



Effect of seed size and fungicide on germination and survival of buried seeds of two grassland species on the Loess Plateau, China

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ABSTRACT

Seed size has important fitness consequences that potentially extend throughout the life cycle of plants. However, the effect of variation in seed size on seed survival in the field is poorly understood. We used two grassland species *Setaria viridis* (physiological dormancy) and *Lespedeza davurica* (physical dormancy) to test the effect of within species seed size variation on seed germination, viability and susceptibility to pathogen attack in the soil. Seed bags with different sizes of seeds for each species were treated with fungicide or water (control) and buried for 4 months in the field on the Loess Plateau, China. We found that field germination of large seeds was consistently higher than that of small seeds for both species. Small seeds of *S. viridis* had higher field mortality than large seeds, but the mortality of seeds of *L. davurica* was not affected by seed size. More small than large seeds of *L. davurica* remained viable during field burial, but the viability of buried seeds *S. viridis* did not differ with size. Our study suggests that within species seed size can influence germination, mortality and viability of buried seeds in the soil, but seeds with different kinds of dormancy shows different patterns in the relationship between seed size and seed survival.

1. Introduction

Soil seed banks play an important role in vegetation dynamics (Thompson et al., 1997; Baskin and Baskin 2014; Wang et al., 2020). Apart from the small fraction of seeds with successful germination and emergence, the majority of seeds in the soil eventually die due to unsuccessful germination, ageing and loss of viability, or attack by predators and pathogens (Grundy et al., 2003; Gómez-Aparicio et al., 2012; Leishman et al., 2000; Westerman et al., 2003). Soil fungal pathogens are one of the important agents causing seed mortality in a variety of habitats (Crist and Friese 1993; Li et al., 2019; Mordecai 2012; O'Hanlon-Manners and Kotanen 2004). Among the seed fungal pathogens, some are generalists, while others are closely associated with seeds of a particular species (Gallery et al., 2007; Sarmiento et al., 2017). Increasing evidence from research suggests that the outcome of pathogen-seed interactions is closely related to seed traits (Beckstead et al., 2007; Chen et al., 2018; Gardarin et al., 2010; Pringle et al., 2007). For example, Beckstead et al. (2007) found that pathogens such as the fungus *Pyrenophora semeniperda* generate selective pressure that influences the timing of germination of *Bromus tectorum* seeds, i.e. if seeds

germinate rapidly they can do so before they are destroyed by the fungus. A recent study by Chen et al. (2018) pointed out that dormancy may protect seeds of *Stipa bungeana* and *Lespedeza davurica* from infection by the pathogen *Fusarium tricinctum*, since nondormant seeds are more susceptible to fungal attack than dormant seeds.

Seed size is an important evolutionary trait that affects seed germination, seedling development, fecundity and fitness throughout the life cycle of plants (Larios et al., 2014; Moles and Westoby 2004). The size of seeds ranges from the dust-like seeds of orchids and some mycoheterotrophic and parasitic species (some of which weigh just 10^{-6} g), across ten orders of magnitude to the 20-kg seeds of the double coconut *Lodoicea seychellarum* (Harper et al., 1970; Moles et al., 2005). Even within a species, the smallest and largest seeds can differ by approximately fourfold (Michaels et al., 1988). Compared to small-seeded species, large-seeded species often have a competitive advantage in terms of seed germination and seedling survival (Moles and Westoby 2006; Susko and Cavers 2008; Winn 1988). In contrast, species with small, rounded seeds tend to have more dormant and persistent seeds than those with larger, more elongate, flattened seeds (Bekker et al., 1998; Thompson et al., 1993; Wang et al., 2011), because the former are

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more easily incorporated into the soil than the latter and are therefore less likely to be attacked by seed predators and pathogens (Foster, 1986; Westoby et al., 1992). However, some studies in Australia and New Zealand found that seed size and shape were poorly related to persistence of seeds in the soil (Leishman and Westoby 1998; Moles et al., 2000).

