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Variation in germination character of *Robinia pseudoacacia* L. (Leguminosae) seeds at individual tree level

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Abstract To demonstrate the seed dormancy and germination characters of *Robinia pseudoacacia* L., an exotic tall tree species in Japan, we applied scarification, cold stratification, diurnal thermal regime, heat shock, and/or winter weathering treatments to the seeds. These characters differed markedly among three seed sources (Trees K, B, and I). Scarification revealed that most seeds from Tree K showed physical dormancy, whereas those from Tree B had no physical dormancy. The seeds from Tree I showed weak physical dormancy so that the seeds eventually germinate without any treatments as time goes by. The physical dormancy in Tree K was broken by a long, high heat shock treatment. The results imply that seeds from Tree K respond to fire in natural conditions. Seeds from Tree I responded to a wide range of thermal regimes, except for long, high heat shock. In contrast, most seeds from Tree B absorbed water during cold stratification and some germinated. However, many seeds from Tree B died in the soil during the winter, presumably because of microbial and fungal infections. Seeds from Trees I and B acquired physical dormancy by weathering during the winter, implying that seedpods that remain in the crown function as an aerial seed bank. *R. pseudoacacia* may be able to produce various levels in dormancy with respect to the winter condition.

Keywords Heat shock · Physical dormancy · *Robinia pseudoacacia* · Seed germination · Wintering

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Introduction

Robinia pseudoacacia L., a leguminous tall tree species native to North America, is one of "the top 100 worldwide woody plant invaders" that threatens native vegetation (Cronk and Fuller 1995). In Japan, in 2006, the Ministry of the Environment listed the species as "Uncategorized Alien Species" that have the possibility to be categorized as invasive alien species, and the Ecological Society of Japan (2002) listed the species as in "the top 100 invasive alien species in Japan". However, little is known about the invasion process of *R. pseudoacacia* to native vegetation. Though rapid spread over a wide area will be attained by seed dispersal (Fukuda et al. 2005; Takahashi et al. 2008), seeds of *R. pseudoacacia* have long been considered to be impermeable seeds specific to legumes (cf. Guppy 1912) that require scarification for germination to take place (Strode 1977; Huntley 1990), and form a soil seed bank (Kostel-Hughes et al. 1998; Lambers et al. 2005; Takahashi et al. 2008). We have much information about the break of dormancy in *R. pseudoacacia* seeds by artificial methods (Toda and Ishikawa 1951; Sato and Shoji 1954; Tazoye 1938; Baskin and Baskin 2001); however, we have little knowledge about the germination ecology in natural conditions.

In the Appalachian Mountains of North America where *R. pseudoacacia* is a major forest component, fire often greatly impacts the forest (Elliott et al. 1997, 1999; Lafon et al. 2005). *R. pseudoacacia* regenerates in open sites, implying that it may have historically been an important colonizer of burned sites (Boring and Swank 1984). In fact, *R. pseudoacacia* often appears after fire (Anderson and Brown 1986; Waldron et al. 2007). Many species in fire-prone ecosystems depend on fire for seed germination (Eyre 1938; Floyd 1966; Keeley 1986; Stone and Juhren

1951). Thus, one of the germination stimulants for *R. pseudoacacia* seeds may be heat shock.

However, there are no fire-prone ecosystems in Japan. *R. pseudoacacia* often regenerates at roadsides, flood plains, declined coastal forests, and so on (Maekawa and Nakagoshi 1997a, b; Sakio 2003; Fukuda et al. 2005; Yamada and Masaka 2007; Takahashi et al. 2008). *R. pseudoacacia* can spread without fire, indicating that some *R. pseudoacacia* seeds are stimulated to germinate by factors other than heat. Studies in the 1930 s indicated the existence of seeds that germinated just after dispersal without any treatments (Burton 1932; Chapman 1936; Wilson 1937; Tazoye 1938). These seeds may show no physical dormancy. Thus, polymorphism in seed dormancy will be observed in *R. pseudoacacia* seeds (Takahashi et al. 2008). Chapman (1936) showed the difference in percent physical dormancy among trees implying that the intensity of physical dormancy also varies among trees. Though long time survival of *R. pseudoacacia* seeds stored in the soil (Toole 1946) suggests that the seeds will show 'strong' physical dormancy, it is doubtful that seeds with no physical dormancy could survive in the soil, because the seeds would absorb water after dispersal before the winter. Seeds with 'weak' physical dormancy, on the other hand, could survive in the soil during the winter and might germinate without strong heat shock. Therefore, germination ecology in natural conditions should be studied at the individual tree level to understand the regeneration strategy in *R. pseudoacacia*.

