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Effects of low temperature and nitrogen addition during cold stratification on seed germination of Korean pine (*Pinus koraiensis*)

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1 **Effects of low temperature and nitrogen addition during cold**
2 **stratification on seed germination of Korean pine (*Pinus koraiensis*)**

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Abstract: *Pinus koraiensis* seeds are primary dormant following dispersal, restricting natural regeneration of populations. The aim of this study was to determine the combined effects of increasing low temperature and nitrogen addition during cold stratification on seed germination. We stratified seeds at 0 °C, 5 °C and 10 °C for 3.5 months. At each cold stratification temperature, seeds were subjected to 0 (0 nitrogen), 0.08 (low nitrogen), 0.16 (medium nitrogen) and 0.25 mol L⁻¹ (high nitrogen) nitrate additions. Seed germination response was tested at 25/15 °C and 10/5 °C. Under 0 nitrogen and high nitrogen levels, increasing cold stratification temperature did not remarkably affect seed germination at 25/15 °C, but significantly inhibited seed germination at 10/5 °C. At medium nitrogen level, seed germination at both 25/15 °C and 10/5 °C significantly decreased. At low nitrogen level, increasing cold stratification temperature significantly decreased germination percentage at 25/15 °C from 86.7% (0 °C) to 50.2% (10 °C), but did not markedly change seed germination percentage at 10/5 °C. We suggest that the effect of increasing cold stratification temperature on seed germination of *Pinus koraiensis* depends on both the level of nitrogen addition during cold stratification and subsequent incubation temperature after cold stratification.

Keywords: *Pinus koraiensis*, nitrogen addition, cold stratification temperature, seed dormancy, seed germination.

45 **Introduction**

46 Seed dormancy is a state in which seeds do not germinate even when the environmental conditions are
47 favorable for germination (Bewley and Black 1994; Hartmann 1997). Dormancy mechanism prevents
48 germination under environmental conditions unsuitable for seedling establishment, and helps coordinate
49 germination with a favorable season. Seed dormancy release in many summer annuals or perennials
50 plants in temperate regions is dependent on low temperature conditions during winter. The germination
51 of seeds in early spring following dormancy release favors seedling growth and subsequent survival
52 owing to the attainment of sufficient size before winter frosts (Grime 2001; Marcante et al. 2012).

53 Temperature is one of the primary environmental factors influencing seed dormancy state.
54 Temperature, via its effects on seed dormancy degree during maturation, dormancy break and
55 germination, influences germination percentage and rate (Roberts 1988). Low temperature conditions
56 during dormancy release are linked to the subsequent temperature range for germination (Derkx and
57 Karssen 1993). Steadman and Pritchard (2004) reported that the range of temperature over which
58 germination can occur gradually widens as cold stratification temperatures decrease.

59 Intensified anthropogenic activities, such as the widespread use of fertilizer and fossil fuel
60 combustion, have caused an increase in atmospheric nitrogen deposition (Erisman et al. 2013; Battye et
61 al. 2017). Global nitrogen deposition will be almost two-fold higher than that at the beginning of the
62 1990s by 2050 (Galloway et al. 2004). The rate of atmospheric nitrogen deposition has been increasing
63 significantly in China (Liu et al. 2013).

64 Many field observational, experimental, and simulation studies have revealed that elevated
65 atmospheric input of nitrogen has potential effects on the relative abundances of individual plants, plant
66 community composition and biodiversity, and ecosystem functioning (Clark and Tilman 2008; Reay et

al. 2008; Yang et al. 2012; Simkin et al. 2016; You et al. 2017). Enhanced nitrogen deposition could affect ecosystem function due to changes in seed dormancy degree during maturation, soil seed bank size, germination capacity or seedling establishment with nitrogen addition (Matakiadis et al. 2009; Basto et al. 2015; Varma et al. 2016; Li et al. 2017). Endogenous nitrate content of seeds negatively affects seed dormancy depth, as evidenced by mother plants with higher nitrate feeding during seed development producing less dormant seeds (Alboresi et al. 2005; Matakiadis et al. 2009; He et al. 2014). Many results have showed that seed germination in many species is enhanced by nitrogen addition (Pill et al. 1991; Plassmann et al. 2008; Shim et al. 2008; Ochoa-Hueso and Manrique 2010). However, an inhibitory effect of nitrogen on seed germination is also reported in some species (Luna and Moreno 2009; Ponert et al. 2015). Experiments have shown that nitrogen deposition did not alter the density or species composition of the seed bank, reduce seed number and species richness of the seed bank, or increase seed richness in an alpine meadow, arid grassland, and temperate steppe, respectively (Basto et al. 2015; Zhang et al. 2019; Miao et al. 2020).

