we predicted (H3), nor were there differences between land-use history (Table 5). The fungicide application trended toward being beneficial compared to no fungicide, especially when litter was present (Figure 7). Looking closer at the soil microbial community, soil fungal community composition differed across sites based on land-use history ( $p = 0.021$ ) but not fire regime ( $p = 0.229$ ; Table 6). Remnant sites were more dissimilar to post-agricultural sites with a broader range of variability (Figure 8). Fungal community composition appears to trend toward separation by fire frequency, but the dissimilarity between these groups is not significant (Figure 9). Conversely, species diversity (Shannon's D) was not affected by land-use history or fire regime (Table 7; Figure 10).

#### **4. Discussion**

Despite their importance in structuring mature plant communities, we found that land-use history, fire regime, and fungicide application did not affect seed germination probability. We determined that greater litter depth significantly improved germination outcomes across species. Soil fungal community composition was impacted by land-use history but not the fire regime. Our findings have several implications: many understory plant species may not be limited by germination in longleaf pine savannas, and mechanisms impeding plant regeneration from seed may lie in later stages of plant development, such as emergence. Thus, other critical environmental filters must be tested. Litter management is vital for seed germination. Although it is common to state that removing litter is essential for seed germination, some litter may be

advantageous, particularly for systems where water may be limited (Facelli et al., 1999). Soil microbes can be essential for plant development. Our findings suggest that soil fungi may hinder germination, and historic disturbances do not negatively affect the interaction between soil fungi and seeds (Figure 7).

The legacy effects of tillage and differences in fire regime did not impact seed germination probability, suggesting that differences in microsite conditions are less critical for the early seed to seedling stage of plant development. Despite lower levels of soil organic matter, soil moisture, and inorganic nitrogen (Brudvig et al., 2013) — which are vital components of soil properties necessary for successful germination (Gallagher, 2015) — post-agricultural sites did not negatively impact whether a seed germinated in the field. A study by Coffee and Kirkman (2006) found similar results for *S. odora* and *S. secundum* but the opposite for an *Aristida* grass species. They found that overall, seeds of most understory plant species survive after one year of burial in a restored pine ecosystem and can germinate. However, many species have little to no seed dormancy and fail to germinate after two years (Coffee and Kirkman, 2006). Our results demonstrate that seeds can successfully germinate, and other studies have elucidated the short timeframe for which seeds are viable post-dispersal. However, this study only tested a small subset of the vast number of native plant species, and understanding which species are constrained by germination in longleaf pine savannas remains limited.

Contrary to our hypothesis, differences in site conditions due to fire regimes, such as soil moisture and light availability, did not impact germination probability. Fire is a heterogeneous disturbance altering site conditions at different rates and spatial scales, even within the same fire event (Lertzman et al., 1998). Our small sampling area and limited replication may not have captured the range at which fire impacts site conditions and indirectly alters germination

outcomes. Overall, seed germination was not impacted by disturbance to soil properties or site conditions by fire or land-use change.

Litter depth was not found to be directly impacted by the differences in land-use history or fire regime, despite our predictions. Although litter depth is strongly correlated with fire history, it can also be determined by vegetation type and density (Ellair and Platt, 2013). A site with high-fire frequency can also have areas of high litter accumulation similar to that of a low-fire frequency site. A broader survey of litter type and density could be a better indicator of how site conditions influence seed germination as the depth of litter was found to be a significant factor in whether a seed germinated. In our study, all seeds were buried under 1 cm of soil with 0–6 cm of litter on top. Seeds need contact with soil for successful germination and establishment (Rotundo and Aguiar, 2005; Shaw et al., 2020); thus, seeds buried deeper into the soil profile ensure proper contact. Litter and soil protect seeds from changes to ambient temperature and light that alter soil moisture and temperature (Loydi et al., 2013). Seeds buried under deeper litter had higher germination probability, suggesting that litter can mediate environmental conditions and act as a protective insulator. These results support other studies that found higher germination and seedling recruitment rates in buried seeds versus seeds at the soil surface or in the litter layer (Rotundo and Aguiar, 2005; Zhang et al., 2017). Despite germinating, seeds could fail to emerge through elevated levels of leaf litter, as it can act as a mechanical barrier (Loydi et al., 2013; Zhang et al., 2017). In addition, growing conditions just below the soil surface can be favorable for germination but may not be suitable for continued growth, hindering establishment and persistence (Fay and Schultz, 2009; Lloret et al., 2009). Our previous study found that seeds were more likely to emerge under 2 cm of leaf litter versus no



litter or higher amounts of litter (Bautista and Damschen, in review), showing that site conditions mediated by litter are essential for seedling establishment but not as vital for germination.

Species differed in their response to disturbance types and removal of litter and fungal communities (Figure 7). Although results were non-significant, species displayed a wide range of germination rates under each predictor variable. Studies have found that many longleaf pine savanna plant species possess a high level of versatility, allowing them to germinate and establish under differing environmental conditions and disturbance types (Harms et al., 2017; Kirkman et al., 2001; Orrock et al., 2023). Because species responses vary significantly, it can be challenging to determine to what level they will be impacted by changes to available microsites. The fungicide application was non-significant yet showed a slight increase in germination probability compared to treatments without fungicide. These findings support previous studies that found non-significant results showing that soil fungal pathogens pose a weaker threat to seed mortality compared to seed predators, desiccation, or germination at inappropriate times (Blaney and Kotanen, 2001; Connolly et al., 2017; Schafer and Kotanen, 2003). Captan is a non-systemic fungicide and was only applied once before burial, impacting the soil fungal community at a small local scale for a short period. Additional applications may increase its effectiveness; however, the potential to reduce beneficial fungi in this ecosystem is largely unknown, and more studies testing its unintended impacts are needed.