In the present study, we addressed the following questions to determine the germination ecology of *R. pseudoacacia* seeds in natural condition at the individual tree level: (1) To what extent does the intensity of physical dormancy in *R. pseudoacacia* seeds differ among trees? (2) To what extent does the degree of physical dormancy influence the wintering in the soil? (3) What diurnal thermal regime influences seed germination (fluctuating temperature that approximated spring or summer weather conditions)? (4) To what extent does the degree of physical dormancy relate to the break of physical dormancy? Some *R. pseudoacacia* seeds experience extreme cold during winter because some seed pods remain in the crowns of some trees. Thus, *R. pseudoacacia* may form an aerial seed bank, and we also addressed the following question: (5) To what extent does weathering during winter influence dormancy?

Materials and methods

Study species

Robinia pseudoacacia L., is a tall tree species native to the eastern United States of America (Surles et al. 1989;

Huntley 1990). The species has been widely planted throughout the world's temperate zone (e.g., Panagopoulos and Hatzistathis 1995; Lee et al. 2004; Groninger and Fillmore 2006), since the species has been available for soil improvement at nutrient poor sites, control of erosion in road cuts, and revegetation at strip-mined area due to its drought-tolerance and ability of nitrogen fixation (Boring and Swank 1984; Bormann et al. 1993). The species has also been used in the honey-production industry (Rédei et al. 2002). Consequently, the planted area of this species is second only to *Eucalyptus* spp. among broadleaved species worldwide (Keresztesi 1980). However, *R. pseudoacacia* rapidly spreads at flood plains, coastal forests, suburban forests, and abandoned fields (e.g., Larsen 1935; Luken et al. 1992; Elliot et al. 1997; Maekawa and Nakagoshi 1997a, b; Sakio 2003; Lee et al. 2004; Rice et al. 2004; Fukuda et al. 2005; von Holle et al. 2006; Yamada and Masaka 2007; Takahashi et al. 2008).

R. pseudoacacia sometimes forms dense stands by intensive root suckering (Beck and McGee 1974; Huntley 1990; Chang et al. 1998). The fragrant, whitish flowers, borne in showy racemes, appear after leaf emergence. In central Hokkaido, northern Japan, we can observe flowers in June. The perfect flowers originate in the axils of current year leaves and are pollinated by insects, primarily bees. According to Marbach and Mayer (1974), seeds of *R. pseudoacacia* can acquire physical dormancy until late July. The fruit is a flattened, oblong pod that ripens during September and October. These pods contain 1–20 seeds, and the number of seeds is 36,000–65,000/kg, and we have found no significant correlation between seed weight and the percent dormant seeds (recalculated from Chapman 1936). Some pods open on the tree, some other pods disperse by wind: we often find the seed pods far away from *R. pseudoacacia* trees in Hokkaido. Seeds and pods are shed from September to April. Some seeds can survive at least 39 years in the soil (Toole 1946), but the survival curve of buried *R. pseudoacacia* seed in the soil is still unknown. *R. pseudoacacia* seeds are eaten by many bird species (Strode 1977), and can be classified occasionally as bird-dispersal seed in the original habitat (Lambers et al. 2005). However, it is also unknown whether *R. pseudoacacia* seed is dispersed by birds effectively, since the seed has no pulp or food body for feed of birds.