Several studies in tropical forests have explored the relationship between seed size and susceptibility to fungal pathogens, but the results vary among the studies. Murphy et al. (2017) found a positive relationship between seed size and the strength of the conspecific negative density-dependent effect (CNDD), suggesting that large-seed species with limited dispersal suffer higher mortality than small seeds when dispersed near conspecific adults. In contrast, Lebrija-Trejos et al. (2016) found that the CNDD was stronger in small-seeded than in large-seeded species, suggesting that small seeds were more likely to be attacked by soil pathogens than large ones. However, Augspurger and Kelly (1984) found no significant relationship between seed size and pathogen attack on newly-emerged seedlings of 18 tree species in a forest in Panama.

In light of the importance of soil seed banks for vegetation regeneration, it is important to understand variation in seed traits, such as size, that influence their survival and dynamics. In particular, how variations in seed size within a species influence seed germination, viability and susceptibility to pathogen attack in natural grasslands are not well understood. To address this gap, we conducted a 4-month field burial experiment on the Loess Plateau, northwest China using two round-seeded grassland species *Lespedeza davurica* and *Setaria viridis* with different seed sizes following fungicide treatment. Specifically, for each species we hypothesized that: (i) large seeds have higher germination percentages than small seeds; (ii) large seeds are more susceptible to fungal pathogens than small seeds; (iii) small seeds are more persistent than large seeds in the soil. By testing these hypotheses, we expected to further our understanding of the relationships between seed size and seed survival in the soil.

2. Materials and methods

2.1. Study area and species

The study site was located near the Tian Shui Grassland Research Station, Huan County, Gansu Province, northwestern China (37.12°N, 106.82°E, 1650 m in elevation). This area has a typical semi-arid monsoon climate. Mean annual temperature is 7.1 °C, and mean annual rainfall is approximately 360 mm, with more than 80% of it occurring from June to September. Dominant plant species in the area are *Artemisia capillaris* Thunberg (Asteraceae), *Lespedeza davurica* (Laxm.) Schindl (Fabaceae) and *Stipa bungeana* Trin. (Poaceae) (Chen et al., 2017).

Two plant species with rounded seeds in this area were selected for use in the experiment: *L. davurica* and *Setaria viridis* (L.) Beauv (Poaceae). These two species were selected because they naturally co-exist and are widely distributed on the Loess Plateau, but they have contrasting seed characteristics. Seeds of *L. davurica* have strong physical dormancy, and those of *S. viridis* have physiological dormancy (Chen 2012). *Lespedeza davurica* flowers in August, and seeds become mature and start to disperse in late September. *Setaria viridis* flowers in the middle of August, and seeds ripen and are dispersed in October. Dormancy of seeds of *L. davurica* can be broken by physically damaging the seed coat. Newly harvested seeds of *S. viridis* have physiological dormancy, and dry storage at room temperature for a few months can accelerate the loss of dormancy (Chen 2012).

2.2. Seed collection and viability test

In October 2004, seeds of *L. davurica* and *S. viridis* were collected from plants from the same population growing on roadsides 1–2 km

from the Tian Shui Grassland Research Station. Mature inflorescences were harvested by hand from many plants and brought to the laboratory, where they were air-dried for one week (RH, 20–35%; 18–25 °C) and then cleaned. Seeds of the two species were categorized into three sizes: small, medium and large using a series of sieves (Table 1).

Each seed lot was stored in water-impermeable packaging at 4 °C until used in May 2005.

Prior to the burial experiment, viability of seeds of each species with different sizes was tested using tetrazolium tests (Peters 2000). Seeds were cut in half and the part containing the embryo was soaked in 1% tetrazolium solution for 1 h. Seeds with embryos that stained red were considered to be viable and those with nonstained embryos nonviable. For each seed sample, four replicates of 50 seeds were tested (Table 1).

2.3. Field burial and retrievals

Seed bags were made from nylon stockings, and each contained 50 seeds mixed with 150 ml of soil; for each species there were 240 bags. The soil was previously collected from the study site and subsequently air-dried and sieved. Prior to field burial, seed bags containing a mixture of 50 seeds and 150 ml of soil were subjected to one of two treatments: saturated in fungicide solution or in water (control) (Blaney and Kotanen, 2001). The fungicide used was commercially formulated Captan (N-trichloromethylthio-4-cyclohexene-1,2-dicarboximide; Tianlong Biotechnology, Hangzhou, China; ai. 80%), which is a non-systemic heterocyclic nitrogen fungicide and is active against a wide range of fungi in the Oomycota, Ascomycota and Basidiomycotina (Neergaard 1977). Captan was diluted in water to a concentration of 1:100, following the manufacturer's recommendations.