Our study found that soil fungal community composition was most impacted by a history of agricultural practices but not by differences in fire frequency. These results are supported by other studies that found tilling can reduce species abundance and richness of soil fungi (Hui et al., 2018; Wagg et al., 2018). As a disturbance, heat from fire impacts the top layers of the forest floor, typically consisting of the organic horizon and a few centimeters into the mineral soil

(Cairney and Bastias, 2007; Lavoie et al., 2010), compared to tilling, which can disturb many layers of the soil profile. So, it is unsurprising that land-use history had a more profound impact than fire as a disturbance. Although the difference was not statistically significant, our results showed a trend suggesting that fungal composition tended to differ between areas with low and high fire frequency. (Figure 9). A more robust relationship could be determined with more replicates and a broader sampling area for each fire frequency. Fungal diversity was not affected by land-use history or fire regime (Figure 10). A 2020 study by Turley et al. found similar results, where a history of agricultural land use had no significant impact on fungal diversity. Perhaps these soil fungal communities, like their perennial plant counterparts, once established, are long-lived and can persist for many years despite experiencing numerous disturbances (Hansen et al., 2019; Kirkman et al., 2001; Coffey and Kirkman, 2006). In fire-dependent ecosystems like grasslands and savannas, frequent low-intensity fire may select for fire-adapted fungal taxa, much like the standing plant community, which could explain the lack of difference between composition and diversity among the fire regime in our study. In addition, having strong host-specific symbiotic relationships may allow for a higher diversity of fungal communities in a biodiversity hotspot that supports a multitude of plant species.

## **5. Conclusion**

We found that site conditions have minimal influence on seed germination below the soil surface. Instead, indirect impacts of leaf litter may create a bottleneck, restricting further seedling development and establishment within the plant community. At the local scale, litter depth increases germination success; however, it also acts as a mechanical barrier if seedlings fail to emerge from the litter. Contrary to expectations, removing soil fungi did not increase

germination rates, suggesting that soil fungal pathogens do not pose a significant threat to germination in this system, unlike more mesic environments. Furthermore, soil fungal composition varied significantly by land use, demonstrating that land-use change can have longer-lasting impacts compared to natural disturbances like fire. Fungal diversity, however, was unaffected by land use and fire. Since disturbance and the availability of suitable microsites are critical for plant regeneration and seedling establishment, the next step in managing longleaf pine ecosystems is to understand the optimal amount and type of litter that supports seed germination and seedling growth. This knowledge will help improve ground cover management to better promote the germination and establishment of native species.

**Author roles:** Both authors designed the study. EID led and AB contributed to experiment set-up. AB led and EID contributed to data collection, analysis, and writing.

### **Acknowledgments**

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## Tables

**Table 1.** The four species used in the experiment and their traits. The days to germination and seed coat thickness are taken from Orrock et al. 2023.

Family	Genus	Species	Days to germination	Seed coat thickness (mm)
Asteraceae	<i>Pityopsis</i>		3	0.45
Asteraceae	<i>Solidago</i>	<i>odora</i>	5	0.31
Poaceae	<i>Aristida</i>	<i>beyrichiana</i>	9	0.40
Poaceae	<i>Sorghastrum</i>	<i>secundum</i>	16	0.40

**Table 2.** Results of a generalized linear mixed-effects model of the effects of land-use history, fire regime, and species on seed germination. Bolded values indicate significance at  $p < 0.05$ .

Main effect	Chisq	DF	Pr(>Chisq)
Land-use history	1.70	1	0.19
Fire regime	0.16	1	0.69
Species	8.87	3	<b>0.03</b>

**Table 3.** Results of a linear mixed-effects model of the effects of land-use history, fire regime, and their interaction on litter depth (cm). Bolded values indicate significance at  $p < 0.05$ .

Main effect	Chisq	DF	Pr(>Chisq)
Land-use history	0.23	1	0.63
Fire regime	1.28	1	0.26
Land-use history: Fire regime	0.09	1	0.77

**Table 4.** Results of a generalized linear mixed-effects model of the effects of litter depth (cm) and species identity on seed germination. Bolded values indicate significance at  $p < 0.05$ .

Main effect	Chisq	DF	Pr(>Chisq)
Litter depth (cm)	6.15	1	<b>0.01</b>
Species	8.22	3	<b>0.04</b>

**Table 5.** Results of a generalized linear mixed-effects model of the effects of litter depth (cm) and species identity on seed germination. Bolded values indicate significance at  $p < 0.05$ .