Seed collection and general conditions

The present study was conducted at the Hokkaido Forestry Research Institute (HFRI) (43°17′N, 141°51′E, 45 m a.s.l.) in Bibai, central Hokkaido, northern Japan. Many *R. pseudoacacia* trees are found in the region because they have spread over strip coal mines, abandoned crop fields, failed conifer plantations, and clear cuts in silviculture

areas (Yamada and Masaka 2007). According to the Japan Meteorological Agency (http://www.data.kishou.go.jp/, 1979–2000), average precipitation in Bibai is 1,155.6 mm per year. The mean monthly temperature during the warmest (August) and the coldest (January) months is 21.1 and −6.7°C, respectively. The forest floor is usually under snow from late November until the following April, and the maximum depth of snow cover is 1,150 mm.

Because *R. pseudoacacia* is a clonal species (Larsen 1935; Gyokusen et al. 1991; Chang et al. 1998) and is a bee-pollinated species (Surles et al. 1989), we selected three trees (Trees I, K, and B) that were located far apart. *R. pseudoacacia* trees were selected from Iwamizawa on 10 October 2006 (43°10′N, 141°48′E, 50 m a.s.l.), Kami-Sunagawa on 12 October 2006 (43°28′N, 142°00′E, 170 m a.s.l.), and Bibai on 15 October 2006 (43°18′N, 141°53′E, 60 m a.s.l.). These trees grew along a road. Seeds were collected in their pods from the crowns. Pods and chalaza were brown and almost dry, and few were open. In the experiments described below, 50 seeds were used as a replicate and three replications were conducted. When Petri dishes were placed in an incubator or oven, each Petri dish was kept in a sealed polyethylene bag to reduce water loss caused by evaporation. No disinfectants were applied to the seeds because we have no knowledge about the effect of disinfectants on seed germination in *R. pseudoacacia*. Protrusion of the radicle was the criterion for germination. Germinated or dead seeds were counted and removed from the dishes at each census. Dead seeds can be distinguished from live seeds because the seeds absorb water before the appearance of mold or disintegration of the organism. A total of 12,600 seeds were used (4,200 seeds/tree).

Seed coat impermeability

Impermeability of the seed coat to water was tested. On 27 October 2006, pierced fresh seeds were placed on filter paper moistened with distilled water in Petri dishes and kept in an incubator at 20°C. As a control, freshly collected seeds were placed under the same conditions for 500 days. Germination of control seeds was recorded two or three times per week, whereas germination of pierced seeds was recorded daily until all seeds had germinated.

Cold stratification

In central Hokkaido, seeds experience a cold winter with heavy snow from late November to early April. Before the germination tests ('diurnal thermal regime' and 'heat shock' experiments), fresh seeds were placed in a polyethylene net bag (seed bag; 2 × 2 mm mesh) and buried under the litter layer in a dense *Picea glehnii* stand in

the HFRI arboretum in Bibai (burial treatment) on 27 October 2006. No vegetation was observed on the forest floor. On 9 April 2007, we removed the seed bags for the experiments explained in the next section and recorded the condition of seeds (imbibition, germination, and survival). The imbibed seeds can be easily distinguished from dormant seeds (Takahashi 2007). As a control, freshly collected seeds were placed on moistened filter paper in Petri dishes and kept in an incubator at 3.8°C for the same period of time. In the control, we recorded seed condition (imbibition, germination, and survival) twice in November, and then once per month until April.

Effect of diurnal thermal regime on seed germination

Germination tests were conducted at diurnal temperature regimes of 25/15, 20/10, and 15/5°C for 12/12 h in an incubator. We started the experiment on 9 April 2007. The cold stratified seeds during winter mentioned above (burial treatment and control) were used in this experiment. These thermal conditions approximate the mean monthly maximum and minimum air temperatures in central Hokkaido from early spring to early summer (April–June). The number of germinated seeds was censused weekly for 180 days (from April to September). To demonstrate the effect of diurnal thermal conditions on germination, one-way analysis of variance (ANOVA) with a least significant difference (LSD) test was conducted for the final number of germinated seeds, dormant seeds, dead seeds, and total seeds within the same thermal regime and within the same tree.