The field burial trial was performed in 20 plots (3 m × 2 m) near the Grassland Station. Within each plot, a 4 × 6 grid was created with points separated by 0.5 m. In the middle of May 2005, one seed bag was buried 5 cm below the soil surface at each grid point, so that each plot contained all treatment × species combinations. All plots were arranged in a randomized complete block design. Thus, the design of the field burial experiment was 2 plant species × 3 seed sizes × 2 treatments (control and fungicide) × 2 periods of burial × 20 replicates, for a total of 480 seed bags.

Half the seed bags were retrieved on July 16, 2005 (2 month trial), and the other half was recovered on September 18, 2005 (4 month trial). Each seed bag was opened, and the soil in the bag was carefully loosened and passed through a 0.5-mm sterilized sieve (the small size of tested seeds cannot pass through this sieve). Seeds retained within the 0.5-mm mesh were picked out using sterile tweezers and examined under a stereo dissecting microscope. Seeds were classified as germinated (alive-seed with living radicle or dead-seed with radicle emerged but shriveled;

Table 1
Seed size category and seed size and mass, proportion and initial viability of the two study species (n = 4).

Plant species	Seed Category	Seed size (mm)	Seed mass (mg)	Proportion (%)	Seed viability (%)
<i>L. davurica</i>	Large	>2	4.31 ± 0.07 a	5.9	69 ± 1.5
	Medium	1.5–2	2.84 ± 0.08 b	84.5	71 ± 1.9
	Small	<1.5	2.07 ± 0.06 c	9.6	69 ± 2.1
<i>S. viridis</i>	Large	>0.8	0.97 ± 0.08 a	6.9	97 ± 1.8
	Medium	0.6–0.8	0.77 ± 0.07 b	85.6	100 ± 0.5
	Small	<0.6	0.43 ± 0.08 c	7.5	98 ± 1.0

Different lowercase letters indicate significant differences between seed sizes (*p* < 0.05).

the radicle >1 mm), intact (hard to the touch), dead (soft to the touch) or missing (no evidence of seed) (Schafer and Kotanen 2004). The number of seeds in each seed bag falling into each of the four categories was recorded. Intact seeds were washed free of soil, surface-sterilized in 75% ethanol for 1 min and in 0.1% sodium hypochlorite for 5 min, followed by three rinses in sterilized water, and then placed in Petri dishes containing two sheets of filter paper moistened with distilled water. Seeds were incubated in light at 20 °C for 14 days, after which the germination percentage was determined. At the end of the germination trial, non-germinated seeds were tested for viability with tetrazolium tests, as described above.

2.4. Calculations and statistical analysis

The proportion of germinated/dead seeds for each seed bag was calculated as the number of seeds found germinated/dead in the field burial divided by the total number of buried seeds. The proportion of viable seeds for each seed bag was calculated as the total number of viable seeds, which was the sum of germinated seeds in the lab test and those that tested positive with tetrazolium divided by the number of intact seeds after retrieval. Considering that some dead seeds may have decayed with increasing burial time and be hard to find, we classified them as missing. Therefore, the proportion of dead seeds may decline with the increase of burial time.

Each species was analyzed separately, as it was expected to vary in its intrinsic germination biology. The proportion of seed germination, mortality and viability was analyzed using generalized linear mixed models (GLMMs) with seed size, fungicide treatment, and length of burial period as fixed effects, and replication as a random effect. Prior to data analyses, all data were checked for normality and homogeneity. Proportional data were arcsine square-root transformed to meet the assumptions for parametric testing and converted to percentage values for data presentation. The differences of individual comparisons were tested using Kruskal-Wallis test at $p < 0.05$. All data analyses were performed in R program (R Core Team, 2016).

3. Results

3.1. Seed germination

Germination of *S. viridis* and *L. davurica* seeds buried in the field was not affected by application of fungicide (Table 2, Fig. 1a and 1d). In contrast, the field germination of both species was significantly affected by seed size and the length of burial period (Table 2). Germination of large seeds of *S. viridis* and *L. davurica* was 28.3% and 15.1%, respectively, and it was significantly higher than that of small seeds, which was 20.4% and 10.4%, respectively (Fig. 1b and 1e). The percentage of field germination of both plant species was enhanced significantly by the length of burial period (Fig. 1c and 1f).