Main effect	Chisq	DF	Pr(>Chisq)
Land-use history	1.75	1	0.18
Fire regime	0.17	1	0.68
Fungicide	5.52	3	0.14
Species	8.98	3	<b>0.03</b>

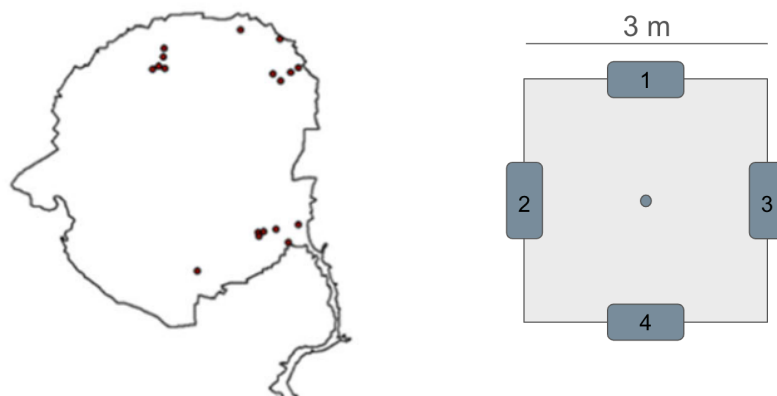
**Table 6.** Results of a PERMANOVA of the effects of land-use history and fire regime on dissimilarity between soil fungal communities. Bolded values indicate significance at  $p < 0.05$ .

Main effect	DF	Sum of Sqs	R2	F	Pr(>F)
Fire	1	0.403	0.033	1.126	0.219
Land-use history	1	0.602	0.050	1.682	<b>0.015</b>
Residual	31	11.088	0.917		
Total	33	12.093	1.000		

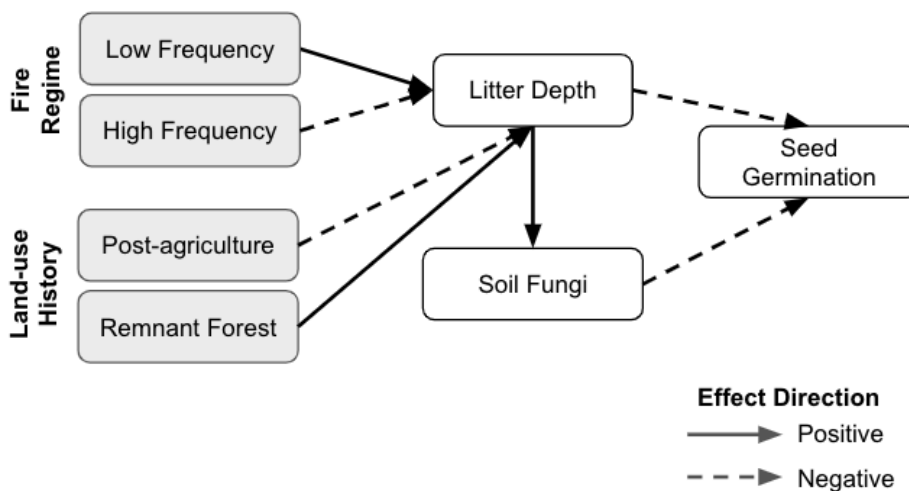
**Table 7.** Results of a linear regression model on Shannon's Diversity Index of fire regime and land-use history, (cm) on the species richness and evenness of soil fungi across sites.

<b>Main effect</b>	<b>Sum Sq</b>	<b>Df</b>	<b>F value</b>	<b>Pr(&gt;F)</b>
Fire	0.012	1	0.317	0.577
Land-use history	0.001	1	0.003	0.957
Residuals	1.145	31		

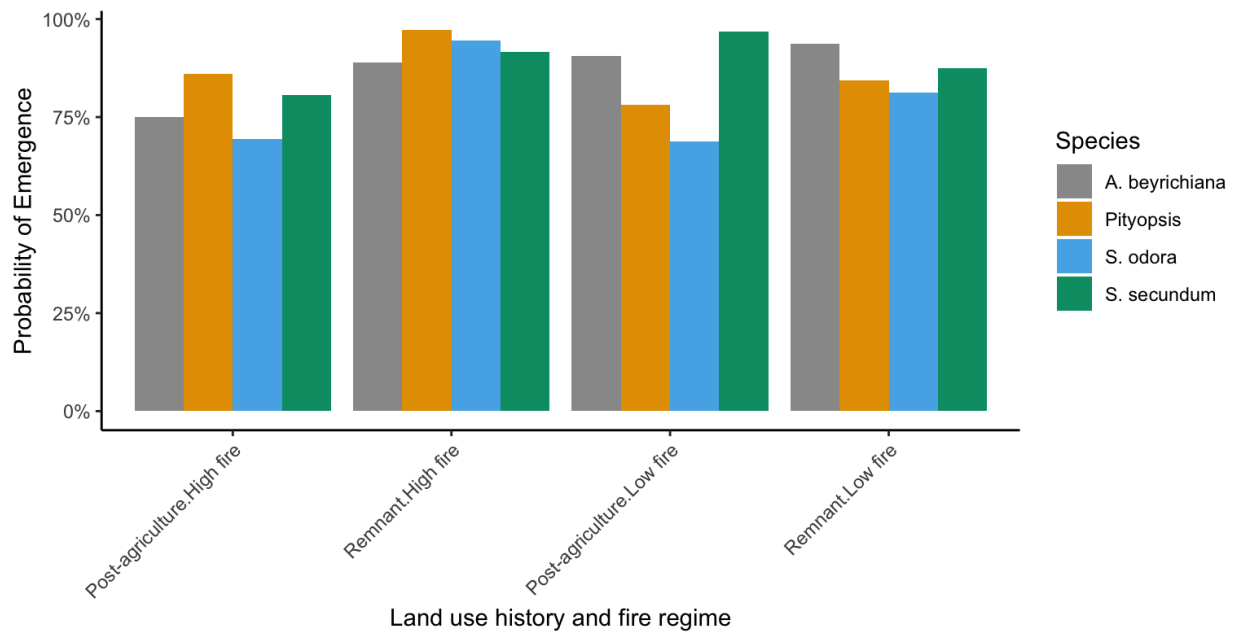
## Figures



**Figure 1.** Study design. Map of SRS consisting of 34 sites, at each site, one 3 x 3 m plot was established with four positions. One seed bag for each species was placed in a single layer in a random order within each tray (tray locations 1-4). Treatment-level combinations (litter present with fungicide, litter absent with fungicide, litter present without fungicide, litter absent with fungicide) were randomly assigned to each position location.