Many studies have focused on the response of seed germination (the most vulnerable stage of plant growth) to increased atmospheric nitrogen deposition. However, for species with seed dormancy, dormancy break is a prerequisite for germination. Despite this, the effects of nitrogen deposition on seed dormancy release, an important factor for plant growth affected by different biotic and abiotic factors, has been poorly studied.

Mixed-broadleaved Korean pine forest (MKPF) is the zonal climax vegetation type in Northeast China. Expansion in agricultural activities and heavy industrial deforestation have already caused large-scale degradation of MKPF. The successful regeneration of Korean pine (*Pinus koraiensis*), the dominant species of MKPF, may play a major role in MKPF restoration. Korean pines grow in moist, deep soil

layer, fertile, well drained, aerated and slightly acidic, mountainous brown forest soil (Editorial Committee of Flora of China). The age of mature Korean pine is 300–400 years (Editorial Committee of Flora of China). Young Korean pines are more tolerant to shade, and the light requirement gradually increases with increasing stand age (Editorial Committee of Flora of China). In the forest industry, the Korean pine is regenerated by tree planting, a forest restoration technique (Wang et al. 2021). A few studies reported that direct seeding afforestation could also be a useful technique for Korean pine restoration (Li et al. 1984). Korean pine seeds have deep primary morphophysiological dormancy when they disperse from the mother plant, affecting seedling production in tree nurseries (Wang et al. 1986). A small proportion of Korean pine seeds germinate in the first spring following seed dispersal, and the germination of most seeds is concentrated in the second spring (Song et al. 2018). Due to a long interval between the two seed germinations, these seeds maybe eaten by rodents, consequently limiting direct seeding afforestation. A cold stratification simulating low temperature conditions in winter is required for the release of dormancy in Korean pine seeds. Seed dormancy release is certainly affected by climate change, especially in the case of the rapidly increasing winter temperature in Northeast China. The atmospheric deposition of nitrogen has rapidly increased in China, ranging from 13.2 kg N ha⁻¹ yr⁻¹ in the 1980s to 21.1 kg N ha⁻¹ yr⁻¹ in the 2000s (Liu et al. 2013). The maximum rate of nitrogen deposition in the Changbai Mountain region of Northeast China can reach 23 kg N ha⁻¹ yr⁻¹ (Lü and Tian 2007). Sun et al. (2015a) reported that wet deposition of nitrogen totaled to 19.1 kg N ha⁻¹ yr⁻¹ between 2010 and 2012 in the Maoershan region of Northeast China. Nitrogen deposition also occurred during winter in Northeast China (from November to following April) (Sheng et al. 2013). Approximately 50.1% of dissolved inorganic nitrogen deposited during winter in the Maoershan region of Northeast China. However, few experiments have focused on its effects on the status of seed dormancy.

The present study was conducted to quantify the potential responses of seed dormancy release in Korean pine to increased temperature and enhanced nitrogen availability during cold stratification. Determining the combined impacts of increasing low temperature and nitrogen addition on seed dormancy release is critical for understanding the response of ecosystem to global change. The findings of our study will also have important implications for MKPF conservation and restoration.

Materials and methods

Seed collection

Fresh seeds were collected in October from primary MKPF from Changbai Mountain Natural Reserve in Northeast China (41°31'–42°28'N, 127°9'–128°55'E). The regional climate is temperate continental monsoon, with a long, cold, dry winter and short, warm, moist summer (Zhang et al. 1980). Mean annual precipitation is approximately 600 mm with 80% falling between June and September (Yu et al. 2011). Mean annual temperature varies between 3.3–7.3 °C, ranging from -23.3 °C in January to 19.3 °C in July (Yang and Xu 2003). Freshly harvested seeds were tested for viability with 2, 3, 5-triphenyltetrazolium chloride (TTC). Seeds were stored at -20 °C in a sealed plastic bag for further cold stratification treatment.