Table 2

Results from generalized linear mixed models for the effects of “fungicide treatment” (FT), “seed size” (SS), and “length of burial” (LB) and their interactions on germination (SG), mortality (SM), and viability (SV) of seeds of *Lespedeza davurica* and *Setaria viridis* during the burial experiment.

Factors	df	<i>Lespedeza davurica</i>			<i>Setaria viridis</i>		
		SG	SM	SV	SG	SM	SV
LB	1	214.41 ***	2.28	133.48***	299.28 ***	384.59***	235.55***
SS	2	33.52 ***	2.56	13.08***	33.71 ***	73.64***	0.60
FT	1	0.08	0.19	<0.01	3.62	33.32***	271.46***
LB × SS	2	0.69	0.31	0.62	10.69 ***	48.44***	10.88***
LB × FT	1	0.13	0.54	0.09	0.71	5.19*	2.05
SS × FT	2	6.54**	0.19	3.60*	1.96	4.89**	0.21
LB × SS × FT	2	1.58	0.68	0.76	0.34	0.30	1.49

Data are represented as F-values and the asterisk indicates significant p -values (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

3.2. Seed mortality

Mortality of *S. viridis* seeds during burial was significantly affected by fungicide, seed size and the length of burial period (Table 2). Seed mortality of *S. viridis* was significantly decreased by fungicide application (Fig. 2a). Mortality of large and small seeds of *S. viridis* was 4.2% and 10.7%, respectively, and differed significantly (Fig. 2b). The mortality of *S. viridis* seeds declined significantly with increased time of burial (Fig. 2c). In contrast, seed mortality of *L. davurica* was not affected by application of fungicide, seed size and length of burial period (Table 2). Indeed, mortality of seeds of *L. davurica* was very low (<1%) (Fig. 2d, e and 2f).

3.3. Seed viability

Application of fungicide significantly increased viability of *S. viridis* seeds, but it had no significant effect on seeds of *L. davurica* (Table 2, Fig. 3a and d). Seed viability of *S. viridis* did not differ significantly with seed size (Table 2, Fig. 3b). In contrast, the viability of small seeds of *L. davurica* was significantly higher than that of medium and large seeds (Table 2, Fig. 3e). The viability of both species declined significantly with increased burial time (Table 2, Fig. 3c and e).

4. Discussion

In this study, we performed a field-based trial to test the effects of within species seed size variation on germination, viability and the susceptibility to pathogen attack in the soil. In support of our first hypothesis that large seeds have higher germination percentages than small seeds, we found that the percentages of germination of large seeds of *S. viridis* and *L. davurica* in the field were significantly higher than those of small seeds. Our results were similar to those from two previous studies performed using the desert winter annual *Dithyrea californica* (Larios et al., 2014) and the forest tree *Pinus thunbergii* (Mao et al., 2019). Muller-Landau (2010) proposed that large seeds often have more nutrients than small ones, which, therefore, can improve seed germination and seedling tolerance under unfavorable conditions. Seedling establishment and growth in the arid and semiarid grasslands of northwest China are severely limited by water availability (Zeng et al., 2010), so the higher germination of large than small seeds of the two species may give their seedlings an advantage to better tolerate drought stress by providing more resources.

Contrary to our second hypothesis that large seeds are more susceptible to fungal pathogens than small seeds, we found that small seeds of *S. viridis* had higher field mortality than large seeds. In agreement with our results, Lebrija-Trejos et al. (2016) reported that large seeds were more tolerant of pathogen attack than small ones. This is probably because large seeds have both greater energy reserves and harder and/or thicker coats than small seeds (Pearson et al., 2002; Pringle et al., 2007; Osunkoya 1994), thus they could better defend against attack by fungal pathogens than small seeds. However, Pringle et al. (2007) found that pathogen susceptibility tended to positively correlate with seed size in

