FUNCTIONAL SEED ECOLOGY: FROM SINGLE TRAITS TO PLANT DISTRIBUTION PATTERNS, COMMUNITY ASSEMBLY AND ECOSYSTEM PROCESSES

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Effect of Nitrogen Addition on Selection of Germination Trait in an Alpine Meadow on the Tibet Plateau

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Seed germination requirements may determine the kinds of habitat in which plants can survive. We tested the hypothesis that nitrogen (N) addition can change seed germination trait-environmental filter interactions and ultimately redistribute seed germination traits in alpine meadows. We determined the role of N addition on germination trait selection in an alpine meadow after N addition by combining a 3-year N addition experiment in an alpine meadow and laboratory germination experiments. At the species level, germination percentage, germination rate (speed) and breadth of temperature niche for germination (BTN) were positively related to survival of a species in the fertilized community. In addition, community-weighted means of germination percentage, germination rate, germination response to alternating temperature and BTN increased. However, germination response to wet-cold storage (cold stratification) and functional richness of germination traits was lower in alpine meadows with high-nitrogen addition than in those with no, low and medium N addition. Thus, N addition had a significant influence on environmental filter-germination trait interactions and generated a different set of germination traits in the alpine meadow. Further, the effect of N addition on germination trait selection by environmental filters was amount-dependent. Low and medium levels of N addition had less effect on redistribution of germination traits than the high level.

Keywords: alpine meadow, germination traits, nitrogen addition, species diversity, Tibet Plateau

INTRODUCTION

Trait-based community ecology is a new approach to understanding community assembly (Díaz et al., 2004; McGill et al., 2006). Functional traits have been considered as common currency to predict the performance of species, species composition of communities and how these things shift over time and space (Larson and Funk, 2016). A trait–filter community assembly theory has been developed and applied to help understand how environmental factors and plant traits act jointly to determine community structure (Cornwell and Ackerly, 2009; Larson and Funk, 2016). Thus, analyses of functional traits have become fundamental tools for predicting the responses of a plant community to environmental change. Although the number of studies on plant functional traits and community assembly is increasing, most of them have focused on easily measured traits (e.g., plant height, specific leaf area, and seed mass) and only a few on seed germination traits (Jiménez-Alfaro et al., 2016; Liu et al., 2018, 2020; Larson et al., 2020).

Seed germination is the earliest expressed life history trait (Donohue et al., 2010), and it can directly or indirectly affect the expression of post-germination traits that are closely related to plant fitness (Simons and Johnston, 2000; Donohue, 2002). Therefore, seed germination can potentially influence the composition of plant communities (Wu and Du, 2007; Saatkamp et al., 2019). Many factors such as light, temperature and soil moisture can affect seed dormancy-breaking and germination (Fenner and Thompson, 2005; Bewley et al., 2013; Baskin and Baskin, 2014). Generally, these factors act together to determine the timing of seed germination and thus play an important role in seedling survival (Westoby, 1981; Densmore and Zasada, 1983; Baskin and Baskin, 2014). The breadth of the temperature niche for germination and how it changes are important determinants of the seed germination season of plants in regions with seasonal temperature changes (Baskin and Baskin, 2014). For example, a wet-cold storage requirement for dormancy-break can prevent seeds from germinating soon after dispersal in autumn, thus avoiding seedling death from freezing in winter (Liu et al., 2011). Further, a fluctuating temperature requirement for germination restricts germination to vegetation gaps, thus avoiding seedling death due to shading by neighboring plants (Thompson et al., 1977; Liu et al., 2013). To some extent, environmental requirements for seed germination of a plant species are important determinants for its existence in a given community and environment (Fenner and Thompson, 2005; Larson and Funk, 2016).

Soil nitrogen (N) is an important factor that can affect plant fitness and interspecific interactions (Hautier et al., 2009; Milbau et al., 2017; Hämmerle et al., 2018). For example, N addition can increase the intensity of competition for light and result in loss of some species in grassland communities (Hautier et al., 2009). Thus, N is an important environmental filter in determining community structure and ecosystem function. Due to human activities, the concentration of atmospheric N and N deposition are increasing (Gruber and Galloway, 2008; Ackerman et al., 2019). The Tibet Plateau is experiencing serious N deposition. For example, data collected from 1990 to 2003 show a mean of 7.55 kg ha^{-1} $year^{-1}$, a minimum of 1.08 kg ha^{-1} $year^{-1}$ and a maximum level of 17.81 kg ha^{-1} $year^{-1}$ of N deposition in Qinghai (on the Tibet Plateau) (Lü and Tian, 2007). It previously has been shown that this level of N deposition in Qinghai can change the species composition of alpine meadows on the Tibet Plateau (Zong et al., 2016).

With increased atmospheric N deposition and increased fertilizer use in agriculture becoming global problems, the mechanism by which of N influences plant community structure has become an important area of research in community ecology (Niu et al., 2008; Hautier et al., 2009; Zhou et al., 2017). In particular, many studies have been done in an attempt to reveal how N addition changes natural plant community composition (Tilman, 1987; Dirkse and Martakis, 1992; Diekmann and Falkengren-Grerup, 2002). Some of them have focused on effects of N addition on interspecific competition for light and soil nutrients (Newman, 1973; Wilson and Tilman, 1993; Hautier et al., 2009) and others on how N addition influences plant functional traits and biomass allocation (Niu et al., 2008, 2009; Li et al., 2013). However, the link between germination traits and change in plant community composition due to N addition has not been explored.

Previous studies have shown that N addition has a significant effect on environmental conditions in the habitat and thus on community structure/composition (Tilman, 1987; Hobbs and Huenneke, 1992; Hautier et al., 2009). Nitrogen addition can increase nitrate ($NO_3{}^-$) and/or $NH_4{}^+$ in soil (Onipchenko et al., 2012), resulting in increased plant height and density (Tilman, 1987; Diekmann and Falkengren-Grerup, 2002). Increased plant cover can decrease daily maximum and minimum soil temperatures, maximum near-ground air temperature and magnitude of soil temperature fluctuations; however, it can increase the minimum near-ground air temperature and soil moisture (Zhao et al., 2011; Song et al., 2013). Furthermore, increased vegetation height and density can decrease solar irradiance and the red/far red ratio of light at the soil surface (Reed, 1977). All of these abiotic environmental factors can have a significant effect on seed germination (Steinbauer and Grigsby, 1957; Auchmoody, 1979; Freijsen et al., 1980; Sendon et al., 1986; Pons, 1989; Liu et al., 2013) and seeding establishment (Grime, 1973; Grime et al., 1981; Fenner and Thompson, 2005). Thus, we hypothesized that N addition can change the seed germination trait- environmental filter interactions and ultimately redistribute germination traits in alpine meadows, thereby causing changes in community composition.

To test our hypothesis, we tested for shifts in seed germination traits along a N addition gradient in Tibetan alpine meadows on the Tibet Plateau. At the species level after N addition, we expected (1) a broad temperature niche for germination, and (2) a positive germination response to temperature fluctuation. At the community level after N addition, we expected germination traits that would restrict germination to time and places (gaps) free from competition for light, would be more common in grasslands but that functional diversity of seed germination would decrease.

MATERIALS AND METHODS

Study Site

The field experiment was conducted in an alpine meadow at the Research Station of Alpine Meadow and Wetland Ecosystems of Lanzhou University in Maqu (N33°40′, E101°52′, altitude 3,550 m), Gansu Province, China, on the eastern Qinghai-Tibet Plateau. Average annual precipitation (1983-2013) is 620 mm, and it occurs mainly during the short, cool summer (Zhang et al., 2014). Mean annual temperature 2°C, and mean monthly temperature varies from −10.7°C in January to 11.7°C in July. The annual cloud-free solar radiation is ∼2580 h (Luo et al., 2006). There is an average of 270 frost days per year. The alpine meadow is mainly dominated by *Anemone rivularis* (Ranunculaceae), *Carex kansuensis* (Cyperaceae), *Elymus nutans* (Poaceae), *Festuca ovina* (Poaceae), *Kobresia graminifolia* (Cyperaceae) and *Poa poophagorum* (Poaceae). All of these six species, except *P. poophagorum,* were included in our study.

Nitrogen Addition Experiment and Vegetation Sampling

Five 10 m × 6 m blocks with the same topography and slope aspect separated by 2–3 m were established in a N-limited alpine meadow (Shen et al., 2002; Luo et al., 2006) in spring 2015. In each block, we established five 2 m × 4 m plots separated by 2 m and randomly allocated a control (N_0, 0 g N m^{-2} $year^{-1}$), low (N_1, 5 g N m^{-2} $year^{-1}$), intermediate (N_2, 10 g N m^{-2} $year^{-1}$) and high (N_3, 20 g N m^{-2} $year^{-1}$) plot. Thus, each of the four fertilization treatments included five replicates. The plots were fertilized on a day with light rainfall in early May 2015, 2016, and 2017 with granular slow-release ammonium nitrate (NH_4NO_3) applied by hand. Vegetation sampling was done at the peak of the growing season (July 2017). One 50 cm × 50 cm quadrat was sampled in each plot, and the presence and abundance (number of individuals of each species) of all species within this quadrat were recorded.

Seed Collecting and Germination Experiments

Mature seeds of the 63 species found in the vegetation sample (Appendix S1) were collected at their natural dispersal time during July to October 2017. In general, seeds of each species were collected from more than 20 plants and from more than five non-fertilized grasslands located near the experimental site. If there were less than 20 plants of a species, we collected seeds from all plants of the species found. The collected seeds were used to test the three aspects of germination traits most related to plant survival in the field: (a) range of temperatures over which seeds would germinate, which can determine time during the growing season on the Tibet Plateau when seeds can germinate; (b) temperature fluctuation requirement for germination, which can restrict seed germination to vegetation gaps; and (c) cold stratification requirement for seed germination, which can determine whether seeds germinate before or after winter. There were two germination experiments. One tested the first two aspects of germination traits (experiment 1), and the other tested the third aspect of seed germination traits (experiment 2). Previous studies on the emergence of seedlings in the field have shown that most species in grasslands on the eastern Tibet Plateau have a long mean emergence time (MET, the period of time from seed dispersal to seedling emergence), indicating that seeds of most species in these communities need a period of after-ripening for germination (Cao et al., 2018). Therefore, seeds treated by after-ripening instead of fresh seeds were used in germination experiment 1. Thus, the seeds were air dried and cleaned, and stored under warm-dry conditions at room temperatures (ca. 15°C to 20°C), until used in experiments.

Experiment 1

Germination of seeds stored under dry-warm conditions was tested in incubators (Conviron E15 Growth Chamber, controlled Environments Ltd., Winnipeg, MB, Canada) at five constant temperatures (5, 10, 15, 20 and 25°C) and at an alternating temperature regime (5/25°C, 12/12h) in darkness. At the study site in the germination season (late March to late May in spring or late August to late October in autumn), the daily temperature range is about 5-25°C near the soil surface. Thus, the above constant temperatures and alternating temperature regime were selected in our study. This germination experiment was started in mid-March (when germination begins in the field) 2018, at which time seeds were 150 to 240 days old, depending on when they were collected, and any dormancy in fresh seeds would have bene broken via afterripening. For each species, there were three replicates of 50 seeds, and they were placed in Petri dishes (9-cm-diameter) on two sheets of filter paper moistened with distilled water. Germination was checked daily, at which time seeds were exposed to light for few minutes; thus, any light requirement for germination most likely was fulfilled during these exposures (Baskin and Baskin, 2014). Germinated seeds (radicle visible) were removed from the Petri dishes at each counting, and water was added to the filter paper as needed. The germination tests lasted for 60 days.

Experiment 2

To evaluate the effect of storage conditions on germination, we compared germination of seeds stored in dry-warm (at 15-20°C for 150-240 days, depending on when they were collected) and those receiving dry-warm + wet-cold (i.e., dry at room temperature for about 110-200 days depending on when they were collected and then on a moist substrate at 3°C for 40 days). For species requiring cold stratification to germinate, their main germinate season is spring (April to late May) (Cao et al., 2018), during which time the temperature ranges from 20°C in the day to 5°C at night (Liu et al., 2011). Thus, following the two storage treatments, seeds were incubated at 20°C (12 h)/5°C (12 h) in darkness for 60 days. The procedures for germination testing/monitoring were the same as those described above.

Data Analysis

For the fertilization experiment, we calculated the response of species abundance to fertilization (R_f). For the germination experiment at the species level, four germination indices were calculated: (1) germination percentage (GP); (2) germination rate (GR); (3) response of germination to alternating temperature ($R_{5/25}$); and (4) germination response to wet-cold storage (R_{wc}) (**Table 1**).

Three indices (**Table 1**) were calculated to describe the seed germination niche: BTN, breadth of temperature niche for seed germination; O_j, the extent to which a given species occupies the *j*th germination temperature condition ($j = 5, 10, 15, 20, 25$); P_j, proportion of temperature niche for seed germination at the *j*th temperature condition (j = 5, 10, 15, 20, 25).

To compare seed germination traits at the community level, community-weighted means (CWM) of these indices of germination traits were calculated by weighting these indices with species relative abundance to avoid amplifying the influence of rare species in the community (Niu et al., 2016a,c; **Table 1**). Germination trait diversity (the range and variation of germination indices in a community) was tested based on seven variables (seed germination percentage at 5, 10, 15, 20 and 25°C and response of seed germination to temperature fluctuation and wet-cold storage) using the 'dbFD' function of the R package 'FD' (Laliberté and Legendre, 2010). Three typical diversity indices (Mason et al., 2005; Villéger et al., 2008; Niu et al., 2016b) were estimated: 1) FRic, functional richness; 2) FEve, functional evenness; and 3) FDiv, functional divergence.

Beta regression, a model for response variable bounded in (0,1), was used to test the correlation of GP, GR, $R_{5/25}$, R_{wc}, BTN, O_j and P_j with R_f (Ferrari and Cribari-Neto, 2004). Simultaneously, to meet the requirements of Beta regression we removed the boundary values (i.e., −1 and 1) in the data and converted the data with (y + 1)/2 to shift our data interval from (−1, 1) to (0, 1) (Douma and Weedon, 2019). The Beta regression was performed with the 'betareg' function of the R package 'betareg' (Cribari-Neto and Zeileis, 2010). Generalized linear mixed model (GLMM) was used to evaluate the effects of N addition, temperature and their interaction on community-weighted mean of each germination trait (i.e., CWM_{GP}, CWM_{GR}, $CWM_{R5/25}$, CWM_{Rwc}, CWM_{BTN}, CWM_{Oj} and CMN_{Pj}) and germination trait diversity indices (FRic, FEve and FDiv), because of the lack of normality and homogeneity of the data. When GLMM was conducted, block was treated as a random variable. GLMM was conducted with the 'glmer' function of the R package 'lme4' (Bates et al., 2015) on communities with different N additions at different temperatures. Comparison of data between groups was performed by Tukey test.

To determine if shifts in seed germination traits occurred along the fertilization gradient, we used Principal Components Analysis (PCA), based on a correlation matrix, to identify species groupings or trends on the basis of comprehensive variables. CWM of 23 variables were included in PCA: CWM_{GP} at 5, 10, 15, 20 and 25°C; CWM_{GR} at 5, 10, 20 and 25°C; CWM of breadth of temperature niche for germination; CWM of occupation of temperature niche for germination at 5, 10, 15, 20 and 25°C; CWM of proportion of temperature niche for seed germination at 5, 10, 15, 20 and 25°C; CWM of $R_{5/25}$; and CWM of R_{wc}.

TABLE 1 | Indices used in this study.

Index	Meaning	Formula	Range
R_f	Response of species abundance to fertilization	$R_f = (RAi_{N3} - RAi_{N0})/(RAi_{N3} + RAi_{N0})$ RAi_{N3}, RAi_{N0}: relative abundance of species i (i = 1, 2, 3 … 63) in N_3 and N_0, respectively.	[−1, 1]
RA_i	Relative abundance of species	$RA_i = n_i/N$ n_i: abundance of species i (i = 1, 2, 3 … 63); N: total number of all species.	[0, 1)
GP	Germination percentage	$GP = (G_{fin}/n) \times 100\%$ G_{fin}: final number of germinated seeds; n: total number of seeds sown.	[0, 1]
GR	Germination rate	$GR = \Sigma G_i/(n \times i)$ G_i: number of germinated seeds on day i (i = 1, 2, 3…); n: total number of seeds sown.	[0, 1]
$R_{5/25}$	Response of germination to alternating temperature	$R_{5/25} = (GP_{5/25}-GP_{15})/(GP_{5/25} + GP_{15})$ $GP_{5/25}$, GP_{15}: germination percentage at 5/25°C and 15°C, respectively.	[−1, 1]
R_{wc}	Response of germination to wet-cold storage	$R_{wc} = (GP_{wc} - GP_{dr})/(GP_{wc} + GP_{dr})$ GP_{wc}, GP_{dr}: germination percentage of seeds stored under wet-cold and dry-room conditions, respectively	[−1, 1]
BTN	Breadth of temperature niche for seed germination	$BTN = 1/R\Sigma O_j$ R: number of temperature conditions; O_j: an index to measure the extent to which a given species occupies the *j*th temperature condition.	[1/R, 1](R = 5)
O_j	Species germination temperature occupation index	$O_j = g_j/g_{max}$ g_j: germination percentage at *j*th temperature condition (j = 5, 10, 15, 20, 25); g_{max}: highest germination percentage at all temperature conditions.	[0, 1]
P_j	Proportion of germination temperature niche	$P_j = O_j/\Sigma O_j$ O_j: species germination temperature occupancy index.	[0, 1]
$CWM_{trait\ i}$	Community-weighted means of germination traits index	$CWM_{trait\ i} = \Sigma trait_i \times RA_i$ $trait_i$: germination trait index (trait = GPi, GRi, $R_{5/25}$, R_{wc}, P_j, O_j, BTN); RA_i: relative abundance of species i (i = 1, 2, 3 … 63).	(0, ∞)

PCA was conducted using the ‘princomp’ function of the R package ‘stats’ (R Core Team, 2021). All statistical analyses were conducted using R version 4.0.4.

RESULTS

Relationship Between Germination (GP and GR) and Response of Species Abundance to Nitrogen Fertilization (R_f)

Relative abundance of species along the fertilization gradient is shown in Appendix S2. The results of Beta regression analysis indicated that germination percentages at the five temperatures (5, 10, 15, 20 and 25°C) were significantly and positively related to R_f. Germination rates of seeds at 10, 15, 20 and 25°C were significantly related to R_f (**Table 2**), whereas at 5°C they were not (**Table 2**).

TABLE 2 | Results of Beta regression models testing correlations of response of species abundance to fertilization (R_f) with germination percentage, germination rate (speed) and response of germination percentage to temperature fluctuation and wet-cold storage.

Germination index	Regression equation	z-value	*P*-value	R^2
GP_5	y = −0.96 + 1.44x	3.286	**0.001**	0.263
GP_{10}	y = −0.83 + 1.51x	3.382	**< 0.001**	0.271
GP_{15}	y = −0.84 + 1.34x	3.037	**0.002**	0.224
GP_{20}	y = −1.05 + 1.41x	3.256	**0.001**	0.256
GP_{25}	y = −1.03 + 1.34x	2.986	**0.003**	0.218
$GP_5/_{25}$	y = −0.64 + 0.79x	1.781	0.075	0.092
GR_5	y = −0.68 + 19.81x	2.323	0.20	0.151
GR_{10}	y = −0.78 + 13.31x	3.505	**< 0.001**	0.292
GR_{15}	y = −0.71 + 7.03x	2.949	**0.003**	0.230
GR_{20}	y = −0.69 + 3.67x	2.678	**0.007**	0.180
GR_{25}	y = −0.83 + 4.81x	3.160	**0.002**	0.257
$GR_{5/25}$	y = −0.63 + 7.05x	2.182	**0.029**	0.129
$R_{5/25}$	y = −0.28 + (-0.23)x	−0.858	0.391	0.023
R_{wc}	y = −0.29 + (-0.19)x	−0.573	0.567	0.011

Significant P values ($P < 0.05$) are indicated in bold. GP_5, GP_{10}, GP_{15}, GP_{20}, GP_{25} and $GP_{5/25}$: germination percentage at 5, 10, 15, 20, 25 and 5/25 °C, respectively; GR_5, GR_{10}, GR_{15}, GR_{20}, GR_{25} and $GR_{5/25}$: germination rate at 5, 10, 15, 20, 25 and 5/25°C, respectively. $R_{5/25}$: response of germination to temperature fluctuation; R_{wc}: response of germination to wet-cold storage.

TABLE 3 | Results Generalized linear mixed model (GLMM) testing the effect of temperature (tem), fertilization gradient (fer) and their interaction on community weighted mean of germination percentage (CWM_{GP}) and of germination rate (CWM_{GR}).

Response/AIC/BIC	Fixed effects		
	χ 2	DF	*P*-value
CWM_{GP}/673.4/728.1	139.76	4	**<0.001**
Temperature	161.49	3	**<0.001**
Fertilization	25.12	12	**0.014**
temperature*fertilization			
CWM_{GR}/351.1/405.8			
Temperature	105.50	4	**<0.001**
Fertilization	16.48	3	**<0.001**
temperature*fertilization	1.97	12	0.999

Significant P values ($P < 0.05$) are indicated in bold.

GLMM indicated that both temperature (5, 10, 15, 20 and 25°C) and fertilization (N_0, N_1, N_2 and N_3) had a significant effect on community-weighted means for germination percentages and rates (CWM_{GP} and CWM_{GR}) (**Table 3**). There were no interaction effects between temperature and fertilization on CWM_{GP} or CWM_{Gr} (**Table 3**). CWM_{GP} increased with increased N addition, and CWM_{GP} was significantly higher in the N_3 treatment than in the N_0 treatment (**Figure 1**). Only under low temperature (5 and 10°C), CWM_{GP} was significantly higher in the N_2 treatment than in the N_0 treatment (**Figure 1**). Similarly, CWM_{GR} increased along the fertilization gradient (except at 15°C), where there was a significant difference between N_3 and N_0 (**Figure 1**).

Relationship Between Germination Response to Alternating Temperature ($R_{5/25}$), Cold-wet Storage (R_{wc}) and R_f

Beta regression analysis showed no significant relationship between $R_{5/25}$ and R_f (**Table 2**). Neither did R_{wc} have a significant relationship with R_f (**Table 2**). However, at the community level GLMM showed that fertilization significantly affected $CWM_{R5/25}$ ($x^2 = 54.39$, $P < 0.001$) and CWM_{Rwc} ($x^2 = 15.29$, $P = 0.002$). The strength of the positive response of seed germination to alternating temperature was significantly higher in N_1, N_2, and N_3 treatments than in N_0 treatment (**Figure 2A**). In contrast, at the community level the strength of the positive response of seed germination to wet-cold storage decreased with increase in amount of N added, and CWM_{wc} was significantly lower in N_3 treatments than in N_0 treatment (**Figure 2B**).

Relationship Between Temperature Niche for Seed Germination (BTN) and R_f

Beta regression analysis revealed a significant positive relationship between BTN and R_f (**Figure 3A**), and it also showed that proportion of temperature niche for seed germination at 10°C (P_{10}) was significantly and positively related to R_f (**Table 4**). However, there was no significantly positive relationship between R_f and proportion of temperature niche for seed germination at the other temperatures (**Table 4**). The results of Beta regression analysis indicated that occupation index of seed germination for all temperature conditions (5, 10, 15, 20 and 25°C) was always significantly and positively related to R_f (**Table 4**). At the community level, the CWM_{BTN} of the community treated with N_3 was significantly higher than that of the control (**Figure 3B**). The CWM_{BTN} of the community with N_1 or N_2 did not differ significantly from N_0 or N_3 (**Figure 3B**).

Principal Component Analysis (PCA) of Seed Germination Strategies at Different Nitrogen Levels

Based on the CWM of 23 variables, PCA revealed some clear trends. The first two principal components (PCs) accounted for 77.98% of the variation in the data set, with the first

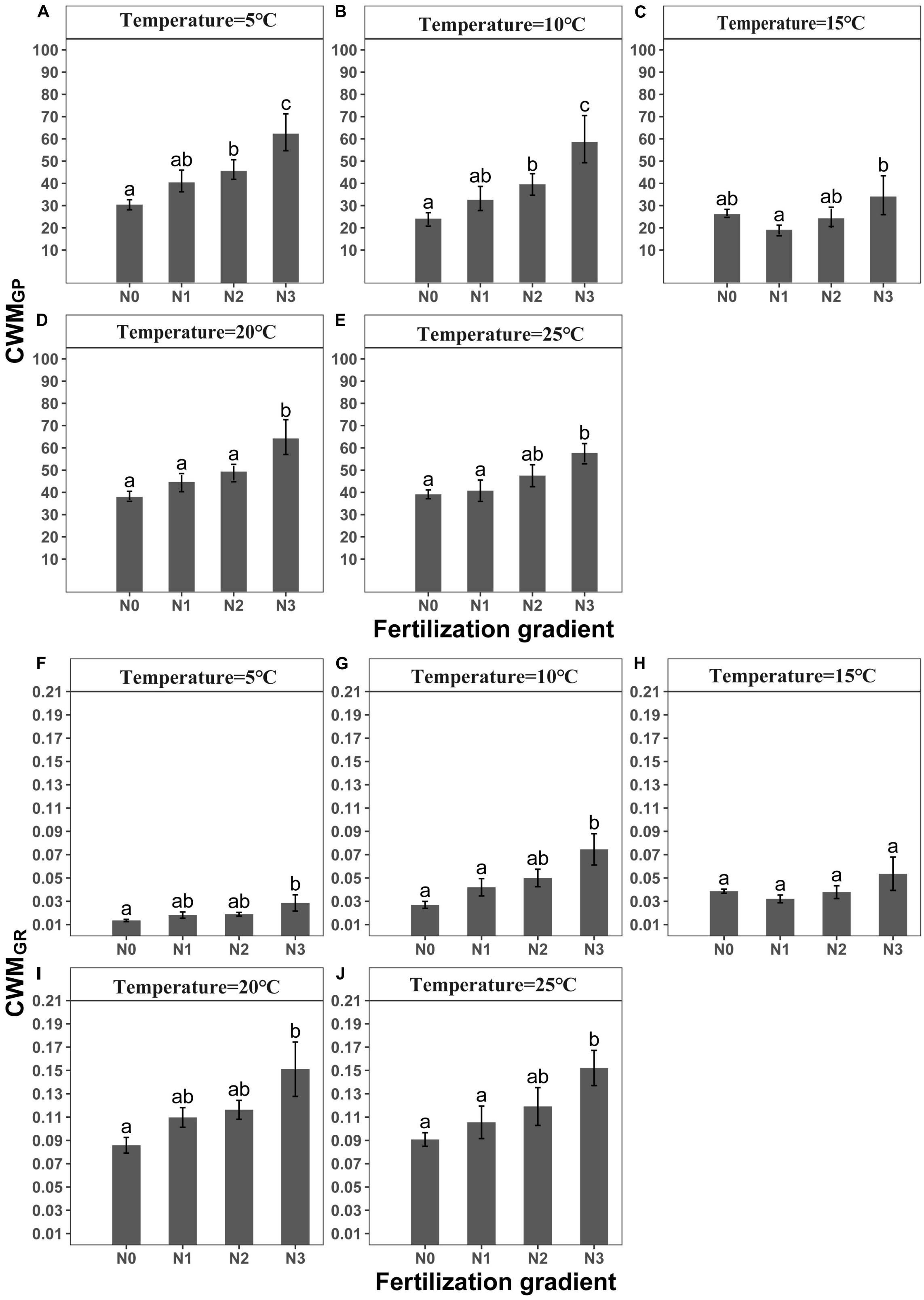

FIGURE 1 | Effects of temperature and level of nitrogen addition (N0, 0 g N m $^{-2}$ year $^{-1}$; N1, 5 g N m^{-2} year^{-1}; N2, 10g N m^{-2} year^{-1}; N3, 20g N m^{-2} year^{-1}) on CWM$_{GP}$ (community weighted mean of germination percentage) and **(A–E)** CWM$_{GR}$ (community weighted mean of germination rate). **(F–J)** Different letters indicate significant differences in level of nitrogen addition ($P < 0.05$) at each temperature. Error bars are ± SE.

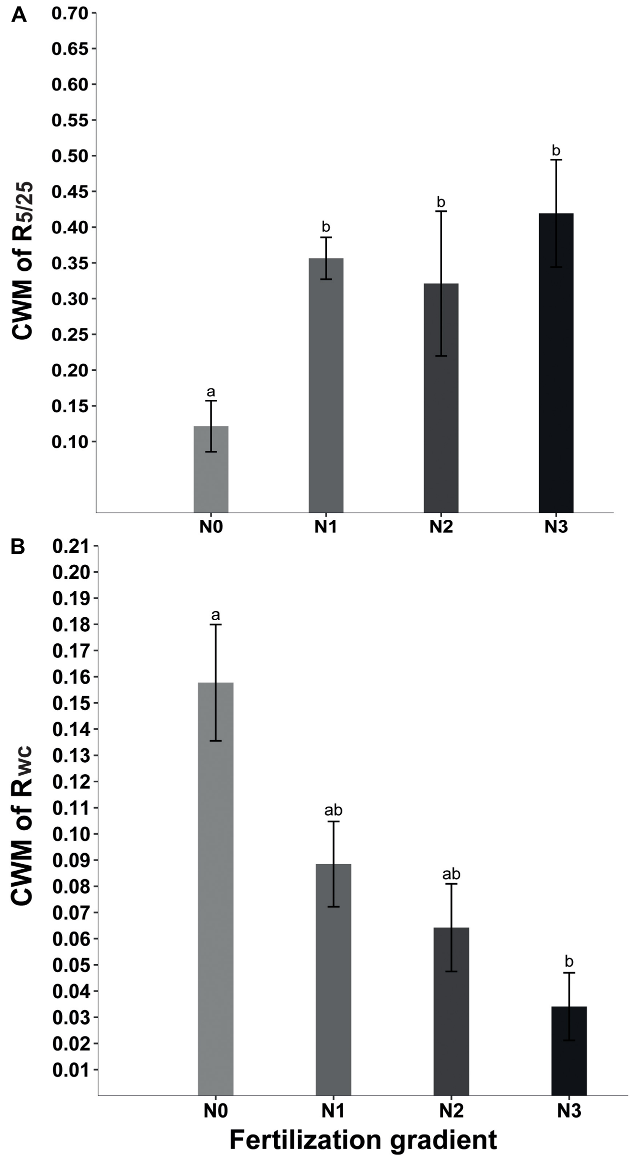

FIGURE 2 | Effect of nitrogen addition (N0, 0 g N m^{-2} year^{-1}; N1, 5 g N m^{-2} year^{-1}; N2, 10g N m^{-2} year^{-1}; N3, 20g N m^{-2} year^{-1}) on CWM (community weighted mean) of $R_{5/25}$ (germination response to temperature fluctuation) **(A)** and CWM of R_{wc} (germination response to wet-cold storage) **(B)**. Different letters indicate significant differences ($P < 0.05$). Error bars are ± SE.

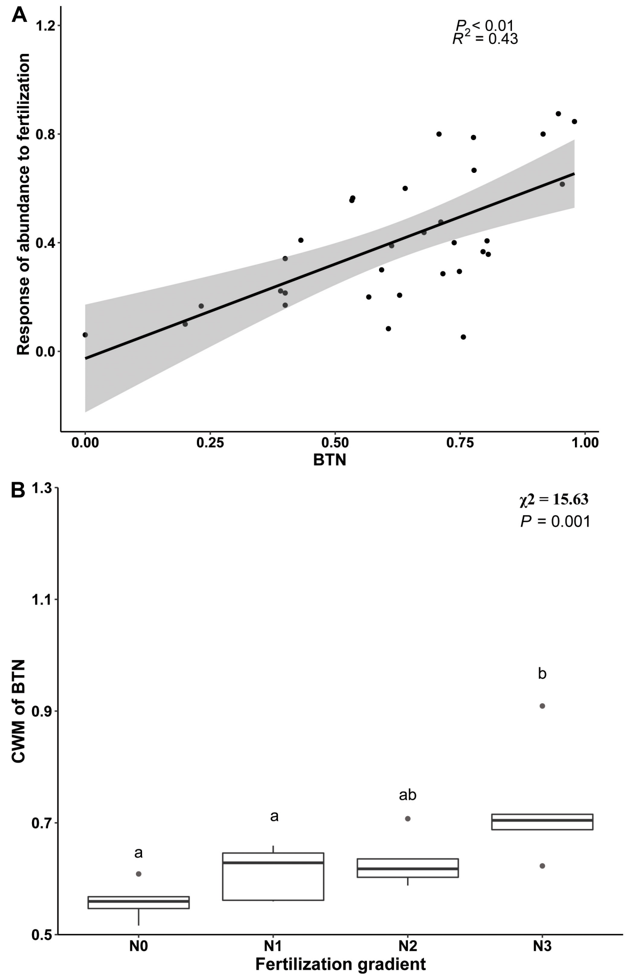

FIGURE 3 | The relationship between response of species abundance to fertilization (R_f) and breadth of temperature niche for germination (BTN) at species level **(A)** and the effect of nitrogen addition (N0, 0 g N m^{-2} year^{-1}; N1, 5 g N m^{-2} year^{-1}; N2, 10g N m^{-2} year^{-1}; N3, 20g N m^{-2} year^{-1}) on CWM (community weighted mean) of BTN **(B)**. Ends of a box represent first and third quartiles; thick line in box, the median; ends of vertical line (whiskers), maximum and minimum; and dots, outliers.

and second PCs explaining 53.02 and 24.96% of the variation, respectively. The two PCs represent the linear combinations of variables that are highly correlated with each other (**Figure 4**). The three variables most positively related to the first PC are CWM_{GP5}, CWM_{P25} and CWM_{BTN}; however, the variable most negatively related to the first PC is CWM_{RWC}. The three variables most positively related to the second PC are CWM_{O15}, CWM_{Gp15} and CWM_{P15}, while the three variables most negatively related to the second PC are CWM_{P5}, $CWM_{R5/25}$ and CWM_{O25} (**Figure 4**). PCA revealed that shifts of germination strategies occurred along the entire fertilization gradient and especially in the high N addition treatment (N_3) (**Figure 4**).

Shift in Diversity of Germination Traits Along the Nitrogen Fertilization Gradient

Germination trait diversity indices along the N fertilization gradient are shown in Appendix S3. GLMM indicated that N addition had a significant effect on FRic ($x^2 = 19.25$, $P < 0.001$). FRic decreased with increased N, and FRic was significantly lower in N_3 treatments than in N_0 treatment (**Figure 5A**). However, N addition did not have a significant effect on either FEve or FDiv ($P = 0.88$ and $P = 0.31$, respectively) (**Figures 5B,C**).

TABLE 4 | Results of Beta regression models testing correlations of response of species abundance to fertilization (R_f) with traits of temperature niche for germination.

Niche traits	Regression equation	*z*-value	*P*-value	R^2
O_{i5}	y = −1.06 + 1.10x	2.530	**0.011**	0.202
O_{i10}	y = −1.12 + 1.68x	4.038	**<0.001**	0.348
O_{i15}	y = 0.81 + 0.85x	1.988	**0.047**	0.111
O_{i20}	y = −1.13 + 1.02x	1.971	**0.049**	0.138
O_{i25}	y = −1.15 + 1.02x	2.111	**0.035**	0.143
P_{i5}	y = −0.63 + 1.45x	1.130	0.258	0.047
P_{i10}	y = −1.02 + 5.51x	3.084	**0.002**	0.244
P_{i15}	y = −0.49 + 0.92x	0.622	0.534	0.013
P_{i20}	y = −0.29 + (-0.26)x	−0.252	0.801	0.003
P_{i25}	y = −0.24 + (-0.43)x	−0.464	0.642	0.010

Significant P values ($P < 0.05$) are indicated in bold. BTN: breadth of temperature niche for germination; O_5, O_{10}, O_{15}, O_{20} and O_{25}: occupation of temperature niche for germination at 5, 10, 15, 20 and 25°C, respectively; P_5, P_{10}, P_{15}, P_{20} and P_{25}: proportion of temperature niche for seed germination at 5, 10, 15, 20 and 25°C, respectively.

DISCUSSION

Changes in Germination Trait Selection Due to Fertilization

Our hypothesis that changes occurring after N addition can change seed germination trait-environmental filter interactions and ultimately redistribute germination traits in the community and alter community composition was supported. At the species level, germination percentage at all temperatures, germination rate at all test temperatures except 5 °C and properties of the temperature niche for germination (BTN, P_5, O_j) were significantly related to R_f. Analyses at the community level also indicated significant differences between CWM_{GP}, CWM_{GR}, $CWM_{R5/25}$, CWM_{Rwc} and CWM_{BTN} of the alpine meadow with high level of N addition and those without N addition. The seed germination trait richness of the alpine meadow also decreased significantly after N addition. These effects of N addition on germination trait selection by the environmental filter is amount-dependent (i.e., low and medium level of N addition had less effect than the high level).

These results indicated that N addition alters filter-trait interactions. The environmental filter in the alpine meadows after N addition tended to select germination traits such as high germination percentage, fast germination rate (speed) and an alternating temperature requirement for germination. However, other germination traits were not selected by the environment filter (N addition), resulting in a decrease of seed germination trait richness in the meadow.

Previous studies indicated that fertilization can cause loss of biodiversity in the grassland community (Niu et al., 2008; Hautier et al., 2009). The decrease in germination traits richness in our study may provide a new explanation for biodiversity loss in grasslands. That is, the environmental filter (N fertilization) does not select for as many germination traits as found in grasslands without fertilization. Thus, grassland species with germination traits not selected by the fertilization filter may be lost from grasslands after fertilization.

Germination Traits and Plant Survival in Alpine Meadows After Nitrogen Addition

Our research provides some evidence that seed germination traits at both the species and community levels play a role in plant survival/persistence in alpine meadows after N addition. Species with high germination percentages and rates are more likely to survive in fertilized alpine meadows than those with low germination percentages and rates. Previous studies indicated that germination traits such as high germination percentage and fast germination speed tend to be selected by a favorable environment (Baskin and Baskin, 2014). The increased plant height and density after N addition can increase soil moisture and reduce exposure to strong sunlight on the soil surface (Bakelaar and Odum, 1978; Ostertag and Verville, 2002; Fenner and Thompson, 2005; Song et al., 2013). In addition, N addition increases soil nutrition (Niu et al., 2008; Li et al., 2013). These habitat changes can promote seedling growth and survival and decrease the possibility of seedling death due to high temperature and drought conditions (Fenner and Thompson, 2005). Thus, favorable germination traits for species to occupy space and acquire resources, such as high germination percentage and fast germination speed, may be selected in fertilized alpine meadows.

Competition for light is a primary determinant of the survival of plant species in the community after N addition (Grime, 1973; Newman, 1973; Tilman, 1988). Since competition for light is plant size- and height-dependent, a newly-germinated seedling in a high-N community is at a great disadvantage with established plants in capturing light. Seed germination traits that can restrict germination to a time and place without competition for light are more likely be selected by the fertilization environmental filter in alpine meadows. Our study showed that the magnitude (slope) of the relationship between seed germination rate and response of species relative abundance to N addition is much greater at low than that at high temperatures. Thus, faster germination speed at low than at high temperature is important for survival of plant species in fertilized alpine meadows. Fast germination in early spring (when the temperature is low on the Tibet Plateau) is conducive to successful seedling establishment before the canopy of established plants becomes dense in the spring. It is a mechanism by which a plant species can avoid seedling death due to competition for light. Furthermore, the magnitude (slope) of the relationship between occupation of temperature niche for germination at low temperature (10°C) and the response of abundance to N fertilization (R_f) is larger than that at high temperature, and a proportion of germination temperature niche can positively relate to the response of abundance to fertilization (R_f), only at low temperature (10°C). These two results also prove that seed germinability at low temperatures is critical for plant species survival in fertilized alpine meadows. Previous studies also found that herbs in dense vegetation usually can germinate in early spring at low temperatures (Baskin and Baskin, 1988; Schütz, 1997; Vandelook et al., 2009).

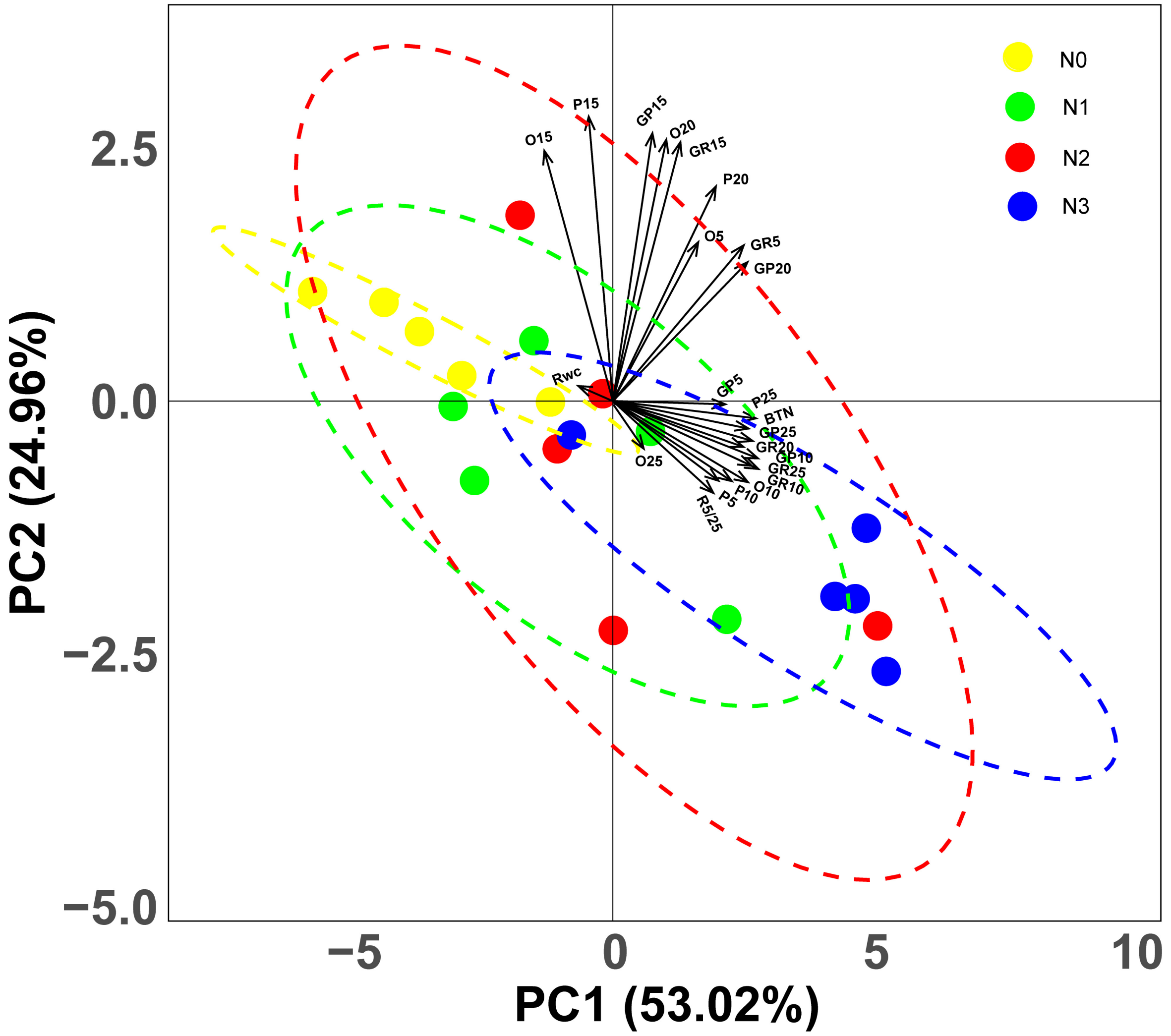

FIGURE 4 | Principal Component Analysis (PCA) representing the two main axes of variation in germination traits, based on community weighted mean (CWM) of 23 variates. GP5, GP10, GP15, GP20 and GP25: germination percentage at 5, 10, 15, 20 and 25°C respectively; GR5, GR10, GR20 and GR25: germination rate at 5, 10, 15, 20 and 25°C, respectively; BTN: breadth of temperature niche for germination; O5, O10, O15, O20 and O25: occupation of temperature niche for germination at 5, 10, 15, 20 and 25°C, respectively; P5, P10, P15, P20 and P25: proportion of temperature niche for seed germination 5, 10, 15, 20 and 25°C, respectively. Dots represent quadrats, different colors represent different levels of nitrogen addition.

As mentioned above, competition for light after formation of a dense canopy in spring is not conducive to seedling establishment. However, in Tibetan alpine meadows many vegetation gaps usually are created by burrowing animals such as pika, zokor and marmot and by overgrazing by sheep and yak (Liu et al., 2011). Light is sufficient for seedling establishment in these vegetation gaps. A fluctuating temperature requirement for germination, which restricts germination to vegetation gaps, would promote survival of plant species in fertilized alpine meadows. Our study indicates that at the community level, N addition significantly increased germination response to alternating temperature; seeds of more species in the fertilized than in the non-fertilized meadow required alternating temperatures to germinate. Since addition of N promotes plant growth and canopy closure, we would expect gap-sensing mechanisms for successful seedling establishment to be more important in fertilized than in non-fertilized alpine meadows. As expected, there was a stronger positive response of seed germination to alternating temperatures of species in fertilized than in non-fertilized alpine meadows.

A broad BTN is another germination trait that can promote survival of plant species in fertilized alpine meadows. In our research, Beta regression analysis revealed a significant positive relationship between BTN and R_f. One reason for this positive relationship is that a broad BTN allows seeds to germinate as soon as a vegetation gap is created, and a fluctuating temperature requirement for germination is fulfilled during the growing season, which results in an advantage for seedlings to occupy vegetation gaps, where the light environment is suitable for growth and establishment. Another reason is that seeds of species

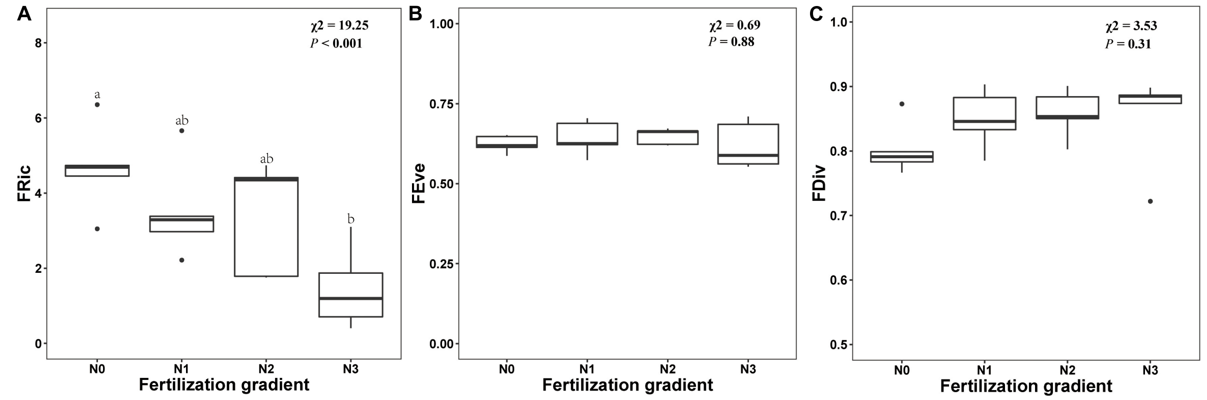

FIGURE 5 | Effects of level of nitrogen addition (N0, 0 g N m^{-2} year^{-1}; N1, 5 g N m^{-2} year^{-1}; N2, 10g N m^{-2} year^{-1}; N3, 20g N m^{-2} year^{-1}) on functional richness (FRic, **A)**, functional evenness (FEve, **B)** and functional divergence (FDiv, **C)** of germination. Ends of a box represent first and third quartiles; thick line in box, the median; ends of vertical line (whiskers), maximum and minimum; and dots, outliers.

with a broad temperature niche for germination usually can germinate to high percentages at 5°C and 10°C, which may be beneficial for seedling establishment before the canopy becomes too dense in early spring.

CONCLUSION

Our study indicates that N addition has a significant influence on filter-trait interactions and can generate a different set of germination traits in alpine meadows. As a result, germination trait richness was decreased by N addition to the alpine meadow. In addition, the effect of N addition on germination trait selection by the environmental filter was amount-dependent (i.e., low and medium levels of N addition had less effect than the high level). A broad temperature niche, a requirement of alternating temperatures for germination, a high germination percentage and a fast germination rate, especially at low temperature, are germination traits more likely to be selected for in fertilized than in non-fertilized alpine meadows. Additional studies on the effect of N addition on seedling establishment and plant clonal reproduction at the species and community levels will help us to further evaluate the extent to which germination traits are responsible for plant survival in alpine meadows that have become N-enriched via use of N fertilizers by humans or via atmospheric N deposition.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

KL, YL, ZZ, and SZ started the project, designed the research, KL, YL, CB, and JB made major revision of the manuscript. KL, YL, ZZ, SZ, HB, TL, SL, and TZ performed the research. KL, YL, XC, and SX analyzed data and drafted the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2021.634850/full#supplementary-material

Supplementary Appendix 1 | Germination percentages of seeds of the 63 species tested in the study. GP$_5$, GP$_{10}$, GP$_{15}$, GP$_{20}$, GP$_{25}$ and GP$_{(5/25)}$: germination percentage at 5℃, 10°C, 15°C, 20°C, 25°C and 5/25°C, respectively; GP$_{dr}$ and GP$_{wc}$: germination percentage at 5/20°C of seeds stored under day-warm condition and wet-cold conditions, respectively.

Supplementary Appendix 2 | Relative abundance of species along the nitrogen fertilization gradient. RaN0, RaN1, RaN2 and RaN3: relative abundance of species (Ra) at positions along the gradient where soil received an application of 0 (N0), 5 (N1), 10 (N2) and 20 (N3) g N m^{-2}y^{-1}.

Supplementary Appendix 3 | Germination trait diversity indices (functional richness, FRic; functional evenness, FEve; and functional divergence, FDiv) at different levels of nitrogen addition along the nitrogen fertilization gradient.

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Fire Seasonality, Seasonal Temperature Cues, Dormancy Cycling, and Moisture Availability Mediate Post-fire Germination of Species With Physiological Dormancy

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Fire seasonality (the time of year of fire occurrence) has important implications for a wide range of demographic processes in plants, including seedling recruitment. However, the underlying mechanisms of fire-driven recruitment of species with physiological seed dormancy remain poorly understood, limiting effective fire and conservation management, with insights hampered by common methodological practices and complex dormancy and germination requirements. We sought to identify the mechanisms that regulate germination of physiologically dormant species in nature and assess their sensitivity to changes in fire seasonality. We employed a combination of laboratory-based germination trials and burial-retrieval trials in natural populations of seven species of *Boronia* (Rutaceae) to characterize seasonal patterns in dormancy and fire-stimulated germination over a 2-year period and synthesized the observed patterns into a conceptual model of fire seasonality effects on germination. The timing and magnitude of seedling emergence was mediated by seasonal dormancy cycling and seasonal temperature cues, and their interactions with fire seasonality, the degree of soil heating expected during a fire, and the duration of imbibition. Primary dormancy was overcome within 4–10 months' burial and cycled seasonally. Fire-associated heat and smoke stimulated germination once dormancy was alleviated, with both cues required in combination by some species. For some species, germination was restricted to summer temperatures (a strict seasonal requirement), while others germinated over a broader seasonal range of temperatures but exhibited seasonal preferences through greater responses at warmer or cooler temperatures. The impacts of fires in different seasons on germination can vary in strength and direction, even between sympatric congeners, and are strongly influenced by moisture availability (both the timing of post-fire rainfall and the duration soils stay moist enough for germination). Thus, fire seasonality and fire severity (via its effect on soil heating) are expected to significantly influence post-fire emergence patterns in these species and others with physiological dormancy, often leading to "germination interval squeeze." Integration of these concepts into current fire management frameworks is urgently required to ensure best-practice conservation. This is especially pertinent given major, ongoing shifts in fire seasonality and rainfall patterns across the globe due to climate change and increasing anthropogenic ignitions.

Keywords: fire regime change, fire severity, heat pulse, Rutaceae, seasonal germination niche, smoke, soil seed bank, seedling recruitment

INTRODUCTION

Global Changes in Fire Regimes

Fire plays a crucial role in the maintenance of biodiversity in fire-prone ecosystems (He et al., 2019). Terrestrial plants have evolved a variety of life history traits that enable their persistence through recurrent fire by promoting the survival of individuals and/or offsetting mortality via *in situ* recruitment or recolonization (Noble and Slatyer, 1980; Keeley et al., 2011; Keith, 2012). Individual species are adapted to suit certain fire regimes defined by the frequency, intensity, severity, type, and seasonality of fire (Gill, 1975; Pausas and Keeley, 2009). In turn, these components of the fire regime filter the species able to persist at a given site (Pausas et al., 2004; Archibald et al., 2017). Climate change and other anthropogenic activities (e.g., land-use change, ignitions and fire suppression) are driving rapid changes in global fire regimes (Bowman et al., 2009; Krawchuk et al., 2009; Pausas and Keeley, 2021) which is increasing the risk of plant population declines and local extinctions (Kelly et al., 2020). A mechanistic understanding of how individual components of the fire regime affect key demographic processes is crucial for accurately predicting species' responses (and resilience) to such regime shifts (Menges, 2000; Enright et al., 2014), and is urgently required to underpin effective fire management for biodiversity conservation (Bowman et al., 2020; Nolan et al., 2021).

Fire seasonality (the time of year of fire occurrence) is a relatively understudied element of the fire regime (Whelan, 1995) despite significant recent advances (Miller et al., 2019; Keith et al., 2020). Historically, wildfires have been more prevalent in warmer or drier seasons, when fuel moisture and weather conditions are most conducive to ignition and fire spread (Krawchuk and Moritz, 2011); however, global increases in annual fire weather due to climate change have led to fire seasons in many regions around the world beginning earlier and lasting longer (Westerling et al., 2006; Jolly et al., 2015). In conjunction with greater anthropogenic ignitions, this is increasing the frequency of unseasonal fires (those outside the historical fire season) in the landscape (Le Page et al., 2010; Balch et al., 2017; Bowman et al., 2020). Variation in fire seasonality can adversely affect plant populations through negative impacts on critical life history stages including adult survival and growth, propagule availability, dispersal, and post-fire seedling establishment (Miller et al., 2019; Keith et al., 2020; and references therein). However, supporting evidence for these mechanisms is relatively limited across most climate and vegetation types—in particular, evidence of how altered fire seasonality affects recruitment from soil seed banks of species with innate seasonal germination requirements (Miller et al., 2019; Cao et al., 2020; Tangney et al., 2020).

Physiological Seed Dormancy

Species with a physiological component to their dormancy [including physiological dormancy (PD) and morpho-physiological dormancy (MPD)] constitute a significant component of the floristic diversity in fire-prone regions (Merritt et al., 2007; Baskin and Baskin, 2014; Collette and Ooi, 2021) and account for a disproportionate number of threatened species in some climate regions (e.g., Collette and Ooi, 2021). However, physiological dormancy remains poorly understood in these ecosystems, with many species reported as difficult to germinate (Merritt et al., 2007). This is largely due to gaps in our knowledge regarding the complex interactions between multiple fire-related cues, and environmental cues, that break dormancy and stimulate germination from soil seed banks (Mackenzie et al., 2016).

Physiological dormancy is overcome in nature by a period of after-ripening and/or stratification during burial (Baskin and Baskin, 2014; **Figure 1**). In fire-prone ecosystems, fire-associated heat and/or smoke, together with sufficient soil moisture, are required to stimulate germination *once dormancy has been alleviated* and this concentrates seedling emergence in the post-fire environment where there are greater resources and reduced competition (Whelan, 1995). An additional seasonal temperature requirement restricts post-fire germination of some PD species to particular seasons (Ooi et al., 2006; Mackenzie et al., 2016; Collette and Ooi, 2017, 2020) and may lead to delayed post-fire emergence following unseasonal fires (Ooi et al., 2004; Auld and Ooi, 2008; Cao et al., 2020). Loss of PD is progressive, with seeds moving from a dormant state (unresponsive to germination cues) to conditional dormancy (able to germinate over a narrow range of conditions) and finally becoming non-dormant (able to germinate over a wide range of conditions) (Baskin and Baskin, 2014). Some species cycle in and out of dormancy in response to seasonal variation in temperature and soil moisture (Merritt et al., 2007; Baskin and Baskin, 2014), resulting in periodic changes in their receptivity to germination cues (Baker et al., 2005c). Even in strongly seasonal rainfall environments where the availability of soil moisture restricts germination to a particular time of year, there are examples of species with innately seasonal germination (de Lange and Boucher, 1993; Roche et al., 1998; Chia et al., 2016).

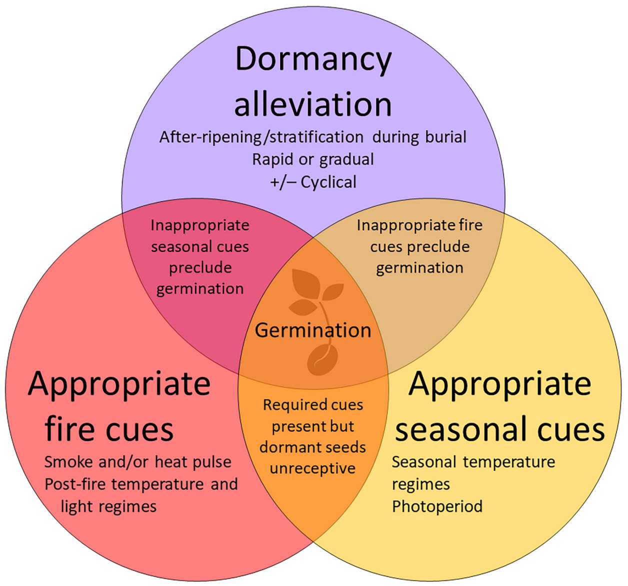

FIGURE 1 | Overlapping conditions required for germination of plant species with physiological seed dormancy in temperate fire-prone ecosystems, given adequate soil moisture. After-ripening and/or stratification are required to overcome primary dormancy in freshly dispersed, mature seeds before they become receptive to one or more fire-related cues required to stimulate germination. Some species also require particular seasonal temperature cues to germinate. The amount and duration of available soil moisture will also influence germination.

Barriers to Understanding Physiological Dormancy in Fire-Prone Ecosystems

Efforts to understand the seed ecology and germination requirements of PD species in natural populations in fire-prone ecosystems have been hampered by a number of factors, including common practices in laboratory germination trials, and complex and variable interactions between multiple fire-cues and other environmental cues (**Table 1**). The poor germination of viable seeds frequently reported in *ex situ* trials of PD species is most frequently attributable to application of germination cues to dormant seeds, or the absence of appropriate fire cues and/or seasonal incubation temperatures (**Figure 1**). Overreliance in seed ecology research on freshly dispersed seeds and/or seeds stored *ex situ* under artificial conditions provides limited reliable insight into the regulation of dormancy and germination in soil seed banks in natural systems. Fresh seeds with PD typically yield poor germination due to high primary dormancy (e.g., Tieu et al., 2001; Ooi et al., 2006; Mackenzie et al., 2016) and are likely to be poor indicators of how seeds in the soil seed bank respond as they after-ripen and undergo stratification and physical deterioration. *Ex situ* storage of seeds may have impacts on dormancy and germination that are difficult to quantify and vary with storage conditions, precluding reliable extrapolation to populations in the field (Baskin et al., 2006).

A lack of data on the combined effects of multiple fire-related cues and environmental cues on germination of PD species is a key knowledge gap. The positive effect of smoke on germination of PD species is well-documented (Dixon et al., 1995; Keeley and Fotheringham, 1998; Brown and Botha, 2004; Moreira et al., 2010); however, as a result, the importance of the heat pulse associated with the passage of fire is an often-overlooked cue in species with this type of dormancy. This is despite a growing number of reports of positive responses to a heat pulse in a diverse range of families with PD [e.g., Apiaceae (Baker et al., 2005c), Ericaceae (Moreira et al., 2010), Lamiaceae (Kazancı and Tavşanoğlu, 2019); Myrtaceae (Auld and Ooi, 2009), and Rutaceae (Mackenzie et al., 2016)]. Where both cues have been investigated in combination, responses are highly variable and species-specific, ranging from neutral to additive (Keith, 1997; Kenny, 2000; Newton et al., 2021), unitive (both cues required (Thomas et al., 2007; Mackenzie et al., 2016; Collette and Ooi, 2020); synergistic (Gilmour et al., 2000; Baker et al., 2005a); and negative (a heat pulse inhibits the smoke response without loss of viability; Keeley and Fotheringham, 1998; Collette and Ooi, 2017). Potential interactions between fire cues and environmental cues such as light (Bell, 1994; Gilmour et al., 2000; Collette and Ooi, 2017) and moisture (Thomas et al., 2010) create additional complexity.

The important role that seasonal temperature cues play in regulating fire-stimulated germination of many PD species has been recognized only relatively recently (Ooi et al., 2006; Mackenzie et al., 2016; Collette and Ooi, 2017, 2020; Hodges et al., 2019). Very few studies have investigated the effect of fire-related germination cues such as heat and smoke in combination with a full complement of seasonal temperatures, limiting our understanding of the germination ecology of PD species in temperate fire-prone regions and predictions of the impacts of altered fire regimes on recruitment. In particular, major shifts in the seasonal occurrence of fires pose a threat to PD species with seasonal germination requirements where seedling emergence is restricted to a particular time of year regardless of the timing of fire occurrence (Ooi et al., 2004). Thus, seedling emergence can be delayed by up to 12 months following fires in certain seasons and may also be diminished in magnitude where such delays reduce or negate the efficacy of fire-related cues. This can have adverse effects on establishment success and subsequent plant performance (Ooi, 2019). However, field evidence for this phenomenon is currently very limited (Miller et al., 2019).