Effect of heat shock on the break of dormancy

According to Washitani (1988), *Rhus javanica* has impermeable seeds and regenerates at open sites, and effective temperatures for seed germination are $55 ± 7.5°C$ (Washitani 1988). Based on these results, we placed *R. pseudoacacia* seeds on moistened filter paper in Petri dishes and exposed them to controlled oven temperatures of 50, 55, or 60°C for 1, 3, or 24 h (total 9 treatments per tree). We started the experiment on 9 April 2007. The cold stratified seeds during winter (burial treatment and control) were used in this experiment. The seeds, kept in the same Petri dish, were transferred to an incubator maintained at 20°C after the heat shock treatment. After the transfer, the number of germinated seeds was censused weekly for 180 days (from April to September). To demonstrate the effect of heat shock on the break of dormancy, one-way ANOVA with an LSD test was conducted for the final number of germinated seeds, dormant seeds, dead seeds, and total seeds within the same tree. To confirm whether seeds died due to the heat

shock treatments, we scarified the remaining non-germinated seeds after the final census.

Effect of weathering during winter on seed dormancy

To demonstrate the effect of weathering during winter on seed dormancy, freshly collected seeds were kept in a polyethylene net bag (2 × 2 mm mesh, 50 seeds per bag) and weathered outdoors from December to April in the HFRI arboretum (weathering treatment). The net bags were separately attached inside a stainless steel box (30 cm in height, 15 cm in depth and 15 cm in width), and the box was bound to the trunk of a metasequoia (*Metasequoia glyptostroboides*) 1.3 m above the ground. To prevent rain damage, the box was bound to the tree on 14 December 2006, after the seeds had been kept in a room with no heater at HFRI. The sides of the box were wire net (1 × 1 cm mesh) and, therefore, porous. As a control, seeds were kept in a polyethylene net bag and dried in an incubator at 3.8°C during the same period (50 seeds per bag). In April, the seeds were placed on filter paper moistened with distilled water in Petri dishes and kept in an incubator at 20°C. The number of germinated seeds was censused weekly for 180 days (from April to September). To demonstrate the effect of weathering during winter on seed dormancy, one-way ANOVA with an LSD test was conducted for the final number of germinated seeds among trees. To confirm whether seeds died due to the weathering treatment, we scarified the remaining non-germinated seeds after the final census.

Results

Percent physical dormancy

Almost all pierced seeds germinated rapidly, within 2 weeks, for each of the three seed sources (mean no. of seeds germinated ± SD: 46.3 ± 1.2 for Tree I, 49.3 ± 0.6 for Tree K and 49.3 ± 1.2 for Tree B; Fig. 1). The remaining seeds seemed to be infected with mold. No empty seeds were observed, indicating that the fresh seeds used were all potentially viable. The seed germination curve differed markedly among seed sources when the fresh seeds were kept in moist conditions after seed collection (Fig. 1). Most of the seeds from Tree K did not germinate in the study period (mean no. of seeds germinated ± SD: 6.3 ± 1.5). In contrast, the seeds from Tree B germinated rapidly, and half the seeds germinated within 24 days. Germination by seeds from Tree B was completed within 177 days (mean no. of seeds germinated ± SD: 48.3 ± 1.5). The remaining seeds seemed to be infected with mold. The seeds from Tree I began to germinate after
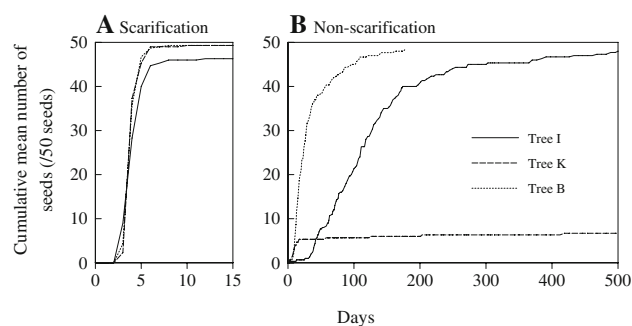
Fig. 1 Germination curves for fresh seeds kept under moist conditions at 20°C in an incubator after collection

1 month, and the number of germinated seeds increased monotonically until approximately 180 days and then the curve gradually approached the upper limit (mean no. of seeds germinated ± SD: 45.3 ± 1.5). No germination was observed during the last 2 months, suggesting that the remaining seeds exhibited physical dormancy.