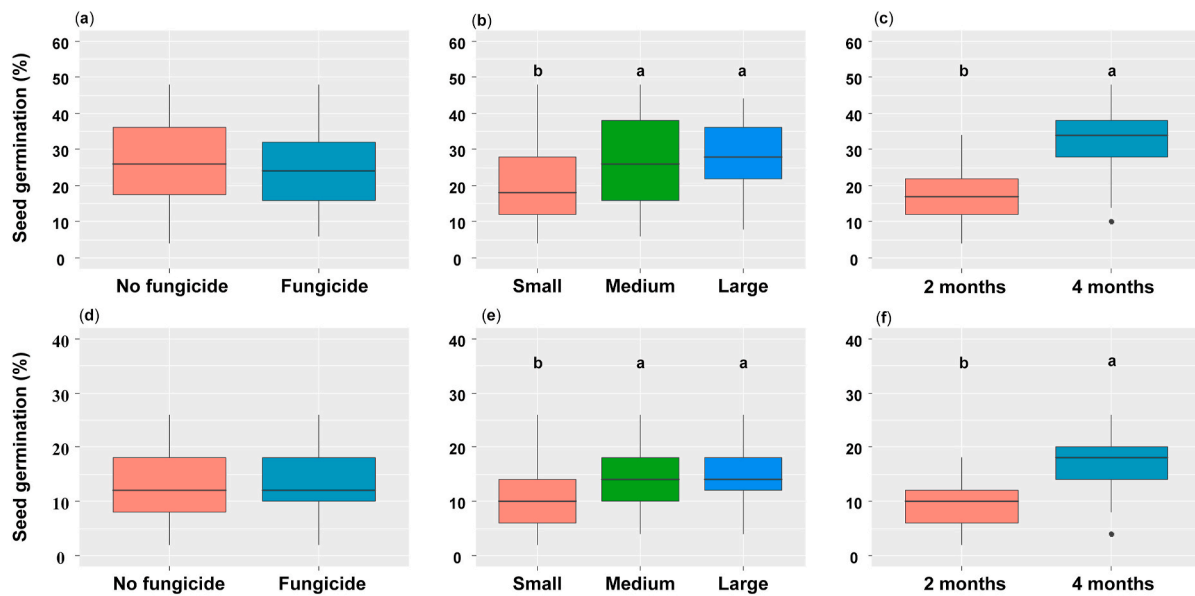


Fig. 1. Seed germination of *Setaria viridis* (a, b, c) and *Lespedeza davurica* (d, e, f) according to seed size, treatment with fungicide and length of burial for 2 and 4 months in the field. The box in each boxplot shows the lower quartile, median and upper quartile values, and the whiskers show the range of the variation; points indicate extreme values. In each panel, different lowercase letters indicate significant differences between treatments at $p < 0.05$ (Kruskal-Wallis test).