Here, we examine mechanisms with the potential to drive seasonal post-fire emergence patterns in PD species within members of the Rutaceae, an important cosmopolitan plant family and one of the most significant families in fire-prone temperate Australia (Collette and Ooi, 2021). Like many other PD species from fire-prone regions, Rutaceae are often reported as difficult to germinate (Brown and Botha, 2004; Martyn et al., 2009; Collette and Ooi, 2020); however, few studies have applied cue combinations that seeds experience in natural populations to viable, non-dormant seeds (Auld, 2001) and, until recently, none had done so in conjunction with a full a complement of seasonal temperatures (Mackenzie et al., 2016; Collette and Ooi, 2017). A poor understanding of the seed and fire ecology of Australian Rutaceae also limits effective management and conservation of

TABLE 1 | Common practices impeding our understanding of how dormancy and germination syndromes regulate post-fire emergence of species with physiological seed dormancy (PD) in fire-prone ecosystems and some practical solutions.

Practice	Potential consequences	Solution
Use of seeds stored *ex situ* under artificial conditions.	Unknown temporal and storage effects on dormancy preclude reliable extrapolation to natural populations.[a] Not an issue if primary aim is maximizing germination to generate seedlings for *ex situ* conservation or horticultural purposes.[a]	Use freshly dispersed seeds as soon as possible after collection for germination ecology studies aimed at understanding regulation of dormancy and germination in natural systems.[a]
Use of freshly dispersed seeds to predict germination responses of soil seed banks.	Germination often poor due to high primary dormancy. Responses unlikely to represent those of seeds in the soil seed bank where dormancy has been partially or fully alleviated.[b]	Follow up with an *in situ* seed burial-retrieval trial to characterize temporal (seasonal) patterns in dormancy alleviation and germination.
Application of germination cues to dormant seeds.	Poor germination and potentially erroneous conclusions about cue inefficacy if cues that break PD (including heat and smoke) are not distinguished from those that stimulate germination once dormancy is broken.[b,c,d,e,f]	Maintain the distinction between mechanisms of dormancy alleviation (after-ripening, stratification) and cues that stimulate germination once dormancy is overcome when interpreting the causes of poor germination.[b,c,d,e,f]
Overlooking fire-associated heat as a potential germination cue.	Poor germination and failure to identify species with a heat response or heat requirement (this includes members of a diverse range of plant families).	Include a heat pulse in studies of fire-stimulated germination of species with unknown germination syndromes.
Application of fire cues such as heat and smoke in isolation from one another.	Poor germination and failure to identify important interactions and species with obligate germination requirements for two or more fire cues in combination.	Investigate factorial combinations of fire cues.
Overlooking certain seasonal temperatures or application of inappropriate diurnal incubation regimes.	Poor germination and failure to identify species with seasonal germination requirements.	Include a full complement of seasonal incubation temperatures appropriate to the study region, noting that fresh seeds are likely to germinate over a narrower range of temperatures than seeds in the soil seed bank. Avoid constant temperatures and continuous light.[a]
Extended duration of germination trials.	Potential alleviation of dormancy via stratification as the trial progresses, overinflating germination response measurements.	Limit trial length to the plausible duration of continuous seed imbibition in natural populations. This is usually poorly known and will vary seasonally so reporting temporal patterns in germination in addition to final cumulative germination is essential.
Overlooking the role of environmental cues such as light and soil moisture.	Poor germination of, and failure to identify, species sensitive to these cues, or, alternatively, overinflated germination in extended trials with continuous moisture availability.	Examine interactive effects of light and/or soil moisture availability.[g]

[a] *Baskin et al. (2006).*
[b] *Baskin and Baskin (2014).*
[c] *Vleeshouwers et al. (1995).*
[d] *Merritt et al. (2007).*
[e] *Thompson and Ooi (2010).*
[f] *Thompson and Ooi (2012).*
[g] *Thomas et al. (2010).*

this family which contains large numbers of rare and nationally threatened species (Auld, 2001).

We investigated the mechanisms of fire-driven recruitment from soil seed banks in species with PD using *Boronia*, the largest Australian Rutaceae genus, as a case study with the dual aims of addressing knowledge gaps in the ecology of this important plant family and of improving understanding of PD in fire-prone ecosystems generally. We employed a combination of laboratory-based germination trials and burial-retrieval trials in natural populations to characterize seasonal patterns in dormancy and fire-stimulated germination over a 2-year period. Our primary aims were to:

i) identify species with seasonal temperature requirements for germination,
ii) quantify seasonal changes in dormancy, and
iii) develop mechanistic models of the effect of fire seasonality on seedling emergence phenology and magnitude of species with PD.

MATERIALS AND METHODS

Study Area and Study Species

The study was undertaken in the Sydney region of south-eastern Australia, with the approval of the New South Wales Office of Environment and Heritage (Scientific License No. SL101105). The regional climate is temperate with no dry season, according to the Köppen classification (Stern et al., 2000). Rainfall is aseasonal (**Figure 2**), meaning that germination can occur year-round cf. Mediterranean-type climates where dry summers usually restrict germination to the cooler, wetter months.

Seven species of *Boronia* were selected for study on the basis of their fecundity, overlap in flowering phenology, and populations within the study region that were large enough to provide the requisite quantities of seed. The study species are all shrubs from fire-prone heaths and woodlands and comprise a mixture of obligate seeders and resprouters, rare and common species, and different evolutionary lineages (sections) within the genus (**Table 2**). Further details on their seed morphology and the

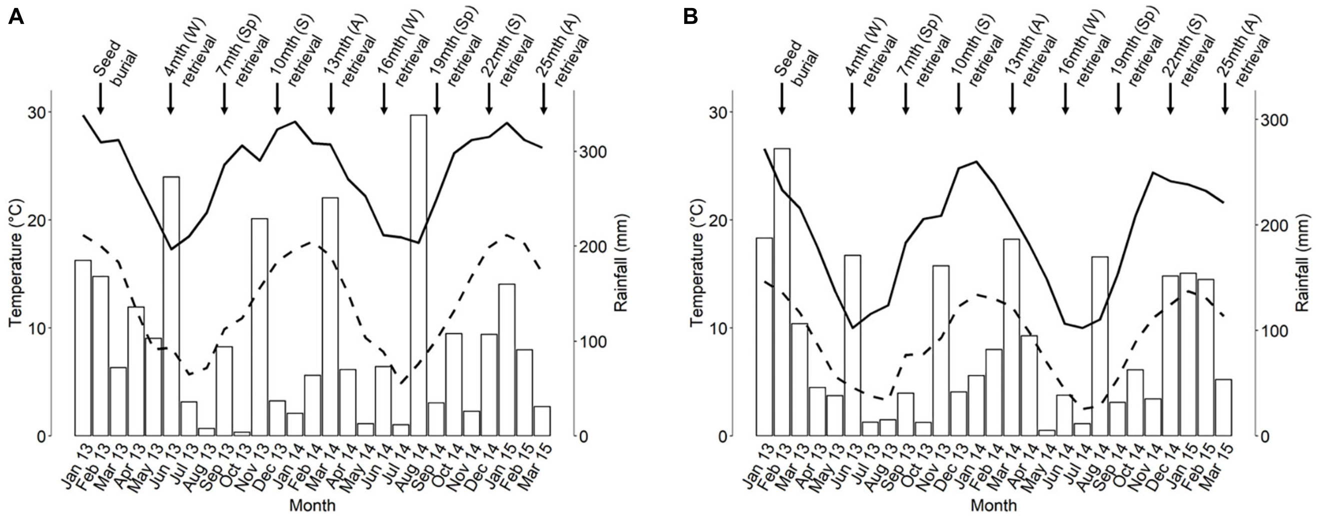

FIGURE 2 | Indicative monthly rainfall (white bars) and mean monthly maximum (solid line) and minimum (dashed line) ambient temperature at the **(A)** coastal and **(B)** upper tablelands study sites during the study period. Data are courtesy of the Australian Government Bureau of Meteorology (2021). Temperature and rainfall data in **(A)** are from the Holsworthy Aerodrome and Audley Royal National Park weather stations, respectively. All data in **(B)** are from the Mt Boyce weather station. S, summer; A, autumn; W, winter; Sp, spring.

TABLE 2 | Study species.

Species	Section[a]	Habitat[b]	Fire response	Regional significance[b]
Boronia anemonifolia subsp. *anemonifolia* A.Cunn	*Cyanothamnus*	Among rocks in open forest and heath	Resprouts[b,c] but can be variable[d]	Widespread on coast and ranges
Boronia floribunda Sieber ex Rchb.	*Boronia*	Ridgetops and rock outcrops in open forest and heath	Resprouts[b,c,d] but can be variable[d]	Local endemic
Boronia fraseri Hook.	*Valvatae*	Gullies in moist eucalypt open forest	Killed by fire[c]	Rare local endemic
Boronia ledifolia (Vent.) DC.	*Valvatae*	Ridges and rocky outcrops in woodland	Killed by fire[b,c,d]	Widespread on coast and ranges
Boronia pinnata Sm.	*Boronia*	Ridges and plateaus in eucalypt forest and heath	Resprouts[b,c,d] but can be variable[c,d]	Chiefly coastal
Boronia serrulata Sm.	*Boronia*	Rock outcrops and platforms in moist heath and woodland	Killed by fire[b,c,d]	Rare local endemic
Boronia thujona A.R. Penfold and M.B.Welch	*Boronia*	Gullies, creeks, cliff lines in moist eucalypt open forest	Killed by fire[b,c]	Northern limit of distribution

[a] *Duretto et al. (2013).*
[b] *Benson and McDougall (2001).*
[c] *NSW Office of Environment and Heritage (2014).*
[d] *B. D. E. Mackenzie (pers. obs).*

locations of the study sites are provided in Mackenzie et al. (2016).

Seed Collection and Field Burial Trials

Fruits are ballistic at maturity, so seeds were captured at dispersal using light-weight polypropylene bags tied around fruiting branches. Seeds were stored in the laboratory at ambient temperature (c. 20–25°C) for 4 weeks [*B. anemonifolia* subsp. *anemonifolia* (hereafter, *B. anemonifolia*), *B. fraseri*, *B. pinnata*, *B. serrulata*] to 7 weeks (*B. floribunda*, *B. ledifolia*, *B. thujona*) prior to the commencement of burial trials.

Replicate burial plots (three for *B. thujona* and four for all other species) were established in mid-summer (late January to early February) at the original seed collection site for each species and positioned up to 15 m apart amongst random stands of mature *Boronia* individuals. Replicate batches of seeds (25 seeds each for *B. ledifolia*; 24 seeds each for *B. thujona;* 20 seeds each for *B. anemonifolia*, *B. floribunda*, *B. fraseri* and *B. serrulata*; and 18 seeds each for *B. pinnata*) were mixed with a spoonful of local topsoil that had been finely sifted to remove any pre-existing seeds. The seed/soil mixture was placed inside 10 cm lengths of nylon stocking and sealed with a knot at each end. This enabled free movement of soil moisture and limited the volume of soil to be searched during later seed recovery. Additional protection from disturbance by animals was provided by placing bags inside durable fiberglass pockets made of 2 mm insect mesh and measuring 5 cm × 10 cm. Replicate pockets were arranged in a contiguous grid and buried 1–2 cm below the soil surface followed by reinstatement of the surface litter.

Commencing in winter (June), random samples of bags were exhumed from replicate burial plots at the beginning of each season for up to 2 years (i.e., at 4, 7, 10, 13, 16, 19, 22, and 25 months post-burial). Retrievals of *B. anemonifolia* were limited to 4, 7, 10, and 13 months due to lower seed availability. The contents of retrieved bags were air-dried in the laboratory for 1–2 weeks and then searched for seeds or seed remains. Recovered seeds were classified as "empty/unfilled," "dead/inviable," "germinated," or "ungerminated and intact," with the latter seeds subjected to germination trials.

Seasonal Germination Trials

Seasonal germination trials were conducted over a 2-year period with the primary aims of (i) measuring seasonal changes in the state of dormancy of buried seeds and (ii) predicting germination responses to fires in different seasons. Seeds buried for 4–13 months were subjected to factorial combinations of fire-associated heat (10 min exposure to 80°C), smoke (10 min exposure to aerosol smoke generated from burning vegetation), and seasonal incubation temperatures (approximating summer, autumn/spring and winter at the study sites), following the methodology of Mackenzie et al. (2016) and using the same species and seed lots. Two species with more limited seed availability (*B. pinnata* and *B. thujona*) were subjected to factorial combinations of smoke and seasonal temperatures only (**Supplementary Material 1**). Germination trials ran for 14 weeks, approximating the length of a season.

Seeds buried for 16–25 months were subjected to species-specific cue combinations that were found to maximize germination during their first year of burial (**Table 3**). Treatments representing fires in different seasons were also applied and these comprised the most effective combination of fire cues (a heat pulse and/or smoke) for each species followed by incubation at whichever seasonal temperature corresponded with field conditions at the time of retrieval.

TABLE 3 | The most effective combinations of germination cues for seven species of *Boronia* (Rutaceae) from south-eastern Australia observed during the first year of a burial trial using freshly collected seeds.

Section/Species	Cue combinations
Boronia	
B. floribunda	S + winter, HS + winter
B. pinnata	S + summer
B. serrulata	S + winter, HS + autumn/spring
B. thujona	S + summer, S + autumn/spring
Cyanothamnus	
B. anemonifolia	HS + summer
Valvatae	
B. fraseri	H + summer, HS + summer
B. ledifolia	H + summer, HS + summer

Fire cue treatments include a heat pulse (H), smoke (S), and a heat pulse plus smoke (HS). Seasons refer to seasonal incubation temperatures. Refer to main text for details.

Analytical Methods

Measuring Dormancy

As dormancy cannot be directly measured, the degree of germinability was used to infer the state of dormancy of buried seeds (Vleeshouwers et al., 1995). Seeds that germinate are, by definition, in a non-dormant state and fire-associated heat and smoke do not break PD—they only stimulate germination once dormancy has been alleviated (Baker et al., 2005b,c; Merritt et al., 2007; Thompson and Ooi, 2010; Mackenzie et al., 2016). Hence, at each seasonal retrieval, a *minimum* estimate of the proportion of seeds in a non-dormant state was inferred from the *maximum* germination response observed across all treatments (**Figure 3**). This approach was robust to an incubator failure at 13 months which limited data for *B. fraseri* and *B. ledifolia* (see **Supplementary Material 2**).

Measuring Seasonal Responses to Fire Cues

Following each seasonal retrieval of buried seeds, the maximum germination observed in response to any combination of fire cues at the incubation temperature corresponding with field conditions at the time of retrieval was used to infer the likely response of seeds *in situ* to a fire occurring in that season (**Figures 2**, **3**). Clear differences in responses between seasons, particularly significant germination vs. no germination, did not require inferential statistics.

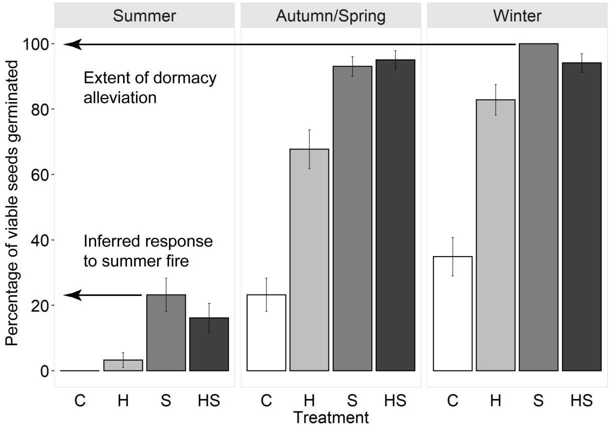

FIGURE 3 | Illustration of how seasonal changes in the state of dormancy and germination responses to fires in different seasons were inferred in the present study. In this example, seeds of *Boronia floribunda* were exhumed in early summer after 10 months' burial and were subjected to factorial combinations of fire cues (C, control; H, heat pulse; S, smoke; HS, combined heat pulse plus smoke treatment) and incubated at one of three seasonal temperatures (top bar: summer, autumn/spring, winter). The minimum percentage of seeds in a non-dormant state after 10 months' burial was inferred from the maximum germination response observed across all treatments (here, 100% after 6 weeks' incubation). The inferred germination response to a summer fire *in situ* was taken as the maximum germination observed in response to any combination of fire cues at summer incubation temperatures (here, c. 24% after 6 weeks' incubation).

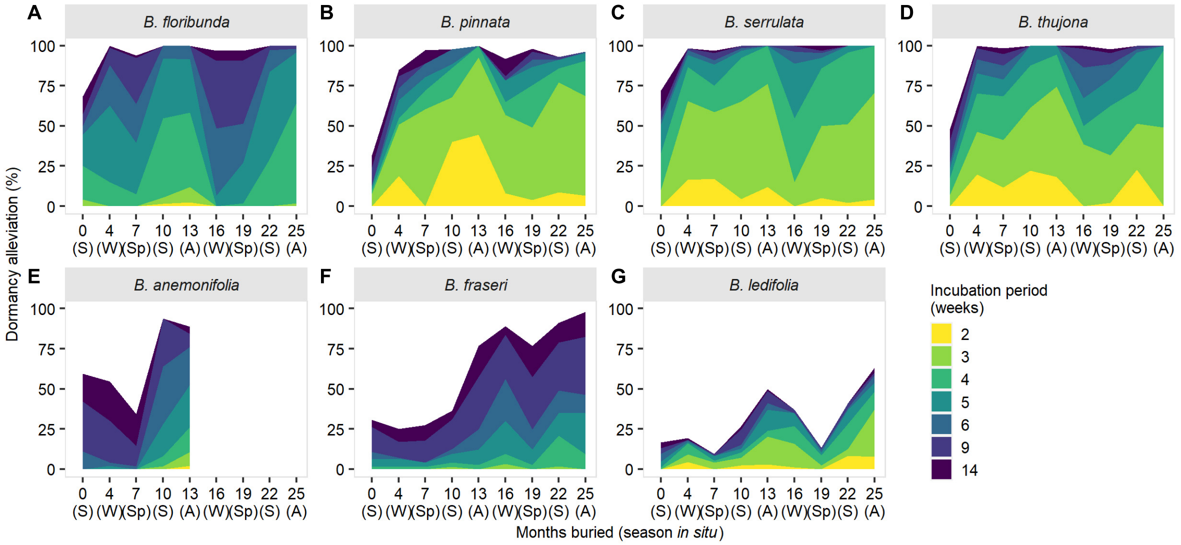

FIGURE 4 | Seasonal changes in dormancy (as measured by maximum germination response) and the effect of incubation period for seven species of *Boronia* (Rutaceae) from south-eastern Australia. S, summer; A, autumn; W, winter; Sp, spring. Species are arranged by sections within the genus: **(A–D)** *Boronia*; **(E)** *Cyanothamnus*; **(F,G)** *Valvatae*. *Boronia anemonifolia* was studied for 1 year only due to limited seed availability. Data for *B. fraseri* and *B. ledifolia* at 13 months have been imputed (refer to **Supplementary Material 2** for details).

RESULTS

Seasonal Patterns in Dormancy

Seasonal patterns in dormancy were evident in all seven species and broad patterns were reasonably consistent within sections in the genus (**Figure 4**). Dormancy estimation was markedly affected by incubation period—a proxy for the duration of sufficient soil moisture for imbibition and germination *in situ*—with longer incubation promoting greater loss of dormancy due to stratification effects (**Figure 4**). Seasonal patterns in dormancy were persistent (i.e., independent of incubation period) in sections *Cyanothamnus* and *Valvatae* (**Figures 4E–G**) but became less pronounced or disappeared altogether in section *Boronia* after 6–14 weeks' incubation (**Figures 4A–D**).

Primary dormancy was highest in sections *Valvatae* and *Cyanothamnus*, and variable in section *Boronia*. Dormancy loss was fastest and greatest in section *Boronia* (substantial alleviation within 4 months of burial/the first winter in the seed bank) while dormancy remained high in other species until the second summer (10 months' burial) or autumn (13 months' burial) (**Figure 4**). Dormancy was almost entirely (94–100%) overcome for all species at some point during the 2-year study; however, *B. ledifolia* proved an exception with a maximum detectable dormancy loss of 64% (**Figure 4**).

Section *Boronia* species were characterized by pronounced reductions in dormancy alleviation in winter or winter-spring (at least in the second in the year of burial) with peak dormancy loss (maximum receptivity to germination cues) in spring-summer (**Figures 4A–D**). These reductions coincided with spring in section *Valvatae* species with peak dormancy alleviation in winter and summer (*B. fraseri*) and autumn (*B. ledifolia*) (**Figures 4F,G**). A similar pattern of peak receptivity was apparent in *B. anemonifolia* (**Figure 4E**); however, more than 1 year of data is required to confirm if dormancy is cyclical in this species.

Seasonal Patterns in Responses to Fire Cues

Seasonal fire treatments had a profound effect on the timing and magnitude of germination in all seven species (**Figure 5**). As per dormancy estimation, seasonal patterns in fire-stimulated germination were highly influenced by incubation period—and, by inference, the duration of soil moisture availability *in situ*—with longer wet intervals leading to marked increases in germination for all species, but not across all seasons for every species. Three section *Boronia* species (*B. pinnata*, *B. serrulata*, *B. thujona*) were able to germinate to appreciable levels (≥50%) in response to treatments representative of a fire within 3 weeks of incubation in at least one season (**Figures 5D,G,J**), while other species required longer periods of imbibition to respond.

High levels of dormancy limited germination of some species in certain seasons (e.g., **Figures 5J,N,Q,U**). However, dormancy loss did not always equate to germination (e.g., **Figures 5B,D,O,R**), with seasonal temperature requirements, or temperature-dependent germination speed (seasonal temperature preferences *sensu* Mackenzie et al., 2016), constraining the timing and magnitude of the response.

An obligate germination requirement for summer temperatures precluded germination of *B. anemonifolia* and *B. fraseri* in response to treatments representative of fire in autumn, winter, and spring, regardless of the extent of dormancy alleviation (**Figures 5M–R**). Other species were able to germinate in response to fire treatments across a broader range of seasons, with the seasonal range and magnitude of responses increasing with the duration of incubation.

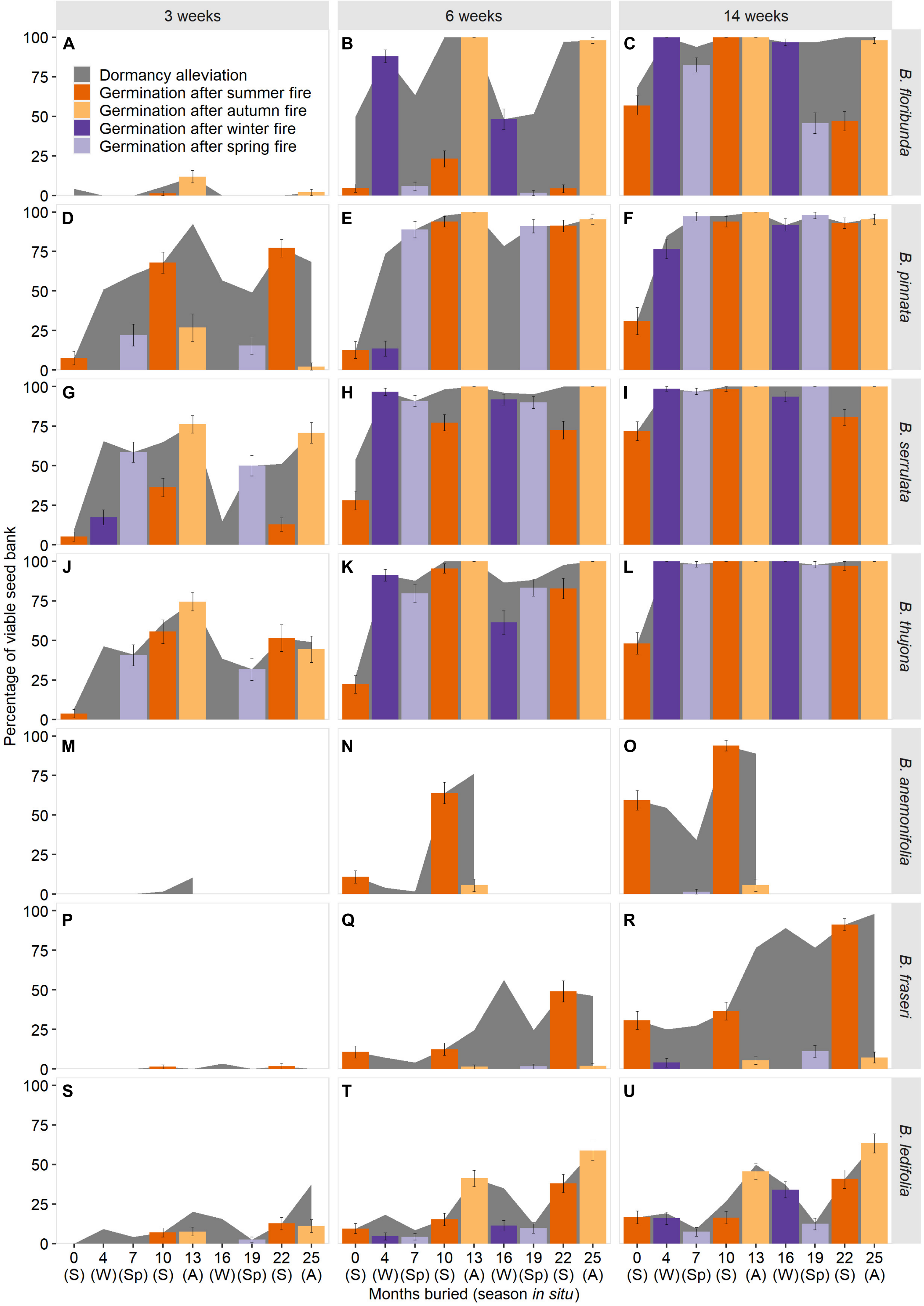

FIGURE 5 | Predicted germination in response to fires in different seasons for seven species of *Boronia* (Rutaceae) from south-eastern Australia as function of incubation period. S, summer; A, autumn; W, winter; Sp, spring. Background shading indicates the proportion of the viable seed bank in a non-dormant or conditionally dormant state and able to respond to combinations of seasonal temperatures and fire-related germination cues. Species are arranged by sections within the genus: *Boronia* **(A–L)**; *Cyanothamnus* **(M–O)**; and *Valvatae* **(P–U)**. *Boronia anemonifolia* was studied for 1 year only due to limited seed availability. Dormancy estimates for *B. fraseri* and *B. ledifolia* at 13 months have been imputed (refer to **Supplementary Material 2** for details).

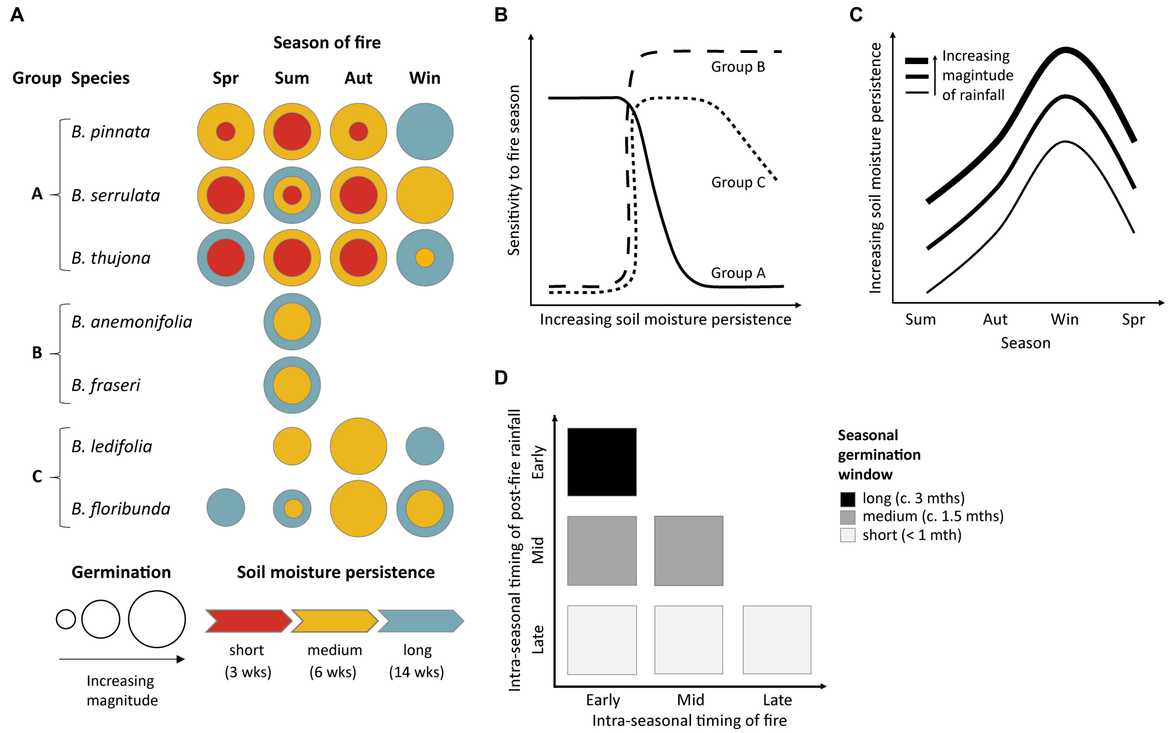

FIGURE 6 | Conceptual models of how the seasonal timing of fire and soil moisture availability affect germination of seven species of *Boronia* (Rutaceae) from south-eastern Australia. **(A)** Functional response groups defined by predicted effects of fire seasonality and soil moisture persistence on germination timing and magnitude. Spr, spring; Sum, summer; Aut, autumn; Win, winter; blank spaces – no germination response with 14 weeks. Where longer durations did not increase germination only the color for the shorter duration is presented. **(B)** Sensitivity to fire seasonality as a function of soil moisture persistence for the response groups identified in **(A)**. **(C)** Seasonal variation in the availability of soil moisture for germination and the effect of rainfall magnitude. Soil moisture persistence increases with greater precipitation (different curves) and is inversely related to evapotranspiration which increases in warmer months (**Supplementary Material 3**). **(D)** Influence of within-season timing of fire and subsequent rainfall on the relative length of the germination window in the season of fire occurrence.

Over shorter periods, non-trivial germination of *B. ledifolia* was restricted to treatments representing autumn fires and—in the second year of burial—a summer fire (**Figures 5S,T**). Extended incubation increased the response to treatments representing winter fires treatments (a function of temperature-dependent germination rates; germination of *B. ledifolia* is slowest at winter temperatures but can occur given adequate time); however, treatments representing spring fires continued to result in negligible germination due to high seasonal dormancy (**Figure 5U**). In section *Boronia*, longer incubation increased germination responses to a broader range of seasonal fire treatments through a combination of increased dormancy alleviation (slower in *B. floribunda* than the other three species) and greater time available for slower germination at certain seasonal temperatures to occur (i.e., cooler seasons for *B. pinnata* and warmer seasons for the remaining species; **Figures 5A–L**).

Conceptual Model of Seasonal Germination

A conceptual model (**Figure 6**) illustrates the importance of both the seasonal timing of fire and soil moisture availability in expected post-fire germination patterns. The study species can be divided into three broad functional response groups based on their germination speed and predicted emergence patterns (**Figure 6A**). Species with slower germination and more restricted seasonal temperature requirements are likely to be more sensitive to fires in different seasons (**Figure 6B**, Groups B and C), although seasonal differences in germination are expected to decrease with increasing availability of soil moisture for species capable of germination over broader seasonal ranges (**Figures 6A,B**, Groups A and C). Interactions between seasonal differences in soil moisture persistence and temperature-dependent germination speed are expected to reduce germination of some species following fires in warmer seasons due to increasingly transient soil moisture (**Figure 6C**; see also **Supplementary Material 3**). We term this phenomenon "germination interval squeeze." Mid- to late-season fires and/or lags in post-fire rainfall further contribute to germination interval squeeze by significantly shortening the potential window of post-fire imbibition and germination within the season of fire occurrence (**Figure 6D**). Slower-to-germinate species and/or those with narrower seasonal requirements are most sensitive to this type of interval squeeze—especially those with obligate germination requirements for summer temperatures (**Figure 6A**, Group B) where late summer fires and/or delayed rainfall may preclude germination for up to 9–10 months until the following summer.

DISCUSSION

Key Drivers of Seedling Emergence Phenology and Magnitude

Seasonal temperatures play an important role in the germination of all seven species investigated, restricting germination of some species to a single season, and concentrating germination of others in warmer or cooler parts of the year. Our models indicate that the timing and magnitude of seedling emergence in natural systems are mediated by seasonal dormancy cycling and seasonal temperature cues, and their interactions with the seasonal timing of fire and soil moisture availability. Fire seasonality effects on emergence patterns vary in their strength and direction, even between sympatric congeners, and are strongly influenced by soil moisture availability (both the timing of availability in relation to fire and the duration that moist soils persist) which is expected to vary seasonally. This greatly increases the stochasticity of seedling recruitment of PD species in these ecosystems due to natural variability in post-fire rainfall and fire ignitions, with climate change and increasing anthropogenic ignitions introducing further complexity and variation via impacts on rainfall patterns and shifting fire seasonality.

Fire Seasonality Effects

Delayed or reduced germination is expected to occur following fires in one or more seasons for all seven species due to mismatches between their dormancy and germination phenology and the seasonal timing of fire. Effects are likely to be exacerbated where soil moisture availability is short-lived and/or delayed post-fire ("germination interval squeeze"; **Figure 6**). Slow-to-germinate species with narrow seasonal tolerances are the most vulnerable to potential impacts of altered fire seasonality on emergence patterns, especially where germination is cued to warmer months where soil moisture is more limiting. Obligate seeders such as the rare *B. fraseri* are most at risk due to reliance on post-fire seedling recruitment for population recovery and persistence. However, resprouters with variable capacity for post-fire vegetative recovery such as *B. anemonifolia* are also susceptible.

Altered fire seasonality, which we define for the study region as fires outside late spring to summer, is predicted to have varied effects on PD species. For the majority of species, unseasonal fires in one or more seasons are expected to delay and/or diminish germination relative to summer fires (**Figures 5**, **6A**). However, our models suggest that certain out-of-season fires may actually reduce the time to emergence for some species. For example, fires outside of summer may increase and accelerate germination of *B. serrulata*; autumn fires may increase and accelerate germination of *B. ledifolia*; and germination of *B. floribunda* may be accelerated by autumn and winter fires. Given that Miller et al. (2019) found no positive demographic effects of altered fire seasonality in their global review, it will be of interest and significance to note whether or not faster emergence of these species following certain unseasonal fires (if it occurs under field conditions as predicted) does in fact lead to increased recruitment success.

Earlier emergence is regarded as advantageous in non-fire prone ecosystems (Verdú and Traveset, 2005). However, there is a relative paucity of quantitative data from fire-prone ecosystems in regions with aseasonal rainfall concerning the consequences of variation in post-fire emergence timing (on a scale of days and weeks to months and/or years) for successful seedling establishment and subsequent plant performance—especially for geosporous species. Nonetheless, late germinants are expected to be disadvantaged if they miss the peak post-fire resource flush associated with ash deposition, or if they are exposed to competition from more rapidly established vegetative and seedling regenerators.

Finally, the effect of increasing time since fire on the stimulatory efficacy of fire-associated heat and smoke on germination of PD species has received little research attention. Where post-fire germination is delayed due to inappropriate seasonal temperatures and/or delayed post-fire rainfall, germination magnitude might be unaffected, diminished, or completely nullified depending on the extent of the delay. The mechanism by which fire-associated heat promotes germination of PD species remains unknown (Mackenzie et al., 2016); however, the active constituents in smoke are water-soluble (Flematti et al., 2004) and, over time, will presumably be leached out of the topsoil where most of the seed bank resides (although Preston and Baldwin (1999) suggest the smoke cue can persist in soil for 7 years or longer). Delayed post-fire rainfall may delay exposure to the smoke cue relative to the heat pulse cue (the latter being coupled with fire passage) which could be important for species that require both cues to germinate. Conversely, smoke may leach into the soil in gaseous form immediately following fire and later in aqueous form via rainfall, and where rainfall is aseasonal as in our study region, delays between fire passage and post-fire rainfall will most often be minimal, even following unseasonal fires. Nevertheless, the effect of time since fire on fire-cue efficacy warrants further investigation in the study of fire seasonality effects on seedling emergence patterns in PD species.

Implications for Fire Management and Plant Conservation

As well as mismatches between seasonal occurrence of fire and germination phenology, fire seasonality may also influence the magnitude of post-fire seedling emergence via seasonal trends in soil moisture and fire severity. Higher moisture content in seeds lowers their lethal temperature thresholds (Tangney et al., 2018); hence, fires occurring in cooler seasons where soil moisture tends to be higher might be expected to increase seed mortality and reduce seedling emergence (Le Fer and Parker, 2005), depending on fire severity. Furthermore, cool-season fires tend to have lower fire severity and hence, lower depth and duration of soil heating due to reduced consumption of fine fuels (Bradstock and Auld, 1995). Dissipation of thermal energy by soil moisture (Stoof et al., 2011) is also generally greater in cooler seasons. This is especially relevant to species where a heat pulse is an obligate germination requirement (e.g., *B. fraseri* and *B. ledifolia* require a heat pulse in combination with smoke) or is required to maximize germination (e.g., *B. anemonifolia*). Contrary to its well-established role in the germination of hard-seeded (physically dormant) species (Jeffrey et al., 1988; Auld and O'Connell, 1991; Keeley, 1991; Reyes and Trabaud, 2009), fire-associated heat has been largely overlooked as a germination cue in PD species due to a focus on the widespread stimulatory effects of smoke (Dixon et al., 1995; Brown and Botha, 2004; Tormo et al., 2014). However, positive responses to heat pulses, including interactions between heat and smoke, have been reported in PD species across a wide range of plant families (see Introduction; reviewed by Mackenzie et al., 2016). Hence, the degree of soil heating during fires may influence recruitment of many PD species and fire severity is thus an important consideration in fire management for their conservation.

Improving Understanding of *in situ* Germination

Reporting temporal patterns in germination is a key way in which to improve the ecological utility of laboratory-based germination studies. Germination responses are routinely censused at multiple timepoints over the course of a trial yet the majority of studies only present final total cumulative germination after a given number of weeks or months of continuous imbibition and incubation. This is sufficient where the primary aim is on maximizing germination (e.g., for horticultural or *ex situ* conservation/restoration purposes). However, for ecological studies that seek to understand and predict species responses *in situ*, data on temporal patterns—including the time to onset of germination and subsequent germination speed and synchrony—are crucial given that moisture and temperature conditions amenable to germination are temporally limited. As demonstrated here, the duration of imbibition has a profound effect on dormancy and germination responses, and studies with an ecological focus should ensure that the length of germination trials and the incubation periods for which results are reported are ecologically plausible, i.e., reflect the intervals and temperatures over which seeds in the upper layers of the soil are likely to remain continuously imbibed in the post-fire environment. Three months seems an appropriate maximum for most temperate fire-prone ecosystems; however, continuous periods of seed imbibition are likely to be much shorter than this in the absence of high or extended rainfall events, especially in warmer seasons. Further studies quantifying *in situ* variation in soil moisture availability in different seasons (e.g., Merritt et al., 2007) across a range of habitat types, and the water potentials across which germination can occur (e.g., Thomas et al., 2010), would greatly improve extrapolation of laboratory-based germination studies to natural populations.

Greater use of seeds aged naturally *in situ* in experimental studies would also significantly improve ecological understanding. Artificial storage may confound natural patterns in seed dormancy and germination responses in unpredictable ways (Baskin et al., 2006), precluding reliable inferences about the responses of soil seed banks *in situ*. Where primary dormancy is low, the responses of fresh seeds to combinations of fire and seasonal temperature cues may help to identify PD species with seasonally sensitive germination. However, as demonstrated here, fresh seed responses are of limited use in predicting the responses of buried seeds due to the effects of burial duration (including seed age) and seasonal cycles in dormancy and germination responses.

Burial trials over long durations are required to accurately characterize soil seed bank dynamics. We followed single cohorts of seeds for 2 years and observed variation in dormancy and germination responses to certain cue combinations between years in some species (**Figures 4**, **5**). A longer experiment could have provided further insights. The distribution of seed ages in the seed bank is unknown for these species but older seeds might be expected to exhibit more stable annual patterns, among other differences. Studies of shorter duration may also be more prone to stochastic events including temperature or rainfall aberrations due to heatwaves, droughts, and/or extreme rainfall events.

CONCLUSION

This study has highlighted seasonal temperature requirements and seasonal patterns in dormancy cycling and moisture availability as key drivers of fire-stimulated germination of PD species in fire-prone ecosystems. The mechanistic models proposed here predict significant effects of fire seasonality, fire severity, and soil moisture duration on post-fire emergence patterns, including the increasing risk of "germination interval squeeze," and call for a more sophisticated and wholistic approach to fire management that explicitly addresses these fire regime and environmental components in addition to the effects of fire frequency (Bradstock and Kenny, 2003). This is urgently required given strong evidence from across the globe of shifting and broadening fire seasonality and reduced seed banks due to interval squeeze (Enright et al., 2015).

The models presented here require field validation and the demographic consequences of any realized delays or reductions in seedling emergence following out-of-season fires need to be evaluated in terms of growth, fecundity, and longevity of recruits. Evidence of adverse effects of delayed emergence on post-fire seedling establishment following unseasonal fires is beginning to emerge (Risberg and Granström, 2009; Ooi, 2010) but is currently scarce. Field studies of post-fire recruitment involving replicated fires across a range of seasons and sites are vital to establish the magnitude of the threat and the extent to which different species (especially rare or threatened taxa), plant families and functional groups are resilient. In the meantime, a precautionary approach to fire management that limits the occurrence of (successive) out-of-season fires is likely to benefit the greatest diversity of species.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

BM conducted the field and experimental work, analyzed the data, and led the writing of the manuscript. All authors conceived the ideas, designed the research, contributed critically to the drafts, and gave final approval for publication.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2021.795711/full#supplementary-material

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Experimental Warming Hastens Physical Dormancy Break and Germination in Tropical Fabaceae

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Climate warming may threaten the germination strategies of many plants that are uniquely adapted to today's climate. For instance, species that employ physical dormancy (PY) – the production of seeds that are impermeable to water until high temperatures break them, consequently synchronizing germination with favorable growing conditions – may find that their seeds germinate during unfavorable or potentially fatal periods if threshold temperatures are reached earlier in the year. To explore this, we subjected the seeds of five species with physical dormancy (from the genera *Abrus*, *Bauhinia*, *Cassia*, *Albizia*, and *Acacia*) to "mild" (+2°C) and "extreme" (+4°C) future warming scenarios and documented their germination over 2 years relative to a control treatment. Under current climatic conditions, a proportion of seeds from all five species remained dormant in the soil for 2 years. A mild warming of 2°C had little to no effect on the germination of four of the five study species. Contrastingly, an extreme warming of 4°C dramatically increased germination in all five species within the first year, indicating a reduction in their ability to persist in the soil long-term. *Cassia fistula* was particularly susceptible to warming, exhibiting a similar increase in germination under both mild and extreme warming relative to control. Our findings suggest that climate warming in the tropics may cause the seeds of species that rely on physical dormancy to stagger the risk of unsuccessful germination across years to leave soil seed banks prematurely – the long-term implications of which remain unknown.

Keywords: climate change, germination ecology, impermeable seed coat, soil seed banks, soil temperature

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INTRODUCTION

The life cycle of plants is intricately linked to the climate. Climate change, therefore, may threaten life history strategies that have arisen over evolutionary timescales. Understanding how plant communities will respond to anthropogenic climate change has become an integral part of ecological research (Warren et al., 2013). Seeds are particularly useful in this endeavor as they enable us to evaluate changes in vegetation at the community level (Walck et al., 2011). Many species have evolved dormancy mechanisms that regulate when germination takes place – an adaptation that enables plants to inhabit environments with volatile climates (Finch-Savage and Leubner-Metzger, 2006; Willis et al., 2014). However, the warming of Earth's climate could cause seeds to germinate at unfavorable times for seedling establishment, potentially leading to local extinctions. Unfortunately, our understanding of how a warmer climate will affect the germination ecology of species with dormant seeds is hampered by a lack of long-term studies.

Seed dormancy can be advantageous in the tropics, where synchronizing germination with the rainy season ensures seedlings are well established before the onset of the harsh dry season (Garwood, 1983; Khurana and Singh, 2001). Tropical plants often avoid dry season germination by either producing dormant seeds or dispersing their seeds in the wet season (Sautu et al., 2007; Salazar et al., 2011; Ramos et al., 2017; Escobar et al., 2018). Thus a trade-off exists, whereby species that disperse seeds during the wet season tend to be non-dormant, germinating immediately following dispersal, while species that disperse their seeds in the dry season tend to be dormant, germinating only when conditions become favorable again in the wet season (Salazar et al., 2011; de Souza et al., 2020). Of the latter, various strategies are used to achieve seed dormancy.

Physical dormancy (PY), caused by a seed/fruit coat that is impermeable to water in some genera of only 18 contemporary angiosperm families is found in 25% of all flowering plants (Baskin and Baskin, 2004; Hudson et al., 2015). It is the second most common class of dormancy after physiological dormancy (PD) and is achieved late in seed development when seeds dry below threshold moisture content (Barrett-Lennard and Gladstones, 1964; Geneve, 2009; Jaganathan, 2016). Impermeable seeds persist in the soil until specific environmental conditions stimulate dormancy-break by opening structures on the seed coat known as “water-gaps” through which water enters and initiates germination (Van Assche et al., 2003; Baskin and Baskin, 2004). PY is typically broken by temperature fluctuations prior to the growing season (Cook et al., 2008; Jayasuriya et al., 2009; Rodrigues-Junior et al., 2018), however, it can also be broken by erratic events such as fire (Jaganathan, 2015) or passage through an animal gut (Jaganathan et al., 2016; Milotić and Hoffmann, 2016). PY can benefit plant fitness not only by synchronizing germination with favorable growing conditions (Baskin et al., 2000), but also by establishing a long-term soil seed bank from which only a portion germinate each year, spreading germination risk across years (Baskin and Baskin, 2014), and protecting seeds against pathogens, soil microbes (Dalling et al., 2011), and seed predators (Paulsen et al., 2013).

While global mean annual temperature is predicted certainly to rise, e.g., 4°C by 2100 (Warren et al., 2013), consequences this change will have for species that employ seed dormancy remain poorly understood. Much of our knowledge of how a warmer climate affects seed dynamics comes from studies conducted in alpine ecosystems (Mondoni et al., 2012; Hoyle et al., 2013); presumably, because they are disproportionately vulnerable to changes in temperature. Tropical ecosystems, on the other hand, have received comparatively less attention (Perez et al., 2016; Stroud and Feeley, 2017). This is partly due to disagreement over the extent to which temperature will rise in the tropics, with predictions ranging widely from 0.26 to 5°C (Corlett, 2012). Regardless of extent, consensus is emerging that warming in the tropics could have serious consequences for plant life (Feeley and Silman, 2010). Soil temperature acts as a bottleneck that controls, not only when seeds break dormancy, but also the proportion of seeds germinating each year. Climate warming may therefore alter the soil environment in which the seeds are present following dispersal. This may lead to increased mortality *via* the excess evaporation of soil water (Ooi, 2012) or the stimulation of dormancy-break (and consequently germination) at unfavorable times, such that seedlings die before establishing (Walck et al., 2011).

Seeds banks are vital to species that inhabit ecosystems with unpredictable climates or short growing seasons (Long et al., 2015; Jaganathan et al., 2019). They can enable species to persist locally, even when seemingly all plant life has been destroyed (Jaganathan et al., 2015). However, while it is becoming increasingly apparent that warming temperatures may significantly affect the regeneration ecology of species with seed dormancy (Orru et al., 2012; Newton et al., 2020), responses appear to be largely species-specific (Seal et al., 2017). Further, despite efforts to understand such effects for species that employ PY (Hudson et al., 2015; Cochrane, 2017), to date, no such study has been done in the tropics. To close this gap, we performed a long-term warming experiment to investigate how a warmer climate will affect the germination ecology of five tropical species with PY. Specifically, we asked:

1. How will climate warming in the tropics affect the germination ecology of species with PY?
2. Do effects differ between mild and extreme warming scenarios?
3. Are responses to climate warming species-specific?

MATERIALS AND METHODS

Study Site

This study was conducted in the Western Ghats of southern India, which is one of the 100 biodiversity hot-spots identified by Myers et al. (2000). The Western Ghats, a discontinuous chain of mountains located on the western side of the Indian peninsular, covers an area of 140,000 km^2 that extends from the Tapti river valley in Gujarat to Kanyakumari in Tamil Nadu. The mountain range is approximately 1,600 km long (Nayar et al., 2014) and has some 8,080 species of flowering plants, of which 1,273 are endemic. The Indian Ministry of Environment and Forestry (MoEF) has estimated that the temperature in the Western Ghats has increased by 1.7–1.8°C since the 1970’s and predicts a further increase of 3–4°C before the end of this century (Sharma and Chauhan, 2011). Our understanding of how this warming will affect the Western Ghats region is hindered by the fact that many of the native plant species are long-lived and are therefore slow to exhibit any changes.

Study Species and Seed Collection

We selected the following five species namely *Abrus precatorius*, *Bauhinia tomentosa*, *Cassia fistula*, *Albizia lebbeck*, and *Acacia* *chundra*, that occur abundantly in the Western Ghats of India but are also distributed across other tropical and sub-tropical regions (see **Table 1**). We selected these species because of their abundance and wide distribution. Understanding the impacts of climate warming on the germination ecology of these species can therefore help inform conservation strategies for not only these five species but also related or ecologically similar taxa. Furthermore, prolific species can serve as models to estimate how other endangered species might be affected.

Seeds of each of the five species were collected directly from 16 (in the case of *Cassia fisula*) to 23 (in the case of *A. precatorius*) individuals during their natural dispersal period in 2017. To ensure fully matured and naturally dispersed seeds were collected, branches were covered with plastic bags containing holes (for air and moisture exchange) that were fixed with nylon thread and left for 11–15 days (**Table 1**). Seeds that fell naturally into the bags were pooled and brought back to the lab on the same day and cleaned and stored in glass jars at room temperature (20 ± 1°C; 50–60% RH) for 2 days before use in experiments. Seeds were extracted from pods by tearing them open by hand or with the aid of a scalpel. Seeds were visually inspected and insect-infected seeds were discarded.

Moisture Content and Imbibition Testing

The moisture content of seeds of all five species was calculated by using three replicates of 15 seeds each and drying them at 103°C for 17 h (ISTA, 2013). The difference in fresh and dry weight is expressed on a percentage of fresh weight basis.

To determine the proportion of permeable and impermeable seeds in the seed lots, we carried out an imbibition test. Seeds that absorbed water were identified as non-dormant (because those with impermeable coats would not absorb water) and excluded from further studies. For imbibition test, seeds from all five species were placed on wet tissue in large plastic trays (60 cm × 50 cm × 12 cm, l × w × h) with perforated lids to minimize water evaporation yet allow gas exchange. Seeds were allowed to imbibe at 20 ± 1°C. Additional water was added when necessary. Seeds that swelled or germinated during the 28-day period were excluded from further analysis. Following the imbibition period, seeds that remained impermeable were dried on cotton towels at room temperature for 48 h and stored in glass jars at room temperature as described above for freshly collected seeds. Experiments with these seeds began within 1 week after storing.

Soil Temperature Measurements

Soil temperature at three locations within the natural dispersal shadow of mother plants was recorded using data-loggers (Rotronic, United Kingdom) for 2 years from 23 January 2017 to 23 January 2019 (see **Table 1** for specific seed collection location). Thermocouples were placed at a depth of 2–4 cm, and temperature was measured at 1-h intervals. The mean highest, mean lowest, and median temperatures for each week were calculated.

Mimicking Current and Future Warming Scenarios

We subjected seeds to three temperature regimes: current temperature (control), mild warming (+2°C), and extreme warming (+4°C). In our control treatment, soil temperatures recorded at the study site were replicated in a temperature control cabinet (Macro Scientific Works Pvt. Ltd., India) in the laboratory (precision ± 0.1°C). Rainfall patterns are also expected to change in the future. Water availability likely influences seed persistence, for example, by breaking dormancy when warm days follow heavy rainfall (i.e., wet heat; Van Klinken and Flack, 2005). However, accounting for rainfall dynamics in germination studies is problematic for several reasons. Firstly, seasonal shifts in rainfall are difficult to predict and are not yet available for our study

TABLE 1 | The sub-family, collection date, location, moisture content at the time of collection (average ± S.D.), percentage of permeable seeds, and distribution and life form of the five legume species studied.

Species	Sub-family	Collection date	Location	Moisture content (%)	% of permeable seeds at the time of collection	Distribution	Life form
Abrus precatorius	Papilionoideae	10/01/2017	11° 5' 46.4712" N 76° 45' 39.2688" E	7.57 ± 3.02	20	Temperate, tropical	Climber
Bauhinia tomentosa	Caesalpinioideae	22/01/2017	11° 5' 34.3428" N 76° 45' 39.2688" E	8.86 ± 0.99	31	Mozamique, Zimbabwe, India and Sri Lanka	Tree
Cassia fistula	Caesalpinioideae	28/01/2017	11° 5' 37.9212" N 76° 46' 27.2352" E	6.63 ± 1.82	23	Indian sub-continent	Tree
Albizia lebbeck	Mimosoideae	04/02/2017	11° 5' 34.2852" N 76° 45' 19.2852" E	5.86 ± 1.29	4	Tropical, sub-tropical	Tree
Acacia chundra	Mimosoideae	06/02/2017	11° 4' 44.5548" N 76° 46' 8.7024" E	9.06 ± 0.86	8	Indian sub-continent	Tree

site. Secondly, it is not known how long soils might retain moisture at elevated temperatures. In this study, we replicated the MoEF's suggestion on rainfall changes published for Western Ghats (Rajendran et al., 2012).

Seeds were sprayed with water whenever rain fell at the collection site. The amount of water sprayed was determined by the amount of water captured by "miniature-plots" – plastic-boxes identical in size to those used in laboratory examination – filled with soil and placed on the ground. We then determined the moisture content of the soil samples and sprayed water in the trays containing seeds. In this way, our experiments enabled seeds to experience rainfall regimes akin to those occurring in the field.

Three replicates of 50 seeds per species were placed in nylon mesh bags (22 cm × 13 cm) and buried 2–4 cm deep in plastic trays (70 cm × 45 cm × 30 cm; l × w × h) filled with natural soil (sieved to remove debris). Twelve trays per species were prepared, each containing three nylon bags with 50 seeds each. Four trays per species were assigned to each temperature regime in germination chambers. For a 24-h cycle of the "current climate" chamber, we used 12 h average low temperature (7 P.M.–7 A.M.) followed by 4 h of median temperature (7 A.M.–11 A.M.) followed by 4 h of average highest temperature (11 A.M.–3 P.M.) and finally 4 h of median temperature (3 P.M.–7 P.M.). In the mild and extreme warming treatments, a similar cycle was applied, however, all temperatures were set 2 and 4°C higher, respectively. Light was provided from 7 A.M. to 7 P.M. at an intensity of 25 and 40 μmol m^{-2} s^{-1} synchronized with the median and highest average temperatures, respectively. Temperatures were adjusted weekly in accordance with data obtained from the field. After 6, 12, 18, and 24 months, one tray per species per treatment was removed, and seeds in the bags were examined. Seeds that had germinated inside the bag were counted. Germination percentage was calculated using the number of germinated seeds upon initial examination of each tray.

Statistical Analyses

To test the effects of retrieval time, temperature regime and species on germination percentage, we performed a General Linear Model (GLM) with germination percentage as the dependent variable and retrieval time, temperature regime, and species as predictor variables. Germination percentage was converted to proportion by multiplying 0.01 and then subject to arcsine-transformation to promote normality. We used reverse stepwise model selection, whereby interactions between all predictor variables were included in the initial model and then removed in a stepwise procedure, using the lowest Akaike's information criterion (AICc for small sample sizes) to determine the "best model" (i.e., the most parsimonious). All statistical analyses were performed in the R environment (v. 3.6.0, R Core Team, 2020), and GLMs were performed using the "lme4" package (Bates et al., 2012).

RESULTS

The moisture content of the seeds of each species ranged from 5.86% for *A. lebbeck* to 9.06% for *A. chundra* (**Table 1**). Impermeable seeds at the time of collection varied from 4% for *A. lebbeck* to 31% for *B. tomentosa* (**Table 1**). Permeable seeds of most of the species swelled and germinated within 10 days after being placed on a moist substrate, although a few seeds of *A. lebbeck* did not absorb water until after 10 days. Soil temperatures peaked during the summer months (May–July) and were lowest during the winter months (Dec–Feb). Mean soil temperature ranged from 63 to 19°C (**Figure 1**).

Overall Effects of Retrieval Time, Temperature Regime, and Species

In the most parsimonious generalized linear model ($\sin^{-1}\sqrt{[0.01 \times \text{germination percentage}]} \sim \text{retrieval time} \times \text{temperature regime} + \text{species}$), an interaction between the fixed factors "retrieval time" and "temperature regime" remained, while interactions of either factor with species did not (**Table 2**). Percent germination varied as a function of retrieval time ($T = 2.319$, $p = 0.024$, **Figure 2**). The effect of temperature regime was primarily seen in the significant interaction between retrieval time and temperature regime ($T = 3.451$, $p < 0.001$), indicating that the relationship between retrieval time and germination differs across temperature regimes. That is, the relationship between germination percentage and retrieval time is steeper in the warming treatments relative to control (i.e., seeds left the soil seed bank earlier, **Figure 2**). This relationship was also identified as significantly different in *C. fistula* ($T = 2.780$, $p = 0.007$). Curiously, *A. lebbeck* exhibited lower germination percentage relative to control in the mild warming treatment after 24 months, however, differences in the responses to control and mild warming in this species were not statistically significant. *Cassia fistula*, on the other hand, exhibited similarly increased germination percentages relative to control in both the mild and extreme warming treatments.

DISCUSSION

The implications of climate change for the regeneration of tropical species are poorly understood, largely because of disagreement about the degree to which the tropics are expected to warm. However, there has been renewed interest in this area (Clark, 2004; Corlett, 2012; Mau et al., 2018). To understand how climate warming might affect the regeneration of tropical legumes with PY, we documented the germination dynamics of five species under two future climate scenarios using a long-term warming experiment. PY was confirmed in all five species using imbibition tests (**Table 1**). While PY has been confirmed in four of these species previously (Jayasuriya et al., 2013), our study appears to be the first to document PY in *A. chundra*.

Many tropical species with PY disperse their seeds at the transition of the wet to dry seasons (Vázquez-Yanes and Orozco-Segovia, 1993; Khurana and Singh, 2001). In the tropics, dormancy is broken by seasonal temperature fluctuations and the extreme soil temperatures (up to 60°C) experienced in summer. In our experiment, a proportion of seeds of all five species incubated under current climatic conditions germinated after 6 months (**Figure 2**). Data-loggers confirmed that soil temperature exceeded 60°C before the 6-month retrieval,

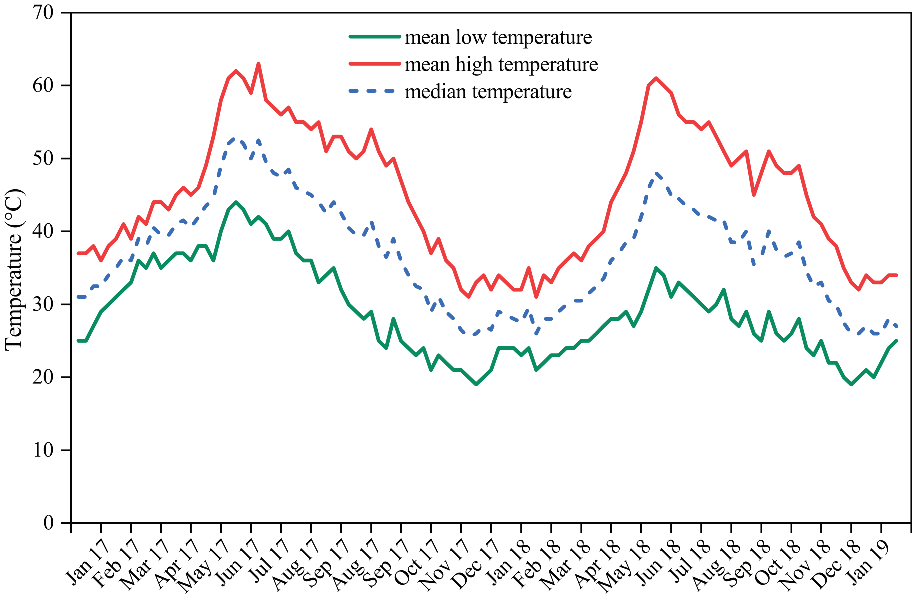

FIGURE 1 | Mean high, mean low, and median weekly soil temperatures recorded in the seed collection site between 23rd January 2017 and 27th January 2019. Values are the mean of measurements made at three locations within a 2 km radius using data-loggers buried at a depth of 2–4 cm in the field.

TABLE 2 | Results of overall general linear model (GLM) on the effects of retrieval time (months), temperature regime (control, +2°C, and +4°C), species, and the interactive effect of retrieval time and temperature regime (Time: temp) on germination percentage.