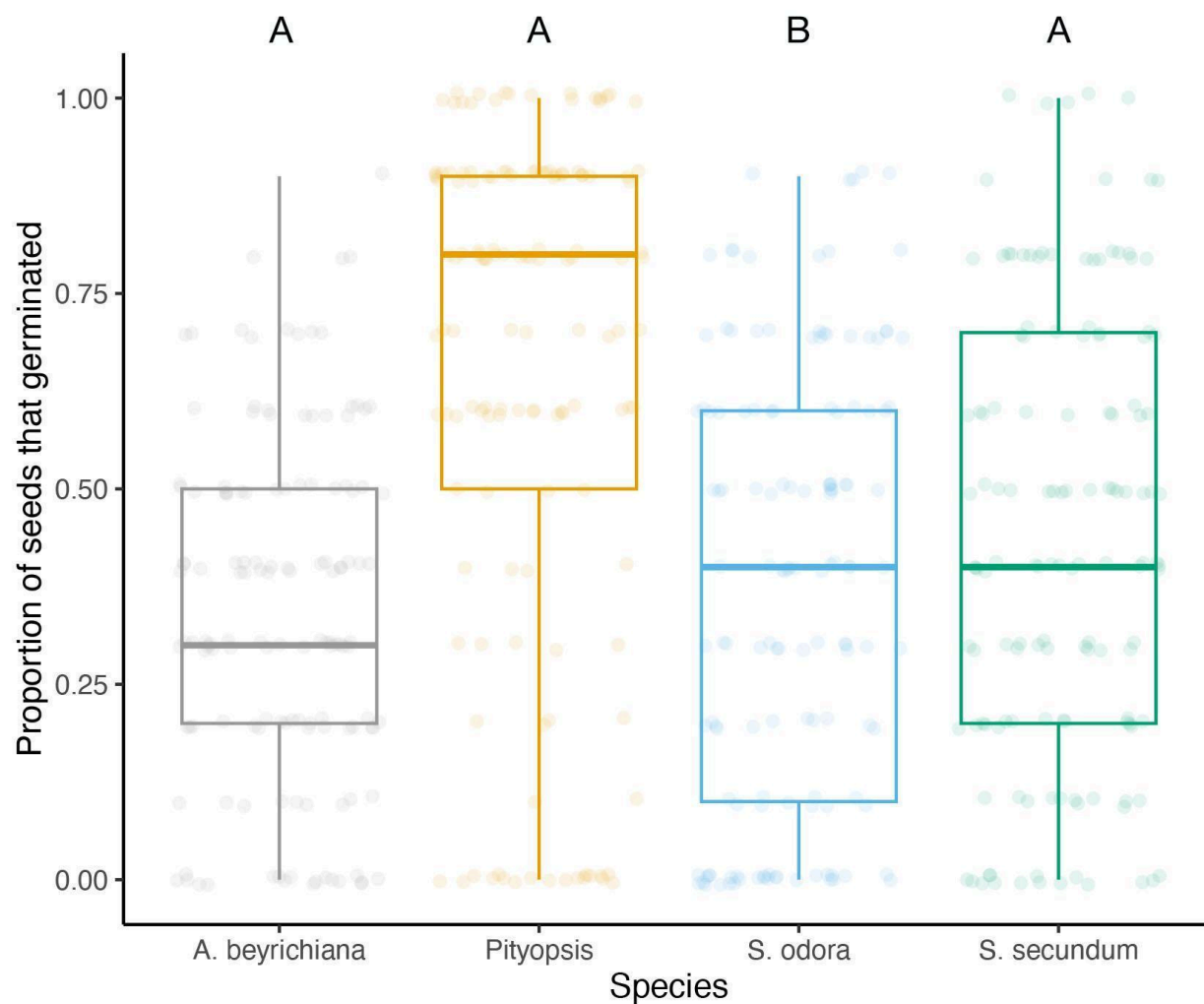


**Figure 2.** Conceptual model shown with direct and indirect pathways of how land-use history and fire regime impact seed germination.