Seed viability test of fresh seeds

Three replicates were used for seed viability test, with each replicate having about 50 seeds. The seed coat was removed and then incubated in a 1% (w/v) TTC solution for 24 h at 30 °C in darkness. At the end of incubation, seeds were cut longitudinally to inspect staining. The number of embryos stained red by TTC were recorded as viable seeds. Seed viability was calculated as the percentage of TTC-stained seeds.

Combined treatments of low stratification temperature and nitrogen deposition

On 14 January 2020, we retrieved seeds from -20 °C conditions. Four groups of 150 seeds, were mixed with sand, moistened with a solution of 0 mol L⁻¹ nitrate, 0.08 mol L⁻¹ nitrate (low nitrogen, LN), 0.16 mol L⁻¹ nitrate (medium nitrogen, MN) or 0.25 mol L⁻¹ nitrate (high nitrogen, HN), respectively. The selection for three nitrogen addition levels was based on following reasons. First, in previous published work that was conducted in Northeastern China, nitrogen added was 5 g N m⁻² year⁻¹, 10 g N m⁻² year⁻¹ and 15 g N m⁻² year⁻¹ in low, medium, and high nitrogen addition treatment, respectively (Sun et al. 2015b). Based on the plot area and times of nitrogen addition, and the amount of water used to dissolve ammonium nitrate in Sun et al.'s study, the corresponding nitrate concentration in three nitrogen addition treatments was 0.12 mol L⁻¹, 0.24 mol L⁻¹ and 0.36 mol L⁻¹, respectively. Second, 10 g N m⁻² year⁻¹ is generally used as the concentration for nitrogen addition in temperate forests in Northeast China (Sun et al. 2016; Wang et al. 2017). Third, Hu et al. (2008) used 0.08 mol L⁻¹ of NH₄NO₃ as the concentration for low level of nitrogen addition. The mixture of seeds and moist sand was then placed into parafilm (PM-996, Bemis Company Inc., USA) bags (25 cm length × 10 cm width). Four seed bags with four different nitrogen levels were placed at three cold temperatures (0 °C, 5 °C and 10 °C), respectively. Three cold stratification temperatures were selected according to following reasons. First, the soil temperature at 10 cm depth above zero changed between 0 °C and 5 °C from November to April in Northeast China (Song and Zhu 2016). Second, we want to determine the possible effect of increasing cold temperature on seed dormancy release. Third, the cold stratification temperature used for seed dormancy release generally varies between 0 °C and 10 °C (Baskin and Baskin 2004). In total, there were 12 combined treatments for low temperature stratification and nitrogen addition. After 3.5 months, seeds were taken from each treatment and the germinated seeds were enumerated and removed. The remainder seeds were used for seed germination test. Approximately six months of cold stratification is reported to

completely release Korean pine seed primary physiological dormancy (Song and Zhu 2016). To illustrate the medium dormancy status of seeds during seed dormancy release under the effects of increasing cold stratification temperature and nitrogen addition, cold stratification treatment was only conducted for 3.5 months.

Seed germination test

Seed germination test was conducted at two alternating temperature regimes (25/15 °C and 10/5 °C) in two growth chambers (MGC-350BP-2, Bluepard, Shanghai, China) with a photoperiod of 14 h at high temperature for each regime. Germination test conducted only at one temperature could not sufficiently illustrate seed dormancy release according to seed dormancy definition. Therefore, 25/15 °C (a favorable temperature for Korean pine seed germination) and 10/5 °C (a mean temperature during moderate spring in Northeast China) were used to detect seed germination performance. Moreover, the germination resulted from a 25/15 °C of test temperature that could also be used to predict the effect of increasing spring temperature on seed germination under the context of global change. For every treatment, there were three replicates of 20 seeds. Twenty seeds were sown on top of five papers with 10 mL distilled water in 10 cm Petri dishes. These Petri dishes were covered with pervious parafilm. We examined the moisture status in the Petri dishes every two days and added distilled water to retard moisture loss. A seed was considered germinated when the radicle protruded from the seed coat. The number of germinated seeds were counted every two days and then removed from Petri dishes. At the end of germination test, the viability of these seeds with no germination occurring for continually three days were determined by TTC. The numbers of seeds germinating and ungerminated seeds (highly viable) were summed to calculate seed viability after 61 days of incubation at 25/15 °C (or 119 days of incubation at 10/5 °C). The calculations of final germination percentage, mean germination time (MGT) and

germination rate index (GRI) were based on the number of viable seeds. MGT represents the mean time that is required for a seed lot to initiate and end germination (Orchard 1977). GRI indicates the germination percentage on each day during germination period (Maguire 1962).