Characteristics of seeds during cold stratification

The activity of seeds kept in an incubator (3.8°C) for cold stratification differed markedly among seed sources (Figs. 2, 3). Most seeds from Tree B absorbed water (mean no. of seeds ± SD: 40.9 ± 3.0; Fig. 2a), and some germinated even at the low temperature (mean ± SD: 1.1 ± 1.5; Fig. 2c). Other seeds died (mean ± SD: 4.6 ± 5.1; Fig. 2b). In contrast, only a few seeds from Trees I and K absorbed water (Fig. 2a). In the burial treatment, most seeds in replicate 2 from Tree B died in the soil (Fig. 3). The seed coat of buried seeds from Tree B that absorbed water during winter was stripped off and a pale-yellowish cotyledon was exposed.

Germination pattern in diurnal thermal regime

The germination curve differed markedly among seed sources when cold stratified seeds were exposed to diurnal thermal regime (Fig. 4). Few seeds germinated from Tree K. In contrast, seeds from Tree I germinated monotonically. The germination curve in Tree I for control is well fitted by linear function ($y = 0.249x$, $r^2 = 0.971$, $P < 0.0001$ for 15/25°C treatment; $y = 0.192x$, $r^2 = 0.991$, $P < 0.0001$ for 10/20°C treatment; $y = 0.154x$, $r^2 = 0.994$, $P < 0.0001$ for 5/15°C treatment; y and x indicate no. of germinated seeds and days, respectively). Though the final number of germinated seeds did not differ significantly among diurnal thermal regime experiment (Table 1), we found a negative relationship between the magnitude of regression coefficient and warmth in the corresponding treatment. This means that the rate of
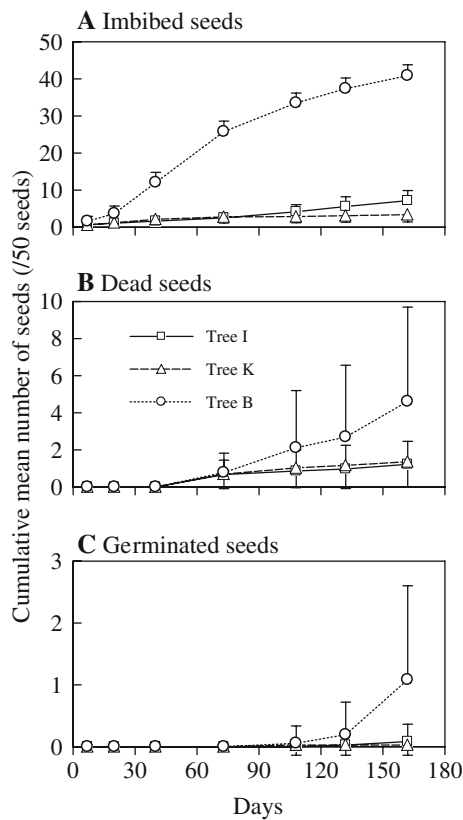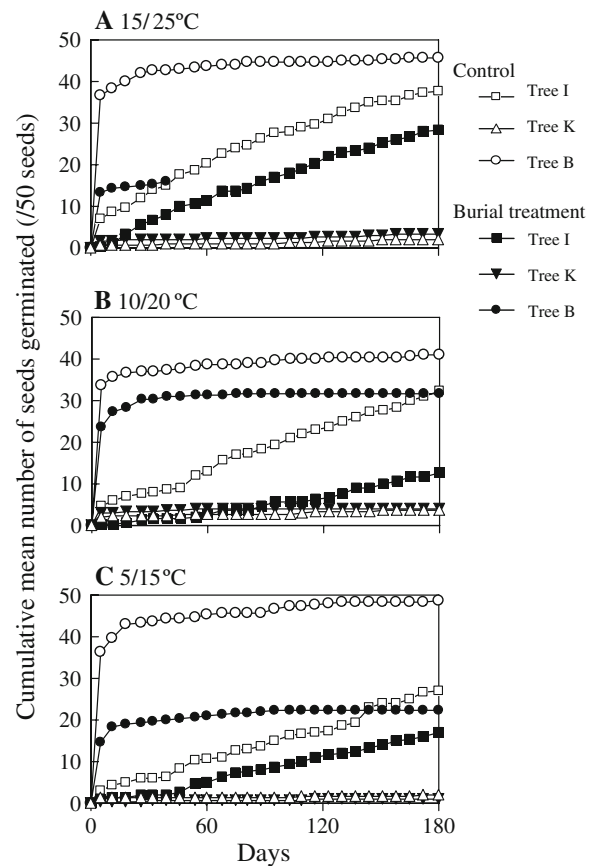
Fig. 2 Aspects of seeds during cold stratification in an incubator (3.8°C; control) versus burial for 6 months, from November to April
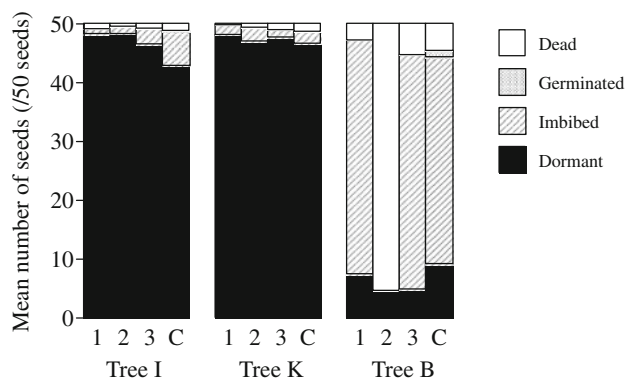
Fig. 3 Aspects of seeds just after 6 months of cold stratification, from November to April. *Numbers* indicate the replicate number in the burial treatment (n = 12) and C indicates the control treatment (n = 36)