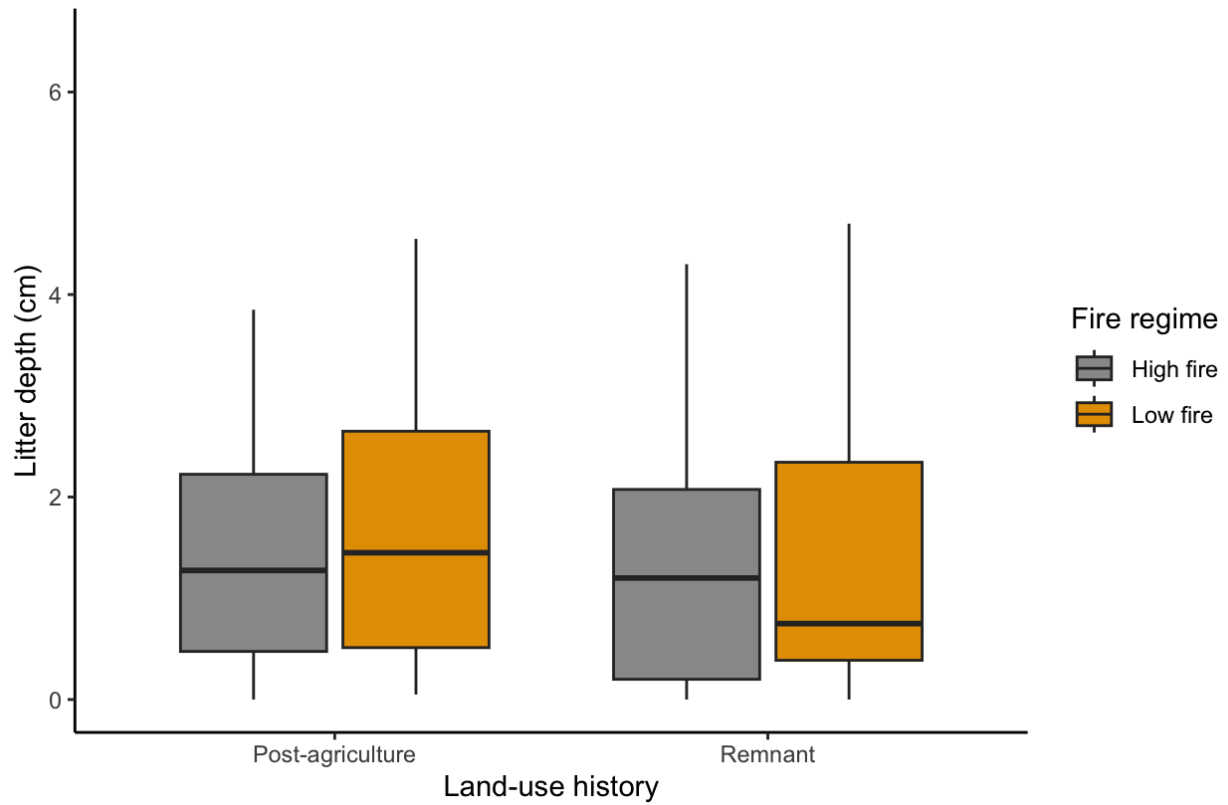


**Figure 3.** Emergence probability was not impacted by land-use history ( $p = 0.19$ ) or fire regime ( $p = 0.69$ ), but did show significant differences between species ( $p = 0.03$ ).