$$\text{MGT (days)} = \sum(n_i \times d_i)/N$$

where n_i is the number of germinated seeds on day i , d_i is the number of days after beginning the experiment, and N is the total number of seeds germinated (Ellis and Roberts 1981).

$$\text{GRI} = \frac{G_{tot}}{p} \sum \frac{g_i}{t_i}$$

where G_{tot} is the total number of germinated seeds at the end of the germination test, p is the total number of seeds, and g_i is the number of seeds germinated between time t_{i-1} and t_i (h) (Steinmanus et al. 2000).

Statistical analysis

The interactive effects of cold stratification temperature, nitrogen addition and incubation temperature on germination percentage, MGT or GRI, and the interaction between the effects of cold stratification temperature and nitrogen addition on seed viability were analyzed with a general linear model of analysis of variance. An independent-samples T test was then performed to test the difference in germination percentage, MGT or GRI between different cold stratification temperatures at a given nitrogen level, or between different nitrogen addition levels at a cold stratification temperature when there was a significant interaction. At each nitrogen level, the liner regressions on germination percentage of cold stratified seeds at 25/15 °C or 10/5 °C against cold stratification temperatures were carried out. All statistical analyses were conducted using IBM SPSS Statistics 19.0 (Chicago, IL, USA).

Results

Seed viability of fresh seeds and seed germination percentage during cold stratification

Seed viability of fresh seeds was approximately 99%. For all combined treatments, germination (about 8%) only occurred when seeds were treated with 10 °C of cold stratification and LN addition.

Germination at two germination test temperatures after cold stratification

There was a significant interaction between cold stratification temperature, nitrogen addition and incubation temperature on germination percentage, MGT and GRI (Table 1). A slight, gradual decline in germination percentage at 25/15 °C was recorded for cold stratified seeds with increasing cold temperature from 0 °C to 5 °C without nitrogen addition (Fig. 1a). 10 °C of cold stratification temperature accompanied with LN and MN additions during cold stratification significantly decreased subsequent germination percentage at 25/15 °C to approximately 50.2% and 57.5%, respectively (Fig. 1a). Following transfer to 25/15 °C, no significant difference in germination percentage of cold stratified seeds with HN treatment was observed among different cold stratification temperatures (Fig. 1a). The germination percentage at 25/15 °C significantly decreased to 76.9% with increasing nitrogen levels from 0N to HN at 5 °C of cold stratification (Fig. 1a).

The germination percentage of seeds at 10/5 °C significantly decreased from 22.8% to 2% with increasing cold stratification temperature in the absence of nitrogen addition (Fig. 1b). In the presence of MN or HN during cold stratification, cold stratification at 10 °C significantly decreased germination percentage at 10/5 °C, compared to 0 °C of cold stratification (Fig. 1b). However, for seeds cold stratified at 0 °C and 5 °C, there was no significant difference in germination percentage between them (Fig. 1b). At each cold stratification temperature, nitrogen addition treatment did not significantly affect germination percentage of seeds at 10/5 °C (Fig. 1b).

The effect of increasing cold stratification temperature on MGT was significantly different only in HN treatment (Fig. 2a). When seeds were transferred to an incubation temperature of 25/15 °C following

cold stratification in the presence of HN, those seeds with a 5 °C (or 10 °C) of cold stratification treatment had a significant lower MGT (14 days) than that at 0 °C of cold stratification (Fig. 2a). After seeds were cold stratified at 10 °C in the presence of HN, the MGT of these seeds at a 10/5 °C of germination test temperature reached a significant maximum of 77 days (Fig. 2b). The MGT of seeds cold stratified at 5 °C in the absence of nitrogen addition was significant longer compared with the three additional nitrogen addition treatments when seeds were incubated at 25/15 °C (Fig. 2a).