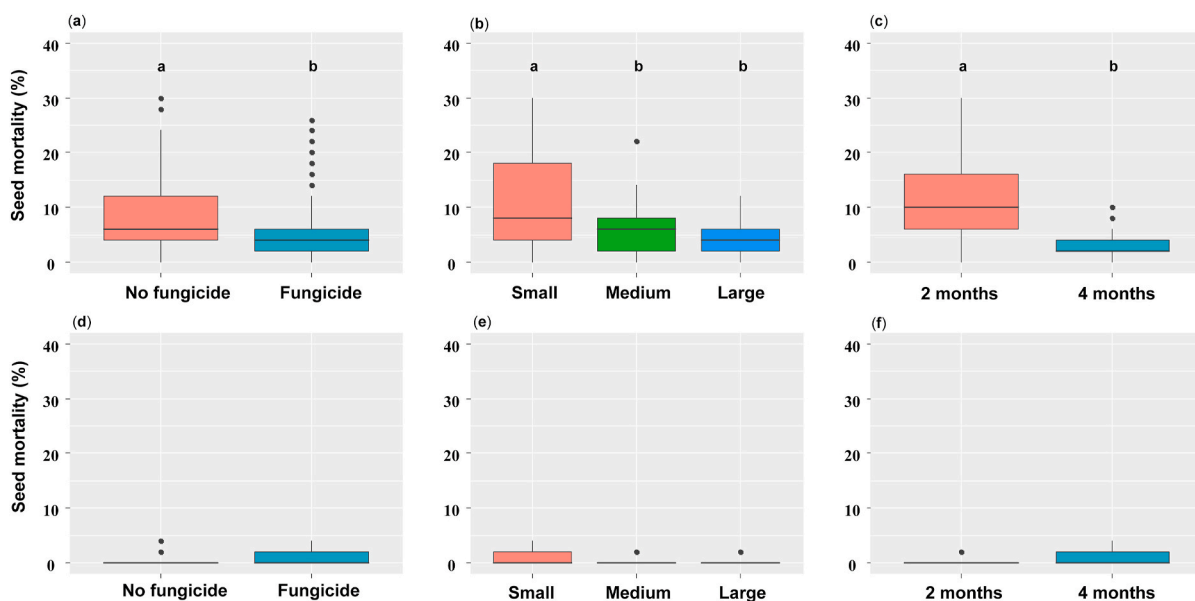


Fig. 2. Seed mortality of *Setaria viridis* (a, b, c) and *Lespedeza davurica* (d, e, f) according to seed size, treatment with fungicide and length of burial for 2 and 4 months in the field. The box in each boxplot shows the lower quartile, median and upper quartile values, and the whiskers show the range of the variation; points indicate extreme values. In each panel, different lowercase letters indicate significant differences between treatments at $p < 0.05$ (Kruskal-Wallis test).

an Amazon forest, which contrasts with our result. Also, Murphy et al. (2017) showed that large-seed species with limited dispersal suffer higher mortality than small seeds when dispersed near conspecific adults. Considering that predation is another important factor causing seeds to die (Moles and Westoby 2006; Westerman et al., 2003), additional experiments that include a larger range of seed sizes and comparison of attack by pathogens and predators are required to better understand the complexity of relationship between seed size and mortality.

However, we were unable to find that field mortality of seeds of *L. davurica* differed with seed size. Also, in our study fungicide application only significantly improved seed survival of *S. viridis*, whereas the mortality of *L. davurica* seeds in the field was very low (less than 1%) and

was not affected by the application of fungicide. In accordance with our results, Schafer and Kotanen (2003) found that fungicide application reduced caryopse mortality of *Bromus inermis* by 43% but did not affect that of *Danthonia spicata*. One possible reason for the variation of fungicide effects on seed survival may be due to a dilution effect imposed on large seeds because we employed identically seed treatments across seed sizes. Nevertheless, another more important reason could be related to seed dormancy, as dormant seeds with a water-impermeable seed coat are proposed to be more resistant to pathogen attack than nondormant (water-permeable) seeds (Dalling et al., 2011; Fuerst et al., 2011). In our study, seeds of *L. davurica* had strong physical dormancy (i.e. the seed coat is water-impermeable) and field germination was only 13%; therefore, the impermeable seed coat of