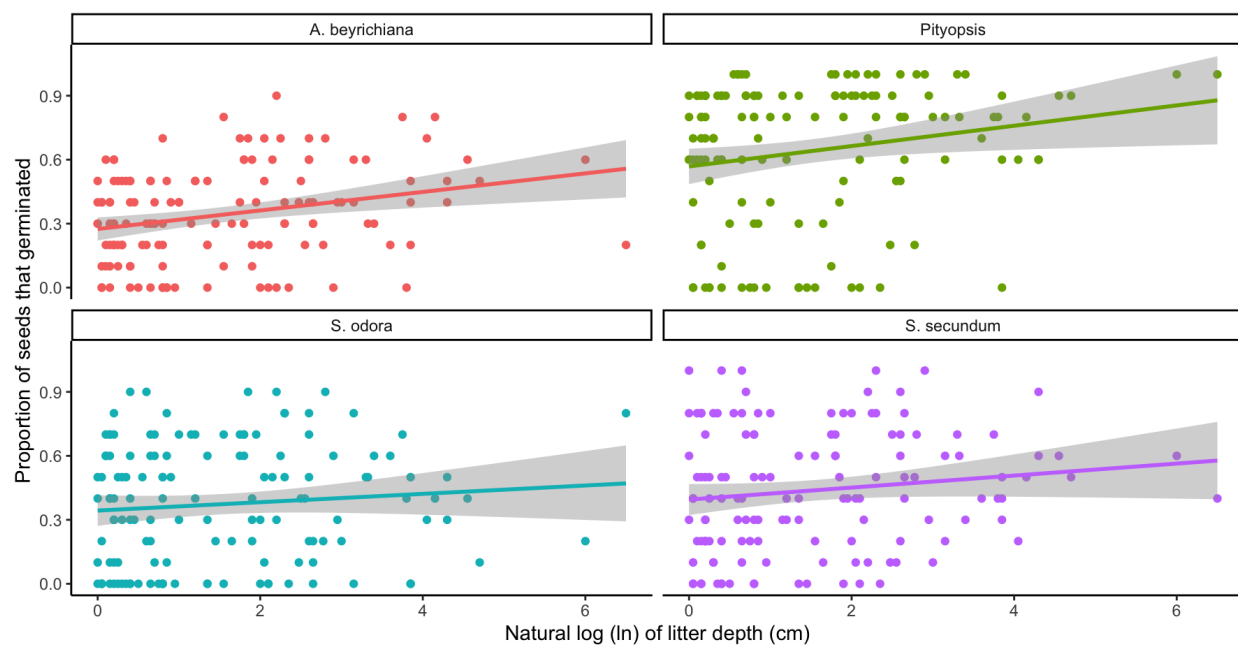




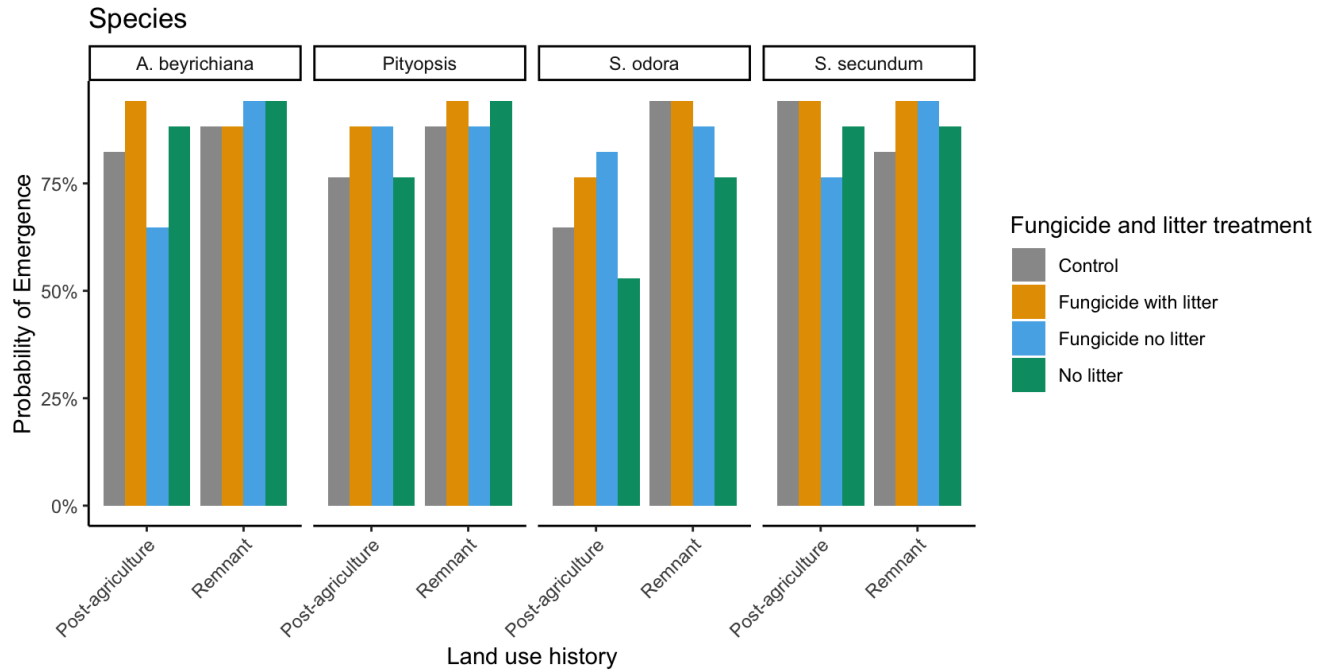
**Figure 4.** The proportion of seeds that germinated by species per seed bag. *Solidago odora* had a significantly lower proportion of seeds germinate compared to the other three species (species main effect,  $p = 0.04$ ). Letters denote significant pairwise comparisons. Error bars represent the first (lower bar) and third (upper bar) quartiles.



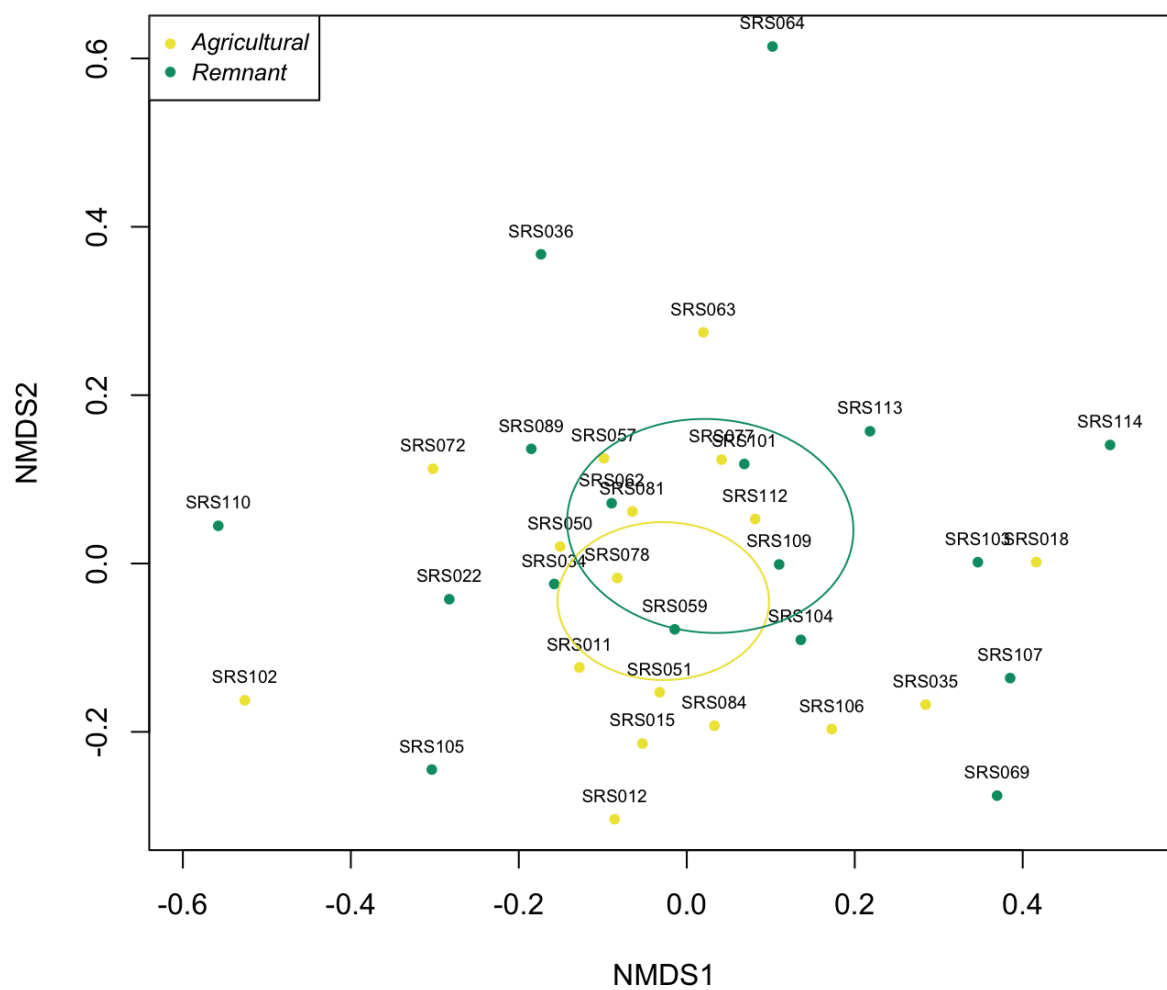
**Figure 5.** Litter depth (cm) was not significantly different between sites with varying land-use ( $p = 0.63$ ) history or fire regime ( $p = 0.26$ ). Error bars represent the first (lower bar) and third (upper bar) quartiles.