No significant difference in GRI at 25/15 °C was detected after seeds were cold stratified under increasing cold stratification temperatures with 0N and HN treatments, whereas 10 °C of stratification temperature in the LN and MN treatments caused a significant decrease in the rate of seed germination at 25/15 °C (Fig. 3a). The GRI of seeds at an incubation temperature of 10/5 °C was not significantly affected by a treatment of increasing cold stratification temperature with or without nitrogen addition (Fig. 3b). When cold stratified seeds were incubated at 25/15 °C, those seeds cold stratified at 0 °C under conditions of HN showed a minimum of GRI among four nitrogen addition treatments (Fig. 3a). For seeds cold stratified at 5 °C, the GRI was significantly higher in LN than that in MN addition treatment (Fig. 3a). At each cold stratification temperature, nitrogen addition treatment did not significantly affect GRI of seeds at 10/5 °C (Fig. 3b).

Relationship between cold stratification temperature and germination percentage

There was a significant linear regression between cold stratification temperature and germination percentage at 25/15 °C for seeds treated with LN or MN addition (Fig. 4). A significant linear regression between cold stratification temperature and germination percentage at 10/5 °C occurred in seeds with 0N, MN or HN addition treatment.

Seed viability after incubation at two germination test temperatures

For cold stratified seeds that were incubated at 25/15 °C, no interaction between cold stratification temperature and nitrogen addition on seed viability was detected (Table 2). There was a significant main effect of cold stratification temperature on seed viability (Table 2); 0 °C significantly decreased seed viability to about 84% compared with that at 5 °C (or 10 °C) (Fig. 5). At a 10/5 °C of germination test temperature, a significant interaction between the effects of cold temperature and nitrogen addition on seed viability, or the main effect of cold stratification temperature (or nitrogen addition) was not detected (Fig. 5; Table 2).

Discussion

Experiments in which mother plants were treated with high nitrates revealed their influence on seed dormancy depth (Hilhorst 1990; Alboresi et al. 2005; Matakias et al. 2009). However, above-mentioned studies concentrated on the impact of high nitrate concentration in mother plant on the degree of dormancy in produced seed, while ignoring the roles of nitrate during dormancy release after seed shedding. Bouwmeester et al. (1994) reported that both the differences in endogenous nitrate contents and in germination percentages among different nitrate treatments gradually decreased during periods when seeds were buried in soil. The effects of nitrate accumulation in mother plant during seed development on subsequent seed germination do not last for long time. It is the soil nitrate content that is important in determining seed germination performance after seed dispersal.

It has been reported that treatment of seeds with KNO_3 followed by moist chilling could improve germination percentage (Shahi-Gharahlar et al. 2012). Few studies have assessed the simultaneous effects of temperature and nitrogen in seed dormancy regulation. However, several studies have revealed the regulation of seed dormancy (or germination) by temperature is involved in the change in the sensitivity of seed germination to nitrate. For example, the sensitivity of seeds to nitrate increased after

seeds were treated with cold stratification (Bouwmeester et al. 1994; Mackenzie and Naeth 2019). For seeds buried in soil, nitrate sensitivity changed during the dormancy cycle, driven by seasonal temperature (Bouwmeester et al. 1994; Finch-Savage and Footitt 2017).

Our results indicated that increased temperature and nitrogen deposition during cold stratification had interactive effects on subsequent germination capacity, while apparently being dependent on the germination temperature experienced by cold stratified seeds. Bouwmeester et al. (1994) reported that when seeds with different endogenous nitrate concentrations were buried in field conditions, they exhibited different germination performance between germination temperatures following transfer to laboratory. This study was conducted for only 3.5 months; however, Korean pine seeds are retained in soil after dispersal at least for six months under low winter temperature conditions. Our findings did not adequately elucidate the regulation of dormancy release by increased cold stratification temperature and nitrogen deposition. Thus, a long-term experiment will be required for future research.