	Estimate	Standard Error	*T*-value	*P*-value
Intercept	0.042	0.118	0.356	0.723
Retrieval time	0.017	0.007	2.319	0.024^{*}
Temperature regime	0.076	0.050	1.511	0.135
Acacia chundra	−0.067	0.075	−0.919	0.361
Albizia lebbeck	−0.120	0.075	−1.613	0.111
Bauhinia tomentosa	−0.088	0.075	−1.174	0.245
Cassia fistula	0.207	0.075	2.780	0.007^{**}
Time: temp	0.012	0.003	3.451	$< 0.001^{***}$

$^{***}p < 0.001$; $^{**}p < 0.01$; $^{*}p < 0.05$.
Significant p values are denoted in bold.

suggesting that current summer temperatures break dormancy in a proportion of seeds (**Figure 1**). Similar patterns of dormancy break have been observed in *Dodonea viscosa* (Jaganathan and Liu, 2014), *D. hackettiana* (Cook et al., 2008), *Delonix regia* (Jaganathan et al., 2017), and *Adenanthera pavonina* (Jaganathan et al., 2018), where seeds artificially buried at a depth of 3 cm–5 cm exhibited 20–40% germination after the first summer.

Physical dormant seeds can persist in the soil for extensive periods of time (Baskin and Baskin, 2014). Indeed, most seeds in the five species we examined remained dormant after 2 years under current climatic conditions. However, the predicted warming in the tropics of 2–5°C by 2100 (Betts et al., 2011; Joshi et al., 2011; Corlett, 2012) may reduce the ability of physically dormant seeds to persist in the soil for so long. Results from our mild warming treatment (+2°C) were idiosyncratic among species. *Bauhinia tomentosa* and *A. chundra* exhibited little to no difference in germination percentages relative to control across the entire 2-year period. Seeds of *A. precatorius* also were not affected by 2°C of warming for much of the experiment, although increased germination was observed in month 24. Interestingly, *C. fistula* seeds exhibited markedly higher germination rates when incubated under both warming treatments, indicating that this species is particularly sensitive to changes in temperature (**Figure 2**; **Table 2**). Such idiosyncratic responses to climate warming might be explained by differences in the conditions required to break dormancy, such as seed moisture content (Jaganathan, 2016), seed coat thickness (Russi et al., 1992; Venier et al., 2012), and seed size (Halloran and Collins, 1974; Rodrigues-Junior et al., 2018), all of which could act independently or in tandem. Why *C. fistula* seeds were particularly sensitive to warming remains unknown and warrants further study.

In contrast to our mild warming treatment, seeds of all species subjected to our extreme warming treatment (+4°C) exhibited considerably higher germination percentages relative to control seeds (**Figure 2**). Cochrane (2017) observed similar results in the seeds four *Acacia* species from Australia that were incubated at 20/65°C for 112 days. Our extreme warming treatment generated very high soil temperatures during both summers (65 and 67°C, respectively), mirroring those of Cochrane (2017). Collectively, these results suggest that temperatures exceeding 65°C deplete the persistence of physically dormant seeds in the soil. Soil temperatures of 65°C are relatively common in tropical soils (Cook et al., 2008; Jaganathan and Liu, 2014), however, temperatures may reach 70°C in some open canopy areas (although direct soil temperature

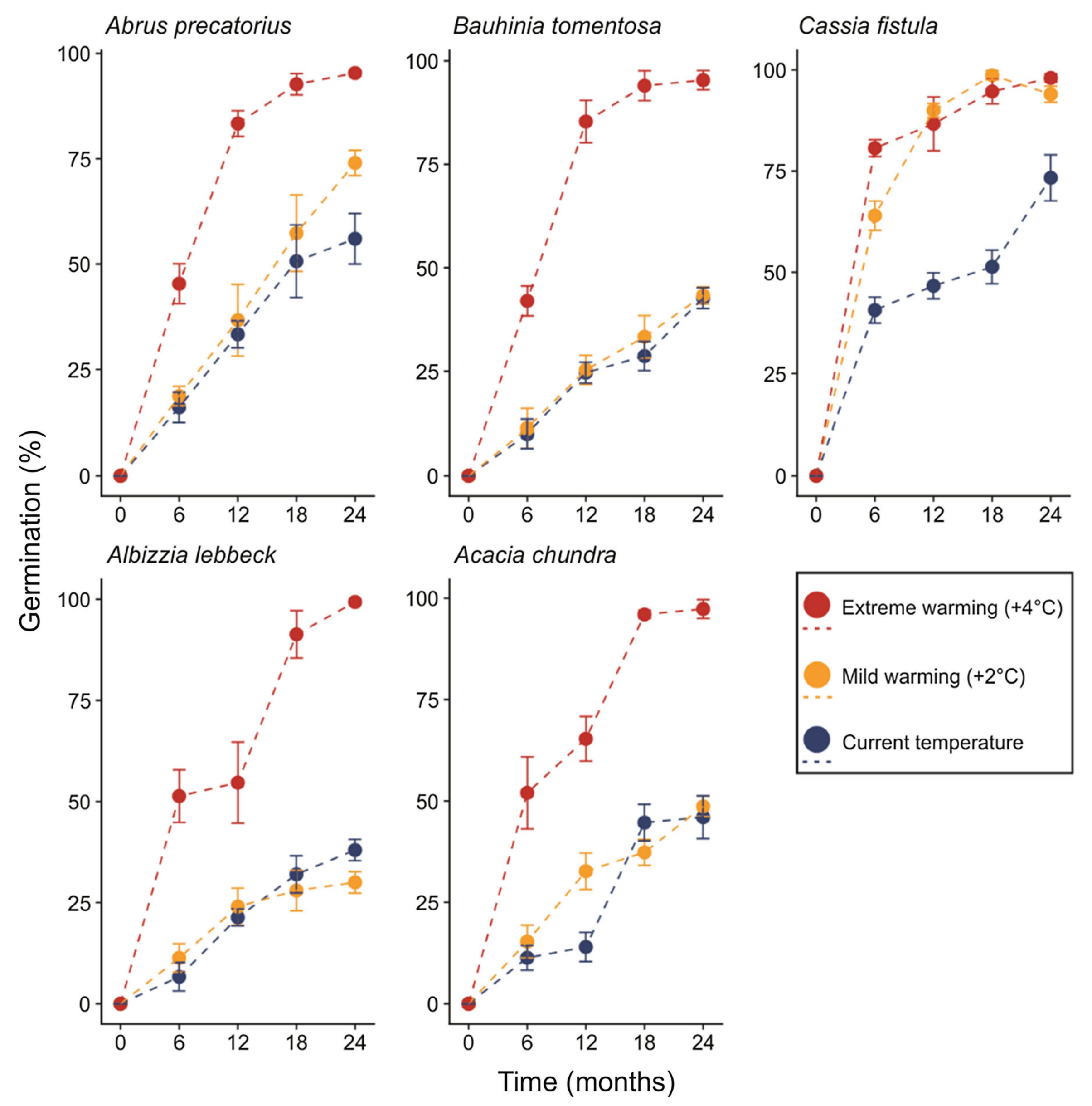

FIGURE 2 | The proportion of seeds germinating over 2 years under control, mild, and extreme warming treatments. Error bars represent the SD.

measurements are lacking). Thus, a threshold temperature appears to restrict the conditions under which PY can effectively operate. How exactly the warming of tropical soils beyond this threshold will affect the distribution of these species (and others) is not known.

The conditions required to break PY in the field have received some attention (Quinlivan, 1961, 1968; Taylor, 2005). Vázquez-Yanes and Orozco-Segovia (1982) demonstrated that impermeable seeds of *Heliocarpus donnell-smithii* matured in Mexico become permeable only when soil temperature exceeds 30°C. Further, more seeds become permeable to water in open canopy sites than under closed canopy, mainly because diurnal temperatures fluctuations in open canopy sites are more extreme. McDonald (2000) similarly found that subjecting seeds to varying temperature regimes of 57/23 and 70/23°C breaks PY, and that this effect was especially pronounced at high temperatures. In the future, a warmer climate is likely to bring more extreme diurnal fluctuations at higher temperatures, potentially rendering PY ineffective as a means of long-term persistence for some species in the soil.

Jaganathan (2016) previously classified PY into two levels: *shallow* and *absolute*. For seeds with shallow PY, their moisture content approaches the range at which impermeability to water is achieved (e.g., 8–10%). Seeds with a much lower moisture content of 5–8%, on the other hand, comprise the absolute PY category. We observed considerable variation in moisture content both within and among species (**Table 1**). Idiosyncratic responses to our warming treatments may, at least in part, be explained by this variation in moisture content. Alternatively, they may represent species-specific adaptations to certain temperature thresholds.

Taylor (1981) proposed that PY loss occurs in two-steps: (1) preconditioning, where seeds are conditioned and made sensitive, but seeds remain impermeable to water; and (2) actual dormancy-break, during which the specialized structures present in seeds (e.g., the lens in Fabaceae) opens, through which water enters and hydrates the embryo. Similar mechanisms of dormancy break have been observed in other families, such as Convolvulaceae (Jayasuriya et al., 2009) and Geraniaceae (Gama-Arachchige et al., 2012). These mechanisms are directly controlled by seasonal changes in temperature. Accordingly, we hypothesize that both stages of dormancy loss will be altered under a warmer climate. Consequently, the bet-hedging mechanism (Philippi and Seger, 1989; Ooi et al., 2009) to spread the risk of germination over time is particularly expected to be altered. One possibility is that warmer temperatures and reduced relative humidity in the future may dry seeds out more, rendering physical dormancy harder to break, and thereby compensating for higher summer temperatures (Bolingue et al., 2010). The effects of climate warming may also occur at the stage of seed maturation on the mother plant. Indeed, the effects of maternal environment on germination dynamics of PY species is gaining attention (Liyanage and Ooi, 2015; Jaganathan and Biddick, 2020), and the ultimate effect of climate warming on species with PY may result from an interplay of processes occurring both before and after dispersal. However, exactly how each stage of dormancy-break will be affected in the future requires further study.

In conclusion, it appears that an increase in temperature of 4°C will significantly increase dormancy loss and alter germination timing of species that employ PY in the tropics, potentially affecting plant establishment and community composition. A milder increase in temperature of 2°C is likely to affect fewer species than 4°C. How severely the germination ecology of species will be affected by climate warming is difficult to ascertain as responses are highly idiosyncratic.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

GJ conceived the idea, performed the experiment, and wrote the manuscript. MB analyzed the statistics and wrote the manuscript. All authors contributed to the article and approved the submitted version.

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Environmental Factors Driving Seed Hydration Status of Soil Seed Banks and the Implications for Post-fire Recruitment

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Changes in fire regimes due to climate change and fire management practices are affecting the timing, length, and distribution of vegetation fires throughout the year. Plant species responses and tolerances to fire differ from season to season and are influenced by species-specific phenological processes. The ability of seeds to tolerate extreme temperatures associated with fire is one of these processes, with survival linked to seed moisture content at the time of exposure. As fire is more often occurring outside historic dry fire seasons, the probability of fire occurring when seeds are hydrated may also be increasing. In this study, we set out to understand the seasonal dynamics of seed hydration for seeds of Banksia woodland species, and how certain seed traits interact with environmental conditions to influence survival of high temperatures associated with fire. We measured the moisture content of seeds buried to 2 cm in the soil seed bank for four common native species and one invasive species on a weekly basis throughout 2017, along with soil moisture content and environmental correlates. We determined water sorption isotherms at 20°C for seeds of each species and used these functions to model weekly variation in seed water activity and predict when seeds are most sensitive to soil heating. Using Generalised additive models (GAMs), we were able to describe approximately 67% of the weekly variance in seed water activity and explored differences in seed hydration dynamics between species. Seed water activity was sufficiently high (i.e., $\geq 0.85\ a_w$) so as to have created an increased risk of mortality if a fire had occurred during an almost continuous period between May and November in the study period (i.e., 2017). There were brief windows when seeds may have been in a dry state during early winter and late spring, and also when they may have been in a wet state during summer and late autumn. These data, and the associated analyses, provide an opportunity to develop approaches to minimize seed mortality during fire and maximize the seed bank response.

Keywords: seed survival, seed hydration, fire season, seed water activity, seed banks

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INTRODUCTION

Post-fire recruitment from seeds is a fundamental mechanism for the persistence, regeneration, and expansion of plant populations in fire-prone ecosystems (Pausas and Keeley, 2014). How plant species respond to, and tolerate the effects of, fire modulates through seasons (Savadogo et al., 2012; Miller et al., 2019, 2020a,b) under the influence of local environmental conditions and seasonal phenological patterns of the species within the ecosystem (Miller et al., 2020a, 2021; Tangney et al., 2020a).

Soil seed banks are the primary repository for seeds within many ecosystems (Thompson, 1987; Saatkamp et al., 2014). The ability of seeds in the soil to persist through fire and sense their environment to time germination, and consequently seedling emergence, to coincide with periods of the year when conditions are most suitable for seedling survival is key to successful regeneration (Baskin and Baskin, 2001). Seeds close to the soil surface have the highest chance of germination and emergence, yet they are also most exposed to the high temperatures generated by fire, where soil temperatures can exceed 200°C in the upper 2 cm (Stoof et al., 2013; Tangney et al., 2018). Soil temperatures during fire moderate with depth due the insulating properties of soil (Tangney et al., 2020a), increasing the chances of survival for seeds buried deeper within the soil. However, increased moisture content of the seeds at the time of exposure to fire temperatures strongly reduces the chances of seed survival (Fer and Parker, 2005; Ruprecht et al., 2016; Tangney et al., 2019).

Seeds with permeable seed coats that reside within the soil seed bank cycle between hydrated and dry states according to the surrounding soil moisture conditions (Turner et al., 2006), and patterns of seed hydration within the soil may be influenced by a range of weather conditions and their influence on the soil conditions (e.g., the amount of rain, prevailing temperatures, or the evaporation rate). Following rainfall, dry seeds passively imbibe water due to the high-water potential gradient between the seeds and their surrounds (Hegarty, 1978; Wuest, 2007). Seeds can remain in a non-germinated but viable state, with moisture content fluctuating seasonally, until environmental conditions are suitable for germination, or until loss of viability (Long et al., 2015).

Seed moisture content drives physiological processes within seeds, and within the soil seed bank, with different physiological processes becoming possible as moisture content varies with changing environmental conditions (Long et al., 2015). For instance, metabolic and cellular processes engage at different thresholds of seed water potential, or water activity, as seeds hydrate (Walters and Engels, 1998; Walters et al., 2005). These thresholds of water activity can be described by constructing water sorption isotherm functions that depict the relationship between seed moisture content and equilibrium relative humidity (at a given temperature; Walters, 2004). The relationship between moisture content and relative humidity is non-linear reflecting the differing properties of water and water activities as seed moisture content increases (Walters and Engels, 1998). Key thresholds of water activity have been defined by Walters et al. (2005): metabolic processes in seeds are greatly limited with water contents in equilibrium with <15–20% RH (hydration level I, <0.15–0.2 water activity a_w), while 85% RH is the threshold for hydration level III, where metabolic processes including respiration become active. In the context of seed survival through the passage of fire, lethal temperature thresholds of seeds are higher at seed moisture contents below region III of the isotherm (i.e., <85% RH). In these drier seeds, measured lethal temperatures exceed 120°C, and can reach as high as 150°C (Tangney et al., 2019). Seeds hydrated to region III (i.e., ≥85% RH or 0.85 a_w) have a significantly reduced tolerance to high temperatures, with lethal temperature thresholds reduced to between 50 and 100°C (Tangney et al., 2019). The risks of seed mortality during fire are therefore higher outside of periods of low soil moisture when seeds are dry.

Changing fire regimes, including shifts in fire season, are motivating improved understanding of mechanisms of plant responses and recovery to fire in different seasons (Miller et al., 2019). In this study, we sought to understand the seasonal dynamics of seed hydration status in relation to prevailing soil and weather conditions in order to identify periods of higher risk of fire temperatures exceeding seed survivability. Our study was conducted within the southwest of Western Australia, which has a hot-summer mediterranean-type climate and unique and highly diverse plant species and communities. Banksia woodlands occur across the Swan Coastal Plain and this vegetation type has recently been classified as a threatened ecological community, due to climate change, invasive species modified fire regimes, and land clearing for urban development, resulting in less than 60% of the original extent of Banksia woodlands currently remaining (Department of the Environment, 2018). Because the current distribution of Banksia woodlands is comprised of remnant fragments, many within and around the Metropolitan area of Perth, local land managers are facing an increasing challenge managing bushfire risk to protect significant economic, cultural, and ecological values in and around Banksia woodlands (Ritchie et al., 2021).

Banksia woodlands occur within a Mediterranean climate, which produces a strongly seasonal climate. Rainfall is generally confined to the late autumn through to early spring (May – September), in which time ~75% of the annual rainfall occurs (Bureau of Meteorology, 2020). While the natural wildfire season is confined to the period of the year when surface and fine fuels are driest, in summer and autumn (November–April; Plucinski (2014)). Options for the management of the fire risk in biodiverse woodlands across the southwest of Western Australia include the use of hazard reduction burns, where fires recur at approximately 10–20-year interval, with few areas remaining unburned for >50 years. The historical fire season is likely summer-autumn (Ritchie et al., 2021), whereas to reduce the intensity of the fire and the risks of uncontained fire events, prescribed burning occurs during cooler and wetter months of March–November (Burrows and McCaw, 1990), predominately during spring months (August–November), which may affect the persistence and recovery of some species following fire (Miller et al., 2020; Tangney et al., 2020a,b; Miller et al., 2021). Further, surface fuels that contribute to soil heating can exceed an average of 10 Mg/ha^{-1} within 10 years following fire and exceed 15 Mg/ha^{-1} in longer unburnt areas of Banksia woodlands (Tangney et al., 2021) which may be sufficient to yield soil temperatures that exceed lethal temperature thresholds in some seeds (Tangney et al., 2020a).

Consequently, the timing of these introduced fires potentially overlaps with periods during which seeds that are within soil seed banks are hydrated and lethal temperature thresholds are lower, with many species forming persistent seed banks, where a large proportion of seeds reside within the top 5 cm of soil (Rokich et al., 2016). Within this context, we measured and modelled the hydration status (specifically seed water activity, a_w) of seeds placed in the soil seed bank over the course of 1 year. Using seeds from five species common in Banksia woodlands, which produce seeds that will be present within soil seed banks at periods when fires may occur, our aim was to quantify the role of soil moisture content and local weather patterns including rainfall and evaporation rate in driving patterns of seed hydration. We focused on identifying periods of seed hydration above and below critical thresholds that are known to influence lethal temperatures for our study species. This allowed us to examine the seasonal variation in seed hydration and infer risks of seed mortality during aseasonal fire.

MATERIALS AND METHODS

Study Species

We assessed variation in seed moisture content in the soil over a 1-year period in five species common in Banksia woodlands: *Anigozanthos manglesii*, *Asparagus asparagoides*, *Banksia prionotes* (weakly serotinous), *Banksia sessilis* (weakly serotinous), and *Conostylis candicans* (**Table 1**). All species, except *A. asparagoides*, are native to Banksia woodlands. Mature seeds of each of the four native species were collected from a minimum of 10 plants within wild plant populations from remnant Banksia woodland fragments within the Perth region during 2015. These species were selected as representative of common Banksia woodlands species that release mature seeds, which readily imbibe water (i.e., seeds do not possess physical dormancy). We included two serotinous species, on the basis that both species are weakly serotinous within the Perth region and readily release mature seeds from their fruits, without need for heat to induce follicle opening (Cowling and Lamont, 1985). Seeds of all species thus commonly reside within the soil seed bank or on or near the soil surface during times of fire. We included *A. asparagoides* which is a common weed species within Banksia woodlands to assess whether native and non-native species were differentiated in their hydration dynamics in a way that might alter their risk of mortality. Following the collection and cleaning of seeds, each seed batch was stored at 15°C and 15% RH prior to use in experiments. To ensure filled seeds were used for experiments, seeds from each species were X-rayed using a Faxitron Specimen Radiography System (MX-20 Cabinet X-ray Unit; Faxitron, Wheeling, IL, United States) and any non-filled seeds were discarded.

Construction of Moisture Sorption Isotherms

Water sorption isothermic functions describe the functional thresholds of water activity within seeds and depict the relationship between seed moisture content and equilibrium relative humidity (at a given temperature). To construct water sorption isotherms, three replicate samples of seeds from each of the five species were first placed inside small, open paper envelopes. The number of seeds per replicate within each envelope was 50 for *A. manglesii*, 20 for *A. asparagoides*, 20 for *B. prionotes*, 20 for *B. sessilis*, and 200 for *C. candicans*. Envelopes were placed inside air-tight polycarbonate electrical enclosure boxes (28 cm × 28 cm × 14 cm; NHP Fibox, Australia), suspended above non-saturated solutions of LiCl, with the concentration of LiCl within each box adjusted to achieve the desired relative humidity conditions of 15, 20, 30, 50, 70, 80, 90, and 95% RH (741, 640, 520, 364, 237, 171, 94, and 48 g L^{-1} of LiCl, respectively; anhydrous, Sigma®, Australia; Hay et al., 2008). To achieve 10% RH, a saturated LiCl solution was used, and to attain 5% RH a saturated $ZnCl_2$ solution was used (Vertucci and Roos, 1990). All boxes were then placed

TABLE 1 | Species used for this analysis and their key attributes.

Species	Family (aceae)	Seed storage syndrome	Dormancy Class	Native	Seed weight (mg)	T_{50w} (°C)	T_{50d} (°C)
Anigozanthos manglesii	Haemodor	Soil	MPD	Yes	1.0	83.5	123.8
Asparagus asparagoides	Asparag	Soil	PD	No	6.5	72.2	75.5
Banksia prionotes	Prote	Weakly serotinous	ND	Yes	23.5	94.2	131.6
Banksia sessilis	Prote	Weakly serotinous	ND	Yes	6.2	98.5	144.0
Conostylis candicans	Haemodor	Soil	PD	Yes	0.3	114.6	130.5

Native data sourced from FloraBase (Western Australian Herbarium, 1998. Florabase), Seed Weight, and T50 values sourced from (Tangney et al., 2019). T_{50} values represent temperatures at which 50% of the seed population is killed. T_{50w} values are presented for wet seeds (> 0.85 a_w), and T_{50d} for dry seeds (0.5 a_w) at 20°C. Dormancy classes: MPD refers to seeds with Morphophysiological dormancy, PD refers to seeds with Physiological dormancy, and ND refers to seeds with No dormancy.

inside an incubator at 20°C. After 3 weeks at respective storage conditions, seeds were retrieved and weighed, before being dried in an oven (Contherm, Korokoro, New Zealand) for 17 ± 1 h at 103°C (International Seed Testing Association, 2021). Seed moisture content was determined gravimetrically on a dry weight basis.

Measuring Seed Moisture Content of Soil Stored Seeds

Filled seeds from each species were partitioned into 153 nylon mesh bags (holes 2 μm in size), in order to retrieve from the soil three replicate bags for each species each week, for 51 weeks. The number of seeds placed into each bag was varied according to seed weight (**Table 1**) to achieve approximately 1 g dry weight per replicate. For *A. asparagoides*, *B. prionotes* and *B. sessilis* there was 17 seeds per bag, for *A. manglesii* 34 seeds per bag, and for *C. candicans* 167 seeds per bag.

Bags containing viable seeds and a small amount of bleached white sand were placed into shallow black seedling trays, with three replicate trays per species. On the 2/1/2017 trays were placed next to each other along the edge of a closed (to public access) sand track within bushland of Kings Park, Perth: like most Banksia woodlands, soil within Kings Park is grey sand. A shallow hole was dug below trays to ensure there was soil contact with the base of the tray. Two centimetres of soil was placed on top of each of the trays, as well as a thin layer of leaf litter, similar to the surrounding undisturbed soil. The 15 trays were buried in groups of five (one species per tray), with trays within groups randomly positioned and each group of five trays spaced 5 m apart.

For each species, in every week for 51 weeks, one remaining seed bag was removed from each replicate tray, placed into an airtight resealable plastic bag and taken immediately back to the laboratory at Kings Park for weighing. Seeds were removed from the nylon mesh bags, separated from any sand and each replicate batch of seeds from each species was weighed separately, before being transferred to a clear plastic container for drying, and seed moisture content was determined gravimetrically after drying seeds at 103°C for 17 ± 1 h.

Measuring Soil Moisture Content

Three replicate soil samples were collected weekly, adjacent to each seed tray using an 8 cm deep × 2 cm diameter plastic cylinder. Each soil sample was placed in an airtight bag before being taken directly for weighing. Soils were weighed in the cylinder and then oven (Contherm, Korokoro, New Zealand) dried at 103°C for a minimum of 17 h. Soil moisture was calculated following ((dry soil weight - wet soil weight)/dry soil weight x 100). Cylinder weight was removed from both wet and dry samples.

Weather Data

Weather data for each day of 2017 were obtained from the Bureau of Meteorology (2020) Perth Metro weather station (Lat: −31.92, Lon: 115.87, elevation: 25 m–Station ID: 009225.); the closest Bureau of Meteorology weather station to the seed burial area (approximately 6 km away). A range of weather variables were collated throughout 2017, all of which were averaged over the 7 days prior to each respective seed collection date. Weather variables included mean maximum temperature, mean daily relative humidity, mean daily pan evaporation, mean daily rainfall, and the sum of weekly rainfall. From the rainfall records, the number of days since last rain >1 mm (DSLR) was also calculated.

Data Analysis

Isothermal functions were plotted from fitted third degree polynomials for each species. Due to the nature of the cubic polynomial function, estimates above 100% relative humidity were limited to 99.9% relative humidity to align with the upper most water activity zone (i.e., >0.99 a_w; Walters et al., 2005). Based upon the measured moisture content of the seeds retrieved from the soil, seed water activity (a_w = RH/100) was determined *via* the third degree polynomial functions. With risks of seed mortality during fire being greatly increased upon hydration of seeds to above 0.85 a_w (Tangney et al., 2019), we focused subsequent analyses on seed moisture contents in terms of water activity and identifying periods of the year when seed moisture contents were above or below this threshold of water activity (i.e., the boundary between hydration level II and III; Walters, 2004).

Generalised Additive Models

To model the influence of weather variables as drivers of seed water activity (a_w) in the soil and understand the environmental requirements for seeds to become "wet" (i.e., moisture contents equivalent to above 0.85 a_w), we engaged a model selection approach, commencing with full subsets generalised additive modelling (GAM) *via* the FSSgam package in R (Fisher et al., 2018). This approach allowed us to construct a set of credible models from a full variable set (mean maximum temperature, mean daily relative humidity, mean daily pan evaporation, mean daily rainfall, and the sum of weekly rainfall, days since last rain > 1 mm and mean soil moisture) and compare these using Akaike's Information Criterion corrected for small sample size (AICc) and AICc model weights (ωAICc), which represent the probability or weight of evidence for each model (Hurvich and Tsai, 1989; Burnham et al., 2011). Models with ΔAICc < 2 were considered as having substantial support (Burnham and Anderson, 2004). Seed water activity was modelled using the gam () function in the mgcv package in R (Wood and Wood, 2015) using a beta (logit link) distribution. All interactions between variables within the model set were allowed. The smoothing parameter was limited to a simple spline (k = 3) to avoid overfitting. The Pearson's correlation cut-off in FSSgam was limited to 0.28 to avoid correlated predictors. Variance explained for GAMs was estimated as adjusted R^2 as reported by gam ().

All statistical analyses were undertaken in the program R (ver 4.0.3, R Core Team, 2020), and visualisations were completed using the ggplot2 (Wickham, 2011), gridExtra (Auguie et al., 2017), ggpubr (Kassambara and Kassambara, 2020), and cowplot (Wilke et al., 2019) packages.

RESULTS

Moisture Sorption Isotherms

There was a sigmoidal relationship between seed moisture content and relative humidity, with patterns in seed hydration effectively described by cubic polynomial models for all species (**Figure 1**: $R^2 = 0.95–0.96$). Seeds of *A. asparagoides* had the highest moisture content at 85% RH, at ~20% moisture content, with all other species reaching c. 15% moisture content at 85% RH.

Seed Moisture Content of Soil Stored Seeds

Across the 51 weeks of burial in soil, relative seed moisture content differed between species, but the patterns of hydration and dehydration were broadly consistent across all species. For example, seed moisture content increased rapidly after a rainfall event during the last week of January across all species, from the 8–10% measured in the first 3 weeks, to 40–60% (**Figure 2A**). Seed moisture content then decreased to 9–11% in the first week of February but increased again following rainfall in the next week to 20–40% across species. A similar rapid wetting and drying event was evident during and immediately following a rainfall event in March (**Figures 2A,B**). After June 12th, seed moisture content remained relatively high (17–50% across all species) until October 30th (**Figure 2A**). This period coincided with consistent rainfall, lower evaporation, and uniformly high soil moisture content (**Figure 2B**).

The best performing model of the weather variables as drivers of seed water activity described ~67% of the variance in water activity, with an interactive function associated with the relationship between mean daily pan evaporation and the number of days since the last rain event >1 mm (**Table 2**; **Figure 3A**). Differences in individual species responses were important (**Figure 3B**) but were not included in the top model (**Table 2**).

Seed water activity was highest when daily average evaporation was low and there were less than 25 days since the last rain event (**Figure 3A**). Seed water activity dropped below 0.85 a_w during extended periods (i.e., >25 days) without more than 1 mm of rain. If rainfall had occurred within the previous 5 days, seed water activity remained high (>0.85 a_w)

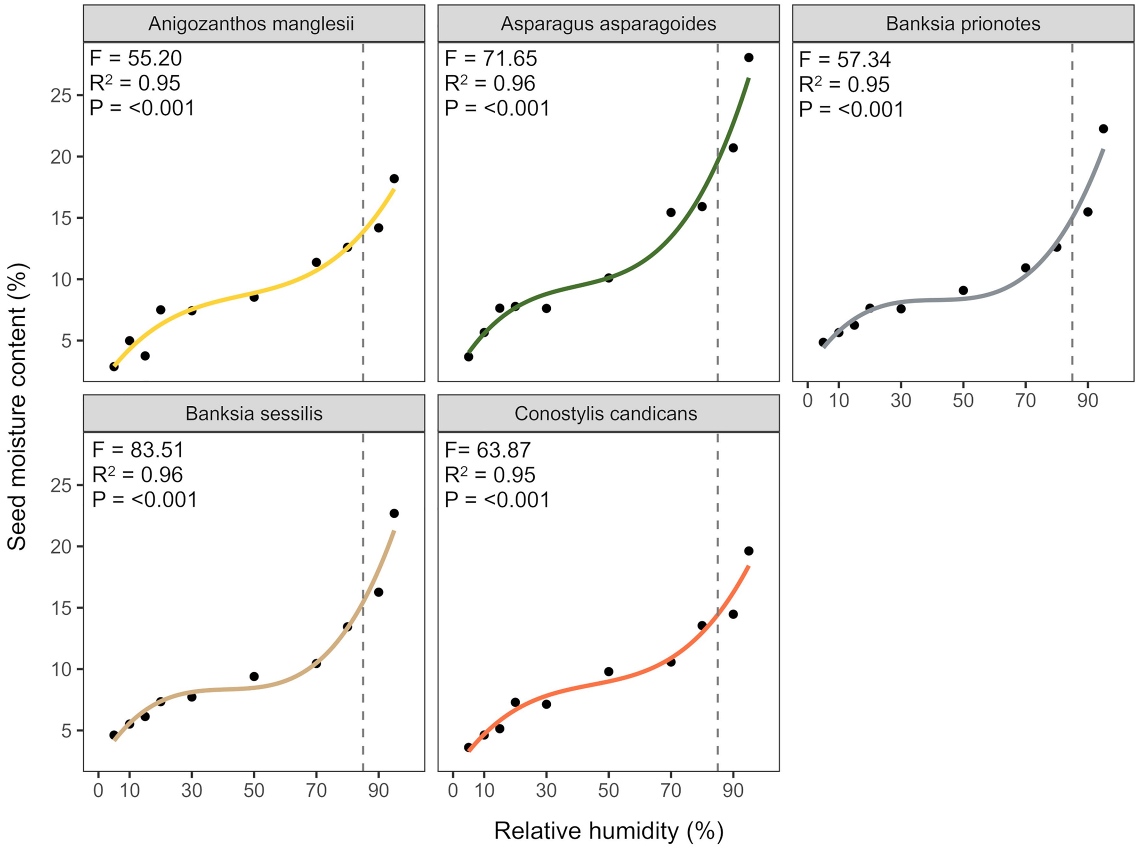

FIGURE 1 | Fitted isotherms of the five study species using cubic polynomial models describing the relationship between seed moisture content and relative humidity of seeds, at 20°C.

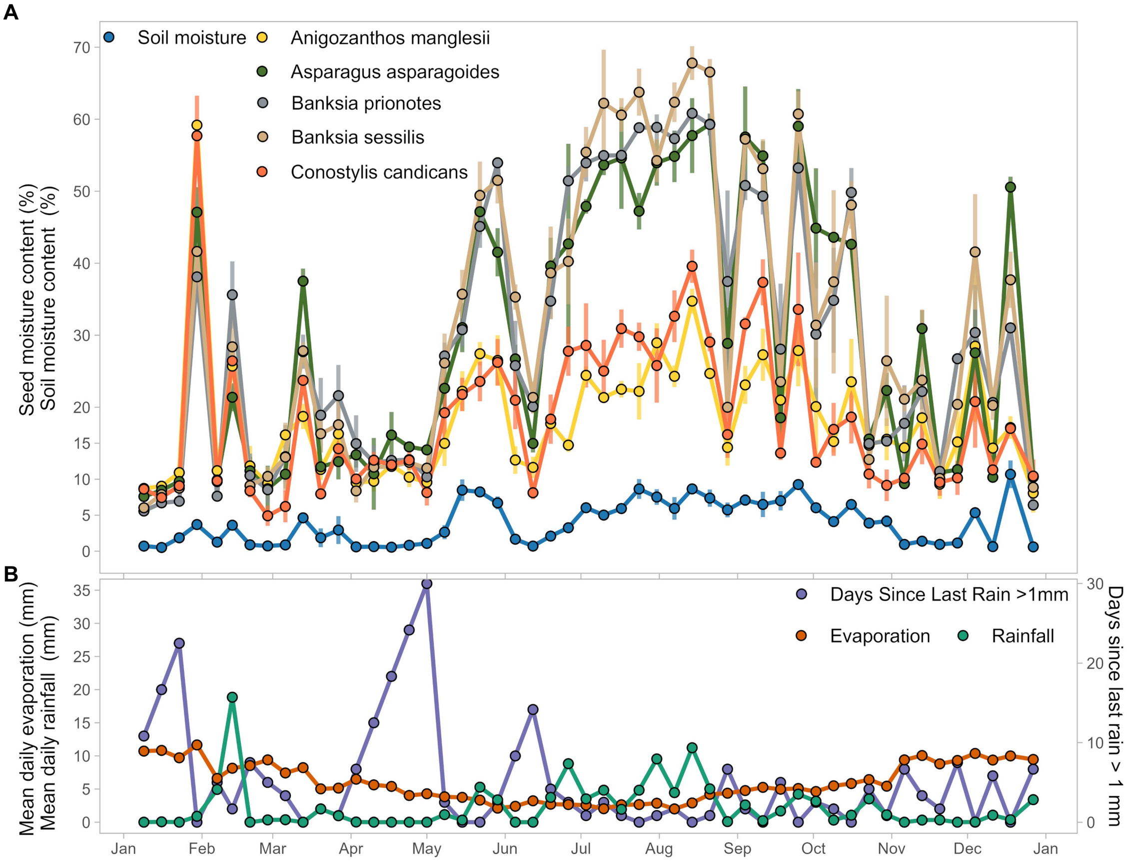

FIGURE 2 | Seed and weather data visualized. Seeds were collected weekly for 51 weeks for the year of 2017. **(A)** Plotted mean seed moisture content, averaged from three replicates for each collection date, and mean soil moisture content averaged from three replicate samples each collection date. **(B)** Mean daily pan evaporation and mean daily rainfall for the 7 days preceeding the seed collection date, and number of days since last rain (>1 mm of rain) over the course of 2017. All weather data extracted from Bureau of Meteorology taken from Perth metro weather station and derived products (Bureau of Meteorology, 2020).

TABLE 2 | Top generalised additive models (GAMS; ΔAICc < 2) from full subsets analysis describing the patterns in seed water activity.

Model	AICc	edf	ΔAICc	ωAICc	R^2	Deviance explained
Mean daily evaporation * Days since last rain	−1389.34	7.84	0	0.52	0.67	80.2
Mean daily evaporation * Days since last rain + Species	−1389.17	11.85	0.16	0.48	0.69	81.3%

Akaike's Information Criterion corrected for small sample size (AICc), estimated degrees of freedom (edf), and difference from lowest reported AICc (ΔAICc), AICc model weights (ωAICc), variance explained (R^2), and deviance explained are reported for model comparison.

even if mean daily evaporation was high (i.e., 10 mm; **Figure 3A**).

Seed water activity was the lowest across all species during the summer months (December–February). Median seed water activity ranged from ~0.65 a_w for *A. manglesii*, to less than 0.40 a_w in *B. prionotes* during summer. Seed water activity varied considerably more during the autumn months (March through to the end of May), but for three out of the five species, median seed water activity remained below 0.85 a_w throughout. Seeds of *A. asparagoides* had the lowest median water activity during autumn ~0.70 a_w. During winter (June through to the end of August) seed water activity was uniformly high, with ~0.99 a_w for all species. The months of spring (September through to the end of November) were similar to autumn whereby seed water activity varied considerably. However, it was evident that median seed water activity was generally higher (by 0.1–0.2 a_w) in spring than in autumn, ranging from ~0.85 a_w for *C. candicans* up to 0.99 a_w for *B. prionotes* (**Figure 3B**).

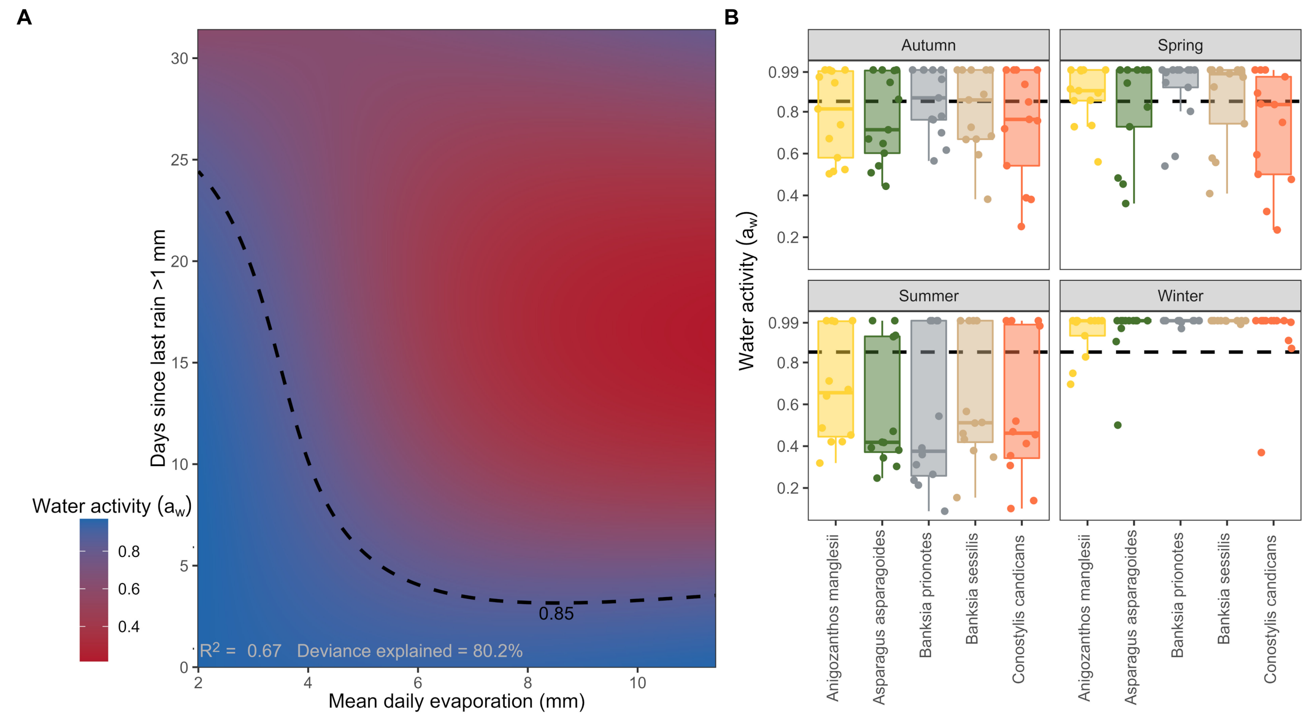

FIGURE 3 | (A) Modelled interactive relationships between seed water activity for seeds within in the soil seed bank, mean daily evaporation, and days since last rain >1 mm. Dashed contour line indicates the threshold water activity of 0.85 a_w. **(B)** Patterns of water activity for each individual species for the year of 2017 stratified by season. Dashed line indicates the hydration threshold where seeds are >0.85 a_w.

DISCUSSION

In this study, we aimed to model potential drivers of seed hydration dynamics in the soil across a 1 year period to identify and predict risks of seed mortality during aseasonal fire. Our previous studies demonstrated that while seeds are dry, their likelihood of surviving temperatures associated with fire is much greater, with lethal temperatures of seeds exceeding 120°C at water contents equivalent to 0.5 a_w (Tangney et al., 2019; **Table 1**). The lethal temperatures of hydrated seeds ($a_w \geq 0.85$) are much lower (c. 31–38°C lower for our study species, **Table 1**). The results of this present study show that seed moisture contents were sufficiently high (i.e., $\geq$ 0.85 a_w) so as to have created an increased risk of mortality if a fire had occurred during an almost continuous period between May and November of the study period (i.e., 2017). The period of apparent increased risk of seed mortality from fire during vulnerable periods in winter and spring is in line with theory and evidence that suggests that susceptible plants may have a reduced recruitment following spring fires within these Mediterranean-climate type ecosystems (Miller et al., 2019, 2020a,b; Tangney et al., 2020). Further, the likelihood that fires that occur during periods of elevated seed moisture content may increase the risk of seed mortality potentially favours those species that produce seeds that remain dry throughout the year, such as seeds with water impermeable seed coats. Seeds with impermeable coats are defined as possessing physical dormancy and are commonly found in the Fabaceae and the Rhamnaceae (Turner et al., 2005), among others. Fires during periods when water permeable seeds are hydrated could contribute to a change in species composition towards these physically dormant species.

Within our study system, our models of the interaction between days since last rain and evaporation were able to describe 67% of the week-to-week variation in seed water activity. The models indicate that when mean daily pan evaporation is low and there has been more than 1 mm of rain in the past 25 days, there is a high likelihood that soil-stored seeds (with permeable seed coats) will be wet (i.e., ≥0.85 a_w). If, however, average daily evaporation is ~4 mm, the window of time over which seeds remain wet reduces to just 8 days following a rain event of >1 mm, and further reduces to just the 5 days following rain >1 mm if the average evaporation is ≥5 mm. This simple metric, based upon the prevailing weather conditions, offers the potential to define those periods during which seed hydration conditions within the soil seed bank are such that the risks of seed mortality during fire are lessened. Periods when seeds are dry likely correlates with periods of elevated fire risk, as soils are drier and fuels that contribute to fire spread are likely to be more available.

Seeds imbibe moisture from their surrounding medium in the form of both liquid and vapour phase water (Wuest, 2007). We found that seed water activity was, on average, highest in winter and spring, and lowest in summer and autumn. During the winter and spring months, regular rainfall and low evaporation rates provided suitable, and relatively constant, levels of available soil moisture for seeds to remain hydrated, but as the time since rain increased and evaporation rates increased through the summer months, the soil moisture conditions became such that seeds were mostly in a dry state. Following long dry periods, rapid imbibition of seeds can evidently occur even upon relatively small rain events, as occurred in January 2017, where seed moisture quickly rose, despite only 6 mm of rain recorded in the week to 31st January. This rapid hydration is due to low water potentials in the dry seeds and the matric and osmotic pressures quickly drawing available soil water into seed tissues (Hadas, 1977). However, seeds dried equally rapidly during summer months, and we did not determine seed moisture contents during summer that exceed our threshold of increased risk of seed mortality (i.e., 0.85 a_w).

The minimal differences in seed hydration between the different species throughout 2017 suggests that these results likely represent the generalised hydration pattern of many Banksia woodland seeds that have water permeable seed coats and spend some portion of their lifecycle in soil seedbanks. Whilst the wetting and drying patterns may be similar amongst species, interactions between seed hydration status and lethal temperatures influence species disproportionately. For example, dry seeds of *A. asparagoides*, a species native to South Africa but invasive across temperate Australia including within Banksia woodlands (Morin and Scott, 2012), have a lethal temperature lower than the examined native species while dry. The *A. asparagoides* seeds thus have a disproportionately greater risk of mortality during fire, and fires while seeds are dry may impede post-fire recruitment (Keeley, 2006), a factor that may be useful for reducing the spread of this invasive species. However, the differences in lethal temperatures between species greatly reduces when they are wet, meaning the relative advantage in survivability maintained by seeds of the natives during historical (dry) fire seasons is lost during burns under high soil moisture conditions.

Two of the species examined (*B. prionotes* and *B. sessilis*) commonly maintain canopy seed banks *via* the storage of seeds within woody fruits (serotiny; Lamont, 1991). Serotiny provides seeds stored within the woody fruits increased protection from fire temperatures, as well as from ambient moisture (Lamont et al., 1991; Huss et al., 2019), with seeds typically released from the fruits after fire (Lamont et al., 1991). However, within our study system of the Banksia woodlands of the Perth region, both *Banksia* species are weakly serotinous and release seeds in the absence of fire – annually and shortly after maturation – into the soil seed bank (Cowling and Lamont, 1985). Thus, the seeds of these species, despite originating from plants with serotinous characteristics, are still exposed to fire temperatures in, or on the surface of soils, if the seasonal timing of fire follows that of seed dispersal. Flowering in both species begins in early autumn and extends through winter, with seeds being dispersed during late spring and summer. Seeds will remain in the soil seed bank until conditions are suitable for germination or until the loss of seed viability, which may occur within 4–8 months following release (Miller et al., 2021). Their seeds are able to tolerate high temperatures while dry (e.g., 131.6 and 144.0°C, respectively; **Table 1**), which may limit their mortality in soil seed banks during naturally occurring fires. But, like the other species in our study, seeds of these *Banksia* spp. will be exposed to greater risk of mortality from aseasonal fire during periods of high soil moisture.

In this study, seeds were buried under 2 cm of loose soil and a shallow layer of leaf litter. As such, soil moisture may be lost more quickly compared to the rate of moisture loss deeper within the soils, or under heavier litter (Tromp-van Meerveld and McDonnell, 2006). While the majority of seeds are buried within the top 2 cm of the soil seed bank, deeper burial, or more leaf litter, may potentially extend the number of days required for seeds to dry below our threshold of 0.85 a_w. Moisture loss from soils is reduced further by the presence of ground cover, including shrub and canopy cover, leading to a reduction in evaporation of moisture from soils (Lamb and Chapman, 1943). Consequently, seeds within soils under shrub or canopy cover may potentially remain hydrated for periods significantly longer than seeds measured in this study. But soil burial depth of seeds may also influence seed mortality *via* increased insulation from fire temperatures as soil depth increases. Soils are strong insulators against heat, able to greatly reduce the temperatures experienced deeper within the soil profile (Tangney et al., 2020a). The insulative properties of soil increases further while soils are wet as available moisture quenches the thermal energy directed into the soil from the fire, converting liquid water into steam and, thus, dampening temperatures (Stoof et al., 2011). Nevertheless, in cases where fuels are dry enough to actively combust while soils are still wet, soil temperatures can rapidly increase once soil moisture is boiled off (Aston and Gill, 1976; Tangney et al., 2018). Therefore, if seeds are wet while surface fuels are dry, seeds stored within the soil seed bank may still be at increased risk of mortality.

We found that days since last rain and mean daily evaporation best predicted seed hydration status, more so than soil moisture and mean daily rainfall. This result was unexpected as soil moisture sampled from adjacent to the seed burial area was anticipated to be closely correlated with seed moisture (Wuest, 2007). Whilst our study design may have influenced the strength of potential contributions of soil water deeper than 2 cm due to the plastic trays potentially influencing water vertical movement of water towards the soil surface, >50% of seeds within the seedbank reside at depths less than 2 cm and the conditions within the trays did resemble that of the upper soil profile (Rokich et al., 2016). Nevertheless, the model described was a powerful predictor of seed moisture, and it has the additional benefit of using commonly reported metrics that are easily calculated from nearby weather stations, rather than requiring more complex *in situ* soil sampling. This study was carried out in an ecosystem with characteristically sandy soils which contains little organic material, limiting the water holding capacity of (Ritchie et al., 2021). The relationships between seed hydration status and weather variables here may likely vary in systems with different soil texture, organic matter content, and moisture retention characteristics, thus influencing how the model predictions perform.

CONCLUSION

During fire, elevated soil temperatures can lead to mortality of seeds stored in contact with soils. Fires that occur while seeds buried within soil seed banks have high moisture contents may further enhance seed mortality. In this study, we have described a model that informs when seeds are most likely to be at greater risk to mortality during fire, *via* simply calculated weather metrics. Our model was able to capture ~67% of the variation in seed water activity across the year, providing key insights into when seeds are most likely to be hydrated, and thus exposed to increased risk of mortality from elevated soil temperatures during fire. Seeds were most likely hydrated shortly after rain, when pan evaporation was low. Seeds remain hydrated following rainfall that occurred within the previous 5 days, even when mean daily evaporation was high (i.e., 10 mm). Seed hydration status was sufficiently high (i.e., ≥ 0.85 a_w) so as to have created an increased risk of mortality if a fire had occurred during an almost continuous period between May and November of the study period (of 2017). There were brief windows when seeds may have been in a dry state during early winter and late spring, and also when they may have been in a wet state during summer and late autumn. Fires that occur while seeds are hydrated may increase seed mortality in soil seed banks, and repeated unseasonal fires may lead to significant community shifts where species that produce water permeable seeds are lost from the ecosystem.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

RT, BM, and DM all equally contributed to the ideas and design of this manuscript and editing of the manuscript. RT led collection, analysis of the data, and the writing. All authors contributed to the article and approved the submitted version.

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Polyploidy but Not Range Size Is Associated With Seed and Seedling Traits That Affect Performance of *Pomaderris* Species

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Ploidy and species range size or threat status have been linked to variation in phenotypic and phenological seed and seedling traits, including seed size, germination rate (speed) and seedling stature. There is surprisingly little known about the ecological outcomes of relationships between ploidy, key plant traits and the drivers of range size. Here we determined whether ploidy and range size in *Pomaderris*, a genus of shrubs that includes many threatened species, are associated with variation in seed and seedling traits that might limit the regeneration performance of obligate seeders in fire-prone systems. We experimentally quantified seed dormancy and germination processes using fire-related heat treatments and evaluated seedling performance under drought stress. We also examined the association of seed size with other seed and seedling traits. Polyploids had bigger seeds, a faster germination rate and larger and taller seedlings than diploids. There was a lack of any clear relationship between range size and seed or seedling traits. The ploidy effects observed for many traits are likely to be indirect and associated with the underlying seed size differences. These findings indicate that there is a higher potential competitive advantage in polyploid than diploid *Pomaderris* during regeneration, a critical stage in the post-fire environment. This insight to the regeneration phase may need to be considered when planning and prioritising management of threatened species.

Keywords: functional trait, early life history, ontogeny, dormancy, germination, rarity

INTRODUCTION

Polyploidy, the condition of having more than two genome copies, is widespread in plants and thought to be a key evolutionary mechanism in the diversification of flowering plants (Hoya et al., 2007; Madlung, 2013). Polyploidy can have significant phenotypic consequences and is therefore likely to influence ecological performance (Ramsey and Ramsey, 2014). Polyploids are thought to have advantages over diploids that allow them to persist in challenging environments (Soltis and Soltis, 2000; Madlung, 2013; Ramsey and Ramsey, 2014). They can be more resilient or competitive under stress than diploids (Godfree et al., 2017; Stevens et al., 2020) and have an increased chance of becoming invasive (Soltis and Soltis, 2000; Hull-Sanders et al., 2009; Pandit et al., 2011; Thébault et al., 2011; Te Beest et al., 2012). In the few studies where the association between rarity or conservation status and ploidy levels has been investigated, diploids and species with a lower number of chromosomes had a higher chance of being threatened than polyploid species and species with a higher number of chromosomes (Pandit, 2006; Pandit et al., 2011). A community-level analysis of a fragmented landscape has shown that diploid plant populations have a much higher chance of facing local extinction than polyploid plant populations (Plue et al., 2018). Overall, polyploidy may affect a species' relative evolutionary capacity, competitive dominance and conservation status.

An understanding of traits that limit performance of threatened or narrow endemic species can help scientists, natural resource managers and policy makers to set priorities for species conservation and population management (Schwartz, 1993; Lavergne et al., 2004; Pandit, 2006). Here we define species listed under conservation legislation as threatened and with a limited geographical distribution of less than 200 km^2 [measured as Area of Occupancy (AOO), IUCN, 2012] as 'narrow range'. We refer to any species with AOO greater than 200 km^2 as 'widespread'. Variation in species ranges is often attributed to the ability of species to tolerate climatic or other environmental conditions (Slatyer et al., 2013; Sonkoly et al., 2017). Comparative studies of threatened/narrow range and widespread species can identify limiting plant traits (e.g., Bevill and Louda, 1999; Burne et al., 2003; Lavergne et al., 2004) across life history stages. Seed and seedling stages are critical to understand because they are under particularly strong selective pressure in disturbance-driven ecosystems where species depend on recruitment from the seed bank for long-term persistence (Keith, 1996; Auld et al., 2000; Ooi et al., 2007; Saatkamp et al., 2019). For example, for fire-killed species in fire-prone regions, the soil seed bank is required for regeneration. Fire-related heat cues often alleviate seed dormancy and promote germination from the seed bank, with dormancy regulating the timing to coincide with a suitable post-fire environment (Keith, 1996; Ooi et al., 2014). While ploidy and range size have been studied in some fire-prone environments or species, there has not been a focus on their role in post-fire regeneration. Numerous phenotypic and phenological seed and seedling traits have been linked with endangered status or narrow range size in comparative studies. For example, endangered/narrow endemic species can have lower seed production (Pavlik et al., 1993; Walck et al., 2001; Lavergne et al., 2004), smaller seed size, slower germination, smaller stature and are less able to form a soil seed bank than more widespread species (Osunkoya and Swanborough, 2001; Walck et al., 2001; Burne et al., 2003; Lavergne et al., 2004; Mattana et al., 2010). Overall, endangered status or narrow range size appear strongly associated with several traits that limit plant performance or regeneration success.

There is still surprisingly little data on the ecological outcomes of relationships between ploidy and key plant traits (Plue et al., 2018), and studies rarely investigate multiple life history stages. Perhaps the most well-supported relationship is that genome multiplication in polyploids is associated with phenotypic traits, such as larger flowers and heavier seeds than diploids (Maceira et al., 1993; Bretagnolle et al., 1995; Hoya et al., 2007; Eliášová and Münzbergová, 2014; Godfree et al., 2017; Astuti et al., 2020; Stevens et al., 2020), which is often attributed to larger cell size. Whether ploidy related seed size variation results in the same well-known ecological benefits of larger seed size (Leishman et al., 1995, 2000; Moles and Westoby, 2004b), or if other factors offset these advantages, largely remains unknown. Heavier seeds in polyploids could enhance fitness as heavy seeds generally produce larger seedlings with a higher chance of survival in competitive environments (Bretagnolle et al., 1995; Liancourt et al., 2009). Another seed trait associated with ploidy is the level of dormancy within a seed lot. A study of meadow fescues in mountain ecosystems showed that tetraploid seeds were dormant whereas diploid seeds were not (Tyler et al., 1978). A study by Hacker (1988) on the African grass species *Digitaria milanjiana* across multiple ploidy levels also suggested a greater proportion of dormancy in populations with higher ploidy levels. While maternal environment may have also influenced the results in previous studies, Stevens et al. (2020) showed that even under common garden conditions, tetraploid plants of *Themeda triandra* (Poaceae) produced more dormant seeds than diploid plants. These examples indicate that ploidy differences within-species may be associated with significant variation in seed dormancy among populations, something that may also influence variation among species. While there are clear relationships between seed size and the speed of germination (rate; Norden et al., 2009) or seedling growth rate (Swanborough and Westoby, 1996), where smaller seeds germinate faster and have a faster seedling relative growth rate (RGR) than larger seeds, there are variable relationships between ploidy and seed germination or seedling growth rates. Polyploidy can be positively related to seed germination rate (Hoya et al., 2007; Astuti et al., 2020) and seedling growth rate (Levin, 1983; von Well and Fossey, 1998). On the other hand, polyploidy can be negatively related to seed germination rate (Levin, 1983) and seedling growth rate (Swanborough and Westoby, 1996). Further investigations of ploidy and growth rates, particularly for seedling traits, would provide critical information on regeneration and establishment potential.

Ecologically, polyploidy is thought to increase a plant's ability to adapt to new environments and enhance stress tolerance, allowing polyploids to be distributed more widely than their diploid relatives (Levin, 1983; Soltis and Soltis, 2000; Čertner et al., 2019). Physiologically, the larger genome size in polyploids increases their hydraulic conductivity by enlarging xylem conduit size and stomata, potentially increasing a polyploid's water retaining capacity and drought tolerance (Maherali et al., 2009). However, there may also be a risk of polyploids being more sensitive to water stress due to an increase in vulnerability to cavitation, resulting from increased xylem conduit size. Many polyploid species possess anatomical features that help reduce water loss, including fewer stomata per unit area and thicker epidermis (Levin, 1983; Li et al., 1996). There is a strong association between tolerance of water stress and survival, as well as numerous traits, such as reduction in growth rate and height, total biomass production (Pérez-Harguindeguy et al., 2016), relative amount of investment in root biomass (Comas et al., 2013; Larson and Funk, 2016) or trade-offs, such as smaller stature in exchange for higher seed production (Eliášová and Münzbergová, 2017). Experimentally, polyploids have been shown to have greater water uptake than diploids (Zaiats et al., 2020) and to be more resilient in drought conditions, for example, seed production was shown to be over four times higher in drought-stressed polyploids than diploids (Godfree et al., 2017).

Ploidy and range size may also impact regeneration in fire-prone ecosystems. A strong negative relationship exists between seed size and dormancy-breaking temperature thresholds, and there is a positive relationship between seed size and heat-related mortality (Hanley et al., 2003; Liyanage and Ooi, 2017a). Therefore, ploidy-driven variation in seed size could influence both the dormancy-breaking temperature threshold and seed survival during fire events. Seed size is also important for emergence as a high-intensity fire can kill seeds at shallow depths (Bond et al., 1999) and larger seeds may have an advantage as they are more capable of germinating successfully from deeper within the soil (Liyanage and Ooi, 2017a). Favourable conditions for seedling establishment tend to be short-lived in fire-prone systems and seeds with faster germination rates and faster seedling growth rates, leading to bigger seedlings at any given time, can have an advantage in capturing the increased availability of resources, such as light, space and nutrients (Moles and Westoby, 2004a; Denham et al., 2011). Factors, such as ploidy and range size, that can be related to traits during early life history stages may have important, perhaps interacting, impacts on regeneration and performance in fire-prone systems.

In this study we asked whether there are detectable effects of ploidy and/or range size on traits associated with seed dormancy, germination and establishment, that may affect performance during early life history in a genus of shrubs from fire-prone systems. We hypothesised that:

i. Driven by trait variation, ploidy and range size would interact to affect seed dormancy, germination and seedling growth.
ii. Polyploid or widespread species would produce larger seeds, and therefore lower dormancy-breaking thresholds, a faster germination rate and larger seedlings than their diploid or narrow range counterparts.
iii. Polyploid or widespread species would display a higher drought tolerance than their diploid or narrow range counterparts.

MATERIALS AND METHODS

Study Species

One of the most effective ways to investigate potentially limiting traits is by using phylogenetically similar species (Burne et al., 2003). *Pomaderris*, with a range of widespread and narrow range/threatened species (Coates and Kirkpatrick, 1999; Kellermann et al., 2005; Messina et al., 2010) and diploid and polyploid species (Chen et al., 2019), is well suited to such investigations. A total of 15 *Pomaderris* species were selected based on seed availability, ploidy levels and range size/conservation status (**Table 1**).

Pomaderris (Rhamnaceae) consists of shrubs and small trees that occur in Australia's fire-prone ecosystems (Kellermann et al., 2005). There are 68 *Pomaderris* species in Australia, most of which are endemic, occurring primarily across southern temperate regions (Kellermann et al., 2005). Most *Pomaderris* species are seeders that have soil seed bank storage (NSW Office of Environment and Heritage, 2014). *Pomaderris* seeds have physical dormancy (Turner et al., 2005; Haines et al., 2007; Ooi et al., 2014; Liyanage and Ooi, 2017a; Wood, 2020), which is alleviated by heat shock (for example fire-related heating of the soil; Liyanage and Ooi, 2017b; Le Breton et al., 2020). Of the 68 *Pomaderri*s in Australia, 17 are nationally listed as threatened (Department of the Environment and Energy, 2018), with further listings at state and territory level (Sutter, 2011; Le Breton, 2016). Many of the species (45 out of 68) occur in NSW, with 16 currently listed as threatened in NSW (Millott and McDougall, 2005; NSW Office of Environment and Heritage, 2018).

We focused the study on *Pomaderris* collections from the state of NSW and therefore used the threat status assigned at State level. The majority of study species have the centre or much of their distribution, occurring within the State of NSW. To assign species as narrow range or widespread we considered the estimated AOO across Australia (**Table 1**). AOO is defined as the smallest area occupied by a species, excluding vagrants (IUCN, 2012). Estimates of AOO were obtained from the literature (Le Breton et al., 2020; Gallagher et al., 2021). Species that have been assigned a threat level (critically endangered, endangered, or vulnerable) in NSW and had a relatively small AOO were categorised as 'narrow range.' Species that have not been assigned a threat-level or had a relatively larger AOO (>200 km^2) were categorised as 'widespread' (**Table 1**).