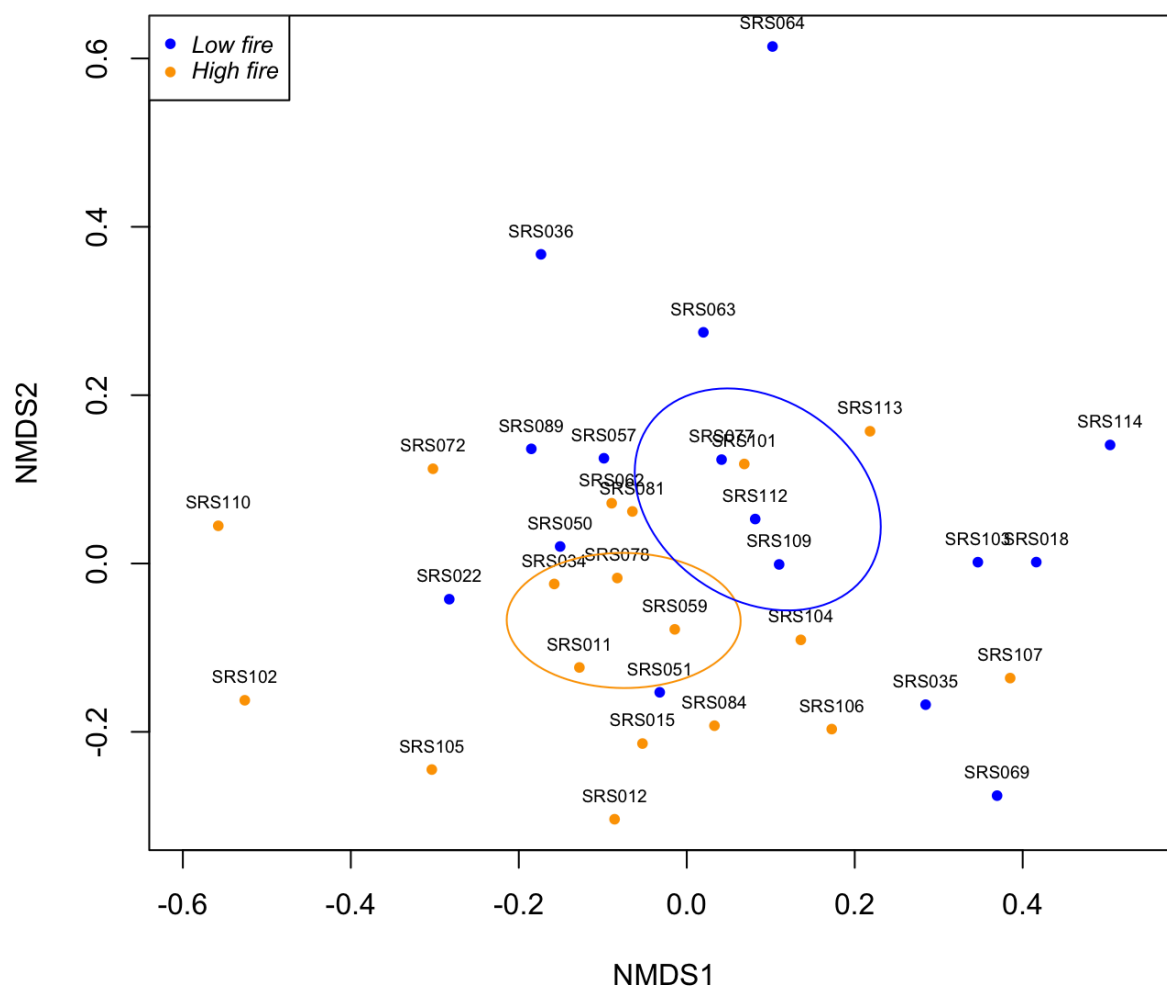
**Figure 6.** Seed germination increased slightly as litter depth (cm) increased in three species ( $p = 0.01$ ; *A. beyrichiana*, *Pityopsis*, and *S. secundum*). Grey area denotes 95% confidence interval of linear relationship between the proportion of seeds that germinated and litter depth (cm). Species identity also impacted germination ( $p = 0.04$ ) with *S. odora* experiencing a negative impact as litter depth increased and all other species displaying increases in germination.