Based on the germination performance of seeds stratified at 10 °C and LN addition, we speculate that if the amplitude of increase in spring temperature is higher under the current context of global change, increasing temperature and LN deposition during winter would inhibit Korean pine seed germination at spring. However, if there is a decreased rise in spring temperature in a climate warming context, Korean pine seed germination during spring might not be inhibited by increasing temperature and LN deposition during winter. But seed germination percentage was still lower and MGT was prolonged. The present study was conducted only for 3.5 months. It is possible that with increasing cold stratification temperature and LN deposition for about 6 months, a high percentage of seeds would germinate at low temperatures during spring in a shorter time period. The germination status of Korean pine seed changes over seasons, with a small percentage germinating in the first spring following seed dispersal and a high percentage

during the second spring (Song et al. 2018). In case most seeds germinate at low temperature in the first spring after seed dispersal in the context of increasing temperature and LN deposition in winter, the time interval from dispersal until completion of germination of most seeds is shorter, thus reducing the chances of seeds being eaten by rodents. Hence, the regeneration of Korean pine would be promoted. Previously, it was reported that Korean pine seeds would enter dormancy in the first late summer and release dormancy in the second autumn and winter; however, the influence of increasing temperature and nitrogen deposition on the seasonal changes of dormancy and germination remains unknown. Thus, this warrants further research.

Even though less than 60% germination at high germination test temperature was recorded for seeds cold stratified with 10 °C under LN or MN additions, seed germination was not further inhibited by increasing nitrogen concentrations. The combined treatments of 10 °C and high levels of nitrogen stimulated approximately 76% of seeds to germinate. Thus, we speculate that increasing temperature and HN deposition during winter after seed dispersal in autumn would not significantly affect Korean pine seed germination during following spring. However, we found that the number of decayed seeds gradually increased as increasing cold stratification temperature and nitrogen additions (unpublished data). Hence, Korean pine seed might have high mortality rates under the effects of high cold stratification temperature and HN addition during winter. Nitrate can provide nutritional resources for soil microbes (Chee-Sanford et al. 2006). Nitrogen deposition increases the probability of seed mortality caused by pathogenic microorganisms (Davis 2007). Given the relative importance of nitrogen on seed dormancy release and germination, we suggest that future studies should consider the effect of nitrogen on seed mortality.

In many species nitrate can act as a nutrient or a signaling molecule that plays a major role in seed

dormancy release and germination promotion (Wang et al. 2003; Alboresi et al. 2005). According to Li (2018), the endogenous total nitrogen contents of Korean pine seeds were not altered regardless of external nitrogen levels applied. Thus, we suggest that nitrate may act on seed dormancy release in Korean pine through nitrogen signaling. Some studies revealed that nitrate induction is correlated with a decline in levels of abscisic acid (ABA) in imbibed dormant seeds or a reduced requirement of gibberellic acid for germination (Wang et al. 1998; Ali-Rachedi et al. 2004; Alboresi et al. 2005). Therefore, it is necessary to determine the ABA content in Korean pine seeds after stratification for 3.5 months under conditions of increasing cold temperature and nitrogen addition. Moreover, the sensitivity of Korean pine seeds to ABA should also be tested. In addition, it is reported that ABA content may increase (or decrease) after seeds are incubated under different temperatures, and seed dormancy is thus maintained (or released) (Benech-Arnold et al. 2006). Thus, we hypothesize that after treatment involving 3.5 months of combined treatments of cold stratification temperature (10 °C) and LN addition, there may be a drastic accumulation of ABA following transfer of seeds to a high germination test temperature of 25/15 °C. In contrast, when seeds are incubated at 10/5 °C, the ABA content may slightly decrease. Altogether, it is possible that cold stratification temperature and nitrogen deposition regulate seed dormancy status via their effects on ABA content (or ABA sensitivity). In addition, LN deposition during cold stratification could be more effective in regulating Korean pine seed dormancy status compared with another two nitrogen addition levels. It has been shown that *Atriplex sagittate* seeds are observed to have various optimum nitrate concentrations required for seed germination, thus allowing adaptation to changing environmental conditions (Mandák and Pyšek 2001).

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Table 1. Variance analysis for the traits investigated of Korean pine seeds in response to cold stratification temperature, nitrogen addition and incubation temperature.