Fig. 4 Seed germination curves during the fluctuating temperature experiment; burial treatment (*closed symbol*), control (*open symbol*)

germination tended to decrease with decreasing warmth. On the other hand, seeds from Tree B had a saturated germination curve and germinated rapidly under all thermal regimes (Fig. 4). We cannot compare the germination curve among thermal regimes for Tree B, because several seeds died in the 10/20°C treatment (Table 1). But we cannot find a visible difference in the curve among thermal

regimes. The final number of germinated seeds was lower for Tree K than for Trees I and B (Table 1).

The number of germinated seeds in Trees I and B for the burial treatment (closed symbol) tended to be fewer than that of germinated seeds for control (open symbol) (Fig. 4). In Tree I, significantly more seeds remained dormant after burial than in the control (Table 1). No significant difference in the number of dormant seeds was observed between the burial treatment and control for Trees K and B. In Tree B, more dead seeds were observed after burial than in the control; there was a significant difference between the burial treatment and the control in the 15/25°C treatment (Table 1), because many seeds died in one or two replicates (standard deviation was much larger than others: see also Fig. 3). No significant difference in the number of dead seeds was observed between the burial treatment and the control for Trees I and K.

Germination pattern after heat shock treatment

The response of seeds to the heat shock treatments differed markedly among seed sources (Fig. 5, Tables 2, 3, 4). Many seeds from Tree I germinated in all treatments except

Table 1 Multiple comparisons by one-way ANOVA with an LSD test for the final results of fluctuating temperature experiments for 180 days in the growing season