Flow cytometry is a rapid and reliable method of identifying polyploid complexes among close relatives due to the relationship between ploidy and nuclear DNA content. The genome size (2C pg) of mature plants of each *Pomaderris* species, sometimes the mother plants for the seed lot, was measured in triplicate using flow cytometry by Chen et al. (2019; **Table 1**). In short, *Pomaderris* leaf samples and a standard were processed in buffer solutions, stained and loaded into a flow cytometer until at least 5,000 events were counted in total per sample. In some cases, if a leaf sample was not available a seed sample was assessed to obtain an approximate estimate of genome size from seed samples and was not replicated (**Table 1**). Chromosome counts were also conducted by Chen et al. (2019) for selected species to inform interpretation of flow cytometry results. For detailed flow cytometry and chromosome count methodology, see Chen et al. (2019). Whether the study species are autopolyploids or allopolyploids could not be determined and is beyond the scope of these

TABLE 1 | List of *Pomaderris* species selected for this study, including information on their State (NSW) threat level, range size (area of occupancy (AOO)), ploidy, genome size, habitat and fire response.

***Pomaderris* species**	**Categorisation**	**NSW threat status**	**Area of occupancy (km^2)$^{\#}$**	**Inferred ploidy level***	**Genome size (2C pg. ± SE)***	**Habitat range**	**Fire response**	**References**
P. adnata	*Narrow range, Diploid*	Endangered	8^{1}	Diploid	0.927 (±0.004)	DSF, WSF	Seeder	Liyanage and Ooi, 2017a; NSW OEH, 2018
P. andromedifolia	*Widespread, Polyploid*	Not listed	328	Triploid	1.547 (±0.004)	DSF, heathland	Seeder	NSW OEH, 2014
P. bodalla	*Narrow range, Diploid*	Vulnerable	80	Diploid	0.966 (±0.004)	DSF, riparian	Seeder	Le Breton, 2016; NSW OEH, 2018
P. brunnea	*Narrow range, Diploid*	Endangered	160	Diploid	0.945 (±0.005)	DSF, WSF, GW, riparian	Seeder	Sutter, 2011; NSW OEH, 2014; NSW OEH, 2018
P. cotoneaster	*Narrow range, Polyploid*	Endangered	136	Tetraploid	1.862 (±0.005)	DSF, WSF, GW, riparian	Seeder	NSW OEH, 2014; NSW OEH, 2018
P. elachophylla	*Widespread^, Polyploid*	Endangered^	476	Triploid	1.393 (±0.007)	DSF, GW, riparian	Seeder	NSW OEH, 2014; NSW OEH, 2018
P. eriocephala	*Widespread, Polyploid*	Not listed	624	Triploid	Approximately 1.3	DSF, GW	Seeder/ Resprouter	NSW OEH, 2014
P. intermedia	*Widespread, Polyploid*	Not listed	1,036	Triploid	1.636 (±0.002)	DSF, forested wetlands	Seeder	NSW OEH, 2014
P. lanigera	*Widespread, Diploid*	Notlisted	1,712	Diploid	Approximately 1.0	DSF, forested wetlands	Seeder	NSW OEH, 2014
P. ligustrina subsp. *ligustrina*	*Widespread, Diploid*	Not listed	284	Diploid	0.998 (±0.003)	Riparian	Unknown	Walsh, 2017
P. pallida	*Narrow range, Polyploid*	Vulnerable	160	Triploid	1.394 (±0.006)	Riparian, shrub communities surrounded by woodland	Unknown	NSW OEH, 2018
P. prunifolia	*Widespread, Polyploid*	Not listed	292	Triploid	Approximately 1.4	WSF	Seeder	NSW OEH, 2014
P. reperta	*Narrow range, Polyploid*	Critically endangered	10^{2}	Triploid	1.497 (±0.005)	DSF, WSF	Seeder	NSW OEH, 2018
P. velutina	*Widespread, Diploid*	Not listed	260	Diploid	0.894 (±0.001)	Riparian	Unknown	PlantNet, 2018
P. walshii	*Narrow range, Polyploid*	Critically endangered	8^{2}	Tetraploid	1.993 (±0.002)	DSF, riparian	Seeder	Liyanage and Ooi, 2017a; NSW OEH, 2018

$^{\#}$Area of occupancy estimates from Gallagher et al. (2021), except ^{1}Le Breton et al. (2020) and ^{2}Conservation Advice for the Australian Government's Environment Protection and Conservation Act 1999.

*Inferred ploidy levels and genome size estimates [2C value in pg (±standard error for triplicate measurements) or approximate estimate based on a single measurement from a seed sample] from Chen et al. (2019).

^Although P. elachophylla is listed as endangered in NSW, its AOO is >200 km^2 and it occurs in greater numbers in other states so was categorised as 'wisdespread.'

Habitat range: DSF, dry sclerophyll forest; WSF, wet sclerophyll forest; GW, grassy woodland.

References: NSW OEH, NSW Office of Environment and Heritage.

studies, and our investigations were therefore based on broadly comparing diploids with polyploids. Species that were diploid were categorised as 'diploid' and species of higher ploidy (triploid or tetraploid) were categorised as 'polyploid' (**Table 1**).

Seed Collections

Pomaderris seeds were obtained from the seed bank facilities at the Australian Botanic Garden, Mount Annan and Australian National Botanic Gardens, Canberra. All seeds were collected from wild populations by botanic garden's staff and Dr. Mark Ooi under relevant permits. For most seed lots assessed in this study, nylon mesh bags were placed over the seeding branches of multiple mother plants per population to ensure the collection of mature seeds. Seeds from mothers within a population were combined into a population sample. Once collected, a vacuum separator was used to separate seeds from chaff. Seeds were then stored in dry dark conditions (15°C and 15% RH) or in dark, dried and frozen conditions (−20°C) at the seed bank facilities prior to use. Such storage conditions have previously been shown to have minimal effects on seed dormancy or viability characteristics of many species with physically dormant seeds, particularly those that require heat shock to overcome dormancy, such as *Pomaderris* (Merritt et al., 2003; Vasques et al., 2014). We used 400–600 seeds for each of the 15 species in this study, depending on seed availability. Not all species were used for all measured seed and seedling traits due to seed availability (See **Supplementary Appendix S1** for the species used in each measured trait).

Seed Traits

Twenty seeds were randomly selected from the seed lots of each species and the individual seed mass measured using a fine-scale balance (A&D GR-202, Japan) with mean mass calculated and expressed in milligrams (mg). Initial viability of seed lots was estimated from seed fill, using three replicates of 20 seeds imaged with an X-ray imaging system (Faxitron MX-20 Cabinet X-ray System, United States).

To identify dormancy-breaking requirements and enable calculation of germination rate (speed), heat treatments were used, and the response was assessed by germination trials. Dormancy is usually overcome by a high temperature heat shock, so seeds were subjected to a range of dry heat treatments simulating temperatures the seed bank would be exposed to in the upper soil profile during fire. Seeds were exposed to four levels of dry heat for 10 min in an oven (LABEC Model ICC36-HT, Australia): 60, 80, 100 and 120°C. The 120°C treatment was chosen as the upper limit as it had been found to be close to the limit for seed mortality in physically dormant seeds (Ooi et al., 2014). Four replicates of 20–25 seeds were used per species for each temperature and for the unheated treatment control (except *P. intermedia* where only three replicates for heat and control treatments were used due to low seed availability), one replicate at a time, to minimise pseudoreplication within each treatment level (Morrison and Morris, 2000). After heat shock treatments to overcome dormancy, seeds were allowed to cool and then put into 90 mm petri dishes on moistened filter paper. Dishes were wrapped with parafilm to reduce evaporation and transferred to an incubator (LABEC Model ICC36-HT, Australia) set at a temperature cycle of 25/18°C on 12 h/12 h light/dark cycle to mimic the approximate natural temperature variation during summer, the fire season in NSW (Gill, 1975). Dishes were randomly placed within the incubator and rotated once every week. Seeds were checked two times a week and germination was scored upon radicle emergence (radicle >1.5 mm). Germinated seeds were immediately removed from the dish for planting, and mouldy seeds were recorded as inviable and removed from the dish to prevent contamination. Distilled water was added to the dishes when necessary to maintain moisture levels. The germination trial for each species was terminated when the cumulative germination reached a plateau.

To assess germination speed, we used data from the temperature with maximum germination for each species. Using the drm function in the *drc* package in R 3.5.1 (Ritz et al., 2015; R Development Core Team, 2018), a log-logistic dose–response curve was fitted, using the number of seeds germinated against the day at which they germinated. Curves were fitted for each species and the time to reach 50% (T_{50}) was calculated by inverse regression.

To compare the minimum dormancy-breaking temperature thresholds, the lowest level of dry heat that produced at least 20% seed germination (G_{20}) for each replicate was recorded. The recorded temperature was then averaged across all replicates within each species to generate the mean G_{20} for each species. As upper threshold or optimum dormancy-breaking temperatures are likely to be very similar (Ooi et al., 2014) we instead used the G_{20} index to estimate the lower-bound variation in dormancy alleviation.

The percentage of seed mortality was calculated at 120°C, the highest level of dry heat treatment and representative of a high-intensity fire. To assess seed mortality after heat treatment, cut tests were performed on ungerminated seeds from the 120°C heat-treated seeds, after the termination of the germination trial. Seeds with white, firm endosperm were scored as viable and black, yellow, brown and spongy seeds were scored as inviable (Ooi et al. 2004).

Seedling Traits and Drought Tolerance

A glasshouse experiment was conducted to compare seedling performance under watered and drought conditions. For each species, 40 to 50 germinated seeds were randomly selected from the germination trial. Each germinated seed was transplanted into a 5 cm × 5 cm × 12 cm pot filled with potting mix, with geotextile placed at the bottom of each pot to hold the soil while allowing sufficient drainage. One cubic metre of potting mix contained one part of Coir peat and two parts of sand with fertiliser (Dolomite: 435 g; Iron Sulphate: 500 g; Micromax: 365 g; Osmocote: 1,500 g). All pots were placed in a random order and rotated once every 3 weeks to capture the potential variation in sunlight exposure inside the glasshouse. All seedlings were grown for 3 weeks with daily watering to field capacity (‘settlement phase’) to reduce the chance of transplantation mortality immediately upon transfer from the petri dish (Maherali et al., 2009). Seedlings were watered for 2 min at 8 am and 1 min at 4 pm in each daily watering. Between 14 and 47 seedlings emerged and survived the three-week settlement phase per species, leaving most species with at least 30 seedlings for the experiment (except *P. velutina*, 20 seedlings and *P. pallida*, 14 seedlings).

After the three-week settlement phase, drought treatments were applied to half of the plants. The drought treatment aimed to stress plants without causing mass mortality and included both a reduction in how often pots were watered and two extended periods (7–10 days at weeks 3 and 7) of no watering. The control group was watered daily for 6 weeks while the treatment group was watered twice a week (‘drought treatment’) in week 1, 2, 4 and 5. The overall water loss was calculated over a 9-day duration of no watering (see **Supplementary Appendix S2**), with a mean change of 0.21 (θ_g).

The temperature range was monitored by a temperature data logger (DS1921G-F5 thermochrons) throughout the experiment for the Control (14.1 ± 0.07°C) and Drought (17 ± 0.07°C) treatments.

To assess the effects of our drought experiment, we measured plant survival and other attributes at the commencement and at the end of the drought treatment. Stem length of individual seedling in both treatments was measured using digital calipers and expressed in millimetres (mm). RGR was then calculated based on stem length, as it is one of the most sensitive stress indicators for water deficits. Mean RGR of each species was calculated by averaging the RGR across all treatment replicates within each species. To calculate RGR for each plant, we used the following formula:

$$RGR = \left(\ln S_2 - \ln S_1\right) / \left(T_2 - T_1\right)$$

where S_1 and S_2 were the stem length of the plants at T_1 (first day of the treatment phase) and at T_2 (final day of the treatment phase).

Dry biomass was also measured at the end of the growth experiment. Shoots and roots from a subsample of five plants per species from each treatment were collected within a week after the final measurement of stem length was taken. Both shoots and roots were washed by hand to remove the soil (Pérez-Harguindeguy et al., 2016), oven-dried at 60°C for 72 h and weighed to estimate total biomass production. The shoot:root ratio (SRR) was calculated for each plant to determine the distribution of dry plant biomass. Mean biomass and mean SRR for each species were calculated by averaging all replicates within each species.

Statistical Analyses

All statistical analyses were conducted in R (R version 3.5.1; R Development Core Team, 2018) using the RStudio integrated development environment (RStudio version 1.1.453; RStudio Team, 2018).

Four seed traits (seed mass, T_{50}, G_{20}, mortality) and five seedling traits (RGR stem length, stem length, SRR, total biomass and survival) were assessed. The two main predictors, ploidy and range size, had two levels, diploid or polyploid and narrow range or widespread, respectively (**Table 1**). Additionally, for the third hypothesis, there was a third predictor (drought treatment) for the seedling traits. The effect of the predictors on continuous response data (seed mass, G_{20}, RGR stem length, stem length, SRR and total biomass) was analysed using Linear Mixed-effects Models (LMM), except for T_{50}, which was analysed using a linear model (only a single value per species). The effect of predictors on proportional mortality data was analysed using a Generalised Linear Mixed-effects Model (GLMM) using a binomial distribution with logit link function. These two sets of models were fitted with the *lme4* package (R Development Core Team, 2018), using species as a random factor. We were unable to directly account for phylogenetic relationships with our analyses because at the time of study a phylogeny was not available for the genus. We therefore accounted for relatedness by including species as a random factor in our analyses. A Generalised Linear Model (GLM) assuming a binomial distribution with logit link function was used to test the effect of predictors on seedling survival. We checked for any potential effects of seed age by assessing storage duration (months) against the germination parameters T_{50}, G_{20} (linear models) and seed mortality (binomial model) and found no significant relationships (data not shown).

All response variable data were checked to ensure normality of residuals and homoscedasticity assumptions. T_{50} values underwent a Tukey ladder of Power transformation to fulfil the homoscedasticity assumption. G_{20} and stem length data were log-transformed to improve the normality of residuals and increase the homoscedasticity of variance. A single outlier replicate was removed from the seed mortality data set as it had three times greater mortality than other replicates, suggesting an experimental anomaly.

Model selection was utilised for each of the seed and seedling traits. For the four seed traits, the initial model contained the two main effects: ploidy and range size and their interaction. For the five seedling traits, the initial model contained three main effects: drought treatment, ploidy, range size and their interactions (interaction between ploidy and range size was excluded due to low seedling availability). All combinations of reduced models (five in total including the null model for the seed germination experiment, 23 in total including the null model for the glasshouse drought experiment) were then produced for each trait (response variable). The models were ranked with Akaike's Information Criterion corrected for small sample sizes (AIC_c) using the MuMin package (R Development Core Team, 2018). The model with the lowest AIC_c was chosen as the final model to capture all potential important terms. Where the null model had the lowest AIC_c and there was competing support ($\Delta\ AIC_c < 2$), the next best model was selected. Values of p were obtained through likelihood ratio tests by comparing the final model against a reduced model excluding fixed effects, using the *anova* function in R.

To further examine the relationship between seed mass and each seed and seedling trait, Pearson's correlation coefficients were calculated, or binomial models fitted (for proportion seed mortality and seedling survival), between log-transformed seed mass and each trait. Note that for seedling traits, data were combined from both watered and drought treatments. Tukey's Ladder of Power transformation was used when necessary to improve the normality of residuals of traits.

RESULTS

There was no interaction between ploidy and range size influencing the four traits measured in the seed germination experiment. Ploidy, often as the sole main factor, had the greatest effect on seed traits (**Table 2**; **Supplementary Appendix S3**) and it was a strong driver of the differences observed between traits at the seed stage. Polyploid seeds were significantly heavier than diploid seeds ($\chi^2 = 11.555$, d.f. = 1, $p < 0.001$; **Figure 1A**) and reached 50% germination (T_{50}) significantly faster than diploid seeds ($\chi^2 = 4.953$, d.f. = 1, $p = 0.048$; **Figure 1B**). G_{20} was similar for polyploids (94°C) and diploids (96°C; $\chi^2 = 0.2684$, d.f. = 1, $p = 0.604$; **Figure 1C**). Although seed mortality overall was relatively low (<15%), significantly more polyploid than diploid seeds were killed after the 120°C heat treatment ($\chi^2 = 4.972$, d.f. = 1, $p = 0.025$; **Figure 1D**).

Ploidy also had an effect at the seedling stage, although for fewer variables (**Table 2**). The full model developed for each seedling trait included interaction terms between ploidy and treatment, range size and treatment, plus their main effects. Removing the interaction terms considerably improved the AIC_c scores across all traits. The main effects of either ploidy or treatment or both were included in most of the best-fitting models (**Table 2**; **Supplementary Appendix S4**).

Polyploids had taller stems than diploids but this difference was not significant ($\chi^2 = 3.373$, d.f. = 1, $p = 0.066$; **Figure 2A**), regardless of drought treatment. The biomass of polyploids was significantly heavier than diploids ($\chi^2 = 4.607$, d.f. = 1,

$p = 0.032$; **Figure 2B**). The shoot:root ratio did not differ significantly between diploids and polyploids (**Figure 2C**).

The drought treatment had a strong effect on seedling traits (**Supplementary Appendix S4**), indicating that it imposed stress; however, the interaction between ploidy level and drought was not in the best-fitting models for any of these traits. Not surprisingly, drought significantly reduced seedling survival compared to watered controls ($\chi^2 = 28.672$, d.f. = 1, $p < 0.001$) and significantly slowed the RGR ($\chi^2 = 4.600$, d.f. = 1, $p = 0.032$). For SRR, the best-fitting model included only drought treatment as the fixed factor, with smaller values under drought (although not significant; $\chi^2 = 3.474$, d.f. = 1, $p = 0.062$), suggesting greater partitioning of resources towards root growth under stressful conditions.

Range size did not appear to be a strong driver of responses for any of the traits measured, although seedlings had a significantly greater RGR than widespread species, as demonstrated by the significant association between range size and the RGR of stem length ($\chi^2 = 5.818$, d.f. = 1, $p = 0.016$). There was also a significant main effect of drought on RGR, with reduced rates under drought conditions ($\chi^2 = 4.600$, d.f. = 1, $p = 0.032$).

Most seed traits displayed significant relationships with seed mass (**Figures 3A–H**). There was a strong negative linear relationship between T_{50} and seed mass ($r = -0.82$, $p < 0.001$; **Figure 3A**). G_{20} also exhibited a significant negative relationship with seed mass ($r = -0.45$, $p = 0.002$; **Figure 3B**). There was a positive relationship with seed mortality, showing a trend of higher mortality for heavier seeds at high temperatures (Binomial model, Pseudo $r^2 = 0.23$, $p < 0.001$; **Figure 3C**).

For seedling traits, RGR and seedling survival did not show any significant correlation with seed mass ($r = 0.008$, $p = 0.881$; Psuedo $r^2 = 0.006$, $p = 0.452$; **Figures 3D,H**), while stem length, SRR and biomass were all positively correlated with seed mass (stem length: $r = 0.31$, $p < 0.001$; SRR: $r = 0.27$, $p = 0.012$; biomass: $r = 0.55$, $p < 0.001$; **Figures 3E–G**). While the results indicate that larger seeds result in larger seedlings (**Figure 3E**) they also demonstrate that RGR was independent of seed size (**Figure 3D**).

TABLE 2 | Summary of results for all traits and factors investigated.

Life stage	Trait	Ploidy	Range size	Drought	Relationship with seed mass
Seed	Seed mass	▨ ***		NA	NA
	T_{50}	▨ *		NA	–ve
	G_{20}	▨		NA	–ve
	Seed mortality	▨ *		NA	+ve
Seedling	RGR (stem)		▨ *	▨ *	ns
	Stem length	▨			+ve
	SRR			▨	+ve
	Biomass	▨ *		▨ *	+ve
	Survival			▨ ***	ns

Shaded cells indicate that the factor was included in the best-fitting model identified by lowest AICc. Asterisks denote significance at less than 0.05 (*), 0.01 (**) or 0.001 (***) for the main factors: ploidy level, range size and drought treatment. Significant relationships with seed mass (mg) are denoted as positive (+ve), negative (–ve) or non-significant (ns). NA denotes factor not tested.

DISCUSSION

Relatively few studies have investigated the ecological consequences of polyploidy (Ramsey and Ramsey, 2014; Plue et al., 2018). Our study has demonstrated that polyploids maintain characteristics in the regeneration stage (faster germination rate and larger and taller seedlings) that contribute to a potentially higher competitive advantage than diploids. Hence, polyploid seeds that survive the passage of fire might have a higher chance of recovering quickly after fire (or other disturbance) than diploids. The larger seed size of polyploid *Pomaderris* may be an underlying mechanism driving some of these key differences in regeneration traits, but seed size is not the only driver. Our investigations showed that range size and ploidy did not interact significantly and, in fact, ploidy alone was significant for most of the seed and seedling traits measured. Overall, these findings indicate that ploidy has a strong influence on performance and several key traits in the regeneration phase of *Pomaderris*, which may have ecological consequences for species persistence in fire-prone habitats.

Polyploid *Pomaderris* seeds were significantly heavier than diploid seeds, consistent with studies of other plant species and ecosystems (Maceira et al., 1993; Hoya et al., 2007; Eliášová and Münzbergová, 2014, 2017; Godfree et al., 2017; Stevens et al., 2020). This pattern is generally attributed to genome multiplication causing an increase in cell size which results in a larger seed size (Bretagnolle et al., 1995; Te Beest et al., 2012). Polyploid *Pomaderris* germinated significantly faster than diploids, adding to the growing body of evidence that demonstrates polyploid germination to be faster than diploid germination (Bretagnolle et al., 1995; Hoya et al., 2007; Eliášová and Münzbergová, 2014). The mechanisms underlying larger seed size and faster germination of polyploids are not yet well characterised. Potentially, the additional genome in polyploid seeds might result in a higher energy content, as well as a larger energy requirement and thus lead to more rapid food reserve mobilisation to support growth (von Well and Fossey, 1998). Ecologically, larger seeds and faster germination, regardless of whether they are effects of seed size or other polyploidy effects (Bretagnolle et al., 1995; Eliášová and Münzbergová, 2014), can be advantageous traits in the post-fire environment.

The high temperatures required to overcome physical dormancy in the relatively small-seeded *Pomaderris* species in this study (a G_{20} mean of approximately 95°C) is consistent with the concept that small-seeded species in fire-prone systems have higher dormancy-breaking temperature thresholds than large-seeded species (Hanley et al., 2003; Ooi et al., 2014; Liyanage and Ooi, 2017a). *Pomaderris* and other small-seeded species close to the soil surface are more likely to be exposed to higher temperatures during the passage of fire. Due to their higher temperature thresholds

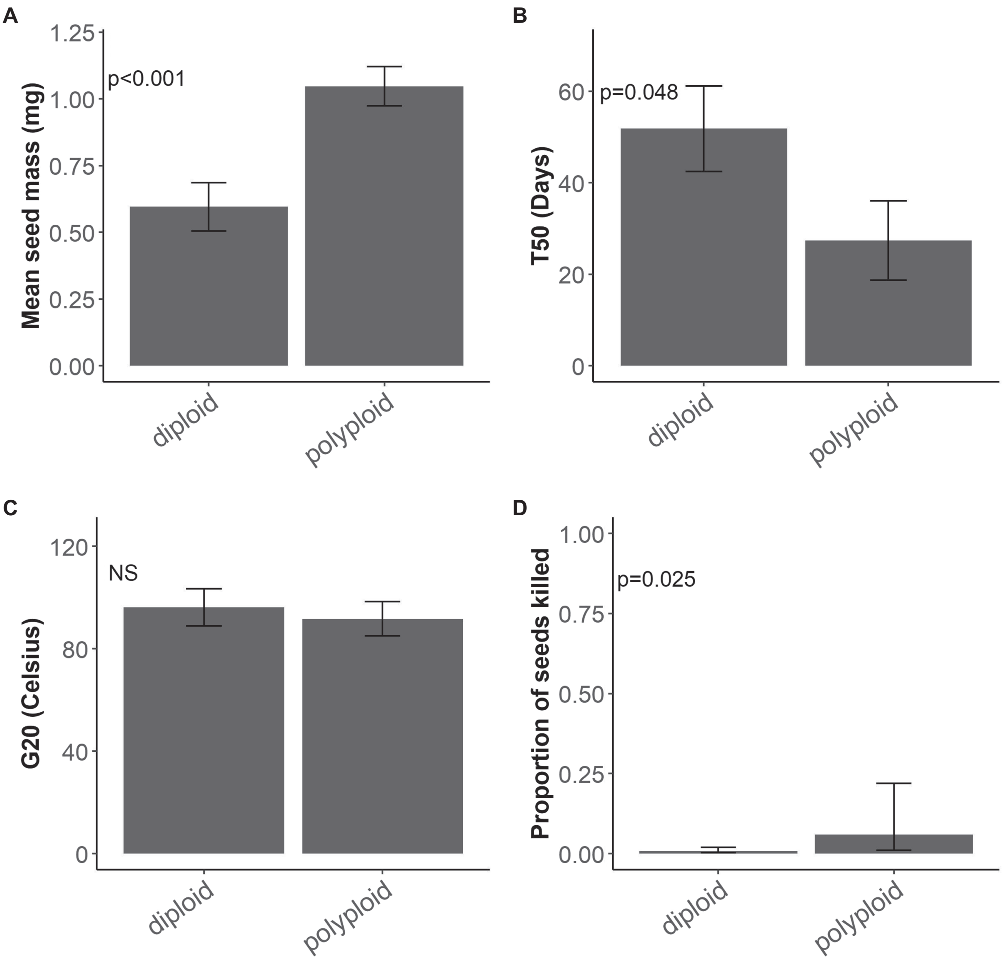

FIGURE 1 | Ploidy and seed traits. Mean and standard error for **(A)** seed mass (mg; $n = 20$ per species), **(B)** germination speed T_{50} (days; $n = 4$ per species), **(C)** minimum dormancy-breaking temperature thresholds G_{20} (°C; $n = 4$ per species) and mean and confidence interval for **(D)** seed mortality (proportion of seeds killed) at 120°C treatment, of diploid and polyploid *Pomaderris* ($n = 4$ per species). NS indicates a non-significant value.

for dormancy alleviation, they emerge from closer to the soil surface and avoid germination from depths too deep for small seeds to emerge successfully (Ooi et al., 2014; Liyanage and Ooi, 2017a). The positive relationship we found between seed size and seed mortality at high temperatures is further evidence of smaller seeds from fire-prone systems being adapted to survive high temperatures in the soil. However, the dormancy-breaking temperature threshold (measured by our G_{20} index) did not differ significantly between ploidy levels. There was significantly higher mortality in polyploid than diploid species as well as a strong correlation with seed mass (smaller seeds had a higher G_{20} and suffered less seed mortality at 120°C).

The hypothesis that polyploid *Pomaderris* would have a higher drought tolerance than their diploid counterparts was not supported for any seedling traits assessed, a finding that differs from other studies investigating water stress response and ploidy (e.g., Li et al., 1996; Eliášová and Münzbergová, 2017). The drought treatment in our study significantly decreased survival, yet did not result in a stress-related advantage for polyploids. Drought tolerance may be reflected in a greater investment in root biomass for better water acquisition (Comas et al., 2013); however, there was no clear difference in the investment of resources between diploids and polyploids, and there was only a relatively weak relationship between SRR and seed mass ($r = 0.27$). Therefore, polyploid *Pomaderris* do not seem to be more stress tolerant or differ from diploids in their root investment under drought. Other studies have found that root investment under water stress is variable or species-specific and could be due to other mechanisms (Larson and Funk, 2016). The stress response of *Pomaderris* seedlings may differ for mature seedlings or adult plants during the plant life-cycle (Satyanti et al., 2021) and could be investigated in future.

The lack of any clear effect of range size on most of the performance traits we studied may be related to the multiple potential causes of narrow range size and threatened status. Most trait relationships with range size or threat have a highly

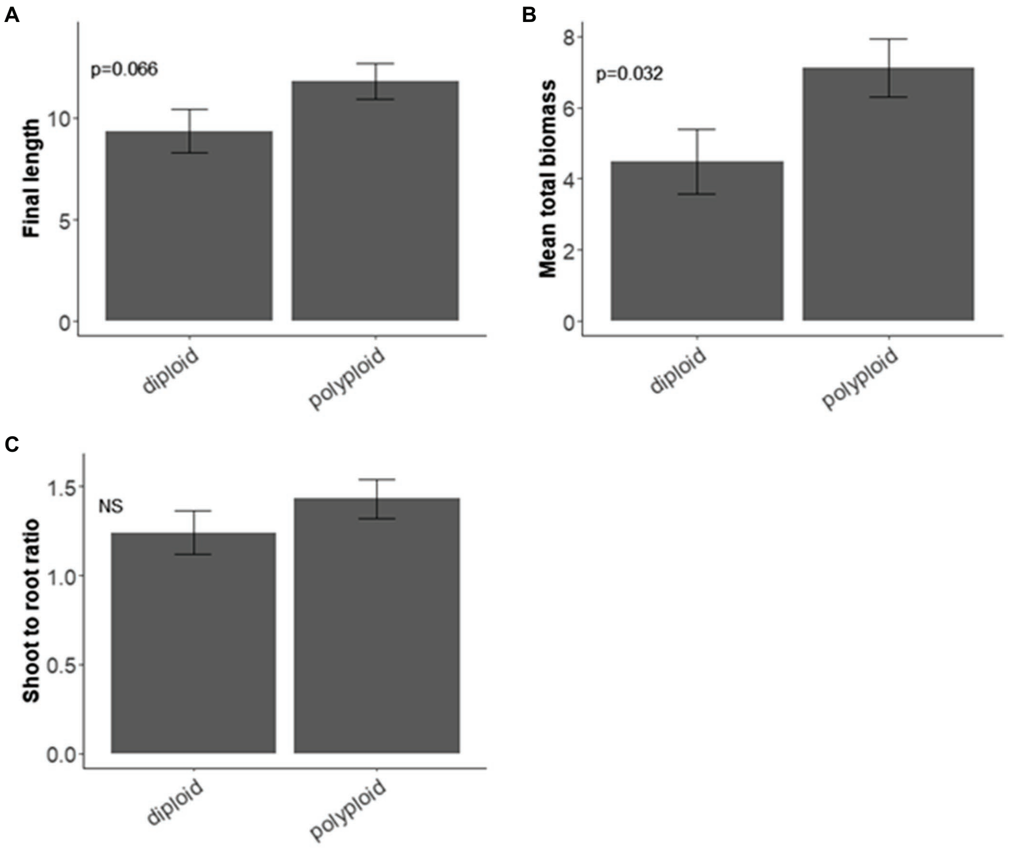

FIGURE 2 | Ploidy and seedling traits of *Pomaderris*. Mean and standard error for **(A)** stem length (mm) at 6 weeks ($n = 25$ per species for each treatment), **(B)** biomass (mg; $n = 5$ per species for each treatment), **(C)** biomass shoot to root ratio (SRR; $n = 5$ per species for each treatment). NS indicates a non-significant value.

context-dependent nature (Bevill and Louda, 1999; Murray et al., 2002). The one significant effect of range size found in our study, faster RGR for stem length, as well as a tendency for lower mortality in narrow range species, showed that narrow range species might not always perform worse than widespread species. However, outside of a controlled environment other external factors, such as competition, can affect species rarity (McIntyre, 1995). Disturbance dependent species in Australia are more likely to be threatened when there is an absence of appropriate disturbances and an increase in exotic plant competition, herbivory or other factors (McIntyre, 1995). Because of the broad range of potential factors driving range size and threatened status, future studies could consider ploidy and range size for a larger number of species and multiple potential drivers (e.g., analysing genome size, range size, niche breadth, latitude, and altitude) which may reveal other relationships (Pandit et al., 2014). The phylogenetic relationships among species may also influence results. At the time of this study, a comprehensive phylogeny of the genus was not available. The recent publication by Nge et al. (2021) of a *Pomaderris* phylogeny will provide opportunities to better account for relationships among species in future studies. Informal comparison of the ploidy results from Chen et al. (2019) and the recent phylogeny (Nge et al., 2021) suggest that ploidy levels do not appear to be phylogenetically clustered. This suggests our results are unlikely to be simply due to relatedness and this could be further explored more robustly by incorporating a larger data set.

Under high-intensity fire, while ploidy may not affect the seed dormancy-breaking threshold, polyploid *Pomaderris* could perhaps emerge from deeper within the soil profile due to their larger seeds (Bond et al., 1999; Hanley et al., 2003; Liyanage and Ooi, 2017a). Taller and larger seedlings enhance the competitive ability of a plant, providing polyploid *Pomaderris* a higher potential to overgrow surrounding vegetation and better compete for light in a post-fire environment. The potential higher competitiveness of polyploid *Pomaderris* species aligns with previous studies that have found a positive correlation between seed mass and seedling height (Liancourt et al., 2009). Our study has demonstrated that polyploid *Pomaderris* may have higher potential competitiveness than diploids in fire-prone habitats. However, there are other stochastic factors that could affect seedling establishment in post-fire environments (Moles and Westoby, 2004a), and the interaction of ploidy with these factors needs further study in the field.

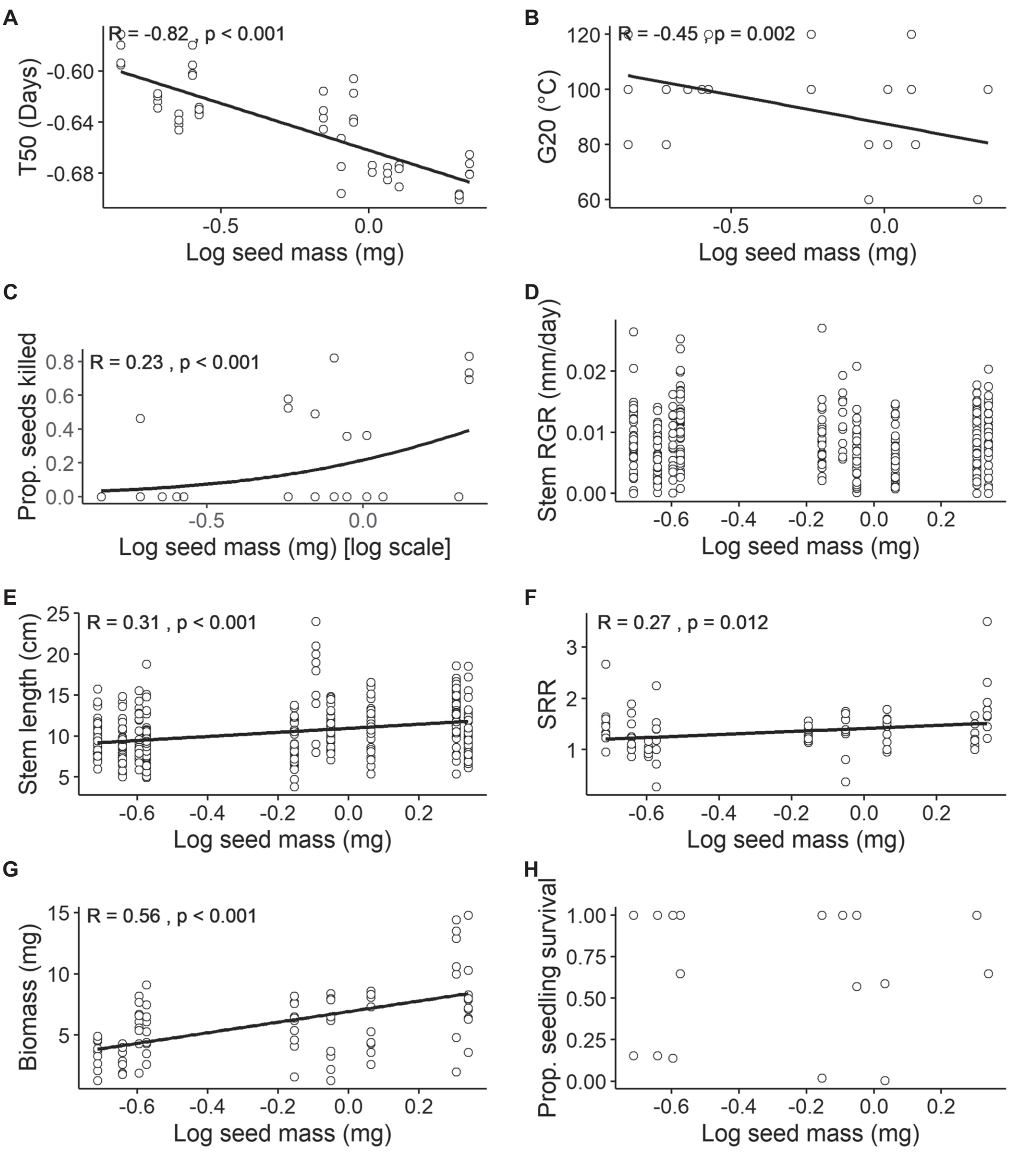

FIGURE 3 | Relationships between mean seed mass (mg) [log scale] and seed and seedling traits. Sample size (*n*) varies within each trait. **(A)** Germination speed T_{50} (days; $n = 4$ per species), Tukey's ladder transformation, **(B)** minimum dormancy-breaking temperature thresholds G_{20} (°C; $n = 4$ per species), **(C)** seed mortality (proportion of seeds killed) at 120°C treatment ($n = 4$ per species), **(D)** Relative growth rate (RGR) of stem length (mm/day; $n = 25$ per species for each treatment), **(E)** stem length (mm; $n = 25$ per species for each treatment), **(F)** shoot to root ratio (SRR; $n = 5$ per species for each treatment), **(G)** biomass (mg; $n = 5$ per species for each treatment) and **(H)** seedling survival (proportion of survived seedlings calculated based on ($n = 25$) sample size per species per treatment) on combined watered and drought treatment. All continuous variables were tested using Pearson's correlation and binomial responses using Generalised Linear Models (GLMs). For significant relationships, the line of best fit has been plotted. No line denotes non-significant associations among traits.

Conservation management has often focused solely on the adult plant persistence niche. However, the long-term recovery and persistence of a species is also dependent on the regeneration niche (Grubb, 1977; Jiménez-Alfaro et al., 2016; Saatkamp et al., 2019). Our study shows that seed and seedling traits exhibited during regeneration of narrow range species did not differ significantly from widespread species. However, ploidy was related to a large difference in overall regeneration performance of diploid and polyploid *Pomaderris*. Among the many threatened *Pomaderris* taxa, the regeneration niche of diploids should be considered as potentially limiting for populations because of their smaller seed size, slower germination rate and smaller seedlings. This insight to the regeneration phase may need to be considered when planning and prioritising management of threatened species.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found online at Figshare at: https://doi.org/10.6084/m9.figshare.15042972.

AUTHOR CONTRIBUTIONS

MO and LG conceived the ideas. JC, MO, and LG designed methodology and led the writing of the manuscript. JC and MO collected the data and analysed the data. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2021.779651/full#supplementary-material

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Functional Traits Drive Dispersal Interactions Between European Waterfowl and Seeds

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Endozoochory by waterfowl is important for a broad range of angiosperms, most of which lack a fleshy fruit. This dispersal function contributes to the formation and maintenance of plant communities and may allow range shifts for plant species under global change. However, our current understanding of what seed or plant traits are important for this dispersal mechanism, and how they relate to variation in waterbird traits, is extremely limited. We addressed this question using a unique dataset identifying the plant species whose seeds are ingested by 31 different waterfowl species in Europe. We used RLQ and fourth-corner analyses to explore relationships between (1) bird morphological and foraging strategy traits, and (2) plant traits related to seed morphology, environmental preferences, and growth form. We then used Generalized Additive Models to identify relationships between plant/seed traits and the number of waterfowl species that disperse them. Although many waterfowl feed intentionally on seeds, available seed trait data provided little explanation for patterns compared to plant traits such as Ellenberg indicators of habitat preference and life form. Geese were associated with terrestrial plants, ingesting seeds as they graze on land. Diving ducks were associated with strictly aquatic plants, ingesting seeds as they feed at greater depths. Dabbling ducks ingest seeds from plants with high light and temperature requirements, especially shoreline and ruderal species growing in or around the dynamic and shallow microhabitats favored by these birds. Overall, the number of waterfowl vector species (up to 13 per plant species) increases for plants with greater soil moisture requirements and salinity tolerance, reflecting the inclination of most waterfowl species to feed in coastal wetlands. Our findings underline the importance of waterfowl dispersal for plants that are not strictly aquatic, as well as for plants associated with high salinities. Furthermore, our results reveal a soil moisture gradient that drives seed-bird interactions, in line with differences between waterfowl groups in their microhabitat preferences along the land-water continuum. This study provides an important advance in our understanding of the interactions that define plant dispersal in wetlands and their surroundings, and of what plants might be affected by ongoing changes in the distributions of waterfowl species.

Keywords: seed dispersal, functional trait, endozoochory, long distance dispersal, waterbirds, Anatidae, migratory birds, feeding behavior

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INTRODUCTION

Long distance seed dispersal (LDD) events have a vital role in determining plant species distribution, genetic flow among populations and the colonization of unoccupied habitats (Levine and Murrell, 2003; De Queiroz, 2014). Dispersal by animal vectors (zoochory) provides longer distance dispersal in comparison to abiotic vectors (Bullock et al., 2017), with migratory birds able to provide extreme dispersal distances (Popp et al., 2011; Viana et al., 2016; Viana, 2017). These birds mainly disperse seeds *via* gut passage (endozoochory), yet their importance for plants lacking a fleshy-fruit has been underestimated because it is ignored by widely used "dispersal syndromes" (Green et al., 2021). Many of the migratory bird species are waterbirds, which are important for the dispersal of a broad range of both aquatic and terrestrial plants between habitat patches, often facilitating dispersal at extreme distances of >100 km (Green et al., 2016, 2021; Viana et al., 2016; Martín-Vélez et al., 2021). The waterbird group best known for seed dispersal is that of the waterfowl (Anatidae: ducks, swans and geese) (Green et al., 2016, 2021; Soons et al., 2016). These birds are well-studied partly due to their exploitation for hunting (Green and Elmberg, 2014), and many species are abundant long-distance migrants (Wetlands International, 2021). Waterfowl are vital vectors for maintaining connectivity between plant populations in wetlands lacking hydrological connections (Amezaga et al., 2002).

Food selection and ingestion are the first stages of seed dispersal and ingestion is the main component of dispersal "quantity" (Schupp et al., 2017). Plant and seed traits, as well as vector traits, may influence seed uptake, so identifying these traits can provide insights as to the mechanisms behind plant community establishment (Schleuning et al., 2020). Interactions between plants and frugivorous birds (i.e., those feeding on fleshy-fruits) in terrestrial ecosystems are known to depend on many traits of both groups of organisms, such as fruit size and palatability and bill morphology (Wenny et al., 2016; Donoso et al., 2017). On the other hand, despite recent advances in identifying the interactions between different waterfowl and plant species in a particular community (Reynolds and Cumming, 2016; Sebastián-González et al., 2020; Silva G. G. et al., 2021), the trait associations underlying the uptake of seeds by different waterfowl species remain unknown. In general, seed ingestion by non-frugivorous vertebrates is not size limited in a manner comparable to frugivores (Chen and Moles, 2015; but see Gurd, 2008).

Finding relationships among traits of seed and waterfowl species may be complicated by intraspecific plasticity and variation. Waterfowl sometimes select seeds or fruits individually, but usually ingest seeds collectively while filtering food items out of water or sediments, while ingesting other plant parts, or even ingest them secondarily when eating animal prey (Goodman and Fisher, 1962; Li and Clarke, 2016; Pecsics et al., 2017). A given waterfowl species may use more than one foraging substrate and different foraging strategies (Li and Clarke, 2016), and encounter a diversity of habitats and flora along their migration routes. On the other hand, aquatic plants have different growth forms and are often amphibious, while terrestrial plants can occur in moist soils where seeds can be ingested by grazing waterfowl, or their seeds may be washed or blown into waterbodies (Wells and Pigliucci, 2000; Lovas-Kiss et al., 2019; Li et al., 2020). Seeds of terrestrial plants also become available to waterfowl during seasonal floods. Nevertheless, waterfowl are traditionally placed into different foraging guilds, and their habitat use (e.g., in relation to depth and shoreline vegetation) is considered to be related to body size and bill morphology (Nudds et al., 2000; Guillemain et al., 2002; Kear, 2005; Gurd, 2008). Therefore, we expect differences in foraging niche between waterfowl species to be reflected in differences in the traits of the plants they disperse.

For the present study, we have established a comprehensive dataset on the plant seeds ingested by European waterfowl. Although there is more information about waterfowl diet in North America, we focus on Europe because of the quality of information on plant traits. Our dataset includes a mixture of data demonstrating seed ingestion from the upper and lower digestive tracts, as well as from the feces of individual birds. The presence of seeds in the upper digestive tract of waterfowl has been shown to be a good proxy for seed dispersal by endozoochory (Kleyheeg et al., 2015; Soons et al., 2016; Costea et al., 2019). Previous studies have used part of this same dataset regarding seven dabbling duck species, including a comparison of traits between European plants whose seeds were ingested, and plants whose seeds were not (Dessborn et al., 2011; Soons et al., 2016).

Here, we investigate if different waterfowl species (including ducks, geese, and swans) tend to disperse plants with different traits. We evaluate how seed and other plant traits determine their chances of dispersal in two different ways. Firstly, to assess whether some plant species have a higher chance of being transported by a particular waterfowl species, we assess whether duck, geese and swan traits act as filters to determine the traits of the seeds they ingest. Then, to evaluate if there are plant characteristics that give them higher chances of being transported, we analyze how seed and plant traits influence the number of different waterfowl species that disperse each plant species.

MATERIALS AND METHODS

Diet Data

We conducted a systematic search on all information on the presence of angiosperm diaspores (hereafter called seeds) in the alimentary canal or feces of European waterfowl and used this as a proxy for dispersal interactions. As a starting point, we took the studies for dabbling ducks compiled by Dessborn et al. (2011) and Soons et al. (2016), together with the accounts for all waterfowl species in Snow et al. (1998). We then added further studies for all waterfowl, by searching in Google Scholar and in the Web of Science up to March 20, 2021. We used as keywords "gut content," "dietary data or diet," and "fecal data or fecal samples" together with the scientific name of each species. We included in our search all 39 waterfowl species that occur in Europe (as both wintering or breeding species) according to the IOC World Bird List (Gill et al., 2021). For species where scientific names have changed (e.g., changes in genera from *Anas* to *Mareca* or *Spatula*) we included all known versions of the name. A list of the literature we used in our study is found in **Supplementary Material 1**. Furthermore, we also used unpublished data from the authors' recent studies of seed dispersal *via* fecal analysis.

We considered only diet data from adult waterfowl and only the presence of seeds from a given plant species in the diet of the waterfowl species, as abundance or frequency data are often unreported, or are not quantified in a comparable manner. As we were looking for trait-dependent relationships, seeds from 85 plant taxa that were not identified to the species level were excluded from further analyses. Domestic plant species (e.g., rice or barley) were also removed from further analyses since they are often used to bait waterfowl, and are less likely to be dispersed (Lovas-Kiss et al., 2018). We did not exclude non-native plants or bird species. From this database, we produced a waterfowl by plant species matrix indicating the occurrence of each seed species in the diet of each waterfowl species (**Supplementary Material 2**). We were unable to account for spatial or temporal factors due to limitations of our technique of analysis and to the low number of studies found for many of the bird species (**Table 1**).

Waterfowl Traits

For both plant species and waterfowl species, we compiled available information on traits that we considered to be possible drivers of the interaction between waterfowl and seeds (**Supplementary Material 3**). Available trait information for waterfowl included morphological measurements and feeding guilds. Waterfowl morphological measurements of bill length (from tip to skull along the culmen, and from tip to nares), beak width and depth at the nares and tarsus length were provided by Pigot et al. (2020). Estimates of mean body mass (g) were sourced from Wilman et al. (2014). Body mass can be a proxy for species' size and, together with tarsus length, may indicate the depth or height reached by a particular species. On the other hand, beak morphology may be associated with differences in seed uptake at a given feeding depth/height (Poysa, 1983a; Kear, 2005). Based on previous studies on waterfowl diet and foraging behaviors (Kear, 2005), we assigned ducks, geese and swans into six feeding guilds: Omnivorous dabbling ducks (OmDab from hereon), Omnivorous diving ducks (OmDiv), Terrestrial plant-eaters (TerPl), Aquatic plant-eaters (AqPl), Marine invertebrate-eating diving ducks (MarInv), and Fish-eating diving ducks (FiEat). In most studies where seeds were recorded in their diet, MarInv were feeding in low salinity habitats such as Lake Myvatyn in Iceland, or the Baltic Sea, where they co-occurred with OmDab and OmDiv.

Plant/Seed Traits

For plants, we considered traits describing environmental preferences, growth form and seed morphology. We sourced Ellenberg Indicator Values (EIVs, Ellenberg and Leuschner, 2010) for soil moisture (F), nutrients (N), salinity (S), temperature (T), and light exposure (L). EIVs indicate the general preference of a plant species for specific environmental conditions, and thus may vary geographically for each plant species with extensive ranges, according to climatic variation. Hence to avoid bias, where possible we used EIV values from the LEDA database (Kleyer et al., 2008). When values for a given species were not available in LEDA, we retrieved them from one of the following sources: PLANTATT (Hill et al., 2004), Pladias database (Chytrý et al., 2021), Baseflor (Julve, 1998), and TRY database (Kattge et al., 2020). Seed morphology is likely to influence the rates at which waterfowl ingest seeds (Guillemain et al., 2002; Green et al., 2016; Lovas-Kiss et al., 2020a). Seed morphological traits used were seed mass (g), seed size (mm^3), seed density (g/mm^3), and seed roundness. We did not use morphological dispersal syndromes because they are not relevant to endozoochory by waterfowl (i.e., the great majority of plants dispersed by endozoochory do not have an "endozoochory syndrome," Green et al., 2021). Seed size was estimated as a multiplication of seed length (Le), width (Wi), and thickness (Th). Seed density was estimated as seed mass divided by seed size, and seed roundness as $(Wi/Le + Th/Le + Th/Wi)/3$. Morphological values were sourced from the same databases used for EIVs. Seed mass was retrieved from the Seed Information Database of Kew Gardens (Royal Botanic Gardens Kew, 2021). We categorized plant species according to the most basic life form as: terrestrial, helophyte, hydato-helophyte (i.e., helophyte species that can have a submerged form), hygrophyte (i.e., amphibious, hydrophyte species that have terrestrial form), and hydrophyte. Finally, we classified hydrophytes, hygrophytes and helophytes into the main growth forms, according to the most typical zone occupied by each species in the aquatic environment. For this, we created four non-exclusive binary variables: submerged plant, floating leaved plant, emergent plant and wet soil plant (**Supplementary Material 4**). Since our analytical methods do not permit missing values, missing plant trait values (2.1%) were substituted for arithmetic means of all values observed for the corresponding trait.

Data Analysis

To assess whether some plant species have a higher chance of being transported by particular waterfowl species, we used RLQ (Dolédec et al., 1996) and fourth-corner analyses (Dray et al., 2014). RLQ has traditionally been recommended to evaluate univariate relationships between environmental variables and species functional traits (Almeida et al., 2018; Schack et al., 2020; Silva J. L. A. et al., 2021). We have utilized the method to look instead at the relationships between waterfowl species traits (substituting environmental variables) and plant species functional traits. In this way, we can ask how waterfowl functional traits might act as filters on plant traits, thus influencing seed dispersal processes and community sorting.

We followed the RLQ protocol using the package *ade4* (Dray and Dufour, 2007), in the R environment, version 4.0.4 (R Core Team, 2021). Correlations between both trait tables and the axes of the RLQ ordination were calculated to evaluate which traits were best represented by the RLQ axes. For the first two axes of the RLQ, we considered those trait-axis correlations exceeding 0.5 to be important. We used three approaches to test waterfowl trait

TABLE 1 | Anatidae species for which seed ingestion data was found, their feeding groups (i.e., guilds), and information on the numbers of published studies found in our literature search (see **Supplementary Material 1** for details), unpublished datasets used, locations for which data were found, sampled individuals, and plant species whose seeds were ingested by each bird species.

Species	Feeding group	Number of published studies	Number of unpublished datasets	Number of sampled locations	Number of sampled individuals	Number of sampled droppings	Number of plant species found in diet
Anas acuta	OmDab	13	4	13	438	95	76
Anas crecca	OmDab	24	6	21	2,997	57	213
Anas platyrhynchos	OmDab	32	7	31	2,999	431	240
Anser albifrons	TerPl	1	0	1	260	0	3
Anser anser	TerPl	3	2	5	0	74 + 5 kg	132
Anserbrachyrhynchus	TerPl	0	0	0	0	0	2
Anser erythropus	TerPl	3	0	3	203	720	9
Anser fabalis	TerPl	1	0	1	175	0	5
Aythya ferina	OmDiv	7	1	7	141	61	38
Aythya fuligula	OmDiv	6	0	7	431	0	40
Aythya marila	OmDiv	2	0	2	463	0	6
Aythya nyroca	OmDiv	2	0	2	2	0	3
Branta canadensis	TerPl	1	0	1	0	50	6
Branta leucopsis	TerPl	0	1	2	0	45	10
Bucephala clangula	OmDiv	5	0	6	201	0	24
Bucephala islandica	OmDiv	1	0	1	48	0	1
Clangula hyemalis	MarInv	2	0	2	528	0	2
Cygnus columbianus	AqPl	2	0	2	60	0	8
Cygnus cygnus	AqPl	0	1	1	0	77	1
Cygnus olor	AqPl	1	0	1	8	0	7
Mareca penelope	OmDab	12	3	13	543	140	75
Mareca strepera	OmDab	9	2	8	475	38	42
Marmaronetta angustirostris	OmDab	5	0	3	105	52	5
Melanitta nigra	MarInv	1	0	1	250	0	3
Mergus serrator	FiEat	1	0	1	143	0	4
Netta rufina	OmDiv	3	0	2	1	8	1
Oxyura leucocephala	OmDiv	0	1	1	17	0	5
Spatula clypeata	OmDab	11	2	10	365	109	46
Spatula querquedula	OmDab	4	0	3	213	0	31
Somateria mollissima	MarInv	1	0	1	50	0	1
Tadorna tadorna	OmDab	6	1	6	663	147	10
Total		63	30	68	11,820	2,104	407

OmDab, Omnivorous dabbling ducks; OmDiv, Omnivorous diving ducks; TerPl, Terrestrial plant-eaters; AqPl, Aquatic plant-eaters; MarInv, Marine invertebrate-eating diving ducks; FiEat, Fish-eating diving ducks.

x plant trait associations. First, we tested the overall significance of the RLQ analysis. Next, we tested the significance of direct trait-trait relationships directly on the raw data tables. Finally, we tested for the significance of the trait-axis relationships, in which those traits correlated with the same axis can be interpreted as associated traits. We used 49,999 permutations and permuted both waterfowl and plant species as a procedure to test for significance ($\alpha = 0.05$) in all three tests, and adjusted P-values for multiple testing using the false discovery rate method (FDR, Benjamini and Hochberg, 1995). See **Supplementary Material 5** for further details on the RLQ and fourth-corner protocol.

To determine if particular seed/plant traits are preferred by a greater number of waterfowl species, we used zero-truncated Generalized Additive Modeling (GAM). GAMs use smoothing curves to model relationships between variables. As GAM allows both linear and non-linear fits, it is very useful for modeling data with non-linear distributions (Zuur et al., 2007). We used zero-truncated models because our data represented counts (number of bird species associated with a given plant species), and only plant species whose seeds were ingested by at least one waterfowl species were included (Zuur et al., 2009). We fitted models of the number of waterfowl species that consumed each seed species (hereafter number of vector species) against the predictor variables of soil moisture, nutrient, salinity, temperature, and light exposure EIVs, and seed roundness, mass and density. We did not include seed volume because this was highly correlated with seed mass (Pearson's $r = 0.78$). We also excluded categorical and binomial variables describing plant life forms, because these were related to soil moisture (EIV F). Seed roundness, mass and density were natural log transformed prior to model construction to reduce the effect of extreme values. The relationships of temperature, light exposure and moisture EIVs with the response variable were assumed to be linear and modeled as such (**Supplementary Table 8**). We modeled the number of vector species against the predictor variables with the negative binomial error distribution to account for overdispersion. We fitted models with all possible combination of factors, and used the Akaike Information Criteria (AIC) for model selection. As we had many models with similar ΔAIC values, we considered as the best model to be the one with the lowest number of terms and ΔAIC <6 (Zuur et al., 2007). We plotted the deviance residuals against the explanatory variables, and compared the observed and fitted counts in a rootogram (a modified histogram plotting the square roots of frequencies) as part of model validation. We fitted our models using the function *vgam* from the package *VGAM* (Yee, 2020), in the R environment, version 4.0.4 (R Core Team, 2021).

RESULTS

Studies containing seed ingestion information were sourced for 31 waterfowl species across Europe. The number of individuals and sites sampled varied widely among bird species and, in general, dabbling ducks (OmDab) were better sampled than other waterfowl species (**Table 1**). After removing the plants whose seeds were identified only to family or genus level ($n = 85$) and domesticated species ($n = 16$), 407 plant species were recorded as being ingested by at least one bird species. The mean number of vectors ingesting each plant species was 2.58 (s.e. = 0.12), with 49.4% of the plant species being ingested by only one vector, and a maximum of 13 vectors ingesting one plant species (for the sea club-rush *Bolboschoenus maritimus* and the mare's tail *Hippuris vulgaris*).

RLQ Analysis

The first two axes of the RLQ analysis explained 80.76 and 10.93% of the total co-inertia, respectively, and were retained for further analysis (**Supplementary Material 5**). Considering only correlation values above 0.5, the first RLQ axis was positively correlated with the foraging guilds marine invertebrate-eating diving ducks (MarInv) and omnivorous diving ducks (OmDiv), and with the Ellenberg indicator value for soil moisture (EIV F), helophytes, hydato-helophytes and hydrophytes and with the submerged, floating leaved and emergent growth forms. This same axis showed negative correlations with the following bird traits: bill length, width and depth, tarsus length, body mass, aquatic plant-eaters (AqPl) and terrestrial plant-eaters (TerPl), and with the terrestrial life form of plants. The second axis presented positive correlations with the foraging guild omnivorous dabbling ducks (OmDab), with EIVs for salinity (S), temperature (T), and light (L), and with helophytes. This axis also showed negative correlations with bill width, tarsus length, body mass, the foraging guilds fish-eating diving ducks (FiEat), AqPl, MarInv, OmDiv, and TerPl and with the hydrophyte life form (**Figure 1** and **Supplementary Material 6**).

Fourth-Corner Tests

The general permutation test showed that there was a statistically significant global link between plant and waterfowl traits, according to the permutations of plant species ($p < 0.001$) and waterfowl species ($p = 0.046$). Furthermore, the fourth-corner tests found significant correlations for both trait-trait and trait-axis relationships. According to the trait-trait test, bird species belonging to the TerPl foraging guild were negatively correlated with soil moisture EIV (i.e., these waterfowl species interacted more with species requiring low soil moisture, see **Figure 2**), while those belonging to the OmDiv guild were positively correlated with submerged plants (i.e., omnivorous diving ducks interact more with seeds of submerged macrophytes, see **Figure 2**). Furthermore, the trait-axis test found that the first RLQ axis showed significant negative correlations with bird tarsus length, bill depth, body mass, with the foraging guild TerPl, and with plants with the terrestrial growth form. This depicts a relationship between geese species and terrestrial plants (**Figures 1–3**). This same axis showed significant positive correlations with the foraging guild OmDiv, with soil moisture EIV, with plants with the hydrophyte life form, and with submerged, floating leaved and emergent plants. This reflects a relationship between omnivorous diving ducks and strictly aquatic plants in all their growth forms (**Figures 1–3**). The second RLQ axis was significantly negatively correlated with OmDiv, but not with plant traits, and significantly positively correlated to OmDab and with the light and temperature EIVs, representing a relationship between dabbling ducks and plants that prefer higher temperature and light exposure (**Figures 1–3**).

Fitted Generalized Additive Modeling

In our best model (the one with the lowest number of terms and ΔAIC < 6), the seed traits of mass and density, and the EIVs for soil moisture and salinity (F and S) were significant predictors of the number of vector species dispersing each plant species (**Supplementary Material 7**). Smoothing terms indicated that log seed mass and log seed density presented unimodal relationships with the number of vector species (**Figure 4**). However, both ends of the smooth line presented very large standardized errors (**Figure 4**), due to the low number of plant species with extremely high or low mass and density values (even after data transformation). The soil moisture EIV smoother revealed a positive linear relationship between moisture and the number of vector species (**Figures 2**, **4**). The salinity EIV smoother revealed an increase in the number of vector species from values 0 to 2, a slight decrease from there to value 5, followed by a slight increase from then on (**Figures 2**, **4**).