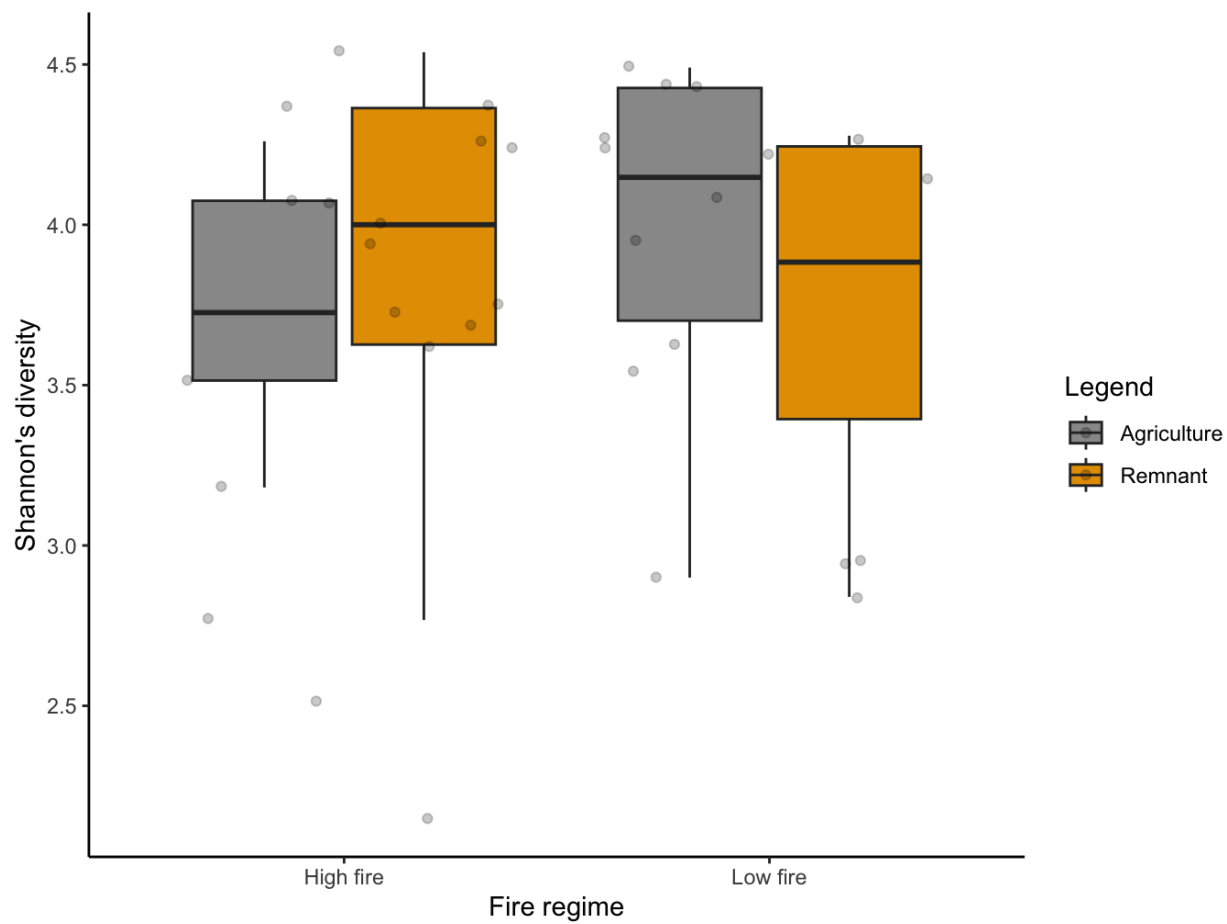
**Figure 7.** Germination was not impacted by land-use history or fungicide application.



**Figure 8.** Land use history alters fungal community composition. Non-metric multidimensional scaling (NMDS) ordination of Bray-Curtis dissimilarities on relative abundances ( $k = 3$ , stress value = 0.13). Post-agricultural sites are significantly different in fungal community composition ( $p = 0.021$ , PERMANOVA)



**Figure 9.** Fire frequency alters fungal community composition. High-fire sites (those with seven fires or more since 1991) do not have significantly different fungal communities than those with few or no fires (those with fewer than seven fires since 1991) (PERMANOVA on Bray-Curtis dissimilarities,  $p = 0.229$ ).



**Figure 10.** Shannon's Diversity index of soil fungal communities across sites by land-use history ( $p = 0.957$ ) and fire regime ( $p = 0.577$ , Table 5)

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