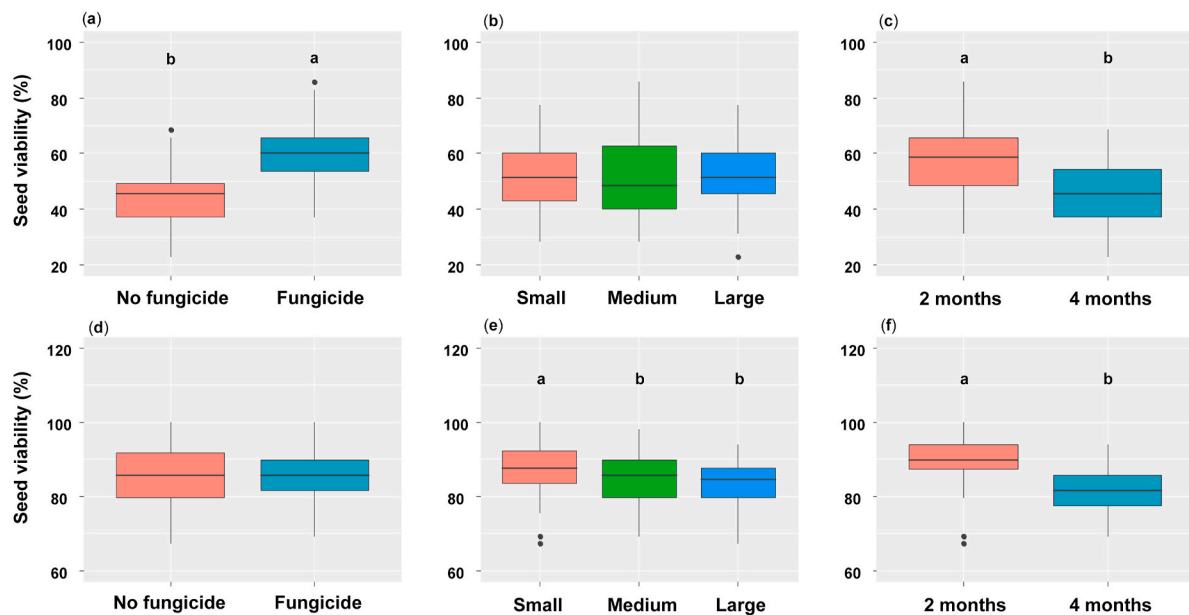


Fig. 3. Seed viability of *Setaria viridis* (a, b, c) and *Lespedeza davurica* (d, e, f) according to seed size, treatment with fungicide and length of burial for 2 and 4 months in the field. The box in each boxplot shows the lower quartile, median and upper quartile values, and the whiskers show the range of the variation; points indicate extreme values. In each panel, different lowercase letters indicate significant differences between treatments at $p < 0.05$ (Kruskal-Wallis test).

L. davurica may provide an effective barrier against pathogen attack. This was further supported by a recent study by Chen et al. (2018) who found that the pathogenic fungus *Fusarium tricinctum* would be unlikely to cause water-impermeable seeds of *L. davurica* to die. Considering the effectiveness of fungicide, our field burial experiment only lasted for 4 months, which may underestimate fungicide effects on seeds of *L. davurica*. Future study involving more species with longer burial time are needed to further investigate the relationship between seed characteristics and susceptibility to pathogen attack.

Seeds of small-seeded species are expected to be more persistent than those of large-seeded species in the soil seed bank, since they are more easily incorporated into the soil and are better protected from seed predators (Bekker et al., 1998; Thompson et al., 1993). We found that the viability of small seeds of *L. davurica* was higher than that of large seeds after burial, whereas the viability of *S. viridis* seeds did not vary with seed size, partly supporting our third hypothesis that small seeds are more persistent than large ones in the soil. Previous studies in Australia and New Zealand have shown that small seeds were not consistently more dormant and persistent than large seeds among a wide range of species (Leishman and Westoby, 1998; Moles et al., 2000). Besides, Garner and Witkowski (1997) showed that seeds of three South African woody species were relatively large but persisted in the soil. In our study, seeds of both *L. davurica* and *S. viridis* are likely to form a persistent seed bank since they can retain their viability in the soil for more than one year (Chen 2012). However, seeds of *L. davurica* with physical dormancy are more persistent than the physiologically dormant seeds of *S. viridis* (Chen 2012), although seeds of *L. davurica* are four times larger than seeds of *S. viridis*. Our study suggests that seed persistence in the soil was not only affected by the size of seed, but it also may depend on the kind of dormancy.

In conclusion, we found that field germination of large seeds was consistently higher than that of small seeds for the two study species. In contrast, mortality that may have been caused by pathogen attack during burial was higher for small than large seeds of *S. viridis*, whereas higher viability of small than large seeds was only found for *L. davurica*. Our results suggested that even within a species large seeds have higher field germination percentages than small seeds, which is consistent with results from previous studies that large-seeded species often exhibit a competitive advantage in terms of seed germination and seedling

survival (Moles and Westoby 2006; Susko and Cavers 2008; Winn 1988). However, the within species effects of size on seed viability and susceptibility to pathogen attack is species-specific and may also rely on other seed traits, such as kind of dormancy. Further experiments that involve a wide range of species with distinct seed traits and comparison of generalist vs. specialist pathogens attack on seeds are required to fully understand the relationship between seed characteristics and seed survival in the soil.

CRediT authorship contribution statement

Jinwei Hou: Formal analysis, Data curation, Writing – original draft, Writing – review & editing. **Zhibiao Nan:** Writing – review & editing. **Carol Baskin:** Writing – review & editing. **Tao Chen:** Formal analysis, Data curation, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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