DISCUSSION

We found evidence that waterfowl traits and seed and other plant traits interact to act as filters for plant dispersal *via* endozoochory. A unique dataset on seed ingestion by European Anatidae species allowed us to elucidate patterns in trait-trait relationships between the two groups, in the first study of its kind. We found that seed ingestion differs between waterfowl

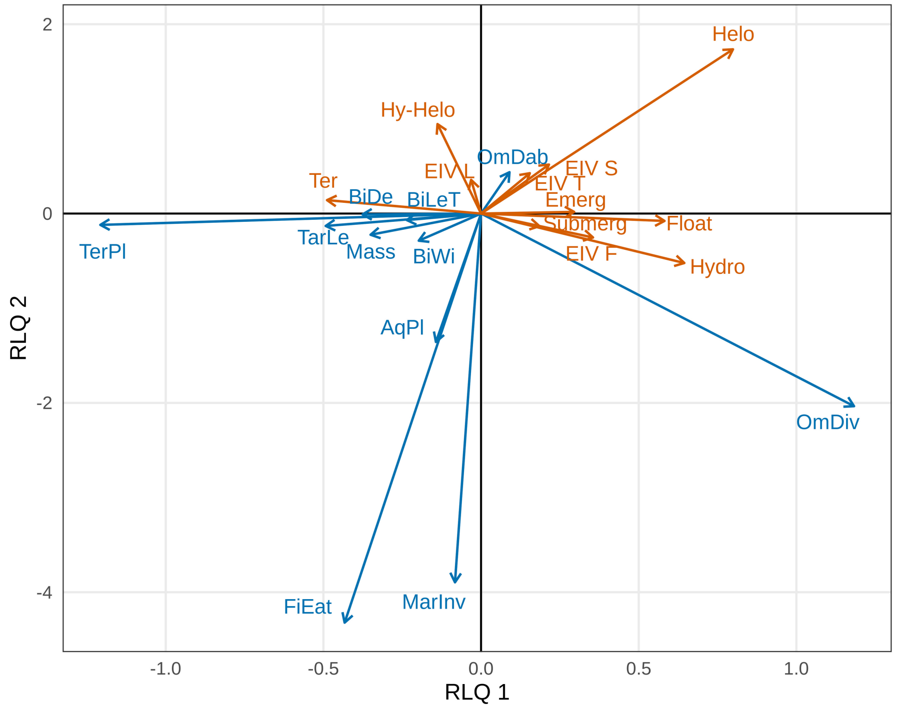


FIGURE 1 | Waterfowl (in blue) and plant traits (in orange) projected onto the first two axes of the RLQ analysis. We only present traits with correlations >0.5 with at least one axis. Traits showing positive (or negative) associations to the same axis are interpreted as being associated. Values of the correlations of each trait to each axis can be found in **Supplementary Material 6**, and their significance according to the fourth-corner test is given in **Figure 3**. BiLeT, Bill length; BiDe, Bill depth; BiWi, Bill width; TarLe, Tarsus length; OmDab, Omnivorous dabbling ducks; OmDiv, Omnivorous diving ducks; TerPl, Terrestrial plant-eaters; AqPl, Aquatic plant-eaters; MarInv, Marine invertebrate-eating diving ducks; FiEat, Fish-eating diving ducks; EIV, Ellenberg Indicator Value for soil moisture (F), salinity (S), temperature (T), and light exposure (L); Helo, Helophyte; Hy-helo, Hydato-helophyte; Hydro, Hydrophyte; Ter, Terrestrial; Submerg, Submerged; Float, Floating leaved; Emerg, Emergent.

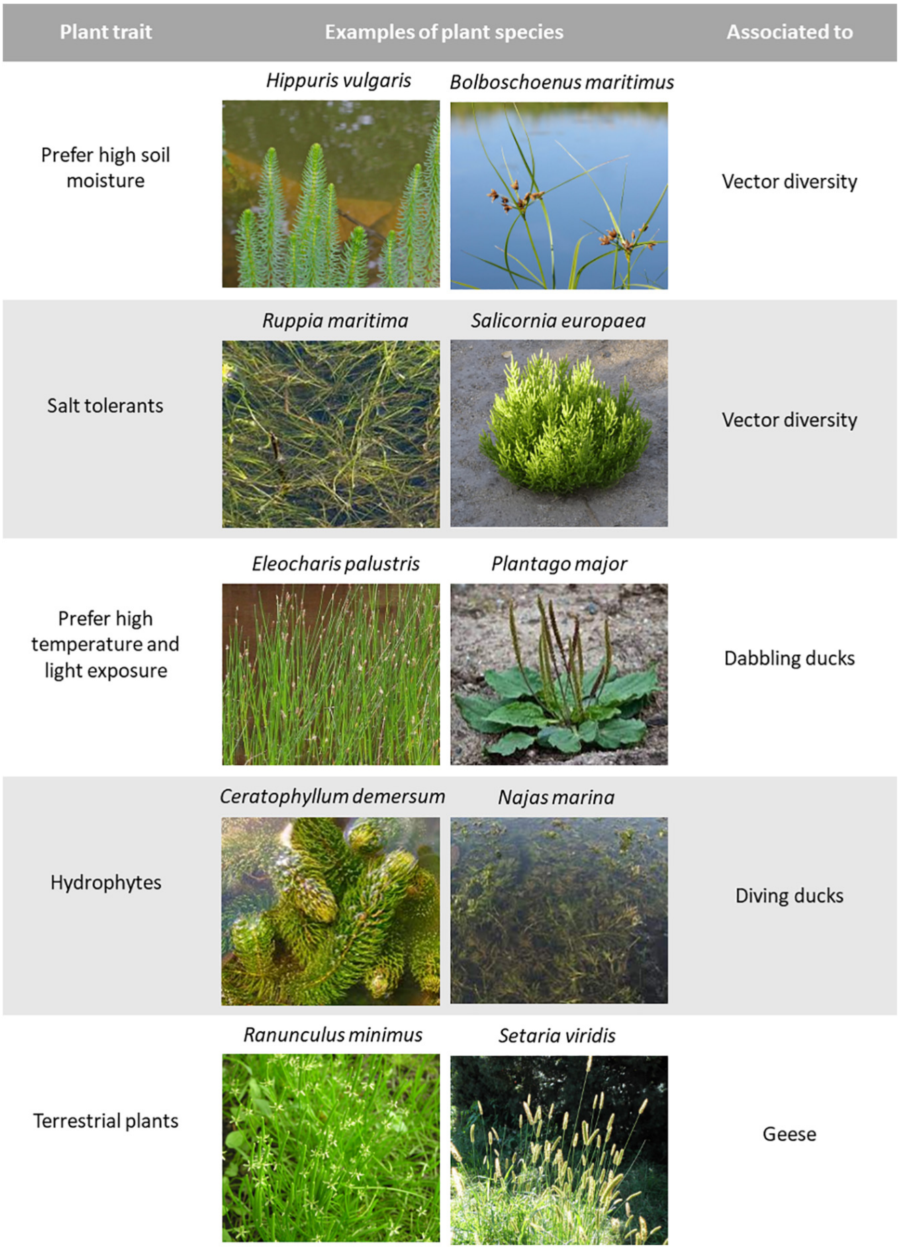
groups in a manner closely associated with differences in their selection of foraging habitat (Kear, 2005). This is particularly clear for herbivorous geese that feed on terrestrial plants and ingest seeds of more terrestrial species, most likely together with green plant material in a manner consistent with the "foliage is the fruit hypothesis" (Janzen, 1984; Green et al., 2016, 2021). At the other extreme, omnivorous diving ducks are typically bottom feeders in deep water, and tend to ingest the seeds of hydrophytes, whatever their growth form. In between are the dabbling ducks associated with shoreline habitats that are typically dynamic with high rates of disturbance, where they consume seeds of plants that prefer higher light exposure and temperature. We also investigated the plant traits that favor seed consumption by a greater number of waterfowl species and found that both higher moisture requirements and greater salinity tolerance favor seed ingestion by more waterfowl species. Seeds of intermediate mass and density (on a log scale) were also ingested by more waterfowl species.

Importance of the Moisture Gradient

Waterfowl feeding groups ingest seeds of plants with different life forms along the soil moisture gradient, with geese and omnivorous diving ducks at the terrestrial and strictly aquatic extremes, respectively (**Figure 2**). Other feeding groups were not associated with this gradient, although this may be due to limited data and diet literature in the case of fish-eating ducks FiEat, marine ducks MarInv and swans AqPl, which presented a very low number of interactions (see **Table 1** and **Figure 5**). Dabbling ducks were the best sampled group in our dataset, and they ingested seeds from plants found along the whole moisture gradient (see **Figure 5**). This suggests that dabbling ducks (exemplified by the ubiquitous mallard *Anas platyrhynchos*) play a more generalist role in the dispersal of different plant life forms, feeding in different habitats with a range of foraging strategies (see also Soons et al., 2016).

Light and Temperature Requirements of Plants Dispersed

Our RLQ analysis indicated that dabbling ducks are more likely to ingest seeds of plants that prefer higher temperature and light exposure. This result is consistent with Soons et al. (2016), who found that dabbling ducks are more likely to disperse plants that prefer higher light exposure than those which don't (a different analysis using part of this same dataset, but comparing it with European angiosperms for which dispersal by ducks has not been recorded). This suggests our result is unlikely to be a consequence of sampling bias, even though waterfowl species were unevenly



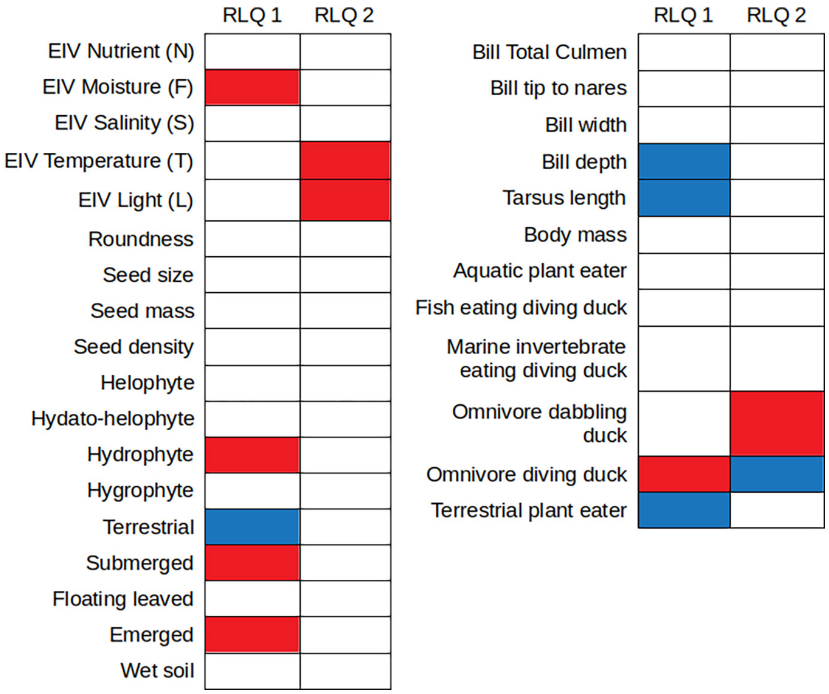
Plant trait	Examples of plant species		Associated to
Prefer high soil moisture	*Hippuris vulgaris*	*Bolboschoenus maritimus*	Vector diversity
Salt tolerants	*Ruppia maritima*	*Salicornia europaea*	Vector diversity
Prefer high temperature and light exposure	*Eleocharis palustris*	*Plantago major*	Dabbling ducks
Hydrophytes	*Ceratophyllum demersum*	*Najas marina*	Diving ducks
Terrestrial plants	*Ranunculus minimus*	*Setaria viridis*	Geese

FIGURE 2 | Plant functional traits involved in the main associations found in our RLQ analysis and GAM. We show examples of plant species that present each trait and with what aspect of waterfowl vectors each trait was associated with. Trait values, waterfowl species that ingest seeds of each plant species and credits for the photographs can be found in **Supplementary Materials 2, 3, 4, 8**.

sampled between latitudes and seasons. A preference of dabbling ducks for ingesting seeds from warmer, more open microhabitats likely reflects their affinity for exposed shoreline habitats and shallow, temporary wetlands such as seasonally flooded grasslands with short hydroperiods (Kear, 2005). Such habitats are created or maintained in response to some extent of stress and disturbance (e.g., hydrological variation, livestock, or urbanization) that do not allow aquatic or terrestrial vegetation to reach a mature late successional stage (Grime, 1977). For this reason, they are typically occupied by annual shoreline or ruderal plant species with high light and temperature preferences.

Different Waterfowl Have Different Roles as Seed Vectors

Our results for ingestion of seeds from plants with different traits confirm that different waterfowl species vary in their roles as seed dispersers. This is consistent with previous studies in South Africa and Brazil (Reynolds and Cumming, 2016;



	RLQ 1	RLQ 2
EIV Nutrient (N)		
EIV Moisture (F)	🟥	
EIV Salinity (S)		
EIV Temperature (T)		🟥
EIV Light (L)		🟥
Roundness		
Seed size		
Seed mass		
Seed density		
Helophyte		
Hydato-helophyte		
Hydrophyte	🟥	
Hygrophyte		
Terrestrial	🟦	
Submerged	🟥	
Floating leaved		
Emerged	🟥	
Wet soil		

	RLQ 1	RLQ 2
Bill Total Culmen		
Bill tip to nares		
Bill width		
Bill depth	🟦	
Tarsus length	🟦	
Body mass		
Aquatic plant eater		
Fish eating diving duck		
Marine invertebrate eating diving duck		
Omnivore dabbling duck		🟥
Omnivore diving duck	🟥	🟦
Terrestrial plant eater	🟦	

FIGURE 3 | Results of the fourth-corner tests on the associations of each plant and waterfowl trait with the first two axes of the RLQ. Blue and red cells represent, respectively, positive and negative significant associations. White cells represent associations not considered as significant by the fourth-corner tests. These results indicate associations between geese species and terrestrial plants, between omnivorous diving ducks and strictly aquatic plants, and between dabbling ducks and plants that prefer higher temperature and light exposure.

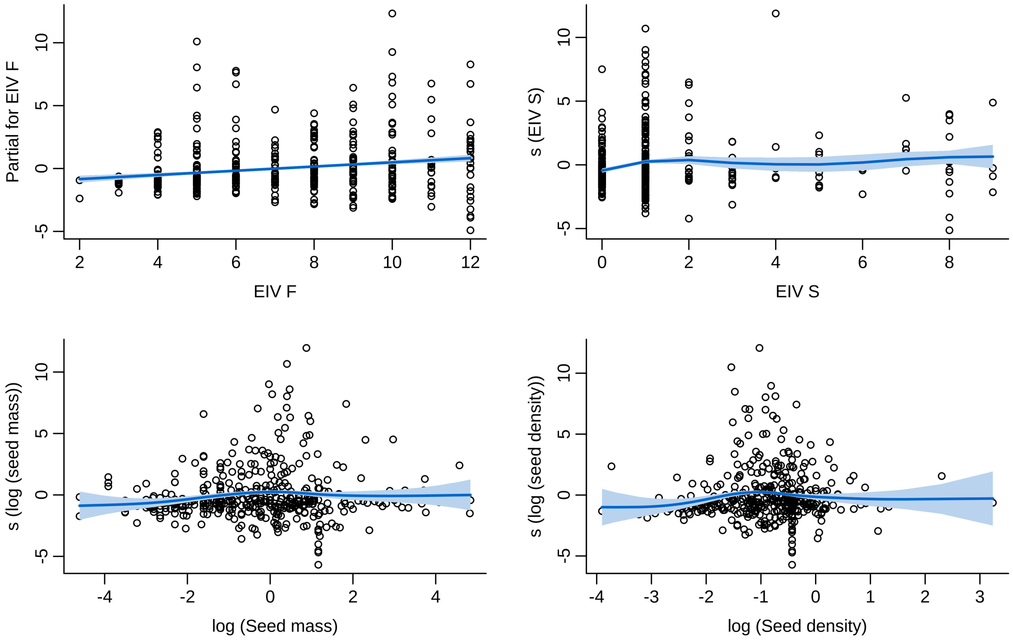


FIGURE 4 | Relationship between plant/seed traits and the number of avian vector species. Log seed mass and log seed density presented unimodal relationships with the number of vector species. The soil moisture EIV was positively and linearly related with the number of vector species, and the salinity EIV had a non-linear relationship with the number of vector species. Fitted lines and their standardized error bands represent the final zero-truncated GAM models. The *Y* Axis represents centered partial residuals from the model fit (the "s" term represents smoothing terms of the variables modeled as non-linear). EIV, Ellenberg Indicator Value for soil moisture (F) and salinity (S). Seed mass and seed density are weakly correlated (Spearman's $\rho = 0.18$, $p < 0.001$).

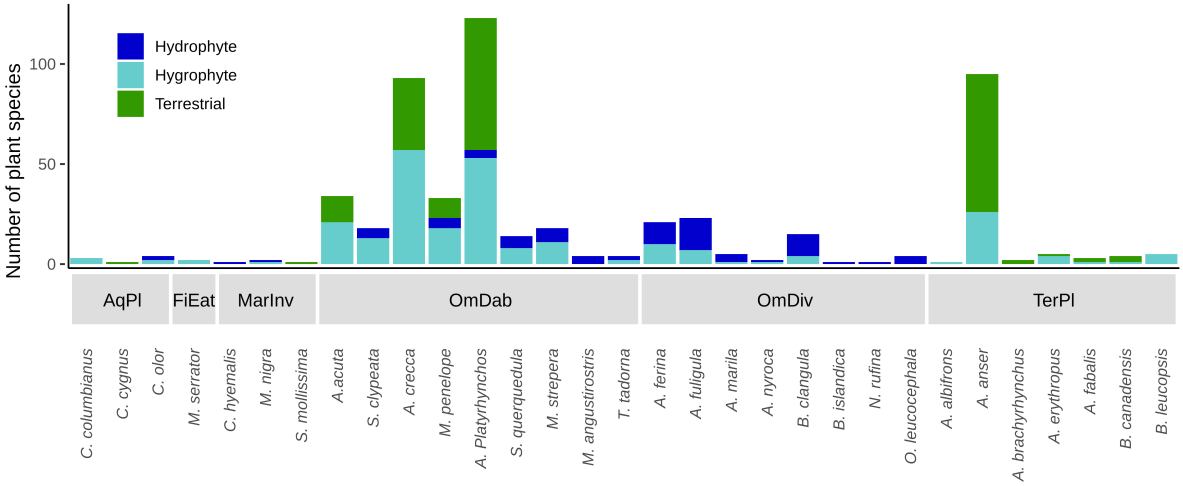


FIGURE 5 | Number of hydrophyte, hygrophyte and terrestrial plant species whose seeds are known to be ingested by each bird species in each feeding group. AqPl, Aquatic plant-eaters; FiEat, Fish-eating diving ducks; MarInv, Marine invertebrate-eating diving ducks; OmDab, Omnivorous dabbling ducks; OmDiv, Omnivorous diving ducks; TerPl, Terrestrial plant-eaters. See **Table 1** for full names of bird species.

Silva G. G. et al., 2021) showing that differences in the species composition of seeds dispersed by waterfowl species are related to their foraging behavior (note, these studies did not consider plant traits). In contrast, a study of other waterbirds in rice fields found no difference between a gull and stork species in the plants they dispersed (Martín-Vélez et al., 2021). Ours is the first study showing that both bird and plant traits drive waterfowl-seed interactions. Our trait-trait analyses showed that amongst waterfowl, feeding group was the trait with the strongest association with seed and other plant traits. Different waterfowl species also vary greatly in their migration and other movement patterns (Scott and Rose, 1996; Wetlands International, 2021), a major driver for long-distance seed dispersal (LDD) patterns. Hence, ongoing changes in abundance and migration patterns of many waterfowl species (Ramo et al., 2015; Pavon-Jordan et al., 2019) will have direct implications for plant dispersal and distributions, although this is outside the scope of the present study.

Plant and Seed Traits That Favor a Diversity of Vectors

Seeds of plants with a preference for higher soil moisture and salinity tend to be ingested and dispersed by more waterfowl species. Even waterfowl that can feed on land will also feed in aquatic habitats (e.g., geese feed in shallow marshes or ricefields, Snow et al., 1998), and so aquatic seeds are those most likely to be encountered by a wider range of vectors. The salinity effects are consistent with general patterns of biogeography and habitat use by migratory waterfowl, as these birds concentrate particularly in larger, low altitude wetlands in coastal plains and deltas that tend to have higher salinity than the smaller, higher altitude habitats holding the highest diversity of aquatic plants and invertebrates (Guareschi et al., 2015; MWO, 2018). Among inland wetlands, salinity is naturally high in closed-basin lakes that are widespread waterfowl habitat in southern Europe, and has increased through water extraction for agriculture and urban use in many areas (Jeppesen et al., 2015; MWO, 2018). Freshwater habitats of lower salinity support a greater diversity of aquatic plant species, but have been destroyed by human activities faster than wetlands of higher salinities (Green et al., 2002).

We also found that more bird species dispersed plants with intermediate values of seed mass and density. As seed mass and density were logged before model construction, these intermediate values were actually closer to the lightest and lowest density seeds than to the heaviest and densest. These results are consistent with differences between the seed size distributions of European angiosperms dispersed by dabbling ducks and those which are not (Soons et al., 2016). Owing to volumetric constraints, more seeds are ingested in a single waterfowl meal as seed size decreases (Mueller and van der Valk, 2002). On the other hand, waterfowl themselves are expected to ignore tiny seeds because of mechanical constraints in their filtering apparatus, and the difficulty of separating food items from unwanted sediments and detritus (Gurd, 2008). Hence, it is not surprising to find that birds are more likely to ingest intermediate-sized seeds, but this does not itself demonstrate selection based on size. Furthermore, we cannot rule out the possibility that the smallest seeds have been underestimated in waterfowl guts because diet studies in the literature may have sometimes ignored them. Since our models of the number of waterfowl species that consumed each plant species are based not on the total diversity of plants that exist in the sampled localities, but instead on the set of plants that have been found in waterfowl diet, it is also possible that the birds tend to ingest intermediate-sized seeds because these plant species might be relatively more abundant than others (an explanation that might also apply for seed density). In principle, waterfowl might be more likely to ingest seeds of intermediate density because those of higher density may be less likely to float or even reach the surface of water where they are most readily ingested, whereas those of even lower density may be of little energetic value as avian food.

Potential Importance of Differences Between Bird Species Within Feeding Groups

Our analysis depended on the placement of waterfowl species into feeding groups. However, we recognize that within feeding groups, different waterfowl species may also have nuanced seed dispersal roles. The variation of particular traits within each feeding group is likely to differentiate species, and may drive species-specific foraging preferences or capacities. The best evidence for this comes from dabbling ducks, where there are interspecific differences within a given locality in the identity of seeds consumed (Brochet et al., 2012) and in how they use their habitat (Green, 1998; Nudds et al., 2000; Arzel and Elmberg, 2004). For example, smaller Eurasian teal *Anas crecca* are more limited in the depth range at which they can extract seeds from sediments than the larger mallard, and also select more small-seeded species (Guillemain et al., 2002). Different traits should be important within each feeding group, as foraging techniques differ between groups. An example of a trait that can be important within a particular group is the density of bill lamellae, which is related to seed size selection in filter-feeding ducks. A higher lamellar density reduces the costs of filtering of smaller items and explains e.g., why Eurasian teal ingest smaller seeds than mallards (Guillemain et al., 2002; Gurd, 2008; Brochet et al., 2012).

Implications for Seed Dispersal Interactions at Different Geographical Scales

Foraging niche separation in waterfowl has previously been shown to partly depend on general food availability, with birds adapting their feeding strategies according to ecosystem productivity, the number of species and/or individuals sharing the same wetland, and seasonal changes in resource availability such as when ducks deplete available seeds gradually during the winter (DuBowy, 2000; Pöysä and Sorjonen, 2000; Guillemain and Fritz, 2002; Tinkler et al., 2009). Niche separation can be reduced at a local scale when food is more available (Pöysä, 1983b; DuBowy, 1988; Guillemain and Fritz, 2002). Our dataset has low temporal and spatial resolution, and is based on data collected in different types of habitats sampled in different seasons. The trait-trait associations found here at a continental scale may not necessarily be reproduced at a local scale, such as in a given wetland ecosystem where interaction networks represent communities subjected to specific conditions of food availability, competition and seasonality (see e.g., Sebastián-González et al., 2020).

Unfortunately, we were unable to control for latitude or season in our analyses due to imprecision and low sample sizes of the available data, and to technical limitations of the RLQ and fourth-corner methods, which currently do not allow the use of continuous covariables. Although our dataset includes all the diet studies we could find on Anatidae species in Europe, it is likely that the actual number of plant species dispersed is underrepresented, even for well-studied bird species. This is suggested by the large fraction of seeds not identified to species level, by major differences in plant community composition between different sites where waterfowl were sampled (Green et al., 2016; Sebastián-González et al., 2020), and by the continuous increase in total number of plant species detected as the number of individual birds analyzed at a given site increases (Soons et al., 2016).

We necessarily adopted a general approach considering only the presence of interaction events, without considering the frequency with which these interactions occurred or separating data by habitat type or seasons. Furthermore, there is a large variability in sampling effort between bird species and feeding groups. Nevertheless, our analyses captured important relationships between the traits of plant and waterfowl species present in Europe. If we had been able to incorporate differences in seed abundance in the diet of each bird species, and if all bird groups were better represented in our dataset, we may have found more and stronger relationships between traits of both groups. In order to improve our capacity to perform future analyses, more sampling of seed dispersal is required, especially to cover the additional eight waterfowl species in Europe that are not represented in this study (due to absence of data), and to provide more data from the spring migration and summer periods. Non-destructive fecal sampling is an ideal method for this (Hattermann et al., 2019; Lovas-Kiss et al., 2019).

CONCLUSION

Seeds have a great diversity of architecture that allows them to survive gut passage, and optimality models explain why waterfowl generally digest only part of their food before egestion (Van Leeuwen et al., 2012; Costea et al., 2019). Even a fraction of soft fish eggs can survive gut passage (Lovas-Kiss et al., 2020b). Therefore, for any plant species a fraction of seeds ingested by waterfowl survives gut passage, with the possible exception of exceptionally large seeds (Brochet et al., 2009; Soons et al., 2016). Thus, our analysis of how plant and waterfowl traits influence seed ingestion can be understood as a good proxy for which seeds are most probably dispersed by which waterfowl species.

Darwin (1859) famously suggested that "the widespread distribution of fresh-water plants . . . depends on the wide dispersal of their seeds by fresh-water birds," although he emphasized epizoochory as the mechanism and overlooked their role in endozoochory (Green et al., 2016). Although modern literature continues to repeat the idea that waterbirds are only important for dispersing aquatic plants (e.g., Viana, 2017; González-Varo et al., 2021), our findings emphasize their importance as vectors for many non-aquatic plants, as well as for plants of saline or brackish waters.

The ingestion of seeds by waterfowl, the first step in the endozoochory process, depends on both plant and bird species traits. Our results suggest that the main mechanism through which duck, geese and swan traits filter the seeds they consume is seed availability in their foraging habitat. Even though we pooled data across seasons, waterfowl feeding groups differed in their function as seed dispersers. Given the exploratory nature of our analysis, our findings need to be tested with other datasets, e.g., from other biogeographical areas. For example, improved plant trait databases are needed for North America (where there is no accessible equivalent to many traits used in our analyses), where waterfowl diet data are particularly extensive (Baldassarre and Bolen, 2006; Callicut et al., 2011). Even for Europe, more extensive data are still required for other traits likely to be important as determinants of ingestion by waterfowl and seed survival during gut passage. Such traits include seed hardness (Lovas-Kiss et al., 2020a) and are also likely to include seed chemistry such as chemical defenses, nutritional content (Petrie, 1996; Dugger et al., 2007), and seed color (Green et al., 2016, 2021).

We hope our study will inspire more research into the importance of seed traits for non-classical endozoochory (i.e., endozoochory by non-frugivorous animals, Green et al., 2021). Waterfowl facilitate LDD events that allow plants to keep pace with climate change (Viana, 2017). Our study provides an important step toward predicting which plants might be affected by changes in the distribution of particular waterfowl species. Further research in seed dispersal by waterbirds should approach how species traits drive seed-bird interactions locally, how these interactions vary through time due to changes in diet and behavior, and how much waterfowl food preferences vs. seed availability influences seed ingestion and dispersal (see also Callicut et al., 2011; Green et al., 2021 for research needs).

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

BA, AG, and CR conceived the idea. BA, ÁL-K, and BL collected the seed ingestion and trait data. BA analyzed the data and wrote the first draft. All authors participated in discussing the idea, revising and approving the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2021.795288/full#supplementary-material

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Soil Seed Bank Persistence Across Time and Burial Depth in Calcareous Grassland Habitats

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Seed persistence in the soil is crucial for population dynamics. Interspecific differences in soil seed mortality could be a mechanism that may stimulate species coexistence in herbaceous plant communities. Therefore, understanding the levels and causes of seed persistence is vital for understanding community composition and population dynamics. In this study, we evaluated the burial depth as a significant predictor of the temporal dynamics of soil seed persistence. We suppose that species differ in this temporal dynamics of soil seed persistence according to burial depth. Furthermore, we expected that burial depth would affect soil seed persistence differently concerning the species-specific type of dormancy, light, and fluctuating temperature requirements for germination. Seeds of 28 herbaceous species of calcareous grasslands were buried in the field into depths of 1, 5, and 10 cm under the soil surface. Seed viability was tested by germination and tetrazolium tests several times for three years. Species-specific seed traits—a type of dormancy, light requirements and alternating temperature requirements for germination, and longevity index—were used for disentangling the links behind species-specific differences in soil seed persistence. Our study showed differences in soil seed persistence according to the burial depth at the interspecific level. Generally, the deeper the buried seeds, the longer they stayed viable, but huge differences were found between individual species. Species-specific seed traits seem to be an essential determinant of seed persistence in the soil. Seeds of dormant species survived less and only dormant seeds stayed viable in the soil. Similarly, seeds of species without light or alternating temperature requirements for germination generally remained viable in the soil in smaller numbers. Moreover, seeds of species that require light for germination stayed viable longer in the deeper soil layers. Our results help understand the ecosystem dynamics caused by seed reproduction and highlight the importance of a detailed long-term investigation of soil seed persistence. That is essential for understanding the fundamental ecological processes and could help restore valuable calcareous grassland habitats.

Keywords: calcareous grasslands, dormancy, longevity index, soil seed bank, light requirements for germination, alternating temperature requirements for germination, burial depth

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INTRODUCTION

Soil seed bank formation is one of the bed-hedging mechanisms for many species because more or less part of their seeds stay ungerminated in the soil for the future years (Venable and Brown, 1988), especially in ecosystems where opportunities for seedling establishment are unpredictable (Wijayratne and Pyke, 2012; Bhatt et al., 2019). At the same time, species-specific differences in the soil seed persistence are known to promote species diversity and co-existence in herbaceous plant communities, as referred to in the storage effect concept (Chesson and Warner, 1981; Warner and Chesson, 1985; Facelli et al., 2005). Understanding the soil seed bank persistence is crucial for understanding the community dynamics in both short- and long-term perspectives (Basto et al., 2018), can improve predictions of species distribution under a changing environment (Ooi, 2012), and has direct implications for successful management and restoration of endangered ecosystems (Richter and Stromberg, 2005; Tatár, 2010; Chapman et al., 2019; Funk, 2021).

One widely used method to study soil seed persistence is determining the depth distribution of germinable seeds in the soil. The depth distribution of seeds is considered indirect evidence of seed longevity (Thompson, 1993; Thompson et al., 1997). Based on these measurements, Thompson et al. (1998) classified soil seed persistence as "transient" or "persistent" and calculated the longevity index to summarize seed-soil persistence data from different studies. On the other hand, direct evidence of seed longevity (i.e., the time elapsed since a species was the last present on the locality or long-term field burial experiments) is rarely used (but see Schwienbacher et al., 2010; Moravcová et al., 2018), although it is known that site-specific conditions can affect both the soil seed persistence (Schafer and Kotanen, 2003; Long et al., 2015) and burial depth (Benvenuti, 2007; Egawa and Tsuyuzaki, 2013). Furthermore, results may be confounded by seed size—seed number trade-off. Smaller seeds enter easier deeper soil layers (Benvenuti, 2007) and are often overrated according to their persistence than larger seeds during indirect longevity estimation (Saatkamp et al., 2009).

TABLE 1 | List of species used in the burial experiment.

Species	Grassland name	Date of burial	Excavation dates	Type of dormancy	Light requirements for germination	Alternating temperature requirements	Longevity index
Antennaria dioica	Eichhalde	1993	O 93–A 94– A 95–A 96	PD	NO	NO	0
Anthericum ramosum	Teck	1992	O 91–A 92–O 92	PY	NO	YES	0
Aster amellus	Surrlesrain	1993	A 94–O 94	ND	NO	NO	0
Brachypodium pinnatum	Teck	1991	O 91–A 92–O 92	ND	NO	NO	0.07
Bromus erectus	Teck	1991	O 91–A 92–A 92	ND	NO	NO	0.29
Bupleurum falcatum	Surrlesrain	1993	A 94–O 94	MD	YES	NO	0
Carex flacca	Teck	1991	O 91–A 92–O 92	PD	YES	YES	0.58
Carlina acaulis	Teck	1991	A 92	ND	NO	NO	0
Carlina vulgaris	Teck	1991	A 92–O 92	ND	NO	NO	0.13
Cirsium acaule	Teck	1991	O 91–A 92–O 92	ND	YES	NO	0
Daucus carota	Teck	1991	O 91–A 92–O 92	PD	YES	NO	0.73
Dianthus cartusianorum	Surrlesrain	1993	A 94–O 94	ND	NO	NO	0
Festuca ovina	Teck	1991	O 91–A 92–O 92	ND	NO	NO	0.19
Gentianella germanica	Eichhalde	1992	A 93–O 93–A 94	MPD	YES	NO	0.17
Globularia elongata	Eichhalde	1993	O 93–A 94–A 95–A 96	PD	YES	YES	0
Hippocrepis comosa	Teck	1991	O 91–A 92–O 92	PY	NO	NO	0.22
Hypericum perforatum	Eselsrain	1992	O 92–A 93–O 94	ND	YES	NO	0.84
Lactuca serriola	Eselsrain	1992	O 92–A 93–A 94	ND	YES	NO	0.88
Leontodon hispidus	Teck	1991 (1993)	O 91–A 92–O 92 (A 94–O 94)	ND	YES	NO	0.36
Linum catharticum	Teck	1991	O 91–A 92–O 92	PD	YES	NO	0.77
Lotus corniculatus	Teck	1991	O 91–A 92–O 92	PY	NO	NO	0.4
Ononis spinosa	Teck	1991	O 91–A 92–O 92	PY	NO	NO	0
Origanum vulgare	Eselsrain	1992	O 92–A 93–A 94	ND	YES	NO	0.81
Pimpinella saxifraga	Teck	1991	O 91–A 92–O 92	MPD	NO	NO	0.05
Pulsatilla vulgaris	Eselsrain	1994	O 94	ND	NO	NO	0.33
Rhinanthus alectorolophus	Surrlesrain	1993	A 94–O 94	PD	NO	NO	0.67
Sanguisorba minor	Teck	1991	O 91–A 92–O 92	ND	NO	NO	0.42
Sedum reflexum	Surrlesrain	1993	A 94–O 94	PD	YES	NO	0

Grassland name = locality of burial; Date of burial indicates the year of seed collection and their immediate burial; Excavation dates indicate time sequence of excavation (A = April in the given year, O = October in the given year); Type of dormancy: MD, morphological dormancy; MPD, morphophysiological dormancy; ND, no dormancy; PD, physiological dormancy; PY, physical dormancy.

Moreover, the deeper the seeds are buried, the lower the chance of seedling reaching the surface (Pearson et al., 2002; Grundy et al., 2003). Different mechanisms which allow seeds to stay ungerminated in the deeper soil layers were evolved (Milberg et al., 2000; El-Keblawy et al., 2018). Three main aspects of germination traits are considered the most important for soil seed persistence, germination timing, and hitting the gap of favorable conditions after disturbances or during the season (Grubb, 1977; Fenner and Thompson, 2005; Saatkamp et al., 2011b). First, delayed germination *via* dormancy mechanisms (Baskin and Baskin, 2014). Second, light requirements for germination, since light can penetrate only an upper layer of soil (Kasperbauer and Hunt, 1988; Mandoli et al., 1990), so seeds can persist deeper until disturbances occur (Baskin and Baskin, 2014; Milberg et al., 2000). Third, alternating temperature requirements for germination may also serve as detection of burial depth and for a gap detection (Thompson and Grime, 1983).

It is unclear whether and how burial depth affects soil seed persistence during a time, and comparison among a higher number of species is almost missing (but see Rivera et al., 2012). Therefore, we investigated the soil seed persistence concerning the burial depth. We address these hypotheses: (i) the deeper the seeds are buried, the longer they will remain viable, (ii) seeds of non-dormant species will survive shorter in the soil without respect to the burial depth, and (iii) seeds of species with light and/or alternating temperature requirements for germination will survive in higher proportions in deeper layers where light is not available and temperature conditions are more constant.

MATERIALS AND METHODS

Study Site and Species Selection

The experiment was located in Baden-Wuerttemberg, Germany. We chose four localities of calcareous grassland—Teck (48.59N, 9.47E), Eichhalde (48.58N, 9.49E), Eselsrain (48.51N, 9.06E), and Surrlesrain (48.84N, 9.05E). All localities are situated on White Jurassic rubble with rendzina soil type. Mild and dry climate and species-rich vegetation are typical. We selected 28 species concerning germinability (Beier, 1991) and aspects of seed-soil bank dynamics (Poschlod and Jackel, 1993). We collected seeds and performed burial experiments between June 1991 and October 1996 (see **Table 1** for details about species, localities, and experiment timing).

Burial Experiment

For the burial experiment, the homogeneous site at each of the localities was selected. We collected diaspores at the same locality where the burial experiment was performed. Harvest was timed to the moment of full maturity, i.e., it was possible to separate seeds from the mother plant with a light touch (except for *Carlina* sp., whose entire inflorescences were collected in mid-October before the achenes were blown off). Random selection of harvested seeds and inflorescences was made to obtain the broadest possible natural spectrum of diaspores (Maas, 1989); maternal effects on the dominant structure of diaspores were not taken into account (Gutterman, 1992). Immediately after harvest, we placed 50 seeds into nylon bags (4 cm × 4 cm, mesh size 300 μm). We made a borehole 10 cm deep and put three nylon bags with seeds of the same species inside the soil core in the depth of 1, 5, and 10 cm. We placed the soil core into a nylon bag (mesh size 2 mm) and inserted it back to the soil. We made five replicates for each species for each excavation time (except for *Festuca ovina*, *Cirsium acaule*, and *Anthericum ramosum* with two, two, and four replicates, respectively because there were not enough seeds available for these species). The position of replicates was randomized inside the site.

We performed several excavations of seeds during the next few years. The first excavation took place at the end of October in the same year as seeds were buried (except for late species whose seeds ripen during September or later). The subsequent excavation was performed during April following the burial (after winter freezing), then during October (after one whole season). The remaining replicates were excavated during April in the following years (see **Table 1** for details).

We took all seeds which were not germinated or molded in the soil, treated them by 2% solution of sodium hypochlorite for 2 min, placed them in the Petri dish with filter paper and sufficient moisture, and kept them in the growing chamber (22°C/14°C at 14 h/10 h light/dark) for 6 weeks. We counted as viable those in which the radicle emerged through the seed testa. We stratified ungerminated seeds in dark conditions at a temperature of 3°C for 6 weeks and then put them again in the growing chamber with the same settings for the next 6 weeks. We tested the viability of remaining ungerminated seeds using the tetrazolium test to distinguish between viable (but dormant) and death seeds.

Seed Traits

We used the longevity index from the LEDA database (Kleyer et al., 2008; Poschlod et al., 2020; unpublished data) and

TABLE 2 | Results of set of mixed-effect linear models with the proportion of viable seeds and proportion of dormant seeds as the dependent variable.

Factor	The proportion of viable seeds		The proportion of dormant seeds	
	Estimate	R^2	Estimate	R^2
Time	–0.28	0.08***	–0.06	0.002*
Depth	0.05	0.04***	–0.003	–
Time:depth	0.001	–	0.004	–
Longevity index	1.21	0.11***	–0.51	–
Dormancy	–0.34	0.11***	0.86	0.19*
Light requirements	0.82	0.21***	0.1	0.003*
Alternating temperature	0.79	0.13***	0.22	–
Longevity index:time	–0.29	0.16***	0.018	–
Longevity index:depth	0.1	0.03***	0.003	–
Dormancy:time	0.18	0.06***	0.02	–
Dormancy:depth	–0.04	0.04***	–0.002	–
Light requirements:time	0.02	0.05***	–0.03	0.003**
Light requirements:depth	0.0	0.05***	0.002	–
Alternating temperature:time	–0.19	0.06***	–0.02	0.001*
Alternating temperature:depth	0.05	0.04***	0.007	–

*Explanatory variables were standardized and log-transformed. Species identity and plant family were included as random effects—indicates non-significant relationships. * indicates significant relationships.*

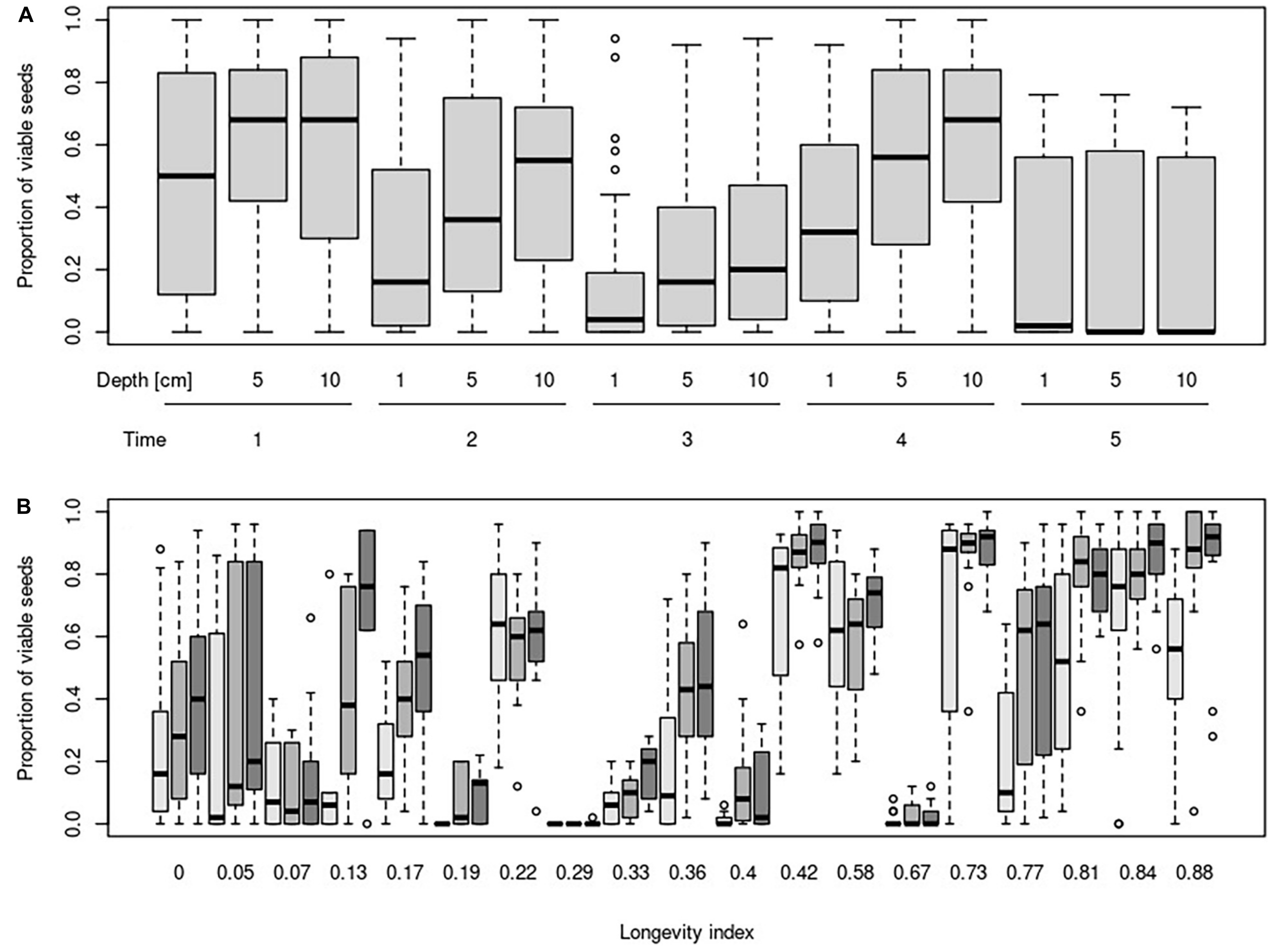

FIGURE 1 | Proportion of viable seeds in different burial depth and their changes according to time **(A)** and longevity index **(B)**. Time steps indicate: 1—October in the same year as seeds were buried; 2—April of the year following the burial; 3—October of the year following the burial; 4—April of the second year following the burial; and 5—April of the third year following the burial. Darkness of boxes indicates burial depth (light gray = 1 cm, middle gray = 5 cm, and dark gray = 10 cm).

information about the type of dormancy, light requirements for germination, and alternating temperature requirements from literature (Kawatani et al., 1976; Grime et al., 1981; Thompson and Grime, 1983; Jones and Turkington, 1986; Pegtel, 1988; Maas, 1989; Beier, 1991; Milberg, 1994; Poschlod et al., 2003; McDavid, 2012; ten Brink et al., 2013; Baskin and Baskin, 2014; Lang et al., 2014; Tudela-Isanta et al., 2018; Leipold et al., 2019; Lopez del Egido et al., 2019; Rosbakh et al., 2020; Holländer and Jäger, n.d.) and online databases ENSCOBASE,[1] Seed Information Database[2] (see **Table 1** for details).

Data Analysis

We used a set of mixed-effect linear models with the proportion of viable seeds (all viable seeds regardless of dormancy) and the proportion of dormant seeds (seeds viable according to the tetrazolium test) as the dependent variable. We used species and plant families as random effects. To avoid the model overfitting, we performed individual analysis for each seed trait and used time, burial depth, one of the seed traits (longevity index, dormancy, light requirements, and alternating temperature requirements), and interaction of respective seed trait with time and burial depth as fixed effects. Furthermore, we performed a model with time, burial depth, and its interaction as fixed effects for investigation of the time × depth interaction. Explanatory variables were standardized and log-transformed to meet the assumptions of normality and homogeneity of variance and take into account the right-skewed distribution of these variables. We classified species with the morphological, morphophysiological, physiological, and physical types of dormancy together as dormant species. Mixed-effect models were performed using the lmer function in R package lme4 (Bates et al., 2015). We tested the random effects using the ranova function from the lmerTest package. We calculated R^2 using Nakagawa and Schielzeth's R^2_{GLMM} (Johnson, 2014) as implemented in the r.squaredGLMM function from the R package MuMIn. For better understanding the behavior of individual species and at the same time for not to overparametrize

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[1] http://enscobase.maich.gr/

[2] http://data.kew.org/sid

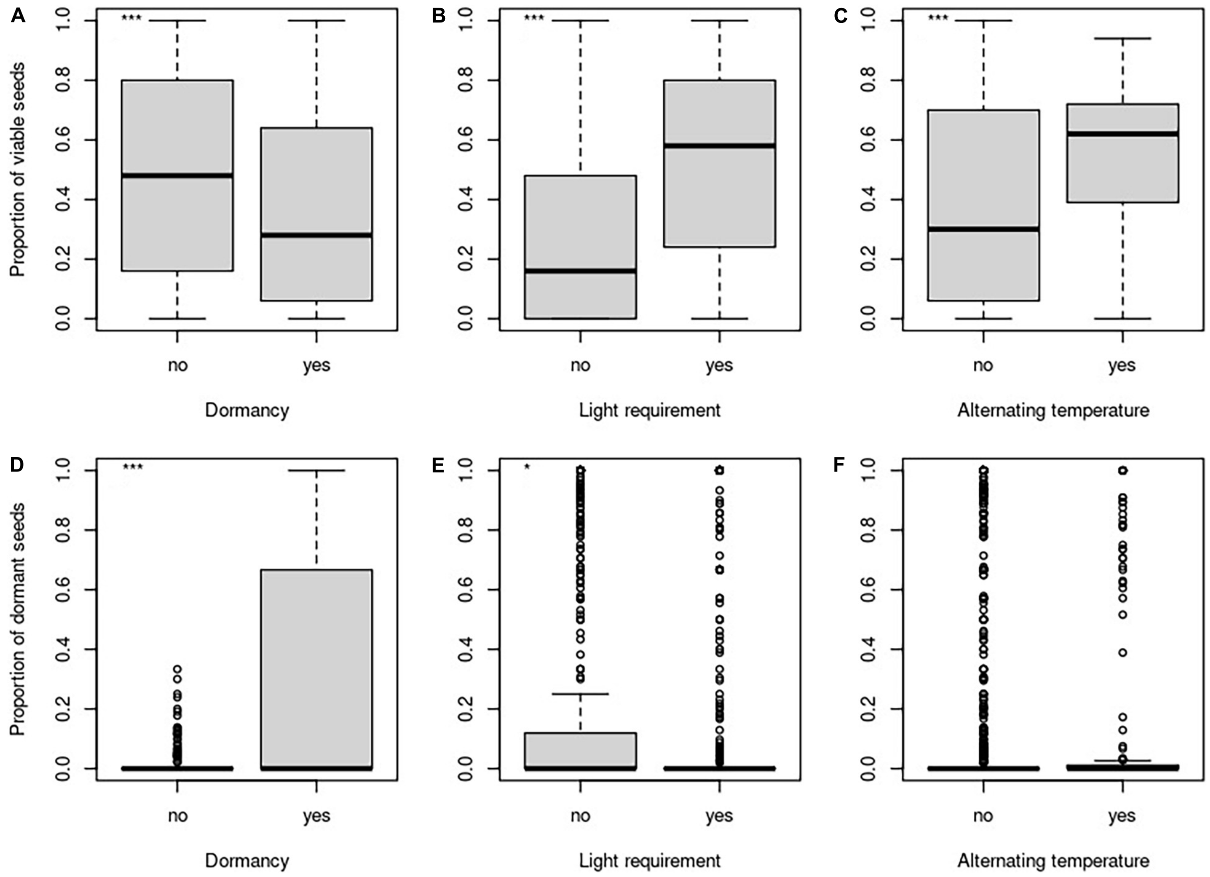

FIGURE 2 | Differences in the proportion of viable seeds **(A–C)** and the proportion of dormant seeds **(D–F)** concerning the dormancy **(A,D)**, light requirements for germination **(B,E)**, and alternating temperature requirements **(C,F)**. *Indicates significant relationship.

the model, we preferred to redo the analysis for each species individually. We fitted linear models for individual species for burial depth, time, and interaction and performed a multiway ANOVA. We used R software (R Core Team, 2021) for performing all analyses.

RESULTS

Time and Burial Depth

The mixed-effect model indicated no very strong main effect of burial depth or time on the proportion of viable seeds. Unsurprisingly, the proportion of viable seeds decreased during the time and increased with burial depth. No significant interaction between time and burial depth was detected (**Table 2** and **Figure 1A**).

We found only a very low negative effect of time on the proportion of dormant seeds; all other investigated factors—burial depth and interaction between time and burial depth—did not affect the proportion of dormant seeds (**Table 2**).

For random effects, we found a strong significant effect of species on the proportion of viable seeds ($p < 0.001$), indicating that the interspecific differences are the most important for the soil seed persistence. We found no effect of a family ($p = 0.14$) on the proportion of viable seeds. On the other hand, both random effects—species and family—affected the proportion of dormant seeds ($p < 0.001$, $p = 0.026$, respectively).

Seed Traits

The proportion of viable seeds was significantly related to all investigated seed traits—longevity index, dormancy, light requirements for germination, and alternating temperature requirements for germination and interacted with both time and burial depth (**Table 2** and **Figures 1B**, **2** for details). The proportion of dormant seeds was significantly related to the dormancy but time and burial depth did not modify it. Furthermore, we found a significant interaction between time and light requirement for germination and time and alternating temperature requirement for germination in the case of the proportion of dormant seeds. Namely, species with some type of dormancy showed a lower proportion of viable seeds than species without dormancy. We determined the opposite pattern in the case of the proportion of dormant seeds (**Figure 3A**).

Surprisingly, a significant negative relationship between the proportion of viable seeds and interaction between longevity index and time was detected. The relationship of the proportion

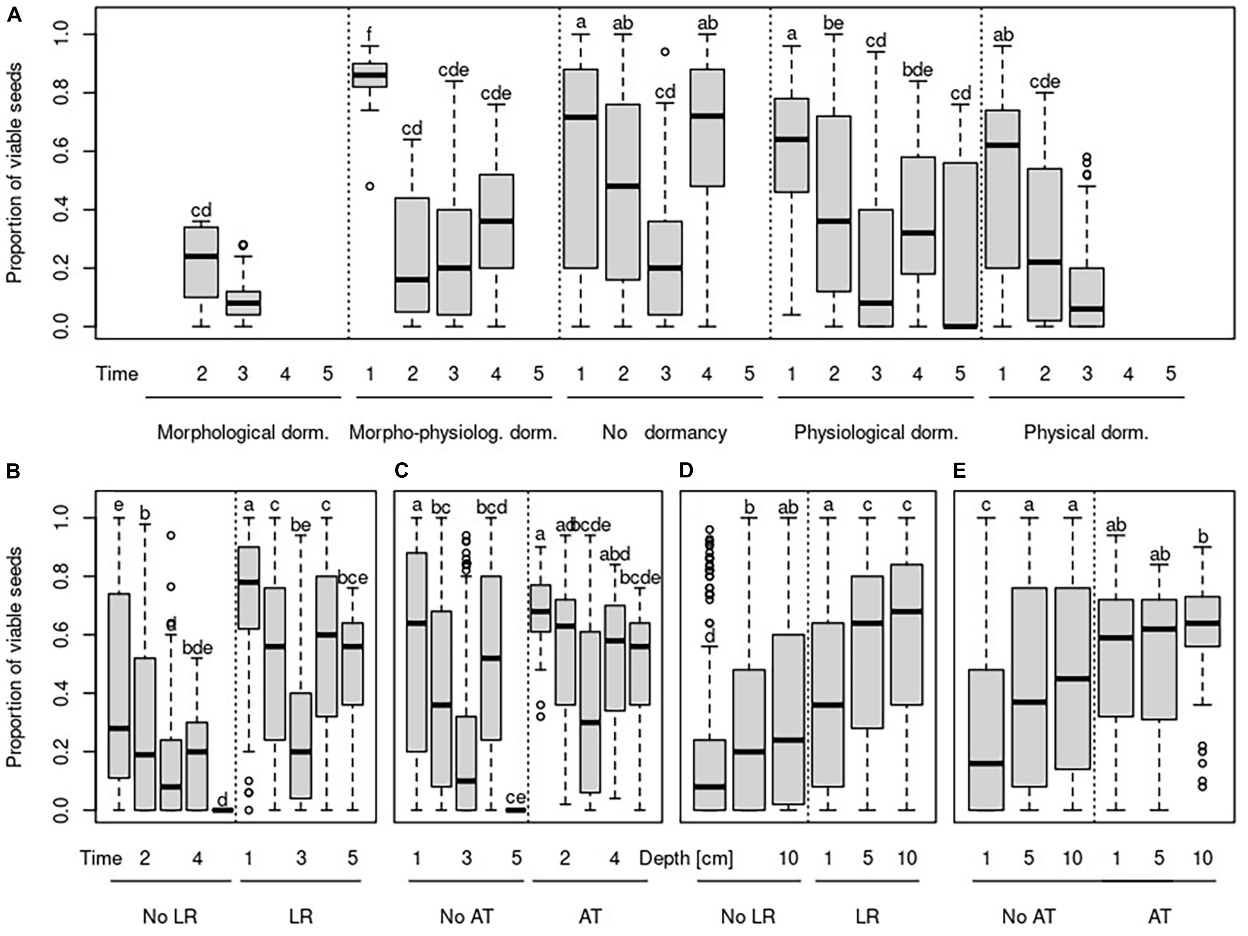

FIGURE 3 | Changes in the proportion of viable seeds over time **(A–C)** and over burial depth **(D,E)** concerning the species-specific type of dormancy **(A)**, species-specific germination requirements to light **(B,D)**, and species-specific germination requirements to alternating temperature **(C,E)**. Time steps indicate: 1—October in the same year as seeds were buried; 2—April of the year following the burial; 3—October of the year following the burial; 4—April of the second year following the burial; 5—April of the third year following the burial. No LR—species without light requirements for germination, LR—species with requirements for germination, No AT—species without alternating temperature requirements for germination, and AT—species with alternating requirements for germination.

of viable seeds with the interaction between longevity index and burial depth was significantly positive.

The species without the requirements to both—light and alternating temperature for germination—had a generally lower proportion of viable seeds (**Figures 3B–E**). Significant interactions between light requirements for germination with both time and burial depth, on the proportion of viable seeds, showed that seeds of species that require light for germination stayed viable longer in the deeper layer of soil (**Figures 3B,D**). We also found a significant interaction between alternating temperature requirements and time and burial depth on the proportion of viable seeds. The proportion of viable seeds increased with the burial depth for species with the requirements of alternating temperature and decreased over time for this species (**Figure 3E**).

Species-Specific Behavior

For most species (21 from 28), the proportion of viable seeds significantly decreased during the time (e.g., *Brachypodium pinnatum*). We also found the relationship between the proportion of viable seeds and the burial depth—the proportion of viable seeds decreased with the burial depth for four species (e.g., *Origanum vulgare*) and increased for 14 species (e.g., *Gentianella germanica*). There was a significant positive interaction between burial depth and time for three species (*Daucus carota*, *Hypericum perforatum*, and *O. vulgare*) and a significant negative interaction for one species (*Bupleurum falcatum*). See **Table 3** and **Supplementary Figure 1** for species-specific details.

Only for *A. ramosum* (the only non-legume species with physical dormancy in our dataset), we found a significant relationship between the proportion of dormant seeds and the burial depth and its significant interaction with time—the proportion of dormant seeds declined during the time in the upper layer but increased in the deeper layer. For three species (*Pimpinella saxifraga*, *G. germanica*, and *Sedum reflecum*), the proportion of dormant seeds significantly decreased during the time (e.g., *G. germanica*) and for *Linum catharcticum*

TABLE 3 | Results of linear models for individual species between the proportion of viable seeds and proportion of dormant seeds and burial depth, time, and its interaction, performed as a multiway ANOVA.

Species	Proportion of viable seeds			Proportion of dormant seeds		
	depth	time	depth:time	depth	time	depth:time
Antennaria dioica	0.03	−0.06				
Anthericum ramosum	0.01	−0.38		−0.06		0.05
Aster amellus	0.03	−0.10				
Brachypodium pinnatum		−0.23				
Bromus erectus						
Bupleurum falcatum	0.05	−0.03	−0.01			
Carex flacca		−0.10				
Carlina acaulis						
Carlina vulgaris		0.16				
Cirsium acaule						
Daucus carota	−0.02	−0.28	0.02			
Dianthus cartusianorum	0.10	−0.06				
Festuca ovina	0.02	−0.03				
Gentianella germanica	0.05				−0.45	
Globularia elongata		−0.05				
Hippocrepis comosa		−0.26				
Hypericum perforatum	−0.01	−0.12	0.01			
Lactuca serriola	0.05					
Leontodon hispidus	0.04	−0.19				
Linum catharticum	0.07	−0.10			0.09	
Lotus corniculatus	0.02	−0.04				
Ononis spinosa	0.05	−0.05				
Origanum vulgare	−0.01	−0.15	0.02			
Pimpinella saxifraga		−0.38			−0.01	
Pulsatilla vulgaris	0.01					
Rhinanthus alectorolophus						
Sanguisorba minor	−0.01	−0.26				
Sedum reflexum	0.01	−0.11			−0.39	

Numbers indicate an estimate of a given relationship; only significant relationships are shown.

significantly increased during the time. All these four species have the physiological or morphophysiological types of dormancy—however, another five species with the physiological type of dormancy did not show this pattern. See **Table 3** and **Supplementary Figure 2** for species-specific details.

DISCUSSION

Our burial experiment with seeds of 28 species from calcareous grassland habitat demonstrates the complexity of the soil seed bank and clearly shows that results of indirect investigation of seed longevity have to be interpreted carefully. Although we found significantly better seed persistence in deeper soil layers for species with a higher longevity index, this correlation was not strong. Our findings confirm the previous investigation of Saatkamp et al. (2009) which found no relationship between soil seed persistence in the burial experiment and seed bank persistence and therefore recommended different use of soil seed abundance and experimental soil seed persistence. Moreover, site-specific conditions, such as rainfall or soil texture, affected both natural seed vertical movement (Benvenuti, 2007; Egawa and Tsuyuzaki, 2013) and soil seed persistence (Schafer and Kotanen, 2003; Long et al., 2015). Therefore, long-term direct investigation under the given environmental conditions is necessary for a precise understanding of the community dynamics.

We found substantial species-specific differences in the pattern of soil seed persistence both over time and depending on the burial depth. These differences were explained mainly on the species level for the proportion of viable seeds and the family level for the proportion of dormant seeds, which is in agreement with our knowledge of seed dormancy as the earliest trait in plant life history (Carta et al., 2016; Liu et al., 2017). Previous investigation in calcareous grassland communities showed essentially similar patterns in species-specific differences for constant burial depth (Pons, 1991). On the other hand, our results emphasized the importance of burial depth for particular species.

In our experiment, we cannot separate if seeds detected as non-viable after the given time of burial germinated in the soil before excavation or were destroyed due to pathogens attack. Mortality *via* fungi attack is both site-specific (Schafer and Kotanen, 2003) and species-specific (Gardarin et al., 2010). Nevertheless, the reason why seeds did not stay viable in the soil has not high importance for answering our questions about soil seed persistence. In both cases, such seeds do not play a role as seed supply in the soil and do not affect the long-term community dynamics.