Thermal regime	Type[a]	Mean ± SD		
		Tree I	Tree K	Tree B
For germinated				
15/25°C	S	28.33 ± 3.79[ab]	3.33 ± 1.53[a]	16.00 ± 14.80[d]
	C	37.67 ± 5.86[a]	2.00 ± 0.00[a]	**45.67 ± 3.51**[a]
10/20°C	S	12.67 ± 1.53[c]	4.00 ± 3.00[a]	31.67 ± 16.50[bc]
	C	**32.33 ± 8.33**[a]	3.67 ± 3.06[a]	41.00 ± 13.00[ab]
5/10°C	S	17.00 ± 2.00[bcd]	1.00 ± 1.73[a]	22.33 ± 15.37[cd]
	C	27.00 ± 6.00[ad]	2.00 ± 1.00[a]	**48.33 ± 2.89**[a]
For dormant				
15/25°C	S	**19.33 ± 4.93**[c]	46.00 ± 1.00[a]	0.00 ± 0.00[a]
	C	8.33 ± 4.51[b]	46.67 ± 0.58[a]	0.67 ± 1.15[a]
10/20°C	S	**34.67 ± 3.06**[b]	45.33 ± 2.52[a]	0.33 ± 0.58[a]
	C	15.33 ± 7.57[a]	44.00 ± 2.65[a]	1.00 ± 1.00[a]
5/10°C	S	**32.33 ± 3.06**[b]	47.00 ± 0.00[a]	6.67 ± 9.87[a]
	C	21.00 ± 4.00[a]	46.33 ± 1.53[a]	0.33 ± 0.58[a]
For dead				
15/25°C	S	1.00 ± 1.73[a]	0.67 ± 1.15[a]	18.33 ± 19.86[b]
	C	2.00 ± 1.73[a]	0.33 ± 0.58[a]	1.00 ± 1.00[a]
10/20°C	S	1.67 ± 2.08[a]	0.33 ± 0.58[a]	5.00 ± 7.81[a]
	C	2.00 ± 1.00[a]	1.00 ± 1.00[a]	4.00 ± 5.20[a]
5/10°C	S	0.67 ± 1.15[a]	1.00 ± 1.73[a]	6.67 ± 7.02[a]
	C	1.33 ± 1.15[a]	0.33 ± 0.58[a]	0.33 ± 0.58 [a]
For total				
15/25°C	S	48.67 ± 0.58[a]	50.00 ± 0.00[a]	34.33 ± 23.69[a]
	C	48.00 ± 1.73[a]	49.00 ± 0.00[a]	47.33 ± 3.06[ab]
10/20°C	S	49.00 ± 1.00[a]	49.67 ± 0.58[a]	37.00 ± 19.08[ab]
	C	49.67 ± 0.58[a]	48.67 ± 0.58[a]	46.00 ± 6.93[ab]
5/10°C	S	50.00 ± 0.00[a]	49.00 ± 1.73[a]	35.67 ± 20.50[ab]
	C	49.33 ± 1.15[a]	48.67 ± 1.15[a]	49.00 ± 1.73[b]

Seeds were cold stratified; burial treatment in the soil (*S*) and storing in a incubator (control; *C*), during winter before experiments. Means that share the same letter did not differ significantly within each section in each tree (*for germinated*, *for dormant*, *for dead*, and *for total*) (*P* > 0.05). Bold values indicate significant differences between S and C within the same treatment. All 3 replications with 50 seeds per replicate

[a] Type of cold stratification before experiment

the 60°C/24 h treatment, and significantly more seeds died in the 60°C/24 h treatment (Fig. 5, Table 2). In Tree K seeds, the maximum percent germination was achieved with a long, high heat shock treatment (60°C/24 h) (Fig. 5, Table 3). The number of dormant seeds from Tree K was significantly reduced by a long, high heat shock treatment, although the number of dead seeds increased slightly (Table 3). In contrast, the maximum percent germination in seeds from Tree B was achieved by the 50°C/1–24 h and 55°C/1 h treatments; many seeds died in the other

treatments (Fig. 5, Table 4). In Tree B, few seeds remained dormant in any treatments.

Compared with the control, the number of germinated seeds that had experienced burial tended to be significantly lower in the 50°C/1 h and 50°C/24 h treatments for Tree B seeds (Table 4). A similar tendency was observed in Tree I seeds in the 50°C treatments, and vice versa in the correspondence of the number of dormant seeds (Table 2). However, few seeds that experienced cold stratification in an incubator (control) germinated after a high, long heat shock treatment (Table 2). A significant difference was observed in the correspondence of the number of dead seeds (Table 2). In Tree K seeds, no difference was observed between the burial treatment and control (Table 3).

All scarified seeds germinated within 1 week, indicating that the remaining non-germinated seeds at the final census were all viable.