Source of variations	Germination		Mean germination		Germination rate	
	percentage		time		index	
	F-ratio	P-value	F-ratio	P-value	F-ratio	P-value
Incubation temperature 'IT'	772.591	0.000	207.608	0.000	444.219	0.000
Cold stratification temperature 'CT'	30.073	0.000	1.170	0.320	7.457	0.002
Nitrogen addition 'NA'	0.418	0.741	3.020	0.040	0.876	0.461
IT × CT	3.974	0.025	0.283	0.755	6.152	0.004
IT × NA	3.474	0.023	2.407	0.080	1.278	0.294
CT × NA	1.576	0.175	4.401	0.001	2.446	0.040
IT × CT × NA	2.949	0.016	4.051	0.003	2.644	0.028

Note: Significant effects ($P < 0.05$) are bolded.

Table 2. Two-way ANOVA of effects of cold stratification temperature, nitrogen addition and their interactions on viability of seeds incubated at two germination test temperatures.

Incubation temperature	Source of variations	F-ratio	P-value
25/15 °C	Cold stratification temperature 'CT'	8.696	0.001
	Nitrogen addition 'NA'	2.896	0.056
	CT × NA	1.136	0.372
10/5 °C	CT	0.982	0.389

NA	1.935	0.151
CT × NA	0.213	0.969

Note: Significant effects ($P < 0.05$) are bolded.

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Figure captions

Fig. 1. Germination percentage of seeds at 25/15 °C (a) and 10/5 °C (b) after seeds were cold stratified at three temperatures (0 °C, 5 °C and 10 °C) and four nitrogen additions (0 nitrogen, low nitrogen, medium nitrogen and high nitrogen). Different small letters indicate significant differences ($P < 0.05$) among different cold stratification temperatures under the same nitrogen level and incubation temperature. Different capital letters indicate significant differences ($P < 0.05$) among different nitrogen levels under the same cold stratification temperature and incubation temperature. Error bars represent one standard error.

Fig. 2. Mean germination time of seeds at 25/15 °C (a) and 10/5 °C (b) after seeds were cold stratified at three temperatures (0 °C, 5 °C and 10 °C) and four nitrogen additions (0 nitrogen, low nitrogen, medium nitrogen and high nitrogen). Different small letters indicate significant differences ($P < 0.05$) among different cold stratification temperatures under the same nitrogen level and incubation temperature. Different capital letters indicate significant differences ($P < 0.05$) among different nitrogen levels under the same cold stratification temperature and incubation temperature. Error bars represent one standard error.

Fig. 3. Germination rate index of seeds at 25/15 °C (a) and 10/5 °C (b) after seeds were cold stratified at three temperatures (0 °C, 5 °C and 10 °C) and four nitrogen additions (0 nitrogen, low nitrogen, medium nitrogen and high nitrogen). Different small letters indicate significant differences ($P < 0.05$) among different cold stratification temperatures under the same nitrogen level and incubation temperature. Different capital letters indicate significant differences ($P < 0.05$) among different nitrogen levels under the same cold stratification temperature and incubation temperature. Error bars represent one standard error.

577 error.

578 **Fig. 4.** Relationship between cold stratification temperature and germination percentage at 25/15 °C (a,
579 b, c and d) and 10/5 °C (e, f, g and h) under different nitrogen addition levels during cold stratification.

580 **Fig. 5.** Seed viability following transfer of seeds that were cold stratified under different temperatures
581 and nitrogen addition levels to 25/15 °C (a) and 10/5 °C (b) of germination test temperatures, respectively.

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Fig. 1. Germination percentage of seeds at 25/15 °C (a) and 10/5 °C (b) after seeds were cold stratified at three temperatures (0 °C, 5 °C and 10 °C) and four nitrogen additions (0 nitrogen, low nitrogen, medium nitrogen and high nitrogen). Different small letters indicate significant differences ($P < 0.05$) among different cold stratification temperatures under the same nitrogen level and incubation temperature. Different capital letters indicate significant differences ($P < 0.05$) among different nitrogen levels under the same cold stratification temperature and incubation temperature. Error bars represent one standard error.

291x204mm (300 x 300 DPI)

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Fig. 4. Relationship between cold stratification temperature and germination percentage at 25/15 °C (a, b, c and d) and 10/5 °C (e, f, g and h) under different nitrogen addition levels during cold stratification.

291x204mm (300 x 300 DPI)

Fig. 5. Seed viability following transfer of seeds that were cold stratified under different temperatures and nitrogen addition levels to 25/15 °C (a) and 10/5 °C (b) of germination test temperatures, respectively.

291x204mm (300 x 300 DPI)