As we expected, seeds of species with light requirements for germination stay viable longer in the deeper layer. Although light can penetrate only a tiny upper layer of soil (Kasperbauer and Hunt, 1988; Mandoli et al., 1990), seedlings, especially of large-seeded species, can emerge successfully from much greater depth (Bond et al., 1999). Generally, germination in light conditions is one of the mechanisms to detect the burial depth. It was shown before that light requirement is essential to keep seeds ungerminated just after entering the soil (Saatkamp et al., 2011b). Our findings of the longer persistence of seeds in deeper soil layers for species with light requirements for germination support this idea. Huge differences between individual species were found, and we can agree with Saatkamp et al. (2011a) that burial depth detection is a highly species-specific mechanism. Different species with light requirements for germination showed different patterns in soil seed persistence. For example, seeds of *B. falcatum* and *Linum catharticum* did not stay viable in the upper soil layer, which suggests that they germinate immediately after burial in light conditions. Seeds of these species stay viable during one season in deeper soil layers, and after this time, the number of viable seeds decreased to the same number as in the upper soil layer. Conditions in deeper soil layers postponed the decline of a count of viable seeds, but they did not guarantee their long-term survival.

Furthermore, we found the group of species with light requirements for germination, namely, *C. acaule, D. carota, H. perforatum, O. vulgare,* and *Leontodon hispidus*, which showed another pattern in soil seed persistence. Seeds of these species survived in a similar amount after the first winter in all burial depths. Later, the number of viable seeds decreased substantially in the upper soil layer. In contrast, at deeper burial depth, their number remained constant throughout the experiment. On the other hand, high seed persistence in the deeper soil layer throughout the experiment and at the same time, constantly low number of viable seeds in the upper soil layer was found for species *G. germanica* and *Lactuca serriola*. That indicates that detecting the burial depth is crucial for these species immediately after burial; they are not able to germinate from deeper soil layers. They can stay viable in the soil seed bank for a long time, although both are often classified as transient soil seed banks (i.e., their seeds should persist in the soil less than one year) in the literature (Pons, 1991; Kleyer et al., 2008). We found around 50% of seeds viable after two winters in the deep soil layer and around 20% of seeds viable in the upper soil layer for *G. germanica*. This inconsistency between classification as transient soil seed bank from literature and our finding of viable seeds after two years of burial could result from the long dormancy, so classical germination experiments cannot detect it, as reported by Fischer and Matthies (1998).

Our results of differences between species with and without alternating temperature requirements for germination match our expectations. The proportion of viable seeds changes with the burial depth and during the time for species with such requirements. This relationship was weak, and we see the main reason for the unbalanced design of our dataset. We have only three species out of 28 with alternating temperature requirements for germination, which differ in other seed traits, so it is challenging to generalize them. Alternating temperature requirements are known as the mechanism which can serve for detection of burial depth but simultaneously also for detection of disturbances (Thompson and Grime, 1983; Saatkamp et al., 2011a). Nevertheless, its role in soil seed persistence in some environments seems to be negligible (Rivera et al., 2012). Therefore, further investigation with the precise selection of species according to this seed trait and careful setup of the experiment is needed to disentangle the role of alternating temperature for soil seed persistence.

Our work showed a broad range of soil seed persistence strategies under the different burial depths. This diversity can potentially promote species coexistence by the storage effect (Chesson, 1994; Facelli et al., 2005) and thereby maintaining a species-rich community that can withstand temporal fluctuations in environmental conditions. On the other hand, it is known that current climatic changes, such as changing temperature and rainfall regimes, can accelerate the decline of seed viability (Chen et al., 2021), compromising the persistence of plant populations dependent on long-lived seed banks (Ooi, 2012) or dry habitats (Basto et al., 2018). Our results from the long-term burial experiment also proved that seeds of some species could survive in the soil much longer than expected from the indirect measurement of seed longevity by the seedling establishment from soil samples. It points out the importance of further direct long-term investigation.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

PP designed the study and performed the experiments. TM analyzed the data. TM and PP interpreted results. TM wrote the text with contributions of PP. All authors approved the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2021.790867/full#supplementary-material

Supplementary Figure 1 | The species-specific pattern in the proportion of viable seeds during the time and different burial depths.

Supplementary Figure 2 | The species-specific pattern in the proportion of dormant seeds during the time and different burial depths.

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No Support for the Neolithic Plant Invasion Hypothesis: Invasive Species From Eurasia Do Not Perform Better Under Agropastoral Disturbance in Early Life Stages Than Invaders From Other Continents

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Pre-adaptation to disturbance is an important driver of biological invasions in human-altered ecosystems. Agropastoralism is one of the oldest forms of landscape management. It surged 12,000 years ago in Western Asia and it was then imported to Europe starting 8,000 years ago. The Neolithic Plant Invasion hypothesis suggests that Eurasian plants succeed at invading agroecosystems worldwide thanks to their adaptation to agropastoralism, which derives from these species' long co-evolution with such practice. Plant species from Western Asia are predicted to have the highest degree of adaptation to agropastoralism, since they have co-evolved with such practice for several millennia more than European plants, and non-Eurasian species should be poorly adapted due to their relatively short exposure. However, this Eurocentric perspective largely ignores that several other cultures around the world independently developed and implemented agropastoralism through history, which challenges this hypothesized superior adaptation of Eurasian species. Here, we tested whether the early-life performance of invasive plants under disturbance depends on their geographical origin and the associated assumed exposure time to agropastoralism. We selected 30 species divided into three groups: exposure long, native to Western Asia; exposure medium, native to Central Europe; exposure short, native to America. Three soil disturbance treatments (control/compaction/tilling) combined with two space occupancy levels (available/occupied) were applied to monospecific experimental units (n = 900), each containing 50 seeds. We predicted that Eurasian species would benefit more from disturbance in terms of germination and seedling performance than species with shorter assumed exposure to agropastoralism, and that this effect would be stronger when space is occupied. Contrary to these expectations, all species groups profited equally from disturbance, while non-Eurasian species were most hampered by space occupancy. For germination success and speed, exposure long species had higher values than exposure short species, regardless of the disturbance treatment. These results do not support that Eurasian species possess a higher adaptation to agropastoralism, but rather that non-Eurasian species can cope just as well with the associated disturbances. We discuss how future experiments that address the complex relationships between species interactions, plant life-phases and the quality of disturbance can help to understand the role of land-use history in plant invasion success.

Keywords: agropastoralism, biological invasions, disturbance, germination, Neolithic Plant Invasion hypothesis, pre-adaptation

INTRODUCTION

Identifying the mechanisms that promote or impede biological invasions remains a challenging goal in plant ecology and evolution research. In recent years, much attention has been paid on the role of species pre-adaptation, particularly for invasions in human-impacted landscapes (Bayliss et al., 2017; Rosche et al., 2018). In fact, human intervention has shaped landscapes on Earth for thousands of years (Ellis et al., 2021), and successful herbaceous plant invaders thus tend towards a more ruderal strategy (*sensu* Grime, 1977) and may have adapted to human-dominated (highly disturbed) environments in their native range (Guo et al., 2018). It has been suggested that agropastoral disturbance and pre-adaptation to such practice are the two key drivers behind the success of Eurasian plants as invaders of agropastoral ecosystems worldwide [Kalusová et al., 2017; Monnet et al., 2020; Neolithic Plant Invasion (NPI) hypothesis, MacDougall et al., 2018]. According to the NPI hypothesis, most plant species outside of Eurasia lack this kind of pre-adaptations as they have experienced agropastoralism only after the first contact with European settlers starting in the 15th century. However, the NPI hypothesis disregards the existence of ancient agropastoral practices in several continents besides Eurasia, and that consequently adaptation of local native plants to such practice might have evolved in parallel at various locations (Clement et al., 2015; Hilbert et al., 2017; Chomicki et al., 2020). In this study, we present the outcomes of a multi-species experiment investigating whether plant invaders from Eurasia indeed perform better under agropastoral disturbance than plant invaders from other regions of the world, as predicted by the NPI hypothesis.

Processes associated with successful invasions display great variability when studied across taxa and contexts (Kueffer et al., 2013). Nevertheless, numerous empirically supported hypotheses highlight the importance of particular species traits such as high reproduction rates, germination success and competitive ability. Such traits can be developed through the individual or combined effects of evolution following introduction (post-adaptation) and fortunate similarity between old and new habitat (pre-adaptation) (Guo et al., 2014). Since human-disturbed habitats seem particularly prone to invasion (Jauni et al., 2015), increasing attention has been posed to pre-adaptation to anthropogenic disturbance and its link to invasions (Hufbauer et al., 2012; Kalusová et al., 2021). Disturbance considerably shifts local abiotic and biotic conditions and consequently resource availability (Pearson et al., 2018), thereby affecting habitat structure and ecosystem function. Plant species that have evolved in disturbance-prone environments can tolerate rapid environmental changes and quickly exploit released resources (Kawecki, 2000; Lee and Gelembiuk, 2008), which is likely a great advantage during an invasion process. Tolerance to anthropogenic disturbance is particularly essential in early life stages including germination and seedling establishment (Hofmann and Isselstein, 2003), which are delicate yet critical for a successful invasion (Hantsch et al., 2013; Hock et al., 2015; Gioria and Pyšek, 2017).

Pre-adaptation to disturbances associated with agropastoral management may be of particular importance when considering that nowadays about half of the planet's inhabitable surface is dedicated to livestock rearing (77%) and agriculture (23%) (FAO, 2021), which is leading to profound landscape transformations in many countries (Carvalho et al., 2020). In addition, such areas are particularly impacted by the ever-increasing exchange of plant species worldwide. The European continent has been one of the hubs of economic and cultural exchange for centuries and has consequently experienced both species introduction and exportation (van Kleunen et al., 2015; Seebens et al., 2017). Numerous European plants that have been introduced worldwide – starting from the 15th century – are now successful invaders of agropastoral ecosystems (La Sorte and Pyšek, 2009; Kalusová et al., 2017). According to the NPI hypothesis (MacDougall et al., 2018), Eurasian plants are particularly successful at invading these ecosystems in other continents because of their assumed pre-adaptation to agropastoralism, a disturbance to which most plants outside Eurasia are poorly adapted. Such agropastoral practices arose around 10,000 BCE in Western Asia from where they were introduced around 6,000 BCE to Europe (Hejcman et al., 2013; Shennan, 2018). In the Neolithic, the introduction of agropastoralism involved an extensive anthropogenic landscape transformation of the European continent. Large stands of forest were cleared over a few millennia in favor of crop fields and pastures, which were exposed to novel anthropogenic disturbance regimes. Crop cultivation required tilling and plowing, which disrupted soil surface layers with tools pulled by domesticated animals. Such practices cause perturbation to root systems and associated microbiota, create open soil patches, and foster the vertical migration of seeds into the soil (Colbach et al., 2014). Livestock rearing is another practice connected to agropastoralism, which affected local vegetation in Neolithic Europe. The enclosure of livestock lead to severe soil compaction and mechanical vegetation damage via trampling, increased nutrient input through excretes, as well as intense and frequent biomass removal via grazing (Cingolani et al., 2003;

Drewry et al., 2008). Along with these practices, many plant species – referred to as Archaeophytes – were introduced from Western Asia to Europe during the Neolithic (Lambdon et al., 2008). Archaeophytes had co-existed with agropastoralism for millennia before being introduced to Europe, and thus they possessed pre-adaptations that likely allowed them to endure the associated disturbances and thrive in their new range (Europe) (MacDougall et al., 2018). The local European species persisting under this severe landscape transformation either adapted to the new anthropogenic disturbance or shifted in their realized niches (Eriksson, 2013). Known adaptations to agropastoral management are, for example, the production of numerous small seeds with high persistence in the soil seed bank (Albrecht and Auerswald, 2009), the ability to germinate rapidly upon the creation of open soil patches, the capacity to successfully germinate in highly compacted/deep soil layers (Place et al., 2008) and compensatory growth following biomass removal. After the 15th century CE, Archaeophytes and disturbance-adapted European species were exported with the agropastoral practices to other continents (Crosby, 1986). In these new regions, they thrived and often outcompeted local plants, that are assumed to have had only little to no contact with agropastoralism until then, according to the NPI hypothesis.

However, the NPI hypothesis does not fully take into consideration that Eurasia is neither the only nor the first region to have implemented agropastoralism on a large scale. Such practices emerged independently in several other areas, such as eastern North America (2,500–2,000 BCE), sub-Saharan West Africa (~ 2,000 BCE), and the Indian sub-continent (3,000–2,000 BCE) (Purugganan and Fuller, 2009). It is therefore questionable whether plant species from outside of Europe have indeed not been in intensive contact with agropastoral disturbance until the arrival of European settlers starting from the 15th century CE. The emergence and broad implementation of agricultural practice in these areas is evinced by the appearance of crop weeds in archaeobotanical remains (Vigueira et al., 2013). In some forested regions of North America, for example, after the introduction of maize cultivation around 500 CE, archaeobotanical remains exhibited higher species diversities as they suddenly included various grasses, legumes and weeds such as *Ambrosia trifida* L. and *Polygonum* spp. (Asch Sidell, 2007). In this particular region, the transition from hunting and gathering to agropastoralism prior to the arrival of European settlers was gradual and the practices coexisted for about 4,000 years, which has likely enabled local species to slowly adapt to novel disturbance (Lemmen, 2013). The fact that species native to such regions, e.g., *Galinsoga parviflora* Cav. are even a nuisance in agropastoral ecosystems in Eurasia (Warwick and Sweet, 1983) support this assumption. Finally, for the particular case of livestock-associated disturbance, one cannot ignore that many regions of the world harbored large native herbivores (bison, deer, vicuñas, etc.) which have maintained species-rich pastoral ecosystems through grazing prior to the introduction of pastoralism by humans (Mueller et al., 2020). In summary, it is highly likely that many non-Eurasian species have adapted to cropping practice, grazing and trampling prior to the "introduction" of the agropastoral lifestyle from Eurasia.

We test the NPI hypothesis by following the premise that invasive plants originating from Western Asia have been exposed for the longest time to agropastoralism, followed by species originating in Europe and then by (other) non-Eurasian species. We used a multi-species experiment involving 30 species that are invasive in agropastoral ecosystems outside their native range by comparing their germination and seedling establishment success under different disturbance regimes associated with agropastoral management. Following MacDougall et al. (2018), our plant species fall into three groups according to their native distribution range and assumed associated historical exposure to agropastoralism: EL – exposure long, native to Western Asia; EM – exposure medium, native to Central Europe; ES – exposure short, native to America. Each individual species was exposed to three types of disturbance (none/soil compaction/soil surface tilling) and two levels of soil space occupancy (full space available/space occupied with the grass *Festuca rubra* L.) in a fully factorial experimental design. We selected *F. rubra* as a grass species to serve as a common ground cover, since the species has a broad native distribution range spanning Eurasia and America (Canadensys, 2021; GBIF, 2021) and thus shares co-evolutionary history with the vast majority of our species. In accordance with the NPI hypothesis, we expect that (i) disturbance and space availability without competitors would benefit early-life performance of all species; (ii) the positive effect of disturbance increases with assumed length of exposure to agropastoralism (EL species performing best, then EM and then ES species); (iii) the positive effect of space availability increases with time of exposure to agropastoralism (EL species performing best, then EM and then ES species), and these differences will be magnified by disturbance.

MATERIALS AND METHODS

Study Species and Seed Material

Among all possible plant species that have successfully invaded agropastoral ecosystems outside their native distribution range, we selected 30 species belonging to three groups: EL (exposure long, native to Western Asia, represented by nine species), EM (exposure medium, native to Europe, represented by eleven species), and ES (exposure short, native to America, represented by ten species) (**Table 1**). The study species belong to the life forms of cryptophytes, therophytes, geophytes, or hemicryptophytes (Raunkiær, 1907), as assessed through the TRY plant trait database (Kattge et al., 2020). Exposure-long species (EL) have an Archaeophyte status in the majority of European countries (Preston et al., 2004; Klotz et al., 2021), and have been reported to be invasive outside of Europe (Swearingen and Bargeron, 2016; CABI, 2020; ISSG, 2021). Exposure-medium species (EM) are native to Europe (Bundesamtes für Naturschutz, 2003; Kalusová et al., 2017; Klotz et al., 2021) and are invasive in agropastoral ecosystems worldwide (ISSG, 2021; Swearingen and Bargeron, 2016; CABI, 2020). Exposure-short species (ES) are plants that have invaded Europe's agropastoral ecosystems and have been introduced from the American continent after the 15th century CE (Kattge et al., 2020).

TABLE 1 | Overview of species composition, seed material accessions and seed pre-treatments of the three target groups of plants: EL, EM, and ES, respectively, long-, medium-, and short- assumed exposure to agropastoralism.

Exposure- length group	Scientific name	Accessions	Geographic origins Country code – City (Number sources)	Collection year	Dormancy-breaking treatment	Habitat in native range	Habitat in invaded range
EL	*Agrostemma githago* L.	4	DE-Bonn (1) DE-Darmstadt (2) DE-Wasbek (1)	2019 2018, 2018 2020	W	Cultivated areas (Klotz et al., 2021)	Cultivated areas, disturbed areas (Invasive.org, 2018)
EL	*Anchusa officinalis* L.	5	AT-Vienna (1) DE-Berlin (1) DE-Bonn (1) DE-Konstanz (1) DE-VWW UG 1 (1)	2017 2019 2018 2016 2019	/	Meadows, urban areas (Klotz et al., 2021)	Grasslands, roadsides, pastures (Fraser Valley Invasive Species Society, 2021)
EL	*Cichorium intybus* L.	7	DE-Berlin (1) DE-Bonn (1) DE-Darmstadt (1) DE-Halle (Saale) (1) DE-VWW UG 1 (1) DE-VWW UG3 (1) KS-Košice (1)	2018 2019 2019 2019 2019 2018 2019	/	Meadows, urban areas (Klotz et al., 2021)	Cultivated areas, disturbed areas, roadsides (Swearingen and Bargeron, 2016)
EL	*Cyanus segetum* Hill	3	DE-Darmstadt (2) DE-Göttingen (1)	2018, 2018 2019	/	Cultivated areas, meadows (Klotz et al., 2021)	Cultivated areas, grasslands, meadows (Invasive Species Council of British Columbia, 2021)
EL	*Dipsacus fullonum* L.	3	DE-Bonn (1) DE-Darmstadt (1) DE-Konstanz (1)	2019 2019 2018	/	Meadows, riverbanks, urban areas (Klotz et al., 2021)	Cultivated areas, grasslands, meadows (CABI, 2020)
EL	*Papaver rhoeas* L.	5	DE-Bonn (1) DE-Darmstadt (2) DE-Wasbek (1) DE-Leipzig (1)	2019 2018, 2019 2020 2019	C	Cultivated areas, meadows, perennial heaps (Klotz et al., 2021)	Cultivated areas (CABI, 2020)
EL	*Thlaspi arvense* L.	3	DE-Darmstadt (2) DE-Halle (Saale) (1)	2018, 2018 2019	W	Cultivated areas, meadows (Klotz et al., 2021)	Cultivated areas (Koop, 2018)
EL	*Tripleurospermum inodorum* (L.) Sch.Bip.	1	DE-Berlin (1)	2019	/	Meadows (Klotz et al., 2021)	Cultivated areas (Koop, 2018)
EL	*Vicia sativa* L.	1	DE-Bruno Nebelung GmbH (1)	2020	/	Cultivated areas (Klotz et al., 2021)	Cultivated areas (Koop, 2018)
EM	*Capsella bursa-pastoris* (L.) Medik.	4	DE-Bonn (1) DE-Darmstadt (1) DE-Kiel (2)	2019 2018 2020, 2020	W	Cultivated areas, meadows, pastures, urban areas (Klotz et al., 2021)	Cultivated areas (CABI, 2020)
EM	*Carum carvi* L.	4	DE-Bonn (1) DE-Darmstadt (2) DE-Halle (Saale) (1)	2018 2018, 2019 2018	C	Meadows, pastures (Klotz et al., 2021)	Forest openings, meadows (Alberta Invasive Species Council, 2021)
EM	*Cirsium vulgare* (Savi) Ten.	3	DE-Kiel (3)	2020, 2020, 2020	W	Forest clearings, meadows, urban areas (Klotz et al., 2021)	Cultivated areas, rangelands, riverbanks, roadsides (CABI, 2020)
EM	*Cynoglossum officinale* L.	4	DE-Darmstadt (1) DE-Göttingen (1) DE-VWW UG 5 (1) DE-VWW UG22 (1)	2019 2019 2020 2020	S+C	Meadows, urban areas (Klotz et al., 2021)	Cultivated areas, pastures, rangelands, roadsides (CABI, 2020)
EM	*Daucus carota* L.	4	DE-Bonn (1) DE-Darmstadt (1) DE-Leipzig (1) DE-Konstanz (1)	2019 2019 2018 2018	C	Grasslands, meadows, urban areas (Klotz et al., 2021)	Disturbed areas, grasslands, meadows (CABI, 2020)
EM	*Hypochaeris radicata* L.	6	AT-Bad Kleinkirchheim (1) DE-Bonn (1) DE-Kiel (2) DE-VWW UG1 (1) DE-VWW UG22 (1)	2016 2019 2020, 2020 2019 2019	C	Meadows, pastures (Klotz et al., 2021)	Cultivated areas, grasslands, meadows, urban areas (CABI, 2020)
EM	*Linaria vulgaris* Mill.	4	DE-Bonn (1) DE-Darmstadt (1) DE-VWW UG1 (1) DE-VWW UG5 (1)	2019 2018 2019 2019	C	Cultivated areas, forest clearings, meadows, urban areas (Klotz et al., 2021)	Abandoned cultivated areas, pastures, rangelands, riparian corridors, roadsides (ISSG, 2021)

(Continued)

TABLE 1 | (Continued)

Exposure- length group	Scientific name	Accessions	Geographic origins Country code – City (Number sources)	Collection year	Dormancy-breaking treatment	Habitat in native range	Habitat in invaded range
EM	*Rumex acetosella* L.	4	DE-Bonn (1) DE-Darmstadt (2)	2018 2017	W	Cultivated areas, forest clearings, meadows (Klotz et al., 2021)	Cultivated areas, meadows, lawns, roadsides (ISSG, 2021)
EM	*Silene vulgaris* (Moench) Garcke	6	AT-St. Lorenzen (1) DE-Bonn (1) DE-Darmstadt (1) DE-Kiel (1) DE-Leipzig (1) DE-Göttingen (1)	2019 2019 2019 2020 2019 2018	/	Grasslands, meadows, urban areas (Klotz et al., 2021)	Cultivated areas, disturbed areas, roadsides (Minnesota Wildflowers, 2021)
EM	*Sonchus asper* (L.) Hill	5	DE-Kiel (4) DE-Wasbek (1)	2020, 2020, 2020, 2020 2020	/	Cultivated areas, meadows, urban areas (Klotz et al., 2021)	Cultivated areas, disturbed areas, pastures, roadsides (ISSG, 2021)
EM	*Trifolium pratense* L.	1	DE-Bruno Nebelung GmbH (1)	2020	/	Lawns, meadows, pastures (Klotz et al., 2021)	Forest clearings, meadows (White, 2013)
ES	*Claytonia perfoliata* Donn ex Willd.	2	DE-Bonn (1) DE-Darmstadt (1)	2019 2019	C	Mountain meadows, grasslands, sagebrush (Matthews, 1993)	Gardens, lawns (Ries et al., 2021)
ES	*Erigeron annuus* (L.) Pers.	3	DE-Berlin (1) DE-Halle (Saale) (1) DE-Konstanz (1)	2019 2018 2019	/	Abandoned areas, cultivated areas, roadsides (Lady Bird Johnson Wildflower Center, 2017)	Disturbed areas, forests, grasslands (Pacanoski, 2017)
ES	*Erigeron canadensis* L.	4	DE-Konstanz (1) DE-Kiel (2) DE-Wasbek (1)	2018 2020, 2020 2020	/	Cultivated areas, grasslands, riparian areas (Tilley, 2012)	Cultivated areas, grasslands (Swearingen and Bargeron, 2016)
ES	*Galinsoga parviflora* Cav.	4	DE-Berlin (1) DE-Halle (Saale) (1) DE-Kiel (1) DE-Wasbek (1)	2019 2018 2020 2020	/	Cultivated areas, disturbed areas, gardens, grasslands (Rzedowski and Calderón de Rzedowski, 2008)	Cultivated areas, urban areas (Damalas, 2008)
ES	*Lupinus polyphyllus* Lindl.	4	DE-Kiel (2) DE-Konstanz (1) DE-Probsteierhagen (1)	2020, 2020 2019 2020	/	Meadows, riverbanks, roadsides (Fremstad, 2010)	Disturbed areas, gardens, roadsides (Fremstad, 2010)
ES	*Matricaria discoidea* DC.	5	DE-Darmstadt (1) DE-Halle (Saale) (1) DE-Kiel (1) DE-Wasbek (1)	2019 2018 2020 2020	/	Ruderal areas (Flora of North America, 2021)	Disturbed areas, pastures, urban areas (Flora of North America, 2021)
ES	*Oenothera glazioviana* Micheli	1	DE-Darmstadt (1)	2019	/	Fallow fields, gardens, railroad tracks, roadsides (Missouri Botanical Garden, 2021)	Open disturbed areas (Flora Digital de Portugal, 2014)
ES	*Oxalis corniculata* L.	2	DE-Wasbek (1) DE-Kolkwitz (1)	2020 2020	/	Cultivated areas, gardens, urban areas (University of California Integrated Pest Management, 2021)	Cultivated areas, gardens, lawns, pastures (ISSG, 2021)
ES	*Phacelia tanacetifolia* Benth.	2	DE-Darmstadt (2)	2019, 2019	/	Open ecosystems in chaparral, sandy slopes, forests below 2,200 m (Smither-Kopperl, 2018)	Disturbed areas, roadsides (Manual of the Alien Plants of Belgium, 2017)
ES	*Solidago canadensis* L.	3	DE-Halle (Saale) (1) DE-Leipzig (1) DE-Konstanz (1)	2018 2019 2019	/	Abandoned pastures and fields, grasslands, forest edges, roadsides, urban areas (Walck et al., 1999)	Abandoned pastures and fields, grasslands, forest edges, roadsides, urban areas (CABI, 2020)

We also report the species' habitat in their native and introduced range. Dormancy breaking treatments: C – cold: seeds were placed in darkness at 5°C for 20 days; W – warm seeds were placed in darkness at 25°C for 15 days; S – scarification: the external seed coating was manually removed. Information on dormancy breaking treatments obtained from information available on Baskin and Baskin (2014) and the Royal Botanic Gardens Kew (2021). EL species are native to Western Asia, EM species are native to Europe and ES species are native to America. Plant species taxonomy obtained from WFO (2021).

We collected the seed material for all three exposure-length groups in Central and Northern European wild populations, as (i) we assume that the pre-adaptations to agropastoral disturbance that support a species' introduction, establishment and spread in a novel habitat will be detectable in its native as well as in its invaded distribution range; and (ii) to select populations that are adapted to the climate present in our common garden. The final composition of the groups (**Table 1**) was driven by our intention of having similar plant families and life forms across groups but also by limitations in the availability of the seed material. Seeds were sampled in wild populations in the field or they were provided by botanical gardens (exclusively wild collections) or regional wild seeds companies. For each species, we gathered seed material from as many accessions as possible. For a detailed list of accessions see **Table 1**. We assigned an equal proportion of seeds from each of the available accessions to each experimental unit in order to avoid that genetic differentiation and maternal effects could confound the effects of out treatments (see next section for details).

Experimental Setup

To investigate how species with different (assumed) histories of exposure to agropastoralism respond in their early life phases to the combined effects of space occupancy and disturbance, we set up a common garden experiment at the campus of Kiel University (54°20′ N, 10°06′ E) in the summer of 2020. Our experimental units consisted of 900 plastic pots with a volume of 7.5 L each (diameter: 25.5 cm, height: 21 cm) filled with a mixture of 50% fine sand, 35% compost, and 15% clay, which resembles the composition of grassland soils that can be found in Germany (LLUR SH, 2021).

To address the effect of space occupancy on the emergence of the target species, we applied two treatments (space available: SA; space occupied: SO), each to 450 experimental units. Experimental units with occupied space were planted with individuals of the grass species *Festuca rubra* L. (cultivar Dipper, originated in Germany; OECD, 2021). We selected this species based on its wide native distribution range, comprising the whole temperate-cold regions of Eurasia and America, as we aimed at avoiding that particular exposure-length groups would be particularly affected by *F. rubra* because of differences in co-evolution with this species. The *F. rubra* cultivar Dipper is not reported to secrete allelopathic chemicals and was therefore preferably selected. The *F. rubra* individuals used for planting were previously germinated and raised under common garden conditions in flowering beds filled with the above-described soil mix. We added 11 young *F. rubra* individuals in a fixed spatial scheme that ensured an equal surface availability within each experimental unit (**Supplementary Material 1**). After the grasses had reached a sufficient size (4 weeks after transplanting; height ∼ 7 cm, diameter ∼ 3 cm), we added 50 seeds of either of our 30 study species to each pot, in order to have monospecific experimental units. All species that required a dormancy-breaking treatment were treated accordingly, following information available in the standard reference Baskin and Baskin (2014) and the Seed Information Database of the Royal Botanic Gardens Kew (2021; **Table 1**). The pre-treated seeds were mixed with 50 mL of fine sand to enable an even distribution on the soil surface. This mixture contained an equal proportion of seeds for all accessions available for a given species. All experimental units were carefully watered with sprinklers immediately after sowing.

We applied three disturbance treatments (no disturbance: DN; compaction: DC; tilling: DT), each to 300 experimental units (ten per treatment per species, half with occupied and half with available space) one day after sowing the seeds into the experimental units. The compaction treatment aimed at reproducing the impact of a cow hoof on the soil. For this purpose, we created a wooden cow hoof (10 cm × 12 cm × 2 cm; impact area ∼100 cm^2) and placed it at the bottom of a metal rod that served as a soil compactor with a weight of 8 kg. The pressure exerted by a cow hoof step can vary between 130 and 250 kPa (adult Friesian cow – *Bos taurus taurus* L.) (Di et al., 2001). We applied a pressure of 200 kPa by placing the compactor on the soil and adding the weight of a 62 kg person, who stepped on the device three times. To cover the whole surface of the soil, we subdivided the surface area of the experimental unit into four sectors and placed a hoof print as described above in each of them (**Supplementary Material 1**). The soil tilling treatment aimed at perturbing the topsoil. We employed a three-tined rake to trace lines that covered the entire soil surface. The rake was pulled through the soil of each experimental unit three times in the same direction with a penetration depth of 5 cm.

In summary, our experimental setup comprised 30 species divided in three groups (EL, EM, ES) × 2 space occupancy treatments (SA, SO) × 3 disturbance treatments (DN, DT, DC) × 5 replicates = 900 experimental units containing each 50 seeds (**Figure 1**). The experimental units were placed on a paved area in 25 rows of 36 elements with a NW-SE orientation and watered with sprinklers when necessary. They were covered with a fine, transparent mesh (0.5 cm openings) to prevent the removal of seeds by animals and minimize the input of seeds from the surrounding environment.

Data Acquisition

We acquired performance data for two different early life phases, namely the germination and seedling stage. We assessed germination performance by counting the number of germinated individuals per pot twice to thrice per week for a period of 8 weeks. We then calculated germination success $\left(\frac{n_{max}}{n}\right)$, speed $\left(\frac{\sum_{i=1}^{k} n_i}{\sum_{i=1}^{k} n_i t_i}\right)$, and synchronization index $\left(\sum \frac{n_i(n_i-1)/2}{\sum n_i(\sum n_i-1)/2}\right)$ where n is the total number of seeds added (50), n_{max} is the total number of seeds germinated, n_i is the number of seeds germinated on the i^{th} occasion, t_i is the number of days from sowing to the i^{th} observation, and k is the last day of germination assessment (Ranal and Santana, 2006). Regarding seedling performance, we calculated seedling survival on the experimental unit level as the proportion of seedlings survived until the end of the experiment out of the total of seeds that germinated. At the end of the experiment, we harvested the total aboveground biomass of all target species individuals within one experimental unit. We did not harvest *F. rubra*. The target species biomass was then dried at 100°C for 24 h. We calculated average target species individual biomass per experimental unit by dividing the cumulative dry biomass by the number of seedlings which survived until the end of the experiment. We can exclude potentially confounding correlations between seed size and survival in our experimental setup. Seed weight, which is a strong predictor for seedling size had a significant positive effect and no negative effect on survival

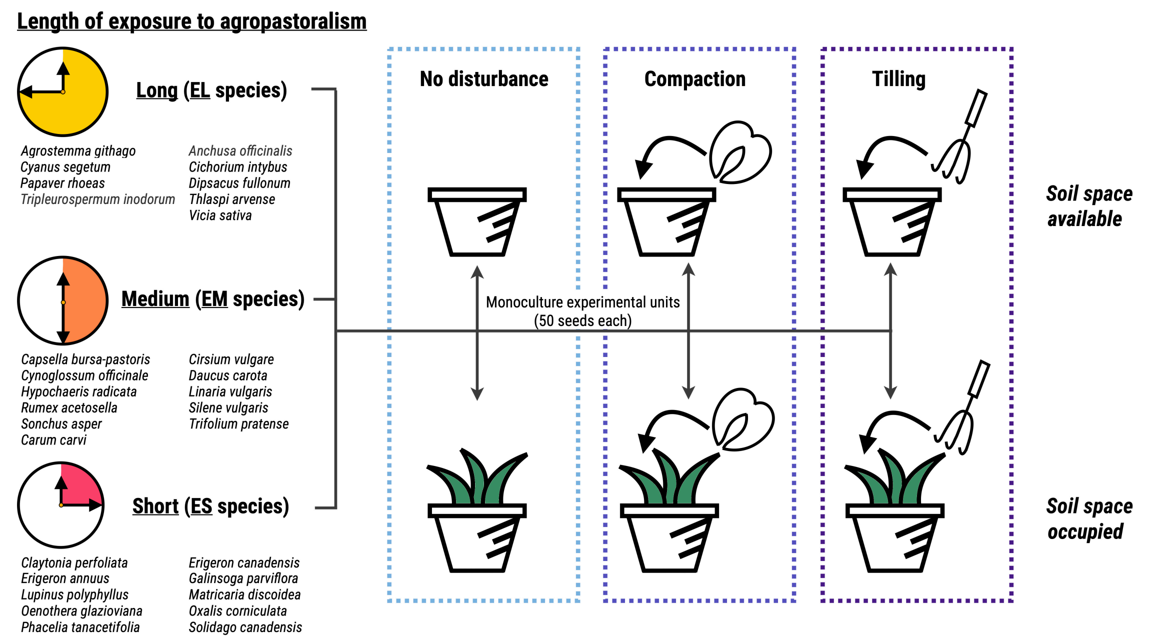

FIGURE 1 | Schematic representation of the experimental setup. We selected species attributable to three groups according to their geographical origin and therefore assumed length of exposure to agropastoralism: yellow – long exposure, orange – medium exposure, red – short exposure. Fifty seeds from a single species were sown into experimental units, which were subject to one of three disturbance treatments (light blue – no disturbance, blue – compaction, purple – tilling), crossed with two levels of space occupancy (white – available, green – occupied). Each species × disturbance × space occupancy combination was replicated with five experimental units. N experimental units = 900.

rates in our experiment. In addition, there was no significant difference between the groups in terms of seed mass [Kruskal–Wallis $\chi^2_{(DF = 2)} = 3.951$, $p = 0.139$, **Supplementary Material 2**].

Statistical Analyses

All statistical analyses were performed in R (v4.0.3, R Core Team, 2019) with (generalized) linear mixed-effects models using the R-package glmmTMB (v1.1.3, Brooks et al., 2017). All models included exposure-length group (factor: EL vs. EM vs. ES), space occupancy (factor: SA vs. SO), disturbance (factor: DN vs. DC vs. DT), and all of their interactions as predictors as well as species (factor: 30 levels corresponding to species identity) as a random effect. Initially, we weighted the species random effect by a matrix of exact phylogenetic relatedness estimates among species as determined with the R-package V.PhyloMaker (Jin and Hong, 2019), based on two recently published phylogenetic trees (Qian and Jin, 2016; Smith and Brown, 2018). A custom script to obtain the matrix and integrate it into a glmmTMB modeling framework was obtained from Li and Bolker (2021) (R-package phyloglmm v0.1.0.9001, 2021). However, the resulting models exhibited dispersion and distribution problems that could not be solved with adjustments of error families, link functions, zero-inflation and dispersion formulas or response data transformations (**Supplementary Material 2**). The same applied for models that included species nested within family as random factor. As these issues likely resulted from unbalanced representation of plant families in our experimental setup, we therefore decided to neglect relatedness effects above and below the species level and maintained the models that included a random effect for species only.

All of our models were fitted with a maximum likelihood approach. We validated them based on residual diagnostic plots and tests provided in the R-package DHARMa (v0.3.3.0, Hartig, 2021). A detailed overview of the chosen distribution families, links and transformations for each model is reported in **Table 2**. Sum-to-zero contrasts were set on all factors for the calculation of type III ANOVA tables based on Wald-χ^2 tests (R-package: car v3.0-10, Fox and Weisberg, 2018). In the case of significant interactions between group, disturbance and space occupancy, we calculated *post hoc* contrasts on the estimated marginal means among levels of a given factor only within levels of other factors involved in the respective interaction (R-package: emmeans v1.5.2-1, Lenth, 2021). Variance components were extracted from all models using the R-package insight (v0.14.4, Lüdecke et al., 2019).

RESULTS

Exposure Length, Disturbance, and Space Occupancy Main Effects

Both germination success [$\chi^2_{(DF = 2)} = 8.752$, $p = 0.013$, **Table 2** and **Figure 2A**] and germination speed [$\chi^2_{(DF = 2)} = 21.399$, $p < 0.001$, **Table 2** and **Figure 2B**] differed significantly among species groups. For both response variables, species with

TABLE 2 | Overview of the structure and the results from the Generalized Linear Mixed Effects Models for early life performance responses.

		Germination success	Germination speed	Synchronization index	Seedling survival	Average seedling biomass
Response families and transformations	Error family	Betabinomial	Gaussian	Gaussian	Betabinomial	Gaussian
	Link-function	Logit	Identity	Identity	Logit	Identity
	Response transformation	None	Log	Logit, then scaled with species as grouping factor	None	Log
Fixed effects	Exposure length (EL, EM, ES)	*	***	n.s.	n.s.	*
	Disturbance (DN, DC, DT)	***	•	*	**	n.s.
	Space occupancy (SA, SO)	n.s.	***	•	n.s.	***
	Exposure length × Disturbance	n.s.	n.s.	*	n.s.	n.s.
	Exposure length × Space occupancy	n.s.	n.s.	n.s.	*	***
	Disturbance × Space occupancy	n.s.	n.s.	n.s.	•	n.s.
Exposure	length × Disturbance × Space occupancy	n.s.	n.s.	n.s.	n.s.	n.s.
% Variance explained by	Fixed effects	4.6	16.3	3.4	3.5	35.2
	Random effects (Species)	12.2	16.6	0	8.8	28.8
	Residuals	83.2	67.1	96.6	87.7	36.0

*The table provides information on the error families, link functions and transformations used for each of the response variables. Moreover, it shows levels of significance for each fixed effect term as obtained from type III ANOVA based on Wald-χ^2 tests as well as variance components for each model. EL, exposure long species; EM, exposure medium species; ES, exposure short species; DN, no disturbance; DC, disturbance compaction; DT, disturbance trampling; SA, space available; SO, space occupied. Significance levels: ns: non significant; • : 0.07 p < 0.05; *p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001.*

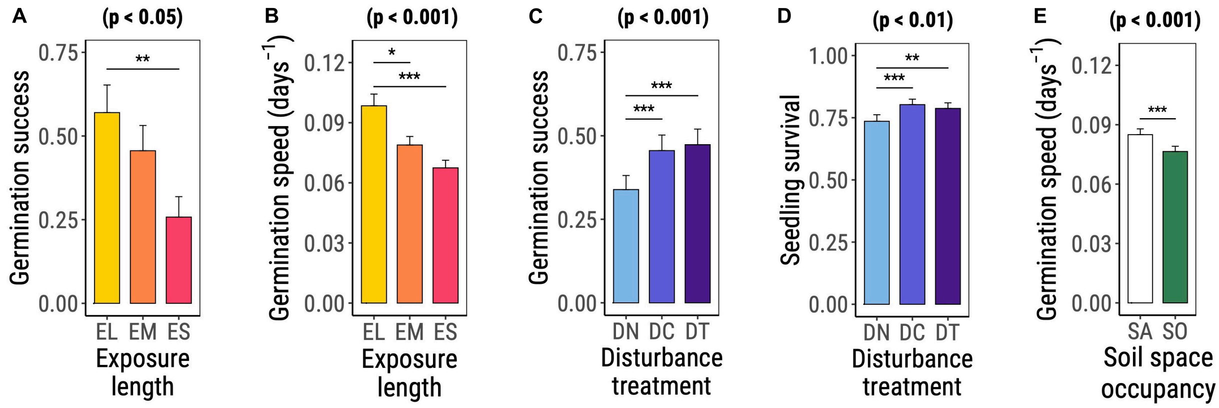

FIGURE 2 | Significant main effects of exposure-length group **(A,B)**, disturbance treatment **(C,D)**, and space occupancy treatment **(E)** on early life performance of the studied invasive plant species as determined based on Wald χ^2 tests on (generalized) linear mixed effects models (significance level noted on top of the plot). Graphs show the marginal means and standard errors estimated by the corresponding model. Horizontal bars with asterisks highlight significant differences among factor levels assessed based on *post hoc* comparisons on marginal estimated means. Abbreviations and color codes: yellow EL: exposure long species; orange EM: exposure medium species; red ES: exposure short species; light blue DN: no disturbance; blue DC: disturbance compaction; purple DT: disturbance tilling; white SA: space available; green SO: space occupied. Significance levels: *$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$.

long exposure (EL) presented the highest values, followed by species with medium (EM) and short (ES) exposure. EL species significantly differed from ES plants in germination success (t-ratio $_{\text{EL-ES}} = 3.010$ with $p = 0.008$) and from both EM and ES species in germination speed (t-ratio $_{\text{EL-EM}} = 2.676$ with $p = 0.021$, t-ratio $_{\text{EL-ES}} = 4.409$ with $p < 0.001$).

Disturbance significantly favored germination success [$\chi^2{}_{(\text{DF} = 2)} = 122.332$, $p < 0.001$, **Table 2** and **Figure 2C**] and seedling survival [$\chi^2{}_{(\text{DF} = 2)} = 10.633$, $p = 0.005$, **Table 2** and **Figure 2D**]. Germination success was equally high under soil tilling (DT) and soil compaction (DC) but significantly lower in the control treatment (DN) (t-ratio $_{\text{DN-DC}} = -7.629$ with $p < 0.001$, t-ratio $_{\text{DN-DT}} = -8.776$ with $p < 0.001$, **Figure 2C**). In comparison with the control treatment, the application of any disturbance treatment significantly increased seedling survival (t-ratio $_{\text{DN-DC}} = -3.831$ with $p < 0.001$, t-ratio $_{\text{DN-DT}} = -2.945$ with $p = 0.009$, **Figure 2D**).

Mean germination speed was the only early life performance trait that was significantly affected by space occupancy [$\chi^2{}_{(\text{DF}=1)} = 21.723$, $p < 0.001$] as a main effect (**Table 2** and **Figure 2E**). Experimental units with available space had faster germination than units containing *F. rubra*.

Interaction Effects of Exposure Length, Disturbance, and Space Occupancy

The interaction between exposure-length group and disturbance significantly affected the synchronization of germination [$\chi^2_{(DF\ =\ 4)} = 9.778$, $p = 0.044$, **Table 2** and **Figure 3A**]. In absence of disturbance, EL species had a significantly more synchronized germination when compared to EM species (t-ratio $_{\text{EL-EM}} = 2.375$ with $p = 0.047$, **Figure 3A**) but did not significantly differ from ES species. The application of a disturbance treatment drastically increased the germination synchronization of EM species (t-ratio $_{\text{DN-DC}} = -3.146$ with $p = 0.005$, t-ratio $_{\text{DN-DT}} = -3.074$ with $p = 0.006$, **Figure 3A**). For ES species, only the tilling treatment significantly increased the germination synchronization (t-ratio $_{\text{DN-DT}} = -2.499$ with $p = 0.034$, **Figure 3A**).

Seedling survival was significantly affected by the interaction between exposure-length group and space occupancy [$\chi^2_{(DF\ =\ 2)} = 7.947$, $p = 0.019$, **Table 2** and **Figure 3B**]. EM species benefited from the presence of *F. rubra*, as shown by the higher survival in experimental units with occupied space (t-ratio $_{\text{SA-SO}} = -2.119$ with $p = 0.035$, **Figure 3B**). ES species were instead hampered by the grass, showing significantly lower survival in the occupied space treatment (t-ratio $_{\text{SA-SO}} = 4.782$ with $p < 0.001$, **Figure 3B**). EM species in presence of *F. rubra* had a significantly higher survival than ES species under the same treatment (t-ratio $_{\text{EM-ES}} = 3.462$ with $p = 0.002$, **Figure 3B**).

Average seedling biomass per experimental unit was also shaped by the interaction between exposure length and space occupancy [$\chi^2_{(DF=2)} = 16.279$, $p < 0.001$, **Table 2** and **Figure 3C**]. All exposure-length groups had smaller seedlings when the space was occupied by *F. rubra*, whereas ES plants were the most strongly impacted in this sense (EL: t-ratio $_{\text{SA-SO}} = 10.358$ with $p < 0.001$, EM: t-ratio $_{\text{SA-SO}} = 14.525$ with $p < 0.001$, ES: t-ratio $_{\text{SA-SO}} = 15.906$ at with $p < 0.001$, **Figure 3C**). In absence of *F. rubra* the three exposure-length groups did not significantly differ in terms of seedling biomass; when the space was occupied, EL species outperformed ES seedlings (t-ratio $_{\text{EL-ES}} = 3.481$ with $p = 0.015$, **Figure 3C**).

None of the investigated early life performance traits were significantly shaped by the three-way interaction exposure length × disturbance × space occupancy.

DISCUSSION

Following the NPI hypothesis, we subdivided a set of invasive plant species into groups of different assumed exposure lengths to agropastoralism. We then investigated their early-life performance responses to simulated management practices, hypothesizing that plants with longer histories of exposure to agropastoral management would benefit more from the associated disturbances than species presumably lacking such co-evolution (and therefore pre-adaptation to disturbance). Our results provide no support for this hypothesis but rather support that the early-life performance of any invasive species is generally fostered by moderate soil disturbance (Catford et al., 2012). The lack of a clear differentiation between the species groups in response to disturbance raises questions regarding the assumption that Eurasian species possess a higher adaptation to agropastoralism. Our results support the idea that non-Eurasian species can too be adapted to such disturbance, as it is a practice developed independently in many regions worldwide before the arrival of European colonizers.

Soil Disturbance and Available Soil Space Generally Promote Early-Life Performance in Plant Invaders

In accordance with the first hypothesis, all species benefited from disturbance and were impaired by space occupancy by *F. rubra*. Experimental units with disturbed soil had higher germination and seedling survival than untreated units (**Figures 2C,D**), while the presence of *F. rubra* slowed down the germination process and reduced seedling biomass in all groups, independent of the assumed length of exposure to agropastoralism (**Figures 2E**, **3C**). These results add to a large body of literature on the facilitating effect of anthropogenic disturbance for biological invasions (Xiao et al., 2016; Meyer et al., 2021). Soil compaction and tilling increase contact between seeds and soil and create favorable germination microenvironments by buffering against mechanical damage as well as temperature and moisture variation (Burmeier et al., 2010; Limón and Peco, 2016; Eichberg and Donath, 2018). A limited availability of free space due to the presence of other species can be an obstacle to a successful early-life phase and it is one of the main drivers of community biotic resistance to invasion (Levine et al., 2004). The presence of grass individuals in the experimental units could hamper germination through several mechanisms, such as modification of the abiotic conditions experienced by the seed (e.g., light, moisture, or temperature regimes) (Weltzin et al., 2005), alteration of microbial community (Miller et al., 2019), and production of allelochemicals (Latif et al., 2017) (see section "Response to Space Occupancy Is Affected by Assumed Exposure Time to Agropastoralism" for further details).

Assumed Length of Exposure to Agropastoralism Does Not Influence Response to Disturbance for Most Traits

Our results provided no support for the prediction that EL species (native to Western Asia, long exposure to agropastoralism) respond more positively to disturbance than EM plants (native to Europe, medium exposure to agropastoralism), and that EM plants respond more positively than ES species (native to America, short exposure to agropastoralism). The vast majority of early life performance responses to disturbance were independent from the assumed length of exposure to agropastoralism. Only germination synchronization responded more positively to disturbance in EM and ES species but had no effect on EL species, which have the presumed highest pre-adaptation to disturbance (**Figure 3A**).

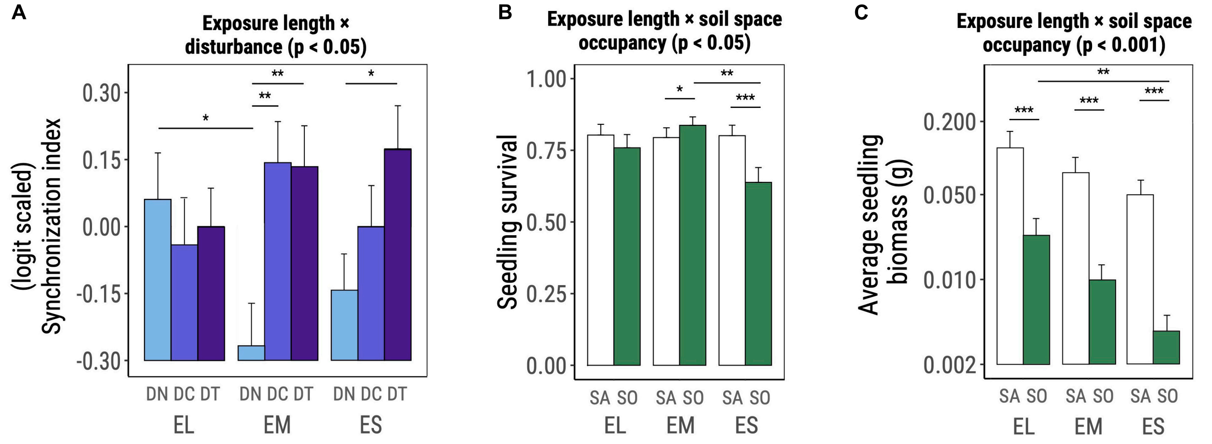

FIGURE 3 | Significant interactive effects of exposure-length group × disturbance **(A)** and exposure-length group × space occupancy **(B,C)** on early life performance of the studied invasive plant species as determined based on Wald χ^2 tests on (generalized) linear mixed effects models (significance level noted on top of the plot). Graphs show marginal means and standard errors estimated by the corresponding model. Horizontal bars denote significant differences among levels of each factor within levels of the other factor involved in the interaction and were assessed based on *post hoc* comparisons on marginal estimated means. Abbreviations and color codes: EL, exposure long species; EM, exposure medium species; ES, exposure short species; light blue DN: no disturbance; blue DC: disturbance compaction; purple DT: disturbance tilling; white SA: space available; green SO: space occupied. Significance levels: $^*p \leq 0.05$; $^{**}p \leq 0.01$; $^{***}p \leq 0.001$.

These findings contradict the assumption that Eurasian species have a superior ability to cope with agropastoral disturbance due to several millennia of co-evolution with such practice. This is in line with other studies: a large-scale manipulative experiment, replicated across several study sites in the world, tested for an interactive effect of nutrient addiction (often connected to agropastoral management) and species' introduction status and demonstrated that the leaf nutrient content of neither European invasive species nor of local native species was influenced by such interaction (Broadbent et al., 2020). Cultivation and animal husbandry are not exclusive to Western Asia and Europe but have surged independently through the millennia in different parts of the world. Researchers have identified eleven centers of plant domestication besides Western Asia, spanning from 8,000 BCE to 2,000 BCE (Purugganan and Fuller, 2009). In the Andes, for example, several edible species have been domesticated (e.g., *Solanum tuberosum* L., potato – Purugganan and Fuller, 2009) and native herbivores such as llama were raised in large numbers as source of sustenance and textile fibers (Flores Ochoa et al., 1994). All these practices must have exerted selection pressures on the local native plant species, leading to adaptation. Even though MacDougall et al. (2018) specify that the NPI hypothesis does not rest "on agricultural land use *per se*, but the specific combination of domesticated grazers, plants and management styles introduced simultaneously by Europeans following colonization," there is no evidence supporting that European style agropastoralism should be profoundly different from practices found elsewhere. In addition, a standard definition of such practice is unrealistic as European settlers could have exported distinct sets of domesticated animals and plants, or even implement different cultivation styles according to the nationality of the exporters, thereby underlining the need for differentiation rather than generalization.

One further aspect to consider is that human landscape management is not the sole driver for adaptation to disturbances in relation to agropastoralism. Open herbaceous ecosystems are often inhabited by large native grazers which can also exert a selection pressure on local plants. In the Argentinian Patagonia for example, the long history of grazing by the native camelid guanaco (*Lama guanicoe* Müller) (Franklin, 1982) lead to the emergence of functional traits mediating grazing resistance in the local vegetation, when compared with a similar ecosystem that lacked large herbivores (Adler et al., 2004). Finally, as all of our seed material was collected in central Europe, we cannot exclude that the positive responses of ES species to agropastoral disturbance result from post-introduction adaptations. However, pre-adaptations are more likely to explain our results, given that ES species occur in cultivated areas and grasslands also in their native habitat (**Table 1**) and that disturbance acts as a strong environmental filter on non-adapted plant species (Huston and Smith, 1987). These pre-adaptations likely arose prior to the spread of agropastoralism with European settlers.

However, further experiments are required to validate these outcomes and conclusions. When monitoring plant response to agropastoral management, it is important to account for the multi-faceted character of disturbance, as determined by quality, intensity, frequency, duration, extent and timing (Zhang and Shea, 2012); which, if varied, can substantially shape the magnitude and direction of responses in plant performance. Mowing during flowering or fruiting periods, for example, is crucial for the management of invasive species, as it can affect plant resource allocation and growth form (Bartoš et al., 2011) but also dramatically reduce reproductive success, and consequently, population growth (Milakovic and Karrer, 2016; Nakahama et al., 2016). Responses to disturbance can also depend on plant life stage. Individuals in the germination, juvenile or maturity phase can be differently affected by environmental stimuli (Knappová et al., 2013; Florianová and Münzbergová, 2018). Jauni and Ramula (2017), for example, found out that for *Lupinus polyphyllus* Lindl. a moderate disturbance (comparable to our tilling treatment) increased germination but did not affect survival, while a stronger disturbance (removal of vegetation, litter and top 5 cm of soil) did not affect emergence but promoted seedling survival. Space occupancy can also affect plant life stages differently. In the case of *Impatiens parviflora* C., germination is impaired by high vegetation cover, which in turn does not affect survival later in life (Florianová and Münzbergová, 2018). Future experiments testing the NPI hypothesis should thus explicitly expand the variety of disturbances applied and consider also mature and reproductive life stages for an even higher number of species.

Response to Space Occupancy Is Affected by Assumed Exposure Time to Agropastoralism

We expected that ES species would be more negatively affected by space occupancy than other groups, and that this would be particularly evident in the presence of disturbance. Even though we did not find support for the latter prediction, our results for seedling survival and final biomass support the former one. In fact, survival in the three exposure-length groups was differently affected by the presence of *F. rubra*, which had a neutral effect on EL species, a positive one on EM species and negative one on ES species (**Figure 3B**). In terms of final seedling biomass, all three exposure-length groups presented smaller seedlings when the space was occupied, with EL species being the least affected and ES species the most affected.

For survival, the identity of the species occupying the space has unlikely played a role, as *F. rubra* is widespread and native to the temperate areas of Eurasia and America. However, we used a cultivar of the species (Dipper) originating from Germany (OECD, 2021). Such cultivar, although not present in nature, has been developed using European populations of *F. rubra*, which can be found together with EM species in various phytosociological classes (Klotz et al., 2021) and therefore shares a particularly long co-evolutionary history with species belonging to this group. The facilitating effect of co-evolved species is assumed to have a great relevance in the context of biological invasions, during which the presence of another non-native species can benefit the establishment and proliferation of others (Simberloff and Von Holle, 1999) through reduction of competition from native plants (Flory and Bauer, 2014), alterations to the soil microbiome or other soil characteristics (Zhang et al., 2020) and allelopathy (Thiébaut et al., 2019). The same may hold for co-evolved species varieties or ecotypes. In a study by Lipińska et al. (2013), allelopathic effects of European cultivars of *F. rubra* ("Dipper" not included) were shown for grass species native to Europe. They observed a mixed outcome of species interactions (positive/negative) between European cultivars of *F. rubra* and the target European grass species investigated. One would not expect systematic variation in the susceptibility of plant species to the allelopathic effects of "Dipper" (in case this cultivar produces allelochemicals) among different exposure-length groups because even within one distribution range there is plenty of variation in the allelopathic potential of cultivars and the effects of particular cultivars on different target species.

The interactive effect of exposure-length group and space occupancy on seedling biomass (**Figure 3C**) could be mediated by a positive correlation between germination speed and seedling size (Grman and Suding, 2010), as individuals from early germinating species (EL) may have had the chance to establish and occupy space before the *F. rubra* cover was excessively dense. In temperate habitats where plant development is constrained during the cold season, an early germination could offer an invasive species the chance to exploit local resources and therefore be detrimental for the establishment and diversity of later-germinating native species (Grman and Suding, 2010). Regarding seedling biomass, it could be argued that large-seeded species would have an advantage as they would have a higher recruitment and generate larger seedlings (Moles et al., 2004). However, this advantage diminishes significantly in ecosystems with sparse canopy such as grasslands (Bruun and Ten Brink, 2008) and it is rather unlikely to apply in the present case as all of our groups possessed both large- and small-seeded species.

CONCLUSION

By testing for the combined effects of space occupancy and disturbance on the performance of introduced plants we found no evidence suggesting that EL and EM species are more adapted to agropastoralism than ES species. In contrast, the latter can cope with the disturbances in early life phases just as well as Eurasian species. However, even though our experimental setup comprised a representative sample of species for the three exposure-length groups that allowed for generalization of the combined effects of soil surface disturbance and space occupancy, a different/larger set of species with a more balanced phylogenetic relatedness may have yielded contrasting results. Further studies are required to expand our findings and evaluate whether EL, EM, and ES species react differently to agropastoral disturbance and space occupancy in later life stages. The complex interplay of pre-adaptation to disturbance, the quality/frequency/intensity of disturbance and the species composition at site is a determinant of invasion success that must be addressed in future studies of this kind. These multifaceted interactions should be assessed employing manipulative experiments in the field, that address plant performance across the entire life cycle (including fitness) and that simultaneously allow modeling population growth rates.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

AE acquired funding and contributed to the final version. GB, AE, and KS conceptualized the study. GB implemented the experiment and collected the data. GB and KS performed the statistical analysis and wrote and revised the manuscript. All authors approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2022.801750/full#supplementary-material

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Recruitment Traits Could Influence Species' Geographical Range: A Case Study in the Genus *Saxifraga* L.

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The reasons why some species occur widespread, while related species have restricted geographical ranges have been attributed to habitat specialization or ecological niche breadth. For species in the genus *Saxifraga*, habitat specialization alone cannot explain the distributional differences observed. We hypothesize that recruitment traits (i.e., germination, emergence, and survival) may account for differences in geographical ranges and that early life stages correlate to survival. We studied recruitment responses in 13 widespread and 12 narrow-ranged *Saxifraga* species in the laboratory and common garden experiments using seeds collected from 79 populations in the European Alps. We found that in the laboratory cold temperature led to higher germination percentages compared with warm temperature for both distribution groups. This represents an exception to the general assumption that alpine species require warm cues for germination. In warm laboratory temperatures, widespread species germinated better than narrow-ranged species, indicating a greater tolerance of warm temperatures for the former. Subsequent to germination, recruitment traits between the two distribution groups were lower or null in the common garden, suggesting that the impact of recruitment on species' geographical ranges occurs at the earliest life stage. Mean time to emergence of narrow-ranged species showed lower variability than that of widespread species. Consistently, intraspecific variation of mean annual temperatures between seed collection sites was lower for narrow-ranged species, indicating a close relationship between home sites and emergence time. Emergence percentage was a strong predictor of survival only for widespread species, underlining that seed and seedling functional traits differ between distribution groups, which require further research. Our results support the view that early life stages are critical to population dynamics and thus can influence species' geographical ranges. The wider responses to climatic conditions in widespread species may have facilitated their spread across the Alps. Our results also suggest that all *Saxifraga* species face a considerable threat from climate warming due to their overall cold-adapted recruitment niche.

Keywords: common garden, phenology, regeneration niche, seed sowing experiment, intraspecific variation, germination

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INTRODUCTION

The reasons why some species occur widespread (e.g., in a variety of habitats or stretched over a wide geographical range) and some closely related species occupy a narrow geographical range (hereafter referred to as narrow-ranged) has been of interest to biologists for decades. Current studies investigated the status of species' rarity (e.g., Rabinowitz, 1982; Broennimann et al., 2005), ecophysiological traits comparing widespread and narrow-ranged species (e.g., Lavergne et al., 2004; Mouillot et al., 2013), habitat preferences of endemic species (Essl et al., 2009), and loss of rare species due to land-use practices globally (e.g., Newbold et al., 2018). However, no sufficiently comprehensive explanation of geographical distribution patterns has been found until the present. In this context, little attention has been given to the role of seedling recruitment (e.g., Münzbergová, 2004; Larson and Funk, 2016; Tudela-Isanta et al., 2018). Seedling recruitment includes key demographic processes such as seed germination (the transition from a quiescent or dormant seed to a germinated seed), emergence (the transition from a germinated seed to an emerged seedling), and seedling survival (the persistence of established seedlings through the first growing season; Fenner and Thompson, 2005). In temperate areas, seedling survival during the first winter period is additionally crucial for seedling recruitment (Niederfriniger Schlag and Erschbamer, 2000; Mondoni et al., 2015, 2020; Margreiter et al., 2021).

Each transition phase plays an important role in population dynamics (Donohue et al., 2010; Fraaije et al., 2015; Jiménez-Alfaro et al., 2016; Rosbakh et al., 2020). The influence of one phase on the subsequent one depends on a set of abiotic (e.g., light, soil moisture, temperature, chemicals, and nutrients) and biotic filters (e.g., fungal pathogens, herbivores, competitors, and facilitators), and interactions among these (see Larson and Funk, 2016), making it hard to disentangle single-trait influences on recruitment. In addition, local adaptation has been noted to impact seedling emergence and survival outcomes (Giménez-Benavides et al., 2007). However, by looking at each life phase separately, the individual contribution to possible distributional differences between widespread and narrow-ranged species may be determined since the transition phases are ultimately interlinked. For example, by using data from 37 dry grassland species and partial regression coefficient calculations, Larson et al. (2015) clearly showed that germination and emergence were useful traits to predict seedling survival.

Early life stages may influence species distribution ranges (Donohue et al., 2010; Larson and Funk, 2016), determined by trait niche breadths (Finch et al., 2018). A general hypothesis states that the environmental niche (i.e., the breadth of any trait) is broad when a heterogeneous environment is inhabited, while a homogeneous environment favors narrow niches (Kassen, 2002; Finch et al., 2018). For example, narrow germination niches were linked to small geographic ranges in geophytes, hemicryptophytes, and shrubs (Ascough et al., 2007; Ranieri et al., 2012), while inhabiting a broader geographic and climatic range was found to be correlated with broad germination niches in hemicryptophytes and chamaephytes (Brändle et al., 2003; Luna and Moreno, 2010). Conversely, germination niches varied among congeneric species of *Asclepias* with similar distribution ranges. A narrow germination niche can be caused by high dormancy cues and slow and low germination that might ensure the survival of seedlings by only germinating when conditions are met (Donohue et al., 2010; Rosbakh et al., 2020). Seed dormancy, operating as a bet-hedging strategy, may prevent germination at the first possible opportunity to spread the risk of seedling mortality over a season or year (Ooi et al., 2009; Donohue et al., 2010; Cotado et al., 2020). On the other hand, when germination thrives under a broad set of conditions with low dormancy cues, seedlings may be occasionally exposed to unfavorable post-germination conditions (Mondoni et al., 2015), but may result in overall higher germination outcomes. On expanding these ideas from the germination niche to the regeneration niche (Grubb, 1977), a narrow niche for early life stages may lead to a limited distribution range of adult species, while in contrast, when early life stages have a wider niche, adults may be able to occupy a wider distribution range.

Species-specific characteristics with regard to germination, emergence, and survival have been widely described (e.g., Münzbergová, 2004; Milla et al., 2009; Fraaije et al., 2015; Mondoni et al., 2015, 2020; Larson and Funk, 2016; Walter et al., 2020; Margreiter et al., 2021), which are expressed as interspecific variations (Albert et al., 2010a,b; Fraaije et al., 2015). On the other hand, intraspecific variability was recognized as a key characteristic in ecology (Des Roches et al., 2018) that acts on different organizational levels (Violle et al., 2012; Chen and Giladi, 2020), meaning that variation is important for functional diversity at species, community, and ecosystem levels (Albert et al., 2010a,b; Larson and Funk, 2016; Des Roches et al., 2018). To occupy a niche within a habitat, a certain degree of intraspecific variability is necessary to respond to conditions of that niche at each life-history stage. High intraspecific variation may allow species to perform similarly under different conditions, thereby reflecting a broad niche; on the other hand, when species lack intraspecific variation, performance is only possible under specific conditions (Finch et al., 2018). Intraspecific variation in seed traits is assumed to increase with increasing species' range size (Luna and Moreno, 2010). However, a screening of recruitment traits of phylogenetically related species has hardly been performed (e.g., Rosbakh et al., 2020; Veselá et al., 2020), and, most importantly, only a few studies covered all transition phases of recruitment, i.e., from germination to seedling survival (see Vázquez-Ramírez and Venn, 2021).

Intraspecific variation of recruitment responses may be especially important in times of an anthropogenic climate change when quick reactions to the environment are essential for species' persistence (Cochrane et al., 2015; Henn et al., 2018; Walter et al., 2020). For example, populations of a single species may be locally adapted to lower levels of soil moisture, and can therefore germinate in years with lower precipitation compared with populations that remain dormant in the seed bank in the same year (Ooi et al., 2009). On the other hand, rapid climate changes have the potential to disrupt existing environmental cues for both seed germination (Donohue et al., 2005; Walck et al., 2011) and seedling survival (Anderson and Wadgymar, 2020). This risk could be especially high for narrow-ranged species as intraspecific variation may be low because fewer populations exist and restricted gene flow may have lowered fitness. Warming (i.e., temperature increase) has been reported to favor seedling emergence for widespread alpine species (Mondoni et al., 2015, 2020; Vázquez-Ramírez and Venn, 2021), but also disfavoring results were obtained due to biotic interactions (Meineri et al., 2013; Margreiter et al., 2021) and local adaptations (Giménez-Benavides et al., 2007).

The European Alps are home to about 60 *Saxifraga* species (spp.), including a high number of subalpine and alpine species (i.e., species at and above the treeline) and a lower number from the montane zone (Aeschimann et al., 2004). *Saxifraga* spp. colonized Europe in the late Eocene and rapidly diversified after reaching the continent from Northeast Asia (Zhang, 2013; Ebersbach et al., 2017). Thus, the species occurring in Europe are viewed as being considerably old and their distributional ranges were influenced by habitat specialization (Hegi, 1923). Species that are rare or endemic in the Alps may therefore be described as "old rare species," meaning that they show a natural patchy distribution as a result of habitat preferences (Meier and Holderegger, 1998). However, for narrow-ranged species in this study, there are suitable habitats not occupied outside the isolated populations, suggesting that the narrow range cannot be explained by habitat specificity alone. For example, *S. squarrosa* grows on calcareous bedrock in rock crevices of the Southeast Alps, and although calcareous bedrock and similar habitats are also abundant in the Northern and Northeastern Calcareous Alps, *S. squarrosa* is not found there. The widespread species *S. caesia*, on the other hand, occurs in the entire calcareous Alps. The fact that all *Saxifraga* spp. have similarly tiny and light seeds that are easily dispersed by wind makes distribution limitation by dispersal unlikely for *S. squarrosa*. Instead, it presses the question of whether distributional differences between narrow-ranged and widespread *Saxifraga* spp. arise due to differences in their recruitment responses. Moreover, investigations on a similar set of *Saxifraga* spp. suggest that the phylogenetic influence on germination niche, expressed by Pagel's lambda (Pagel, 1999), is only low to moderate (Porro et al., submitted; $\lambda < 0.55$ for seed germination in response to temperature and cold stratification, and their interactions with Landolt ecological indicator values). Thus, the *Saxifraga* genus represents an ideal model for the study of the distribution range, intraspecific variations and their drivers.

The general objective of this study is to assess whether traits of the early life stages of *Saxifraga* spp. can explain the species distribution of this genus in the Alps. Seeds of 25 *Saxifraga* spp. from 79 populations were collected and used in the laboratory and common garden experiments. As each transition phase can restrict recruitment outcomes, each phase was studied separately. We tested the hypotheses that (1) compared with narrow-ranged *Saxifraga* spp., widespread species are characterized by (a) higher levels of recruitment traits [germination, emergence, and survival (both during the first growing season and overwinter)] and (b) greater intraspecific variations in germination and emergence and that (2) early transition stages (i.e., germination and emergence) correlate to survival.

MATERIALS AND METHODS

Study Species and Seed Collection

In total, 25 *Saxifraga* spp. (**Table 1**) from 79 populations (**Figure 1** and **Supplementary Table 1**) were collected. Life forms (Landolt et al., 2010) include therophytes, hemicryptophytes, and herbaceous chamaephytes (**Table 1**). Nomenclature refers to Aeschimann et al. (2004); however, two explanations on species shall be made. First, according to phylogenetic studies of Zhang (2013) and Ebersbach et al. (2017), *S. stellaris* may belong to the section/genus *Micranthes*, but we treated *S. stellaris* as part of *Saxifraga*. Second, S. *blepharophylla* is listed as a subspecies of *S. oppositifolia*, but Holderegger and Abbott (2003) declared it as a separate species after applying chloroplast DNA analyses, and so we treated *S. blepharophylla* as a species. Of the 25 species, 13 were classified as widespread and 12 as narrow-ranged species, based on an "IUCN Species Area of Occupancy" (AOO) approach, where AOO is defined as the smallest area of occupancy of a species. To this end, we determined the AOO of each species within the geographic region of the European Alps via the open-source tool GeoCAT,[1] with data points taken from the Global Biodiversity Information Facility (GBIF). If AOO was smaller than 1,000 km^2, species were classified as narrow-ranged and $> 1{,}000$ km^2 as widespread ("wide" and "narrow" in Figures and **Table 1**).

Seeds were harvested at the time of natural seed maturity, i.e., Jun–Oct 2016 and 2017. Collections were done across the whole European Alpine arc at elevations from 400 to 3,020 m a.s.l (**Figure 1**). In addition, seeds from two populations were received from the Botanical Garden in Vienna (S. *caesia*, S. *tridactylites*) situated at 196 m a.s.l. Every population was harvested from at least five individuals. Seed collection was done as a joint effort of the "Alpine Seed Conservation and Research Network" (Müller et al., 2017),[2] collecting seeds in their countries and sending them to Innsbruck, Austria. The seeds were then cleaned and stored at room temperature (∼20°C, ∼60% relative humidity, up to 6 months) until further processing, i.e., preparing seeds in paper bags for the garden experiment and placing the seeds on Petri dishes for laboratory germination tests. Seed storage in this approach is considered to have a negligible effect on germination potential, as the seeds have subsequently undergone a stratification period to release seed dormancy.

Laboratory Experiment: Germination

Prior to germination tests, seeds were cold-wet stratified on moist filter paper for 3 months (∼4°C refrigerator). Petri dishes were wrapped in aluminum foil to guarantee darkness. This stratification served to simulate a winter period and break a possible seed dormancy (Schwienbacher et al., 2011; Tudela-Isanta et al., 2018). Petri dishes were checked twice under green-light conditions (to avoid a light impulse) for watering and to replace filter papers in case of fungal infections. After stratification, the seeds were transferred to growth chambers (SANYO MLR-350H, Sanyo Electric Biomedical Co., Ltd., Japan)

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[1] http://geocat.kew.org/editor

[2] http://www.alpineseedconservation.eu/

TABLE 1 | Study species of the genus *Saxifraga* (Saxifragaceae) having a wide or narrow distribution range (decending order by AOO), and number of populations (No. Pop) collected in the European Alps.

***Saxifraga* spp.**	**No. Pop**	**AOO Alps (km^2)**	**Distribution**	**Elevations of collection**	**Lab test (No. Pop)**	**Common Garden**	**Seed mass (mg)**	**T°**	**K°**	**L°**	**F°**	**W°**	**R°**	**N°**	**H°**	**D°**	**LF°**	**KS°**
bryoides	5	1,948	wide	2,300–2,703	x	x	0.053	1	3	5	3.5	1	2	1	1	5	c	cs
rotundifolia	6	1,840	wide	918–2,048	x	x	0.039	2.5	2	2	4	3	3	4	3	1	h	cs
paniculata	5	1,784	wide	1,487–2,244	x	x	0.04	2	3	5	2	1	4	2	1	5	c	ss
caesia	6	1,572	wide	196, 1,564–2,449	x	x	0.029	1.5	4	5	2	1	5	1	3	5	c	ss
aspera	6	1,568	wide	1,415–2,740	x	x	0.016	2	3	5	2.5	1	2	2	1	5	c	cs
stellaris	4	1,504	wide	1,520–2,235	x (3)	x	0.04	2	2	5	4	3	3	2	3	1	c	cs
androsacea	3	1,368	wide	2,150–2,698	x	x	0.036	1	2	4	3.5	1	4	2	3	3	c	ss
seguieri	2	1,268	wide	2,150, 2,698	x	x	0.036	1	2	5	4	1	2	2	3	3	c	cs
moschata	5	1,220	wide	1,503–2,601	x	x	0.038	1	4	5	3	1	4	1	3	5	c	ss
exarata	4	1,204	wide	1,900–3,020	x	x	0.053	1	4	5	3	1	2	1	3	5	c	ss
aizoides	6	1,140	wide	1,560–2,479	x (5)	x	0.067	2	2	4	4	3	4	2	1	1	c	cs
biflora	3	1,140	wide	2,601–2,730	x	x	0.117	1	2	5	3.5	1	4	2	1	5	c	cs
oppositifolia	5	1,116	wide	2,096–2,601	x	x	0.118	1	4	5	3.5	3	4	2	1	5	c	cs
rudolphiana	1	528	narrow	2,601	x	x	0.07	1	2	5	3.5	3	4	2	1	5	c	cs
cotyledon	2	468	narrow	1,390, 1,762	x	x	0.035	2.5	3	4	3	1	2	2	1	5	c	ss
mutata	2	468	narrow	1,300, 1,435	x	x	0.036	2.5	2	3	4	3	4	2	1	1	h	ss
tridactylites	3	452	narrow	196, 846, 1,050	x	x	0.012	4.5	4	4	2	1	4	2	1	5	t	rs
hostii	3	304	narrow	1,900–1,969	x	x	0.048	2	3	5	2	1	5	2	1	5	c	ss
sedoides	1	296	narrow	2,300	x	x	0.077	1.5	2	5	3.5	1	5	2	3	5	h	cs
squarrosa	1	288	narrow	2,365	x	x	0.029	2	4	5	2	1	5	1	1	5	c	ss
adscendens	2	244	narrow	2,458–2,617	x	x	0.011	1.5	4	4	3.5	1	4	4	3	3	t	rs
blepharophylla	1	208	narrow	2,150	n.a.	x (2 pots)	n.a.	1	3	5	3.5	1	2	2	1	5	c	cs
cochlearis	1	168	narrow	610	x	x	0.05	3	4	4	2	1	5	2	1	5	c	ss
cernua	1	28	narrow	2,700	n.a.	x	0.214[#]	1.5	2	4	3.5	1	4	4	3	5	h	ss
burseriana	1	n.a.	narrow	1,788	x	x	0.052	2	4	5	1.5	1	5	1	1	5	c	ss

The distribution range was classified by the area of occupancy (AOO), determined using the GeoCAT tool with data points from the Global Biodiversity Information Facility (GBIF) for the European Alps. Elevations of seed collection (see details for each population in ***Supplementary Table 1****). Columns six and seven list the laboratory test or the common garden experiment and the number of populations in parenthesis in case of diverging from the text. Seed mass (mg/seed) and ecological indicator values per species are listed in the last 12 columns. Landolt et al. (2010) ecological indicator values. T, Temperature value (1–4.5; alpine-nival to warm-colline); K, continentality (1–5; oceanic to continental); L, Light requirement (1–5; strong shade < 3% light to full light); F, soil moisture (1–5; dry to flooded); W, alternating soil moisture (1–3; low variability to non-variable moisture); R, soil reaction (1–5; pH 2.5 to > 6.5); N, Nutrients (1–5; nutrient poor to nutrient rich); H, Humus (1–5; no/little humus to high humus content); D, Aeration of soil (1–3; poor aeration/wet soil to good aeration/sandy soil); LF, Life form (t, therophyte; c, herbaceous chamaeophyte; h, hemicryptophyte); KS, simplified Strategy types (rs, ruderal stress tolerant; cs, competitive stress tolerant; ss, stress tolerant).*

[#] *Breman et al. (2019); n.a., not available.*

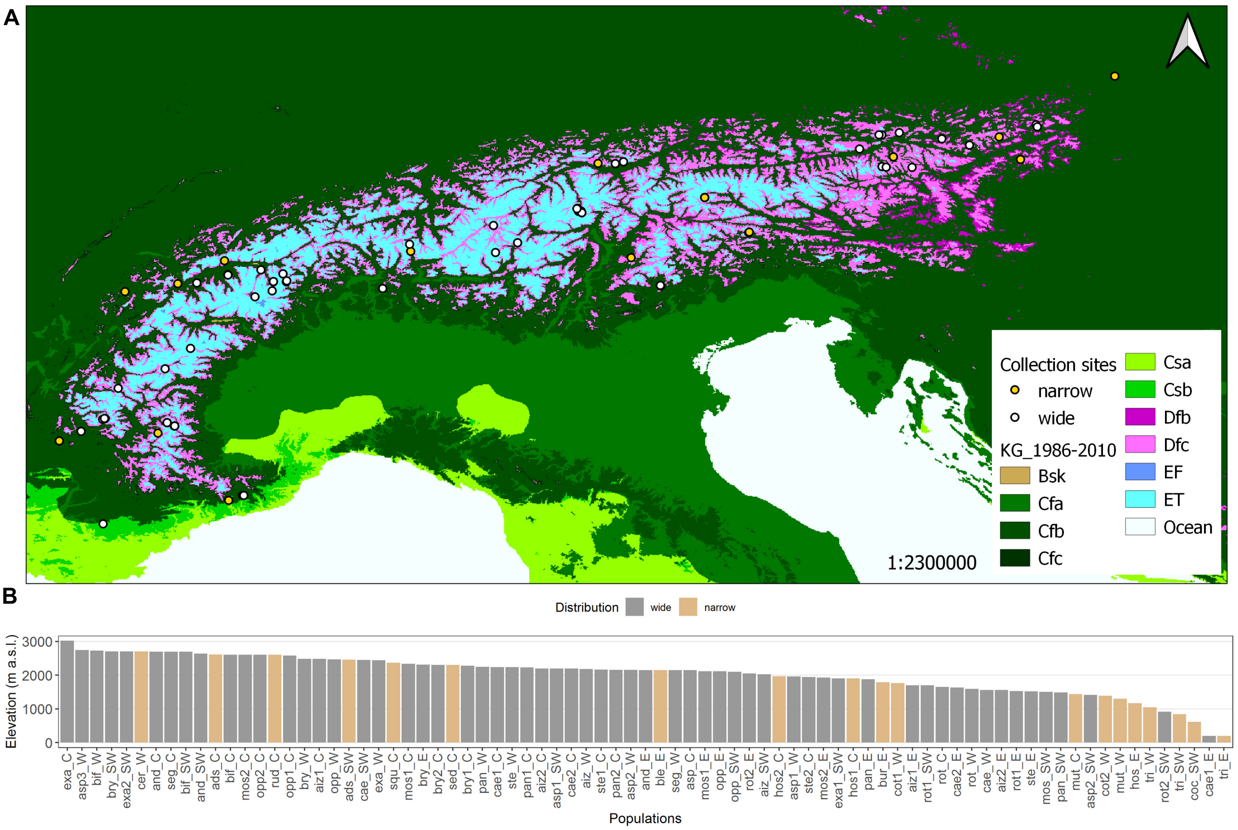

FIGURE 1 | (A) Köppen-Geiger climate map (open source) of the European Alps and seed collection sites (points). In total, 79 populations of *Saxifraga* spp. were collected from Southwest (France) to the East (Austria). As some points overlap in the overview, detailed location maps are provided in **Supplementary Figure 1**. The climatic zones can be classified as arid (Bsk), warm temperate (Cfa, Cfb, Cfc. Csa, Csb), boreal (Dfb, Dfc), and alpine (ET, EF). **(B)** Histogram of elevations of seed collection sites per population, in descending order of elevation.

with two alternating temperature conditions: warm at 25/15°C and cold at 15/5°C. Growth chambers were set to 16 h light (20,000 lx) and 8 h dark (0 lx) with 60% air humidity. The maximum photosynthetic photon flux density was 180 μmol $m^{-2}s^{-1}$. These settings were chosen due to the previous knowledge on warm cues for the germination of alpine species (Schwienbacher et al., 2011; Walder and Erschbamer, 2015; Fernández-Pascual et al., 2020) and previous studies showing good germination results with 15/5°C in other *Saxifraga* spp. (e.g., Giménez-Benavides and Milla, 2013).

Ideally, for each species and population, four Petri dishes with 25 seeds per Petri dish were prepared on three layers of filter paper (Ø 90 mm) and moistened with deionized water. Depending on seed availability, the number of seeds placed per Petri dish was adjusted; exact numbers of seeds sown and germination per population are given in **Supplementary Table 2**. A germination test period lasted 50 days. The Petri dishes were checked every 3–4 days for germinated seeds (i.e., radicle at least as long as the seed) which were removed from the Petri dishes. Seeds infested with fungi were subjected to a pressure test using tweezers, and in the case of softness, they were removed from the Petri dish and counted as non-germinated. After the test period, the cumulative proportion of germination was calculated per Petri dish, i.e., germinated seeds divided by the number of seeds sown (Ranal and Santana, 2006), given as % ± standard error in the text. The calculation based on the number of seeds sown was done for comparability with the garden experiment.

For the species *S. cernua* and *S. blepharophylla*, no laboratory tests could be carried out due to seed scarcity. The same holds for single populations of *S. aizoides* (population aiz_W) and *S. stellaris* (population ste_W; **Supplementary Table 2**).

Common Garden Experiment: Emergence, Survival of Growing Season and Overwinter Survival

Seeds were sown in gardening pots (7 × 7 cm) which were filled with a fine-grained alpine soil mixture (5:2:2:2:1 leaf mold:ground earth:peat:silicate sand:lava) obtained from the Botanical Garden Innsbruck. Pots were placed in the Botanical Garden on the 15 November 2016 and 6 January 2018 following seed collections in the summers of 2016 and 2017, respectively. Seed collections from both years experienced a winter period in the pots, i.e., natural cold-wet stratification to break seed dormancy (Schwienbacher et al., 2011; Tudela-Isanta et al., 2018). During the growing season, the pots were regularly watered and randomly reshuffled. Recordings on emergence (i.e., cotyledon development) in the common garden began after snow-melt on 17 March 2017 and 11 April 2018, respectively. Emergence was then checked once every 2 weeks during the whole growing season (see **Supplementary Table 3** for numbers of emerged seeds). As the cotyledons of *Saxifraga* spp. are tiny, we used magnifying glasses to ensure that all emerged seedlings were recorded. Recording further included removing seedlings from pots using a soft tweezer and putting them into separate multi-pots (Ø 2.5 cm; same soil mixture and saturated with water) so that the emerged seedlings were not double-counted. The emergence experiment ended on 22 September 2017 and 25 September 2018, respectively, lasting for 187 days in 2017, and 168 days in 2018, respectively. The proportion of cumulative emergence was calculated from the amount of seeds sown (given as % ± standard error in the text), as it was not possible to gather the number of non-emerged seeds in the soil-filled pots.

The emerged seedlings were arranged in multi-pots with a maximum of five seedlings per multi-pot, originating from matching populations, and regularly watered. Here, the survival of the seedlings (i.e., survival until the end of the growing season; Fenner and Thompson, 2005) was monitored (counting of seedlings every 3 weeks, i.e., 14 times) until the end of September of the same year as emergence events (**Supplementary Figure 2**). Survival in the growing season ("gs") is expressed as the proportion of seedlings that survived from the number of seeds that emerged (% ± standard error in the text) on 19 October 2017 and 22 November 2018, respectively.

The seedlings that survived across the growing season in the multi-pots were repotted into bigger pots (7 × 7 cm) due to natural seedling growth (on 19 October 2017 and 22 November 2018, respectively). A maximum of 50 individuals per population was repotted due to space limitations. Five individuals per population were pulled together in one pot, which was buried underground, and watered regularly until the first snowfall at the experiment site. The seedling survival of a winter period (i.e., overwinter "ow") was determined after snowmelt in the following year (6 April 2018 and 4 April 2019, respectively). Survival "ow" is expressed as the proportion of survived seedlings from repotted survivors (% ± standard error and numbers in the text).

Average mean soil temperatures of the growing seasons (2-cm depth) were 17.3°C (2017) and 19.6°C (2018); average minimum temperatures were 10.8°C (2017) and 13.0°C (2018); and average maximum temperatures were 27.4°C (2017) and 29.9°C (2018; **Supplementary Figure 2**).

Data Analyses

Responses of Widespread and Narrow-Ranged *Saxifraga* spp.

Seeds from 23 species and 75 populations were available for testing germination ($N = 584$ Petri dishes). We used generalized- and linear-mixed models (package "lme4" Bates et al., 2019) to analyze germination percentage and mean time to germination (MTG), respectively, in response to the fixed-factors distribution ("wide," "narrow"), temperature ("warm," "cold"), and the interaction term; species and population nested within species were set as the random effects. The model for germination percentage was set assuming a binomial distribution (Ranal and Santana, 2006) with a logit link. The model was not overdispersed or zero-inflated as tested via the package "DHARMa" (Hartig, 2020). MTG was analyzed with a Gaussian link; model assumptions were met.

The time that seeds need to germinate gives insights into the dynamics of the germination process. The MTG per Petri dish was calculated in EXCEL via formulas in Ranal et al. (2009) as the following:

$$MTG,\ MTE = \sum_{i=1}^{k} n_i t_i \ / \ \sum_{i=1}^{k} n_i$$

where t_i is the time from the start of the experiment to the ith observation; n_i: number of seeds germinated in the time i (not the accumulated number, but the number corresponding to the ith observation), and k: last time of germination. Due to mathematical reasons, MTG cannot be calculated when germination is zero for a whole Petri dish (Ranal and Santana, 2006), which was excluded from the analyses.

Seeds from all 25 species and 79 populations were available for testing emergence ($N = 236$ pots). Subsequently, we calculated the mean time to emergence (MTE) using the same approach proposed for the calculation of MTG, but based on the number of seedlings that emerged in the particular time interval i instead of germinated seeds (Ranal et al., 2009). Emergence percentage and MTE were analyzed following the same approach as proposed for germination percentage and MTG data analyses, without the temperature factor. Emergence can be zero if no seedling is able to emerge, which was the case in the eight pots.

To test if the survival of seedlings until the end of the growing season ("gs") differed between distributions, we built a generalized linear mixed model (binomial distribution and logit link) with distribution and year (2017 and 201') as a fixed factor; species and population nested within species were set as a random effects; and model assumptions were met. We observed strong seedling mortality in the summer of 2018, and we had to include the year of observation to account for this effect. Survival percentage of the winter period ("ow") was analyzed using a generalized linear mixed model (binomial distribution and logit link) with distribution as the fixed effect, species and population nested within species as random effect; model assumptions were met.

To assess information on the ecology of the species, we used ecological indicator values of Landolt et al. (2010) (Eco-values in **Table 1**). Ecological indicator values describe ecological and biological characteristics of species by values that represent a species' preferred climatic (T, K, L; **Table 1**) and soil conditions (F, W, R, N, H, D; **Table 1**), and species' life form strategies (LF, KS; **Table 1**). The values are based on long-term compilations of expert knowledge and experiments (field or laboratory). We analyzed each response variable (germination percentage, MTG, emergence percentage, MTE, survival "gs" and survival "ow" percentages) as described above and included the 11 ecological indicator values (**Table 1**) individually in the models; likelihood ratio tests were used to assess a significant contribution of the values in the model. We then included those values that were significant in individual models to combined models. Ecological indicator values that were found to have a significant effect in the combined models were kept.

To determine the variance explained by fixed effects in the models, R^2 for the null model (R^2m0) and the conditional (full) model (R^2m1), respectively, were calculated, according to Nakagawa and Schielzeth (2013). Furthermore, we extracted the variance components of the models (i.e., random factors) to determine the intraclass correlation coefficient [ICC; variance of random terms divided by variance of random term plus residual variance; Nakagawa et al. (2017)]. For more detailed information on model building and calculation on ICC (see **Supplementary Text**).

Intraspecific Variation

To assess whether or not the intraspecific variability of recruitment traits differs between widespread and narrow-ranged species, we used the coefficient of variation (CV), which is used as a measure for variation within or between groups (ratio of the standard deviation to the mean; Pearson, 1894; Lande, 1977; Meier and Holderegger, 1998). The CV is here expressed in its ratio form but can be interpreted as a percentage of variation (ratio times 100). Low values are considered as low variation whereas high values indicate high variation. The CVs of the response variables, namely germination percentage, MTG, emergence percentage, and MTE were calculated for each species. The basis for calculations were Petri dishes and pots, respectively, representing the variation within the Petri dishes or pots. Subsequently, the means of these population CVs reflect the variation within a species (i.e., intraspecific variation). If the initial response variable was zero, a CV cannot be calculated. The species-level CVs were then used as variables in simple linear regressions, with distribution as an explanatory factor variable, using separate datasets for warm and cold temperature conditions in the case of germination percentage and MTG. Species that have only single populations were excluded from these analyses. We used a square root transformation of the CV as it can be zero (Pearson, 1894; Lande, 1977). The CV for MTG and MTE were calculated according to Ranal et al. (2009). In our dataset, it was not possible to calculate the CV for germination percentage and MTG for enough number of species in the warm temperature to perform a meaningful analysis for this temperature setting.

To evaluate variations in temperature at the initial seed collection sites, we extracted mean annual temperatures from raster datasets available from the University of East Anglia Climatic Research Unit (Harris et al., 2021) for the years of seed collection 2016 and 2017, respectively (**Supplementary Table 1**). We used QGIS to extract values per location (via latitude and longitude). From these mean annual temperatures per population, we calculated CVs of temperature for individual species. These CVs were analyzed in a linear regression with distribution as the explanatory factor variable to assess if seed source temperature variation differs between distributional groups. Species with only single populations were excluded from these analyses.

Links Between Early Life Stages

To estimate the influence of the earliest transition phases on the subsequent one, we performed correlations on germination-emergence and emergence-survival "gs" (Spearman Method) on means per species of widespread and narrow-ranged species, i.e., mean germination (warm and cold) and the emergence of 23 species (data from 2017 and 2018); and emergence and the survival "gs" of summer 2017 of 17 species.

We used R software (R Core Team, 2020) and RStudio (RStudio Team, 2020) for analyses and graphs ("pastecs" Grosjean et al., 2018; "cowplot" Wilke, 2018; "GerminaR" Lozano-Isla et al., 2019; packages within "tidyverse" Wickham et al., 2019; "scales" Wickham and Seidel, 2020).

RESULTS

Responses of Widespread and Narrow-Ranged *Saxifraga* spp.

Seed germination was similar between widespread and narrow-ranged species under both temperature conditions (**Figures 2A,B**). Average germination percentage under the warm temperature was lower (wide: 22.3 ± 2.3%; narrow: 14.9 ± 3.2%) than under the cold temperature conditions (wide: 39.0 ± 2.5%; narrow: 43.6 ± 5.0%). Distribution *per se* did not affect germination percentage; however, the interaction of distribution × temperature did ($p < 0.001$, df = 1; **Table 2A**). Under cold temperature, total germination percentage was significantly greater than under warm temperature ($p < 0.001$, df = 1; **Table 2A** and **Figures 2A,B**). The MTG between the distribution groups was similar (**Figures 2A,C**). Under the warm temperature, average MTG was higher (wide: 27.3 days ± 1.1 standard error; narrow: 24.6 ± 2.8 days) than under the cold temperature (wide: 24.3 ± 0.7 days; narrow: 22.1 ± 1.0 days). Distribution was very weakly responsible for these results ($p = 0.076$), while temperature had a strong significant effect on MTG ($p < 0.001$, df = 1; **Table 2B**).

In the common garden, the two distribution groups emerged similarly ($p = 0.646$, df = 1; **Table 2C** and **Figures 3A,B**). Widespread species' emergence was 42.3 ± 1.7% on average, while narrow-ranged species' emergence was 39.0 ± 3.9% (**Figures 3A,B**). The MTE also did not differ between the distribution groups ($p = 0.295$; **Table 2D** and **Figure 3C**). Widespread species emerged on average after 38.1 ± 1.3 days (MTE) and narrow-ranged species after 36.1 ± 2.0 days (MTE).

No differences occurred between the two distribution types in survival "gs," i.e., to the end of the growing season ($p = 0.174$, df = 1), but the year of observation made a strong difference ($p < 0.001$, df = 1; **Table 2E**). Seedling survival "gs" in the common garden was on average 36.2 ± 4.2% in 2017 for widespread and 54.7 ± 7.3% for narrow-ranged species (**Figure 4A**). In summer 2018, only 2.6 ± 0.7% of seedlings from wide and 7.11 ± 4.3% of seedlings from narrow-ranged species survived the growing season (**Figure 4A**). The survival of the winter period "ow" was also similar for both the distribution groups ($p = 0.062$, df = 1), i.e., 58.4 ± 4.6% for widespread

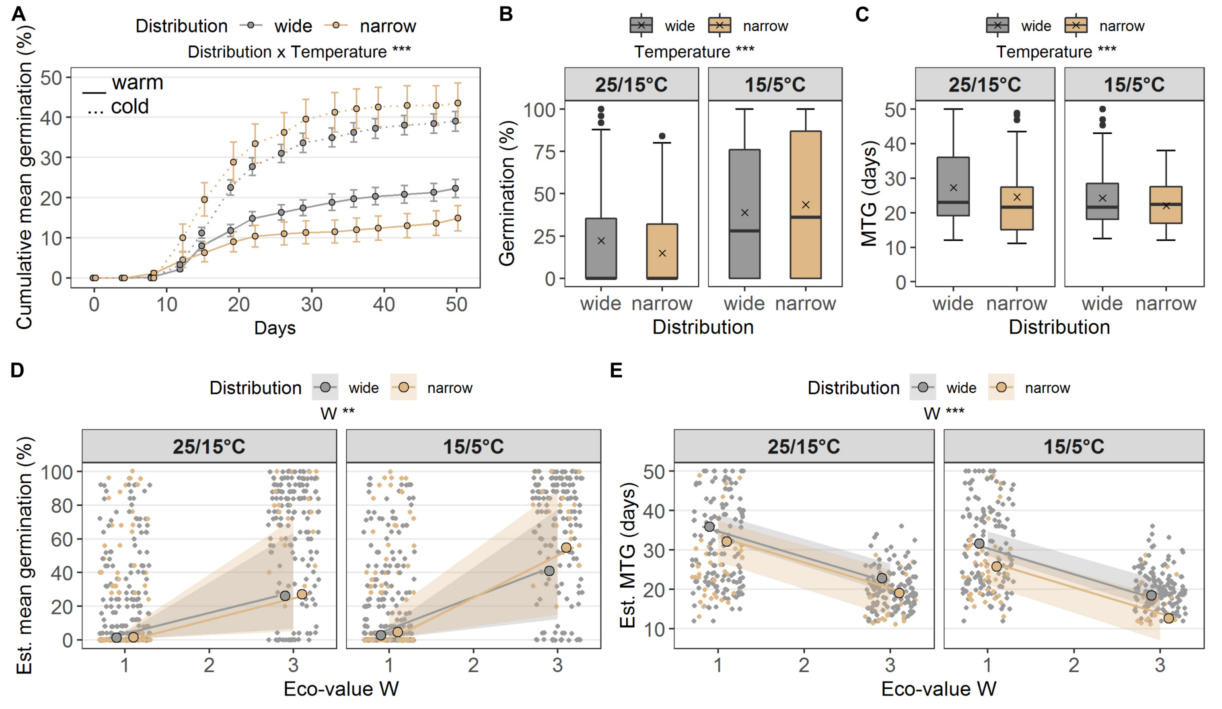

FIGURE 2 | (A) Cumulative germination (%) in the laboratory at two temperature regimes (warm 25/15°C, cold15/5°C), grouped according to distribution ranges (wide, narrow). Points denote the cumulative mean on each day of observation, error bars denote ± standard errors. Boxes in **(B)** germination (%) and **(C)** MTG (days) illustrate the interquartile range with the median as line; whiskers illustrate the minimum and maximum; points denote outliers; x denotes the mean. Points without border in **(D)** germination (%) and **(E)** MTG denote the observed values colored according to the distribution range along the ecological indicator value W (alternate soil humidity); points with border denote the estimated means and colored ribbons denote confidence intervals from the models. Note different maxima on *y*-axis between panels. Stars denote significances at $\alpha = 0.01$ (**) and $\alpha = 0.001$ (***).

and 54.6 ± 10.1% for narrow-ranged species (**Figure 4B**). Recruitment responses at the species level are presented in **Figure 5**.

The ecological indicator value W (alternating soil humidity) significantly affected the germination percentage ($p < 0.01$, df = 1, **Table 2A**), with species assigned to higher *W*-values showing a higher germination percentage (**Figure 2E**). Alternating soil humidity (W) also significantly impacted MTG ($p < 0.001$, df = 1, **Table 2B**), which decreased for species having higher *W*-values (**Figure 2E**). Moreover, species with lower emergence tended to have high indicator value H (humus content; $p < 0.01$, df = 1; **Table 2C** and **Figure 3D**). Furthermore, the emergence differed among life forms (**Figure 3D**), with therophytes (two species) significantly emerging less than herbaceous chamaephytes and hemicryptophytes (**Table 2C** and **Supplementary Table 4**). The MTE was significantly enlarged in response to the ecological indicator values T (temperature; $p < 0.01$, df = 1) and H (humus content; $p < 0.01$, df = 1, **Table 2D** and **Figures 3E,F**). None of the ecological indicator values were significant in the model for survival "gs" (**Table 2E**), but the ecological indicator value F (soil moisture) played a significant role for survival "ow" ($p < 0.001$, df = 1; **Table 2F** and **Figure 4C**), i.e., species with high *F*-value had low survival "ow."

Variance components of the models are listed in **Supplementary Table 5** and are illustrated in **Supplementary Figure 3**. The full model of germination percentage accounted for 67.30% (R^2m1) of the variance and 22.63% was due to the fixed factors (R^2m0; **Supplementary Table 5A**). The random factor level species had a variance of 40.3% and a population of 17.5% (**Supplementary Figure 3** and **Supplementary Table 4A**). The full model of MTG accounted for 84.4% of the variance and 42.1% was due to fixed effects. Random effects, i.e., species (6.7%) and populations (66.4%), also accounted for a substantial fraction of variance (**Supplementary Figure 3** and **Supplementary Table 5B**). The full model of emergence percentage accounted for 26.4% of the variance, but only 0.5% was due to fixed-factor distribution (**Supplementary Figure 3** and **Supplementary Table 5C**). The random factor level species had 6.1% of the variance in the model and a population of 8.0% (**Supplementary Figure 3** and **Supplementary Table 5C**). The full model survival "gs" accounted for 46.9% of the variance, and 28.7% was due to fixed factors (**Supplementary Table 5E**). The random factor level species made up for 16.7% of variance, and population 8.9% (**Supplementary Figure 3** and **Supplementary Table 5E**). The full model of survival "ow" accounted for 2.7% of the variance, and 2.4% was due to the fixed factors (**Supplementary Table 5F**). The random factor level species had 0.0% and a population of 0.3% of the variance in the model, while the largest portion of the variance in survival

TABLE 2 | Results of mixed models of studied recruitment traits (A) germination percentage, (B) mean time to germination (MTG), (C) emergence percentage, (D) mean time to emergence (MTE), (E) survival percentage after the growing season "gs," and (F) overwinter survival percentage ("ow"), in response to distribution, temperature conditions, the interaction term, and ecological indicator values (see **Table 1**) that were found to be significant when individually added to the models.

Model terms	Full model (m1)	Chisq (m1)	*p*-value (m1)
(A) Germination percentage	Log-Odds: Est. (95% CI)		
Distribution	0.05 (−1.63, 1.72)	0.12	0.732
Temperature	0.68 (0.59, 0.77)	378.72	<0.001
Eco-value W	1.60 (0.69, 2.51)	9.42	<0.01
Distr × Temp°	0.51 (0.31, 0.71)	26.64	<0.001
(B) MTG	Est. (95% CI)		
Distribution	−3.77 (−9.39, 1.85)	3.15	0.076
Temperature	−4.30 (−5.46, −3.15)	46.02	<0.001
Eco-value W	−6.56 (−8.82, −4.30)	16.81	<0.001
Distr × Temp°	−2.05 (−5.00, 0.90)	1.85	0.173
(C) Emergence percentage	Log-Odds: Est. (95% CI)		
Distribution	−0.14 (−0.75, 0.47)	0.21	0.646
Eco-value H	−0.43 (−0.72, −0.15)	7.70	<0.01
Eco-value LF (h/t)	0.14 (−0.65, 0.92) / −2.71 (−3.78, −1.65)	17.96	<0.001
(D) MTE	Est. (95% CI)		
Distribution	−0.32 (−0.92, 0.28)	1.10	0.295
Eco-value T	0.61 (0.26, 0.96)	9.35	<0.01
Eco-value H	0.49 (0.22, 0.75)	9.32	<0.01
(E) Survival percentage "gs"	Log-Odds: Est. (95% CI)		
Distribution	0.62 (−1.23, 1.48)	1.85	0.174
Year garden (2017)	−2.70 (−3.16, −2.24)	80.08	<0.001
(F) Survival percentage "ow"	Log-Odds: Est. (95% CI)		
Distribution	−0.25 (−0.50, 0.00)	3.48	0.062
Eco-value F	−0.38 (−0.51, −0.25)	11.25	<0.001

All models were set with distribution "wide" as reference, temperature "warm" in cases (A,B), LF "c" in case of (C). Est., Estimate; CI, Confidence intervals; Chisq, test statistic and p-values of full model (m1). Chi-square and p-values were estimated with maximum likelihood; °in cases of interactions we used contrast-coding (Levy, 2018).

"ow" remained unexplained (**Supplementary Figure 3** and **Supplementary Table 5F**).

Intraspecific Variation

Intraspecific variation of response variables per species is illustrated in **Figure 6A**. Intraspecific variation of germination percentage under warm temperature ranged from lowest CV = 0.13 (*S. aizoides* and *S. stellaris*) to highest CV = 2 (*S. seguieri*); the only one value for narrow-ranged species (CV = 0.31 *S. mutata*) prevented further analyses. Under the cold temperature, intraspecific variation of germination percentage ranged from lowest CV = 0.06 (*S. aizoides*) to highest CV = 1.49 (*S. adscendens*). For the MTG under warm temperature, CV ranged from 0.14 (*S. moschata*) to 0.40 (*S. stellaris*) and under the cold temperature from 0.14 (*S. cotyledon*) to 0.29 (*S. stellaris*). Intraspecific variation of emergence was lowest in *S. mutata* (CV = 0.06) and highest in *S. tridactylites* (CV = 1.13). Intraspecific variation of MTE was lowest for *S. cotyledon* (CV = 0.31) and highest for *S. oppositifolia* (CV = 0.64). When analyzing CVs to assess intraspecific variation between distribution groups (**Figure 6B**), narrow-ranged species had a significantly lower intraspecific variation in MTE ($p = 0.02$; **Supplementary Table 8**) than widespread species. There were no significant intraspecific variations in germination percentage, MTG, and emergence percentage between the two distributional groups ($p > 0.4$; **Figure 6B** and **Supplementary Table 6**).

Intraspecific variation of the average annual temperatures at seed collection sites ranged from lowest CV = 1.04 (*S. mutata*) to highest CV = 3.79 (*S. biflora*). It differed significantly between distribution groups, with narrow-ranged species having significantly lower temperature variation at their sites of seed collection ($p = 0.017$; **Supplementary Table 6**) than the widespread species.

Links Between Early Life Stages

Seed germination and seedling emergence followed a trend of a positive correlation, but only germination percentage in cold conditions and emergence of narrow *Saxifraga* spp. was significant ($R = 0.718$, $p = 0.019$). Conversely, emergence and survival "gs" were significantly positively correlated in widespread species ($R = 0.685$, $p = 0.014$; **Supplementary Table 7**).

DISCUSSION

In this study, we investigated early life stages of 25 *Saxifraga* spp. from the European Alps, representing species with wide

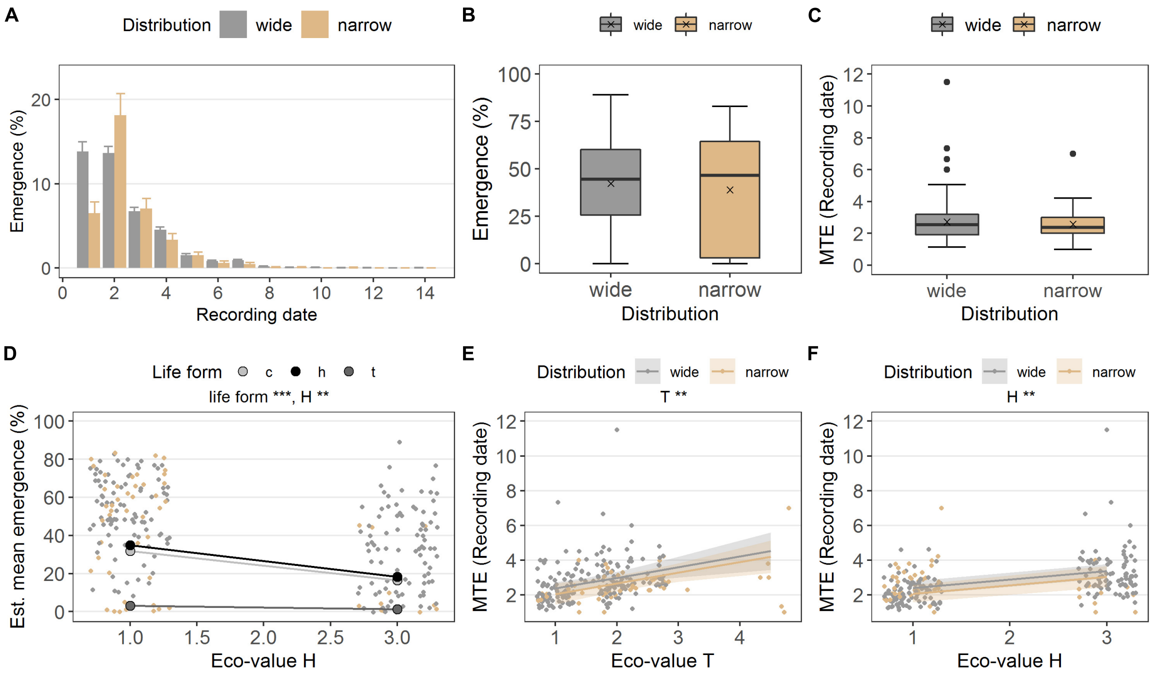

FIGURE 3 | (A) Emergence percentage in the common garden, grouped in distribution (wide, narrow), at each recording date (i.e., every 2 weeks). Bars denote mean at each recording date, error bars denote ± standard errors. Boxes in **(B)** emergence percentage and **(C)** MTE (recording date) illustrate the interquartile range with the median as line; whiskers illustrate the minimum and maximum; points denote outliers; x denotes the mean. Points without border in **(D)** emergence percentage **(E,F)**, MTE, denote the observed values colored according to distribution along ecological indicator values H (humus) and T (Temperature value); points with border denote the estimated means per ecological indicator value life form (c, chamaeophyte; h, hemicryptophyte; t, therophyte), and colored ribbons denote confidence intervals from the models [missing in **(D)** to simplify the graph]. Note different maxima on *y*-axis between panels. Stars denote significances at α = 0.01 (**) and α = 0.001 (***).

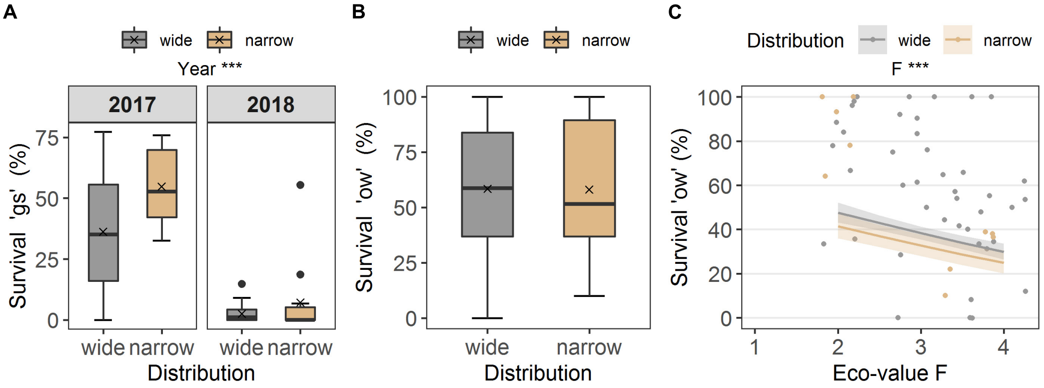

FIGURE 4 | (A) Seedling survival of the growing season ("gs") in the common garden, grouped according to distribution (wide, narrow), for the years of observation (2017, 2018), and **(B)** overwinter seedling survival ("ow"). Boxes in **(A)** survival "gs" and **(B)** survival "ow" illustrate the interquartile range with the median as line; whiskers illustrate the minimum and maximum; points denote outliers; x denotes the mean. Points without boarder in **(C)** survival "ow" denote the observed values colored according to distribution along the ecological indicator value F (humidity), and colored ribbons denote confidence intervals from the model. Note different maxima on *y*-axis between panels. Stars denote significances at α = 0.001 (***).

and narrow geographic ranges. By conducting laboratory and common garden experiments, we studied recruitment transition phases, i.e., germination, emergence, and survival (first growing season and overwinter).

Germination was triggered by the cool temperature conditions in the laboratory in species from both geographic ranges, leading to higher germination percentages with faster MTG in this setting. However, because of the limited options for studying temperature requirements for germination (i.e., only two regimes), further research is needed to better define the germination temperature niche. Yet, these results are in line with Giménez-Benavides and Milla (2013) who

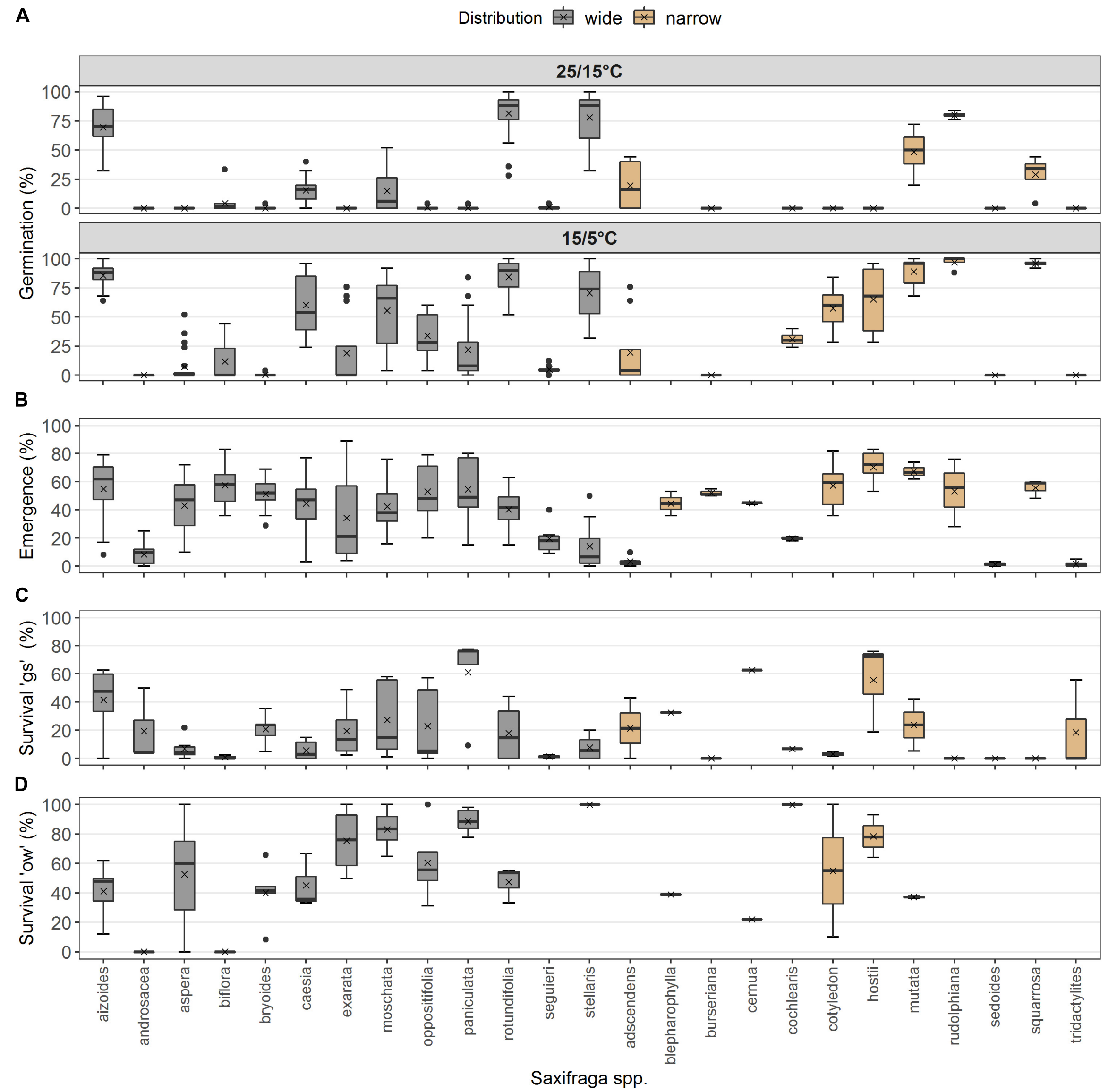

FIGURE 5 | Recruitment responses at species level. **(A)** Germination percentage under warm and cold laboratory temperature (*N* = 584). **(B)** Emergence percentage in the common garden under natural seasonal climate (*N* = 236). **(C)** Survival percentage "gs" (*N* = 79), and **(D)** survival percentage "ow" (*N* = 52). Species are listed in alphabetical order within each distribution. Boxes illustrate the interquartile range with the median as line; whiskers illustrate the minimum and maximum; points denote outliers; x denotes the mean.

reported that cold temperatures favored the germination of two altitudinal vicariant *Saxifraga* spp., independent of their altitudinal occurrence. Similarly, Di Cecco et al. (2019) recorded increased germination under cool temperatures compared with warmer ones in *S. italica*. These results contrast with the general assumption that germination of alpine species is enhanced by warm temperature cues (Walder and Erschbamer, 2015; Fernández-Pascual et al., 2020; Vázquez-Ramírez and Venn, 2021). As germination is a key process for population dynamics (Donohue et al., 2010; Larson et al., 2015; Rosbakh and Poschlod, 2015; Rosbakh et al., 2020), enabling populations to migrate and colonize new habitats, it is crucial for population persistence (Cochrane et al., 2015). Current and future scenarios of warming (IPCC et al., 2021) pose a threat to Alpine *Saxifraga* spp., i.e., warming may lead to reduced germination, which in the long-term may cause a decline in *Saxifraga* populations.

In our laboratory experiments, there was a significant distribution x temperature interaction, with widespread species germinating more at warm temperatures than narrow-ranged species. In combination with their germination in the cold setting, we may interpret these results as widespread species having an overall broader germination temperature niche. A wider germination niche may expose seedlings to the

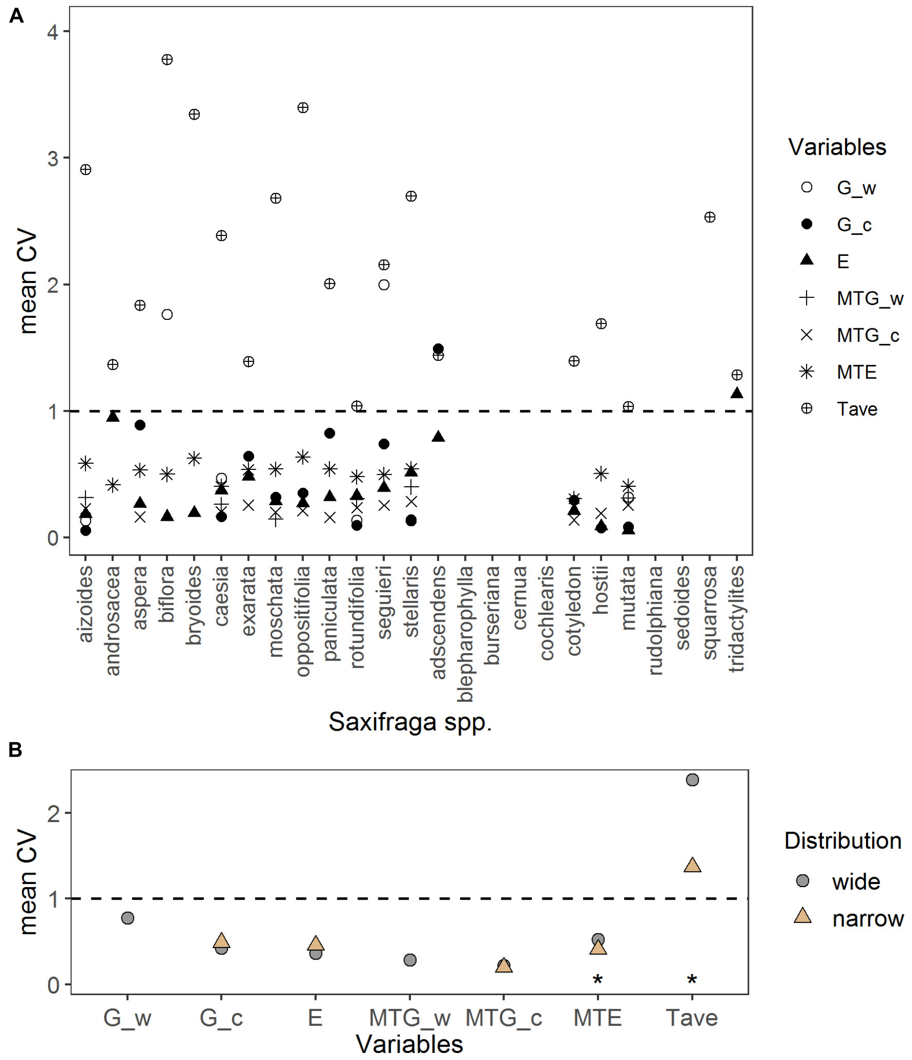

FIGURE 6 | (A) Intraspecific variation per species represented by the coefficient of variation (CV) of response variables: Germination percentage in warm (G_w) and cold (G_c) temperature in the laboratory, emergence percentage (E), mean time to germination in warm (MTG_w) and cold (MTG_c) temperature, mean time to emergence (MTE), and average annual temperature at the seed collection sites (Tave). **(B)** Comparison of mean CV of response variables per distribution range, done by linear regressions. Note different maxima on *y*-axis between panels. Stars denote significances at α = 0.05 (*).

unfavorable post-germination conditions (Donohue et al., 2010; Mondoni et al., 2015; Rosbakh et al., 2020), but may also increase opportunities for recruitment in a diversity of habitats. Consequently, a wider regeneration niche (beginning with germination) could have contributed to a wider distribution range of widespread *Saxifraga* spp., thereby supporting our first hypothesis. In the literature, contradicting results regarding niche breadth and distribution ranges are found (Finch et al., 2018). For example, Thompson et al. (1999) tested the germination niche of 263 herbaceous plants in central England (United Kingdom) and found no correlation of germination niche breadth with range size, although the range size correlated with the temperatures under which species could germinate. In contrast, more recently, Rosbakh et al. (2020) could define five distinct germination niches along a hydroperiod gradient, showing that seeds were able to select a niche that favors seedling survival.

Cold temperature triggered a faster germination in the studied *Saxifraga* spp., and the ecological indicator value for alternating soil humidity (W) made a significant contribution in the models for germination percentage and MTG. Species with high values of W germinated faster and to higher extents, regardless of their distribution range. This can be an important survival strategy in alpine species, especially for those inhabiting bare ground soils that have a low water-holding capacity, but where many *Saxifraga* spp. normally thrive. Under these conditions, species with a fast germination may benefit from the available soil moisture after snowmelt (Giménez-Benavides et al., 2007; Mondoni et al., 2015), meaning a reduced risk of seedlings emergence under possible drought conditions in summer (Garnier et al., 2021).

Widespread and narrow-ranged species had similar emergence percentages and MTE in the common garden experiment. On the other hand, emergence phenology differed in species depending on their ecological indicators for humus content (H), with a higher and faster emergence for those with low H-values. This suggests that *Saxifraga* spp. assigned to medium humus content (i.e., $H = 3$; $n = 10$) could have had difficulties to recruit under the fine-grained alpine soil mixture. However, these latter species have their main occurrences in upper subalpine and alpine vegetation belts (see ecological indicator values for temperature in **Table 1**), where fine-grained soils occur naturally. Another result was that emergence differed among life forms. Surprisingly, the two therophytes in the dataset emerged at significantly lower percentages than herbaceous chamaephytes and hemicryptophytes, although therophytes usually emerge well due to their short life cycle (Fenner and Thompson, 2005; Landolt et al., 2010). As a possible explanation, we suggest that light requirements for germination and subsequent emergence were not met in the common garden. Indeed, although seeds in the garden were exposed to daily sunlight fluctuations, the possibility that they were buried and, therefore, experienced darkness, cannot be ruled out. Photoinhibition of seed germination was noted as a prominent feature in therophytes and small-seeded plants as a strategy to build a permanent soil seed bank (Saatkamp et al., 2011; see Carta et al., 2017 and references therein). Furthermore, some therophytes need disturbances for seed germination and emergence, such as an indirect effect altering the light conditions (Saatkamp et al., 2011).

The MTE for narrow-ranged and widespread species did not differ (i.e., 36–38 days, respectively), thereby showing a similar emergence phenology across the species tested, and reflecting similar emergence times as previously found in other *Saxifraga* spp. in the wild (Mondoni et al., 2015). Emergence times of +30 days do not reflect fast germination after snowmelt, rather it indicates seed dormancy. This slow germination may reflect a bet-hedging strategy for spreading the germination overtime, and with it the risk of seedling mortality (Ooi et al., 2009; Saatkamp et al., 2011). However, in the model of MTE, the ecological indicator value for temperature (T) was significant, with truly alpine species (low T-values) emerging faster than species assigned to higher T-values. These results support previous findings that alpine species germinate and emerge fast after snowmelt to make the best use of soil moisture and avoid the onset of summer drought (Giménez-Benavides et al., 2007; Mondoni et al., 2015; Garnier et al., 2021). Another aspect is that the fast emergence of alpine species could be a strategy to cope with the shorter growing seasons compared with that at lower elevations (Mondoni et al., 2020).

Seedling survival of the first growing season did not differ between narrow-ranged and widespread species, and also no ecological indicator values were found to be significant in the model. Hence, our findings for this transition stage do not support our hypothesis. Seedling survival was significantly low during the second experiment onset (i.e., summer 2018). We attribute the high seedling mortality of this year to drought stress induced by methodological aspects, as seedlings are small and particularly vulnerable to drought stress in the first growing season (Mondoni et al., 2020; Garnier et al., 2021). The multi-pots used for survival monitoring were regularly watered, but also dried out fast in the exceptionally hot summer of 2018 in Europe (soil temperature measurements showed +2.3°C annual mean temperature in the Botanical Garden compared with summer 2017). Although we did not monitor soil water potential, we believe that the species faced drought stress in the garden experiment in the second onset in 2018. Drought stress has been identified to severely reduce the emergence and increase seedling mortality (Garnier et al., 2021; Vázquez-Ramírez and Venn, 2021), especially in unprotected open microhabitats without shelter from surrounding vegetation (Stöcklin and Bäumler, 1996; Margreiter et al., 2021), as found on bare ground or in rock crevasses.

Subsequently, survival into the second growing season ("ow") was similar between distribution ranges, and the ecological indicator value for soil moisture was significant, with species assigned to higher levels of soil moisture surviving significantly less. These results certainly reflect unsuitable water conditions for the species in the garden, but this part of the study should be viewed with caution due to the repeated transplanting of seedlings.

All transition stages varied species-specifically, and it was not possible to draw a solid pattern regarding the distributional range of the species. Accordingly, many studies recorded interspecific variation of seedling emergence in the field (Münzbergová, 2004; Meineri et al., 2013; Mondoni et al., 2015, 2020; Margreiter et al., 2021), and differences in plant performances occurred even if species were phylogenetically closely related (e.g., Lavergne et al., 2004; Milla et al., 2009; Rosbakh et al., 2020).

We found substantial intraspecific variation in germination, emergence, and their mean times to germinate/emerge (**Figure 6A**). At the intraspecific level, narrow-ranged species had a significantly lower variation of MTE than widespread species. Such time constrain for seedling emergence of narrow-ranged species could reflect narrower suitable climatic conditions for this stage. In this regard, mean annual temperature variations between collecting sites were significantly lower in narrow-ranged compared with widespread species, indicating a link between the timing of emergence and climate variability at the species' home sites. Intraspecific variation in seed traits may be linked to a set of climatic conditions that possibly reflect local adaptations (Cochrane et al., 2015). For example, Giménez-Benavides et al. (2007) reported favoring effects of local adaptation for seedling emergence in remnant populations of *Silene ciliata*, and Cotado et al. (2020) found that intraspecific variation in dormancy levels of *Saxifraga longifolia* (Pyrenees) was linked to climatic conditions at the seed collection sites. Furthermore, intraspecific variation in seed traits was found along germination temperature gradients from different locations (Walter et al., 2020). Clearly, the timing of emergence (and germination) is driven by temperature (Walck et al., 2011; Cochrane et al., 2015; Anderson and Wadgymar, 2020). A diverse emergence timing can lead to the spread of emergence events over a broader time scale, spreading the risk of hazards in post-germination phases (e.g., frost, drought, herbivores;

Larson and Funk, 2016). Variation in germination timing was recognized as an important variable for natural selection in *Arabidopsis* (Donohue et al., 2005). Genotypes that had a high variation of germination timing showed a higher fitness due to the postponement of germination toward an optimum for growth, while genotypes with low variation were lacking such opportunities for selection (Donohue et al., 2005). Carrying forward these ideas of germination timing to emergence timing, the greater variation in emergence timing of widespread species found here could have favored their wider distribution range in the past and may be advantageous while facing climate changes when rapid adaptations are needed to cope with novel climatic conditions. Our hypothesis on a greater intraspecific variation of germination and emergence responses for widespread species can, however, only be partially confirmed (i.e., emergence timing).

Research to date suggests that recruitment success may be driven by different seed-trait relationships, showing that, for example, seed mass positively correlates with seed germination (Pearson et al., 2002; Kahmen and Poschlod, 2008) or that dispersal traits positively correlate with seedling emergence (Chen and Giladi, 2020). Relationships between transition stages within recruitment are less studied but suggest that recruitment success may depend on early demographic processes, such as germination and emergence (Fraaije et al., 2015; Larson et al., 2015). In our study, germination and emergence differently affected subsequent transitions phases depending on species distribution range, with emergence probabilities being a strong predictor of survival only for widespread species. Further research is therefore needed to identify seed and seedling functional traits driving such variations between distribution groups.

Finally, we address a methodological point that may have influenced the outcome of this study. The number of populations between widespread and narrow-ranged species was unbalanced, but to the best of our knowledge the mixed models used are well equipped to deal with unbalanced or missing data in the data matrix (Nakagawa and Schielzeth, 2013; Bates et al., 2019). Nevertheless, narrow-ranged species are by definition less common, and we assumed that narrow-ranged species have a narrower niche, reflected by lower recruitment responses and lower intraspecific variability. Our hypotheses were partly supported by the results; hence the outcome of analyses may change to a clearer picture if we had a greater number of populations of narrow species.

CONCLUSION

In summary, recruitment traits of widespread and narrow-ranged species were similar in five out of six cases (i.e., MTG, emergence percentage, MTE, survival "gs" and "ow"). Germination percentage in warm laboratory temperature was higher for widespread than for narrow-ranged species (interaction of distribution × temperature), indicating a higher tolerance of warm temperatures for the former, which may have facilitated their occupation of a greater variety of habitats or habitats across a larger geographical range, respectively. However, these results should be viewed with caution as we only tested germination in two temperature regimes. Intraspecific variation of MTE differed between widespread and narrow-ranged species, and so did the mean annual temperatures between the respective seed collection sites. These results indicate a relationship between climatic cues and recruitment responses. Ecological indicator values did not contribute to explaining the recruitment differences between species distribution ranges, but indicators of soil components underlined that they act as driving factors, which need further investigation in *Saxifraga* spp. In conclusion, our findings on recruitment traits of widespread and narrow-ranged *Saxifraga* spp. suggest that the differences between the distributional groups are mostly due to germination differences, while subsequent life-transition stages play a minor role. The overall recruitment niche of *Saxifraga* spp. shown here, characterized by low-temperature requirements and sensitivity to drought stress, highlight severe challenges in a warmer climate, especially for narrow-ranged species.

DATA AVAILABILITY STATEMENT

The data used in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

VM and BE designed the study. VM collected experimental data. VM performed data analyses with contributions of FP. VM led manuscript writing with substantial contributions from BE and AM. All authors performed seed collections and contributed to the interpretation of results.

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SUPPLEMENTARY MATERIAL

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Seed Size Variation of Trees and Lianas in a Tropical Forest of Southeast Asia: Allometry, Phylogeny, and Seed Trait - Plant Functional Trait Relationships

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Seed size is a key trait for understanding and predicting ecological processes in a plant community. In a tropical forest, trees and lianas are major components driving ecosystem function and biogeochemical processes. However, seed ecological research on both components remains limited, particularly phylogenetic patterns and relationships with other traits. Here, we compiled a unique dataset of seed size (seed mass and geometrical size metrics) based on collections of more than 5,200 seeds of 196 woody plant species, covering >98 and 70% of tree and liana stems, respectively, located on a 30-ha plot in a tropical evergreen forest in central Thailand. We aimed to (1) develop allometric equations among seed size metrics to predict seed mass; (2) examine phylogenetic influence on seed size variation; and (3) examine relationships among seed traits and several other functional plant traits. Our allometric equations relating seed mass, seed volume, and width were well-fitted with data ($R^2 = 0.94$, 0.87 respectively). A phylogenetic signal test found that seed size was randomly distributed across the phylogeny. To study the functional trait relationships, we separately tested seed size data of the tree and liana communities (146 and 50 species, respectively), against mean body size of frugivores, successional niches, leaf, and structural traits. For the tree community, seed size was significantly related to mean body size of frugivores, which we believe is a basic driver of seed size because it is related to the gape width affecting dispersal effectiveness. Nearly all leaf traits were significantly positively correlated with seed size ($p < 0.03$). The significant positive correlation of leaf area and greenness suggested the high-energy demand of large-seeded species. We found a strong positive correlation between seed size and leaf toughness, suggesting a coordination between seed size and leaf defense. However, all these patterns disappeared in the same analysis applied to the liana community. Liana seed size variation was lower than that of trees, perhaps because lianas grow in relatively more uniform conditions in the forest canopy. Frugivore size was the strongest driver of seed size variation. Our study shows a surprising contrast between trees and lianas that is worth further investigation.

Keywords: functional traits, leaf toughness, plant height, seed dispersal, successional niche, seasonal tropical forest, liana, leaf trait

INTRODUCTION

Seed size (here is defined by mass or geometric size metrics, such as volume, length, width, and thickness) is a trait central to many aspects of plant ecology. It is a key trait for understanding and predicting ecological processes in the early phases of a plant's life cycle, for example, dispersal, persistence or dormancy, germination, and establishment (Leishman et al., 2000; Fenner and Thompson, 2005; Moles et al., 2005; Saatkamp et al., 2019). Seed mass is a widely applied metric of seed size in many studies of plant functional traits (Moles, 2018). However, geometric metrics, such as seed volume and seed length, are measured in most studies relevant to dispersal ecology (De Jager et al., 2019) and animal foraging behavior (Yi and Wang, 2015). Often, both seed size metrics and seed mass are not reported in the same studies; hence, we do not have enough information to accurately interchange between them (Kitamura et al., 2002; Moles et al., 2005; Pérez-Harguindeguy et al., 2013; Bello et al., 2015; Díaz, et al., 2016). We propose to develop an allometric equation that can predict seed mass from seed size and vice versa. Generally, the best-fit allometric model is the relationship between seed mass and volume, whereas the relationship with geometric measures, such as seed length or shape, vary among studies (Brewer, 2001; Wright et al., 2007; Casco and Silva Dias, 2008; Wang and Chen, 2009; Ganhão and Dias, 2019). Furthermore, most studies are biased toward Neotropical forests (Wright et al., 2007).

Ecologists have sought to explain variation of seed size within and between plant communities (Coomes and Grubb, 2003; Moles et al., 2007), because variation in seed size is crucial for understanding traits, such as competitive ability (Leishman and Murray, 2001; Turnbull et al., 2004), tolerance to environmental stressors (Baker, 1972; Westoby, 1998), seed predation (Thompson, 1987), seed dispersal (Greene and Johnson, 1993), and dormancy (Harel et al., 2011). In a tree community, seeds of pioneer species are normally smaller than those of shade-tolerant species (Ghazoul and Sheil, 2010). Seed size variation between pioneer vs. shade-tolerant species needs to be reinvestigated based on an objective classification, and systematically assessed *via* the probability of species occurrence in second- versus old-growth forests (e.g., Chazdon et al., 2011).

In fleshy-fruited plants, seed dispersers are regarded as important drivers of seed size, especially in tropical forests, where >70–80% of plant species are dispersed by animals (Osuri et al., 2016; Chanthorn et al., 2019). Generally, the achievement of seed dispersal is determined by whether a seed can pass into the mouth of a frugivore and be taken away from a fruiting tree. A key disperser trait is gape width, which should be wider than seed width (Fleming et al., 1993). Although many physiological and ecological factors may produce selection on seed size, the availability of effective dispersers may place an upper limit on seed size. Strong evidence that gape width of frugivores exerts selective pressure on seed size comes from a study showing a reduction of seed size in Neotropical forests with high defaunation intensity of large-bodied dispersers compared to non-defaunated forests, a phenomenon that has occurred only within the last 100 years (Galetti et al., 2013). However, since gape width data are difficult to collect, body size is often used as a proxy. The study of "anachronisms," for example, demonstrated the consequences of large animal defaunation during the Pleistocene era that affected the dispersal of ill-suited plants with very large seed size (Janzen and Martin, 1982; Pires et al., 2017; Lim et al., 2020). As this topic has been studied mostly in Neotropical forests (Fuzessy et al., 2018; Lim et al., 2020), studies from other tropical regions are needed to gain a global perspective on this subject.

Coordination (positive correlation) and trade-off (negative correlation) of functional traits are common features in plant communities (Díaz, et al., 2004; Moles, 2018), including tropical forests (Wright et al., 2010). Coordination between tree height and seed size has been reported in a global study (Díaz et al., 2016) and in a study focusing only on trees in Neotropical forests (Bello et al., 2015). For the trade-off, species with a high leaf area normally have lower wood density, which supports fast growth in high-light environments (Chave et al., 2009). Intuitively, large seeds tend to generate large seedlings and provide an adaptive advantage to seedlings growing in shade environments, but the relationship between seed and leaf size lacks direct causation and is predicted to be weaker compared to that between seed size and plant height (Díaz et al., 2016). Alternatively, another global study showed a strong correlation between seed size with leaf life span (Moles, 2018). Interestingly, a cross-continental study found a strong relationship between seed size and leaf toughness (fracture resistance), suggesting another functional trait coordination of importance (Kraft et al., 2015).

Here, we compiled data on seed size for a majority of species in a tropical forest community, which was challenging because of the irregular fruiting or masting of most tree species (Suwanvecho et al., 2017; Kurten et al., 2018). Lianas are equally important and typically comprise about one-third of woody plant diversity in tropical forests (Schnitzer, 2018). Studies of lianas have lagged those of trees, and only a few studies have examined seed size or mass in more than a minority of liana species (e.g., Gallagher and Leishman, 2012).

Here we collected a seed size dataset of >5,000 seeds measured in five metrics: seed mass, seed volume, seed length, seed width, and seed thickness, covering >98 and > 70% of tree and liana stems, including many liana species not previously reported in TRY global databases (see **Supplementary Table 3**). We have, in addition to the Mo Singto plot, which is covered with relatively old-growth forest, a chronosequence of smaller plots covering all successional stages in the same landscape, allowing us to classify all tree species into pioneer, shade-tolerant, and generalist types (Chazdon et al., 2011). The life-history traits available for analysis fall into four groups: leaf traits (leaf area, leaf toughness, specific leaf area, and leaf greenness), structural traits (maximum plant height and wood specific gravity), successional niche, and seed dispersal agents or mean body size of frugivores.

The objectives of our study were to create allometric equations among seed size metrics, to examine phylogenetic influence on seed size variation, and to evaluate the relationship (coordination or trade-off) between seed size and plant traits by testing four hypotheses: (1) Large seed size is coordinated with other plant traits that are associated with shade tolerance and longer leaf life span, as opposed to faster growth; (2) Large seed size is associated with old-growth forest as opposed to early successional forest; (3) seed size is correlated with disperser size, as effective dispersal is the initial major hurdle to overcome in a plant's life history; and (4) trees and lianas are expected to show different seed size–life-history trait relationships, because although reproductive lianas occupy similar light environments in the forest crown, they have different growth trajectories and means of support. We compared models with and without phylogenetic correction to examine the independence of variables and perhaps gain insight related to evolution.

MATERIALS AND METHODS

Study Site

This study was carried out on the 30-ha Mo Singto forest dynamics plot (Brockelman et al., 2017; **Figure 1**), which is a member of the ForestGEO network of the Smithsonian Institution, Washington DC (Davies et al., 2021).[1] The plot was established around 2001 in Khao Yai National Park (101°22′E and 14°26′N), a UNESCO world heritage site of Thailand. All trees with DBH ≥1 cm have been fully identified, tagged, and mapped. A re-census has been conducted every 5 years, the latest during 2020–2021. This plot is imbedded in a gibbon (*Hylobates lar*) study site sthat has been monitored since 1980 (Brockelman, 2013). The forest type is tropical seasonal evergreen, representing upland forests across Southern China, Laos, Cambodia, and much of Thailand (**Figure 1**, Map; Ashton, 2014; Brockelman et al., 2017). The altitude of the Mo Singto plot is 720–815 m above sea level, and average precipitation and temperature during 1994–2014 were 2,200 mm and 23°C, respectively (Brockelman et al., 2017). The Mo Singto plot has 264 species in 67 families (2011 census). Family Annonaceae ranks highest in terms of number of stems (25.5% of total), and Rubiaceae and Lauraceae in terms of numbers of species (25 and 22, respectively). The most abundant tree species were *Polyalthia khaoyaiensis* (31.5%of stems), *Cinnamomum subavenium* (9.7% of stems), and *Knema elegans* (8.4% of stems). Inventory of liana species on the Mo Singto ForestGEO Plot in Khao Yai National Park began in 2000. An individual liana is categorized as a "ramet"—the climbing stem on a host tree—or a "genet," a stem rooted in the ground. All ramets with DBH ≥ 2 cm were measured 1.3 m vertical distance from the ground. The most abundant liana species were *Uncaria macrophylla* and *U. scandens* (Rubiaceae), common species specializing in tree-fall gaps.

Seed Sampling and Measurement

Plant voucher specimens, including seeds, are stored in the BBH herbarium at BIOTEC of the National Science and Technology Development Agency, Thailand. Many seeds (38.3%) have been collected from gibbons' feces during gibbon monitoring. Most seeds of species dispersed by gibbons (through defecation) have hard seed coats and do not change in their morphology during gut passage. However, most seeds (46.4%) were collected while monitoring plant phenology. Rarely, when seeds were not available in the herbarium, mostly from rare or non-fruiting species (15.3%), we used information from field-recorded and unpublished data of Mo Singto plot.

Seed mass refers to the dry weight of endosperm and embryo including the seed coat but excluding any morphological structures, such as wings, that aid dispersal (Leishman and Westoby, 1994b). Before measuring and weighing, seeds were oven-dried at 60°C for at least 3 days. After drying, we immediately measured each seed in three dimensions: length (L), the longest linear dimension; width (W), the widest orthogonal direction to L; and thickness (T), the largest orthogonal side to both the L and W, using a Vernier caliper with 2-digit micrometer precision. Finally, we weighed the same seed on a microgram balance (PB303, Mettler, Toledo). Although water loss from the drying process may shrink seed size, it does not affect our categorized dispersal modes. The categorization is justified from swallowed seeds observed in the forest, which are slightly larger than oven-dried seeds.

Plant Functional Trait Data

Leaf Traits

We followed the protocol of Pérez-Harguindeguy et al. (2013) and aimed to sample at least 32 individual plants of different sizes per species. We used at least three non-damaged leaves per individual tree to measure leaf economic spectrum traits. We collected sun-exposed leaves with a long pole, whereas trained tree climbers collected liana leaves. We scanned each leaf with a commercial scanner setting for A4-paper size with the 300-PPI resolution (points per inch) and measured leaf greenness (chlorophyll content) using a portable greenness meter, SPAD-502 (Konica Minolta, Tokyo, Japan). We measured leaf thickness and toughness three times at the upper, middle,

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[1] www.forestgeo.si.edu

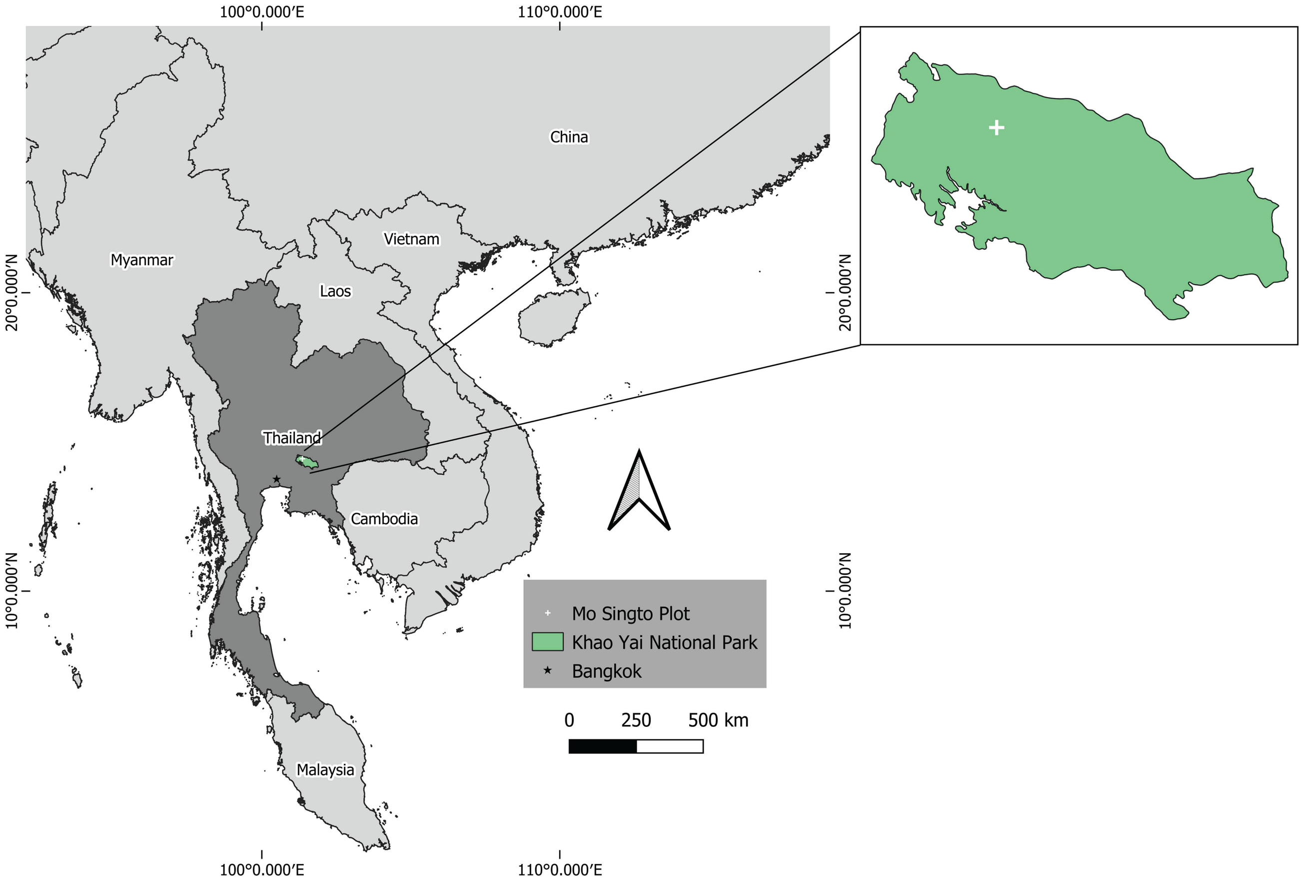

FIGURE 1 | Map of Khao Yai National Park and the Mo Singto plot.

and lower parts of each leaf blade. We used a digital micro mm Vernia Caliper and the puncture test with a Penetrometer (digital force gauge DST-5 N: Imada Co., Ltd., Aichi Japan) for the thickness and toughness measurements. Finally, we dried the same leaves at 70°C for 3 days and weighed them to obtain specific leaf area (leaf area per mass unit). To find the area of each individual leaf, we automatized leaf areas of all pictures scanned using the R package "LeafArea" (Katabuchi, 2015). Overall, we measured leaves of 146 tree species and 46 liana species (**Table 1**).

Succession Niche Traits and Structural Traits (Wood Density and Maximum Tree Size)

We assigned succession niche, wood density, and maximum plant height traits for tree species only. To assign the succession niche, we used the multinomial model of occurrence in two different habitat types (Chazdon et al., 2011), in which our two habitat types were secondary forest (the chronosequence of small secondary forest plots) and old-growth forest (on the Mo Singto plot; Brockelman et al., 2017; Chanthorn et al., 2017). The result allowed us to recognize four types of successional niche: (1) generalist, (2) secondary forest specialist, (3) old-growth specialist, and (4) too rare to be classified (density in both secondary and old-growth forests was too low; for 13, 8.2, 50, 15.1 and 13.7% of tree species, respectively). However, we also assigned "unclassified" for species with inadequate information for the previous classification analysis. Wood density values were derived mainly from the global wood density database (Chave et al., 2009). When wood densities were not available at the species level, we used genus level and family level (for 43.2 and 17.8% of species, respectively). Maximum tree heights were calculated using the DBH–height allometric equation developed based on the dataset of Southeast Asian tropical forests (Feldpausch et al., 2011). We used the BIOMASS R package to calculate tree height (Réjou-Méchain et al., 2017).

Dispersal Agents and Mean Body Size of Frugivores

We used the seed dispersal modes of trees from Brockelman et al. (2017) and Chanthorn et al. (2019), whereas the data of lianas were compiled from unpublished long-term observations on the plot. However, dispersal agent information is lacking for some species, and so we assigned a dispersal mode based on fruit morphology and seed ornamentation or appendages using information available in the Flora of Thailand.[2] For example, we considered seeds or fruits with wings, hairs, or

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[2] www.dnp.go.th/botany/eflora

TABLE 1 | Mean and ranges of values of all traits with available data (*N*: number of species).

Group	Traits	Mean (range)			
		Trees	***N***	**Lianas**	***N***
Structure	Maximum height (m)	28.3 (2.9–55.1)	147	–	
	Wood specific gravity (g cm^{-3})	0.55 (0.21–0.87)	147	–	
	Leaf area (mm^{3})	370 (44–4,235)	146	39.3 (4.1–106.7)	46
Leaf economic spectrum	Leaf toughness (newton)	0.49 (0.16–1.32)	146	0.36 (0.10–1.24)	46
	Specific leaf area (cm^{2} g^{-1})	1,144 (230–10,821)	146	167 (321–74)	46
	Leaf greenness	54.2 (41.7–66.1)	146	49.3 (68.9–32.6)	46
Dispersal	Mean body size of frugivores (kg)*	6.28 (0.48–24.00)	156	10.24 (0.48–24.00)	37
Succession niche**	Pioneer, generalist, shade-tolerant, unknown	–	156	–	–

**We assigned zero for anemochory and unknown species. **This is a categorical variable.*

pappus as wind-dispersed (Note that this classification may be reasonable only for tropical woody plants, but not for other plants or biomes). Some species that we were unable to classify were assigned to an unknown dispersal mode group (see **Supplementary Table 3**). To avoid problems of high variation of body size among frugivores in each tree species, we determined the mean body size of all animal fruit consumers from the body mass data of Kitamura et al. (2002) and Khamcha et al. (2014) (**Supplementary Table 1**). Intuitively, given that one fruit species is equally favored by two frugivores, we assumed the frugivore with larger body size consumes more fruit than the smaller one. For example, *Prunus javanica* is consumed and dispersed mostly by primates and hornbills, whereas large-bodied pigeons consume relatively little (McConkey and Brockelman, 2011). On the other hand, elephants are an opportunistic seed disperser of *Choerospondias axillaris* despite having very large body size (Chanthorn and Brockelman, 2008). Thus, we used an average of body size to avoid this bias. Despite highly skewed data of frugivore body sizes, using mean is better than using median in this context because of the high redundancy of smaller frugivores (e.g., many more species of small birds compared). For the rest of species whereas, we assigned zero. Thus, dispersal agents became a single continuous variable.

Data Analysis

Seed Size Allometry

We estimated seed volume using the geometric formula for a 3-dimensional ellipsoid (Equation 1) or a 2-dimensional spheroid (Equation 2):

$$V = \left(\pi\ LWT\right)/6 \qquad (1)\ \text{or}$$

$$V = \left(\pi\ LW^{2}\right)/6 \qquad (2)$$

where V represents seed volume; L, W, and T are the length, width, and thickness of a seed (mm). For the most species ($N = 188$ species), we used the ellipsoid equation, whereas the spheroid equation was used for seeds in which only length and width were measured (eight species). Because seed mass and volume were skewed, we transformed data with natural logarithm prior to analyses. We fitted the seed mass data and all four seed geometric variables (volume, length, width, and thickness) using a linear regression model. We also visualized the distinction between models for trees and lianas. We expected that wind-dispersed species should have created negative error in the residuals, in which anemochorus seeds should have lighter weight in order to gyrate while at equal volume.

Phylogenetic Analysis, Trait Coordination, and Trade-Offs

We conducted comparative phylogenetic analyses to account for the influence of phylogeny in the allometric analysis. We used the phylogeny compiled by Smith and Brown (2018), which is resolved completely at the family level and contains 80% of the genera of seed plants (Spermatophyta) in the world. This phylogeny provided the best overlap with our data, demonstrating the best match at the genus level (98% of the 144 genera). We used Smith and Brown's megaphylogeny at genus level implemented in the software V. PhyloMaker (Jin and Qian, 2019) as a backbone to generate a phylogeny. Additionally, we tested for phylogenetic signal in seed size using the function "phylosignal" in the package "*picante*" (Kembel et al., 2010). Phylogenetic generalized least square method (PGLS) was used to build regression models in phylogenetic context with the "gls" function in R-package "nlme" and phylogenetic correlation structures from the R-package "*ape*" (Paradis and Schliep, 2019). We created two candidate models—with and without accounting for phylogeny (PGLS). We performed AIC-based model selection to find the best fitting model of all trait variables (**Table 1**). The predictor variables of tree species included six continuous traits, that is, maximum height, wood specific gravity, leaf area, leaf toughness, specific leaf area, leaf greenness, mean body size of frugivores, and the regeneration niche (a categorical variable). We separated the analysis of lianas because of their contrasting life history and lack of data on height, wood density and regeneration niche (**Table 1**). We tested for correlation (Pearson's coefficient) among all predictor variables (see **Supplementary Table 2**). All statistical analyses were conducted using R 4.0.2 (R Core Team, 2016). The ancestral states of seed mass were reconstructed by maximum likelihood using the function ace in ape under a Brownian motion of continuous trait evolution. The observed values for the recent species were first log10-transformed, and then the reconstructed ancestral values were back-transformed to the linear scale before plotting on the phylogenetic tree.

RESULTS

Allometric Equations Among Seed Size Metrics

Across all 196 species, seed mass ranged from 0.7 mg in *Lasianthus biflorus* (Rubiaceae) to 24.5 g in *Mucuna macrocarpa* (Fabaceae). Seed volume ranged from 2.62 mm^3 in *Diplectria barbata* (Melastomataceae) to 21,511 mm^3 in *Mucuna macrocarpa* (Fabaceae; **Supplementary Table 3**). Overall, the distribution of $\log_e$-transformed data was symmetrical without obvious skewness in both trees and lianas (**Figures 2A,B**). The mean $\log_e$-transformed seed volume and mass of trees were larger than those of lianas [log seed volume of trees and lianas: 5.21 ± 0.03 and 4.66 ± 0.04; log seed mass of trees and lianas: 4.91 ± 0.03 and 4.39 ± 0.41 (mean ± standard error); **Figures 2A,B**]. Across all species, the mean seed mass of zoochorous seeds was greater (mean 0.65 ± 1.22 g) than that of anemochorus seeds (mean 0.37 ± 1.16 g). More than 87% of tree species were zoochorous (**Figure 2C**), whereas >74% of liana species were zoochorous (**Figure 2C**). Note that almost all unknown species had fleshy fruit, but we have never seen any frugivores remove the fruit, and some species had toxic fruit (e.g., *Excoecaria oppositifolia*.)

Our fitting of the allometric equation showed the strongest positive correlations between seed volume and seed mass ($R^2 = 0.9414$, $N = 196$; **Figure 3**), with random and Gaussian-distributed patterns of residuals. Thus, the equation to interchange between seed mass (g) and volume (mm^3) was: $\log_e$ (seed volume) = 0.97 × $\log_e$(seed mass) − 7.1 (**Figure 3**). Surprisingly, the fitted equations with simpler geometrical metrics (**Supplementary Figures 1A–C**) showed the poorest fit for seed length ($R^2 > 0.73$, $p < 0.001$; **Supplementary Figure 1A**),

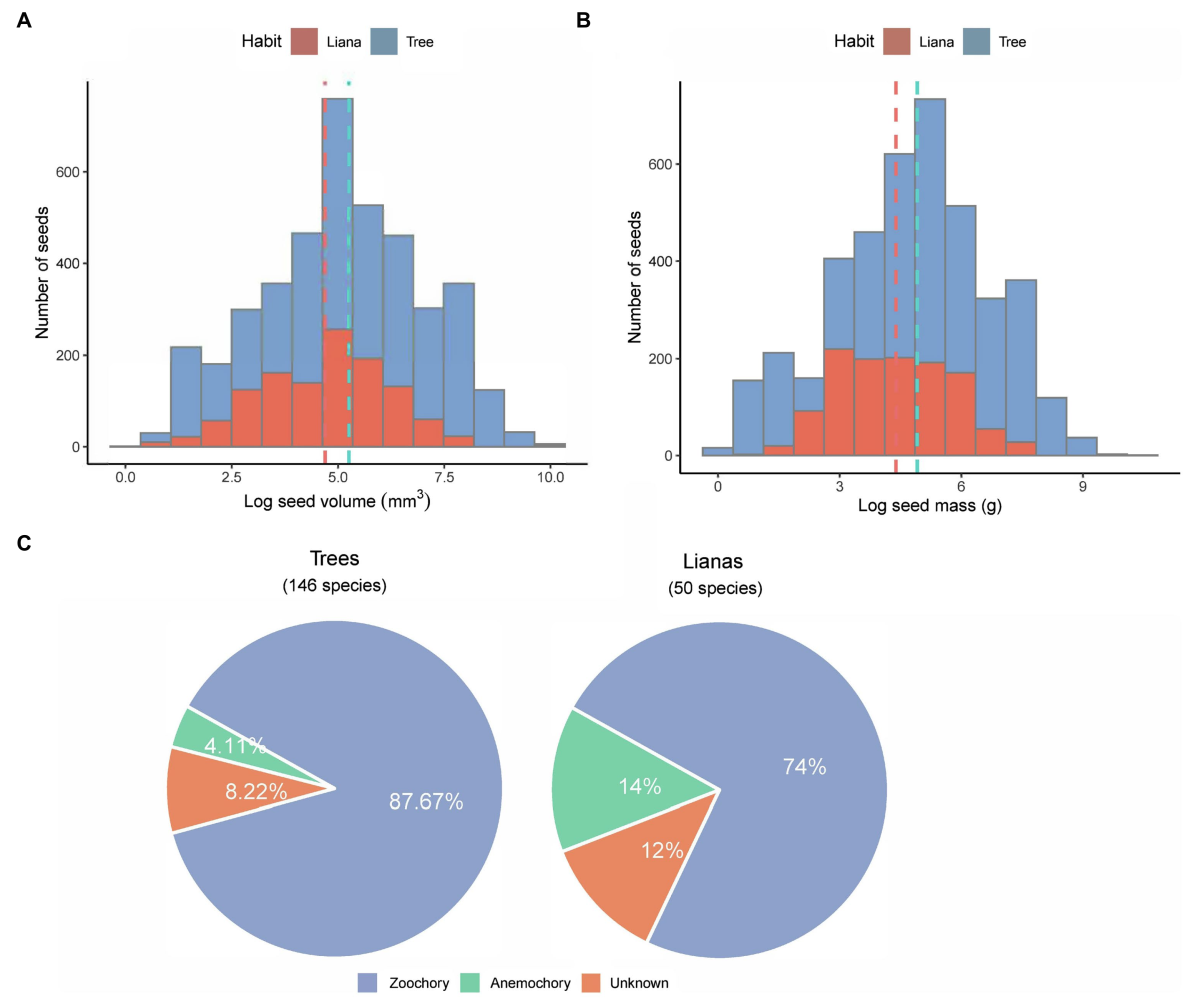

FIGURE 2 | Overview of the seed size between trees and lianas. Histogram distributions of **(A)** seed volume and **(B)** seed mass. All bin widths are logarithmically scaled to facilitate comparison. **(C)** pie charts showing number of species and their dispersal modes in the study.

whereas the best-fit equation was with seed width ($R^2 > 0.87$, $p < 0.001$; **Supplementary Figure 1B**).

Phylogenetic Structure and Seed Size Variation

Seed size variation was randomly distributed across the phylogenetic tree, without any specific lineages clustering with small or large seed size (**Figure 4**). We found no evidence of significant phylogenetic signal in different seed size measures using either mean seed volume ($K = 0.002$, $p = 0.8$), mean seed volume ($K = 0$, $p = 0.9$), or mean seed mass ($K = 0.01$, $p = 0.7$). Interestingly, most species were from different genera, suggesting that analysis at the genus-level phylogeny is reliable for ours. Note that our phylogenetic tree was constructed based on the genus level as the finest phylogenetic level. Therefore, increasing resolution at the species level is not expected to have a dramatic effect.

Seed Trait-Plant Functional Trait Relationships

For tree species, the normal linear model (AIC = 411.05) provided a better fit than the PGLS model (AIC = 481.35). This result implies that phylogeny did not influence the observed patterns. For the analysis of tree species, the results of the normal linear model showed that seed size was significantly correlated with the group indicating seed dispersal (the mean body size of frugivores), three leaf traits (leaf toughness, leaf area, and leaf greenness) and the single structural trait, maximum tree height (**Figure 5**, **Supplementary Table 4**). **Figure 6** shows the component residual plots of significant variables. In contrast with the liana species tree, the PGLS model fitted the seed data slightly better than the normal linear model (AIC values were 124.12 and 122.58, respectively; **Supplementary Table 5**). Nonetheless, there were no significant covariates.

DISCUSSION

Allometry and Phylogeny

Seed size is a major trait in two large research topics: functional traits and seed dispersal (Jansen et al., 2004; Wang and Chen, 2009; Wang and Yang, 2014). However, data are generally created from different seed size metrics,

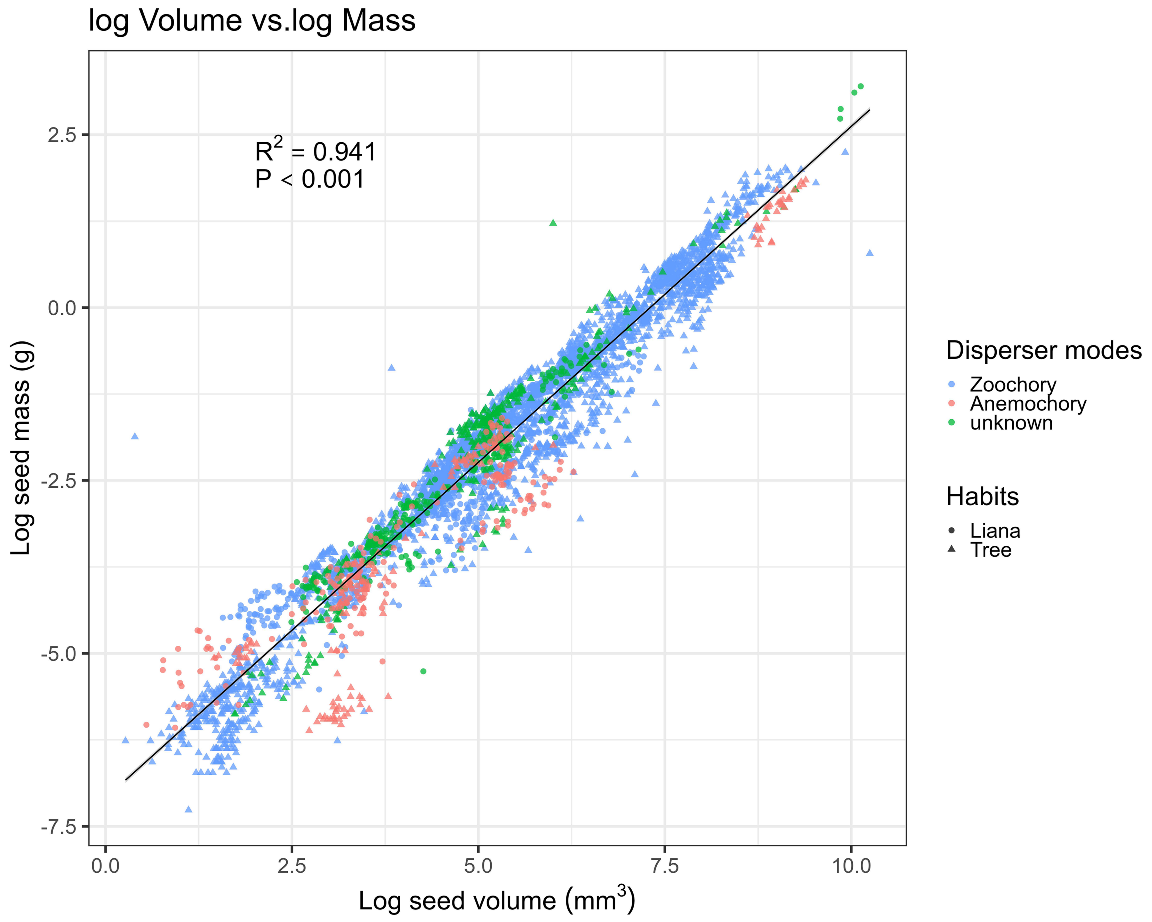

FIGURE 3 | Relationship between seed mass and volume-related traits with the predicted line (black solid line) fitted by the linear regression model ($N = 5,219$). Samples are categorized into zoochory (blue), anemochory (red), and unknown (green), and tree and liana species are categorized by triangular and circular shapes.

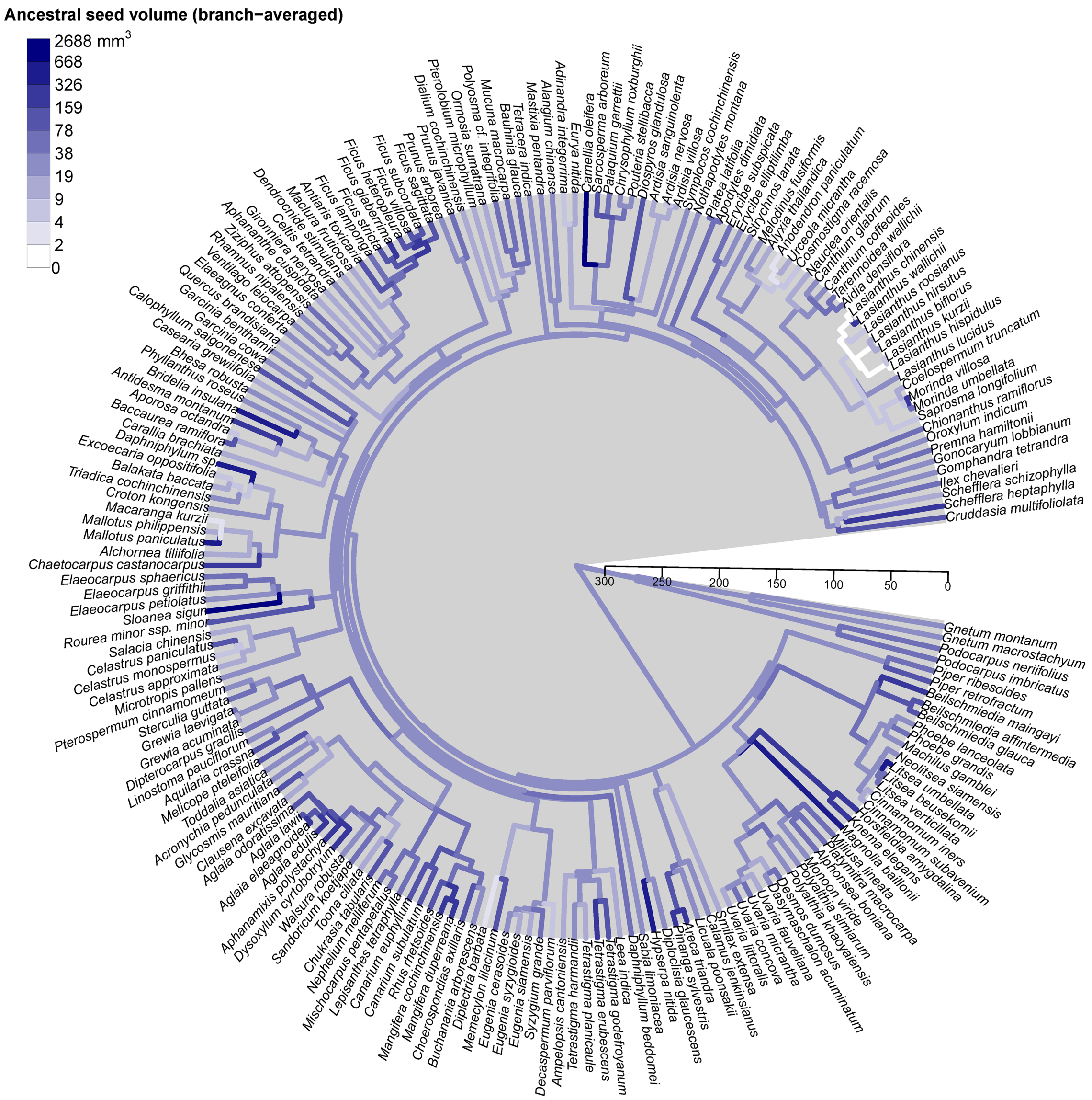

FIGURE 4 | Phylogenetic tree of the 196 species found in Mo Singto plot, illustrating the evolution of mean seed size (seed volume, mm^3). The ancestral states of this trait were reconstructed under a model of Brownian motion fitted by residual maximum likelihood (REML); the values between the two nodes of each branch were averaged to define the color as shown on the plot. The time scale units are Ma (ranging from 0 to 300 Ma).

for example, seed mass and geometric size (volume, length, width, thickness). Measuring two metrics of seed size together can be a laborious task, but our allometric equation helps to reduce this problem. We found high interchangeability of our models because of well-fitting log-linear models using seed mass and volume. Studies in Neotropical forests show well-fitted models similar to ours (Wright et al., 2007). Contrary to our expectation, wind dispersal did not create systematic error. However, based on our visualization, there was a cluster of highly negative residuals from the single species, *Toona ciliata*. Moreover, our best selected model was the one without any phylogenetic effects, suggesting that our allometric equations could apply in other seasonal tropical forests of Southeast Asia where our forest is well represented and shares similar phylogenetic structure (Ashton, 2014; Brockelman et al., 2017).

Species with similar seed size were not clustered in certain lineages on the phylogeny tree. They were distributed among several lineages and groups of lineages across the phylogenetic trees. In our visualization (**Figure 4**), we did not see any clear pattern between Gymnosperms and Angiosperms. This contrasts with the discovery of large differences between both groups in the global study of Moles et al. (2005). However, note that our Gymnosperms comprise only four species belonging to two genera, a significant limitation of our study.

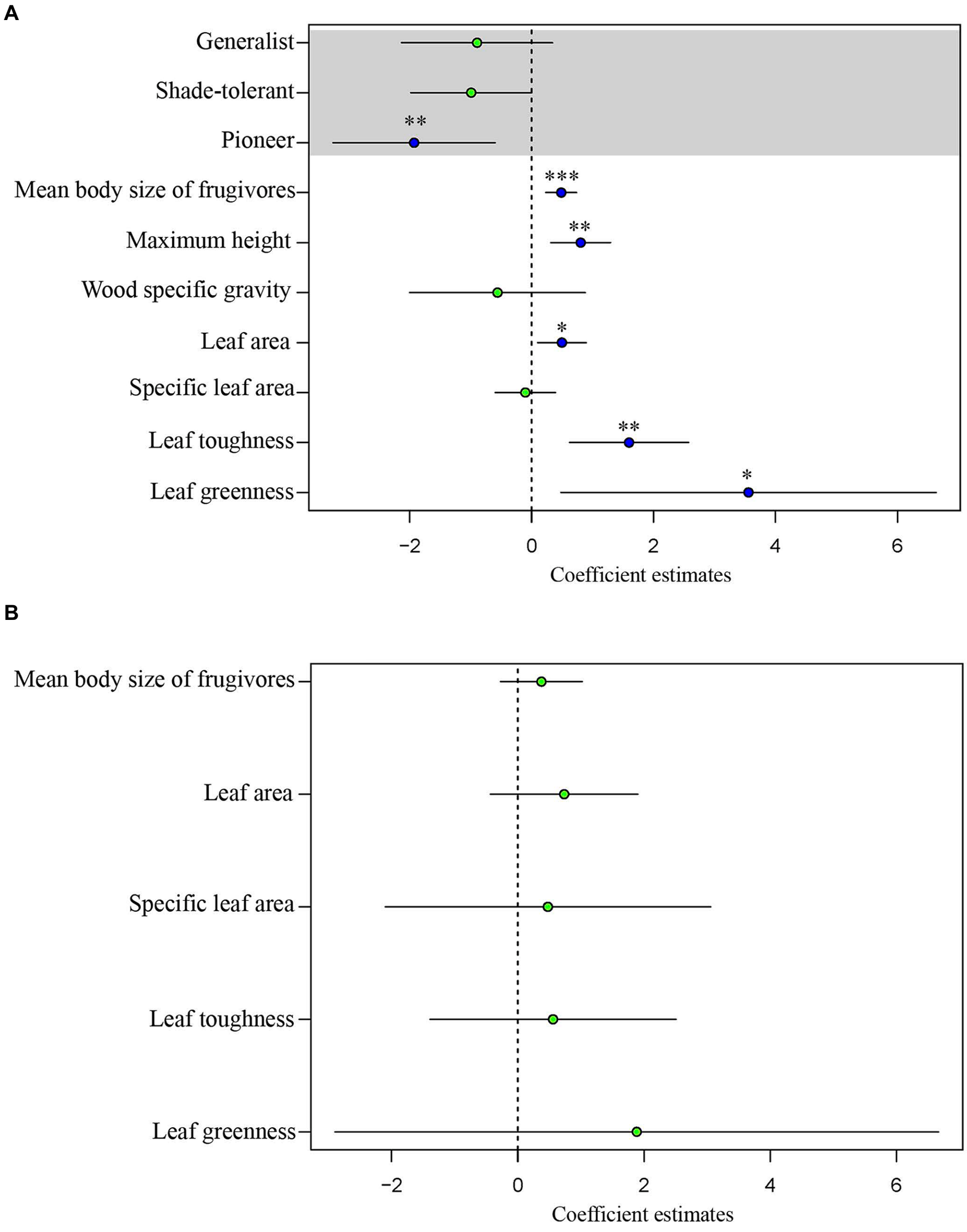

FIGURE 5 | Regression coefficients estimated through generalized least squares (GLS) analysis of seed volume of **(A)** trees and **(B)** lianas. The blue dots indicate statistically significant coefficients (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$); the green dots indicate coefficients which are non-significant ($p \geq 0.05$). The horizontal bars show the 95% confidence intervals.

Trait–Trait Relationships: Driver and Trait Coordination

We interpret a significant positive correlation as either a driver or trait coordination, but interpret a negative correlation as a trade-off. Our results for tree species found only significant positive correlations. The strongest positive relationship was between mean body size of frugivore dispersers and seed size, without any phylogenetic effect. We interpret this trait as a major driver of seed size variation, acting as a constraint. This is a consequence of natural selection in which frugivores tend to feed on fruits with the largest seeds that they can swallow, for single-seeded fruits, such as drupes. Selection on seed size driven by frugivore gape size may occur within a few centuries (Galetti et al., 2013; Dirzo et al., 2014). In Southeast Asia, the seed size of *Choerospondias axillaris*, a primarily deer-dispersed species (Brodie et al., 2013), is smaller in the north of Thailand where deer have been extirpated for *ca.* 80 years compared to our study site, where deer are still abundant. A possible consequence is secondary extinction (Brodie et al., 2014), which may also effect ecosystem services, such as carbon sequestration (Osuri et al., 2016;

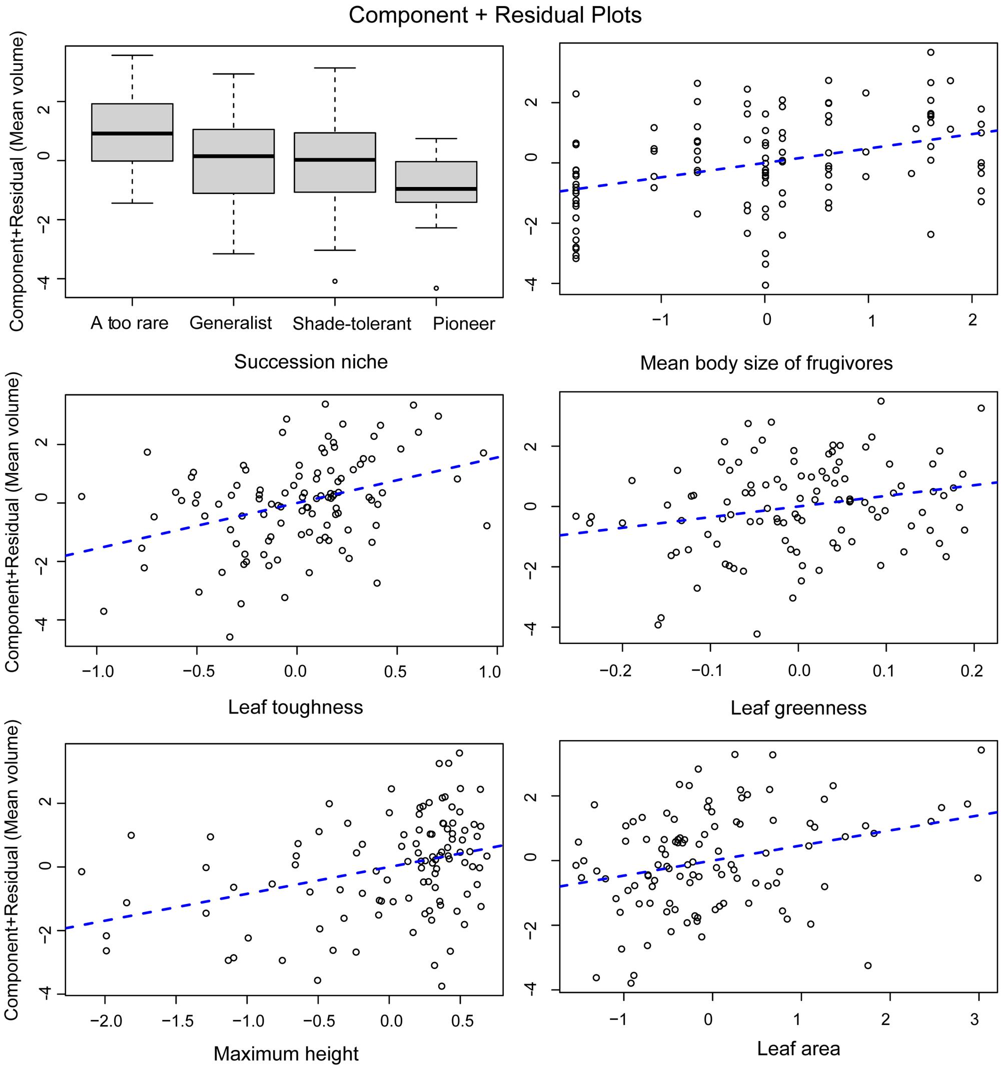

FIGURE 6 | Component residual plots for the main effects in the multiple regression model. Points represent mean seed volume when all factors are held at their median values (partial residuals) and regression lines are derived from the multivariate regression model.

Chanthorn et al., 2019). Evidence from Neotropical forests, that is, "anachronisms," suggests that the extinction of large-sized frugivores during the Pleistocene era may have led to reduction of opportunities for long-distance dispersal in many Neotropical trees with very large-sized fruit (Janzen and Martin, 1982).

The positive correlation between nearly all traits with seed size suggests that traits may be physiologically co-adapted either with or without any mechanistic links. The positive significant relation between leaf area and chlorophyll content (indicated by leaf greenness) may be explained as co-evolved traits which support the energy demand of large seed size. Normally, a large leaf has a high photosynthetic rate, indicating a fast-growing strategy, especially in pioneer species (Poorter and Bongers, 2006; Reich, 2014). In our old-growth forest, species with large-sized leaves may have been selected to produce more energy to sustain large seeds. Although this trend is similar to observations in the global study (Díaz et al., 2016), there has been little explanation for it. Species with large leaves are typically pioneer species (Reich, 2014), which may have been selected to produce large numbers of small seeds more rapidly. In Southeast Asia, *Macaranga* spp. are examples of pioneer trees that support this hypothesis. However, leaves of dominant or common species in our secondary forests are not as large as those of *Macaranga* or *Hibiscus* (Chanthorn et al., 2017). The leaves of our pioneer species seem to be smaller and tend to have lower specific leaf area (see **Supplementary Figure 2**). Moreover, there was a strong negative correlation between seed size and successional niche in the secondary forest, suggesting that many pioneer species produce small seeds coordinated with small leaf size. Also, in meta-analyses of functional traits, plant size and seed size pattern correlate positively along the same principal component axis (Díaz et al., 2004, 2016). Additionally, this relates to vertical niche position in the forest canopy, or to the expectation that a canopy tree may have larger leaves to take advantage of greater exposure to the sun. We also found a positive correlation between maximum tree height and seed size. This is because the old-growth forest has taller trees, and trees that require larger seeds that have a better chance to survive in the shady understory. Although the energy demand of large seeds is supported by high leaf chlorophyll content, the exclusion of specific leaf area (SLA) among all leaf traits from the regression model suggests that larger leaf size is required by a higher photosynthetic rate rather than needed for storing substances or strengthening leaf structure. Furthermore, SLA is largely independent of leaf area (Díaz et al., 2016). Studies at regional and community scales have found that SLA is weakly related to plant height and seed size (Díaz et al., 2004) and that it often shows no relationship with wood density and leaf size across many species and community types (Wright et al., 2010). Consistent with our result that large seeds coordinate with large leaf area and high chlorophyll content, the relationship with SLA is very weak.

Interestingly, our strongest leaf economic trait was toughness (justified by the *p*-value). Our result confirms the trend found in other studies which compared forests in three continents including a tropical rainforest of Southeast Asia (Kraft et al., 2015). This trait coordination reflects the syndrome associated with a slow life-history/reproductive strategy characteristic of large-seeded species adapted to resource-limited environments, with prolonged leaf lifespans and well-defended leaves (Moles et al., 2005). These findings provide supporting evidence for correlated components of the life-history strategy, between seed size and mechanical leaf defense (Dalling et al., 2020).

However, all these relationships were absent in lianas. Leaf trait variability was generally lower in lianas than in tree species in the plot. This is likely because most mature liana species grow to the top of forest canopy, where their leaves are exposed to the same high-light conditions as tall trees (Schnitzer, 2018). Lianas are structural parasites, which germinate on the ground, stay rooted until finding other plants to use as structural support (Visser et al., 2018). Due to this growth form, lianas can acquire resources faster than trees, because they invest fewer resources in structure, and more into productive leaves and reproductive biomass (Collins et al., 2016; Schnitzer, 2018; Werden et al., 2018). For structural traits, such as height, the strong positive correlation was also similar to that found in the global study (Díaz et al., 2016). The explanation is the fecundity trade-off throughout the plants' lifetime, in which large trees compensate low fecundity with larger seed size and high survival throughout their longer life spans (Moles, 2018). In addition, the seed volume of second-growth species was smallest, which may also be explained by the fecundity trade-off. Smaller seeded species have the fecundity advantage which allows them to disperse widely and recruit in light gaps and forest edge environments (Rose, 2005).

Wood density has been proposed as one of the most important functional traits organizing tropical forests at the population level, affecting species survival and competitive ability (Chave et al., 2009). We expected that species with large seed size would tend to have a high wood density (stem specific gravity) There is a well-supported tendency for large hardwood species to have larger fruits and seeds (Wright et al., 2007; Bello et al., 2015). However, this relationship was not significant in our study, suggesting that more studies from Southeast Asia are required to support a pattern different from that in Neotropical forests. For instance, the study by Krishnan et al. (2019) showed that tropical dry-forest trees with large seeds and low wood density had greater survival rates under drought conditions in the Indian subcontinent, which should allow them to cope with global change more efficiently. Several studies have demonstrated the impact of defaunation on carbon storage, which is based on an underlying coordination between wood density and seeds size in many tropical forests, including Asia (Osuri et al., 2016). In our forest, the wood density of trees dispersed by large-bodied frugivores tends to be higher than that of species dispersed by small birds (Chanthorn et al., 2019). However, some species with large seeds are not dispersed by large-bodied frugivores, for example, species belonging to the family Fagaceae and those with unknown dispersal mechanisms, *Excoecaria oppositifolia* (Euphorbiaceae) and *Gonocaryum lobbianum* (Cardiopteridaceae; Chanthorn et al., 2019).

CONCLUSION

We created seed size allometry equations for converting between seed mass and seed volume, which is not primarily a consequence of phylogenetic effects. In addition to seed size, we compiled metadata for other functional traits and introduced a new trait, the mean body size of frugivores. The seed trait and plant functional trait relationships revealed both a driver (mean body size of frugivores) and trait coordination between seed size and other leaf traits of tree species, but there were no trade-offs (negative relationships) with other traits. This pattern was not due to any phylogenetic effect. In contrast, the results of the corresponding analysis of lianas revealed the absence of all patterns. These results do not support all findings from other or global studies (Díaz et al., 2016; Moles, 2018). Our study therefore highlights the importance of local community studies, where all species are subject to the same ecological conditions.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding authors. The data supported this study is openly available at the following URL/DOI: (https://zenodo.org/deposit/6466998).

AUTHOR CONTRIBUTIONS

PP and WC conceived and designed the study with input from EP. PP, WB, TK, NL, AN, and RS collected, weighed, and measured seed size. PT and WC collected liana functional traits. PP, EP, PT, and WC manipulated and analyzed the data. PP and WC wrote the first draft of manuscript. All authors read the manuscript, provided feedback, and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2022.852167/full#supplementary-material

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