### Germination pattern after weathering treatment

The seeds in the weathering treatment frequently experienced a sub-zero environment during winter, and the lowest temperature (−21.6°C) was on 26 January 2007 (http://www.data.kishou.go.jp/). In Tree K seeds, weathering outdoors or in an incubator (3.8°C) had no effect on the break of seed dormancy (Fig. 6, Table 5). Only a few seeds germinated from Tree K. All scarified seeds germinated within 1 week, indicating that the remaining non-germinated seeds at the final census were all viable. This means that the remaining seeds were still dormant. In seeds from Trees I and B, the germination curve in the weathering treatment (closed symbol) differed markedly from that in the control (open symbol) (Fig. 6). Though a few seeds died in Tree I (mean ± SD was 1.3 ± 1.5), other all scarified seeds in Trees I and B germinated within 1 week, indicating that the remaining non-germinated seeds at the final census were viable. Significantly fewer seeds germinated in the weathering treatment than in the control, except for Tree K seeds (Table 5). Many seeds that kept in an incubator during winter germinated successively (Fig. 6), and it implies that the dry condition did not necessarily contribute to the induction of physical dormancy in Trees I and B.

Discussion

Seed dormancy variation in *Robinia pseudoacacia*

The germination characters of *R. pseudoacacia* seeds used in the present study differed markedly among seed sources (trees). Most seeds from Tree K exhibited physical

Fig. 5 Response of seeds to the heat shock treatment. The *upper three panels* are seeds from Tree I, the *middle three panels* are seeds from Tree K, and *lower three panels* are seeds from Tree B. *S* and *C* indicate burial treatment in the soil and storing in a incubator (control), during the winter before experiments, respectively
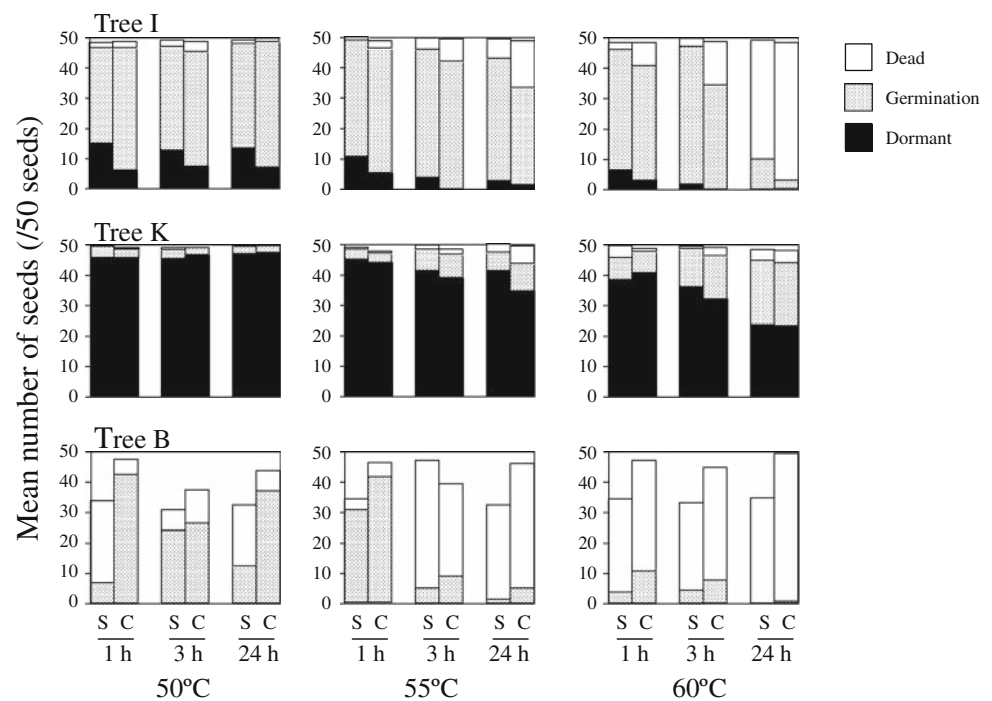
Table 2 Multiple comparisons by one-way ANOVA with an LSD test for heat shock experiments for Tree I

Thermal regime	Type[a]	Mean ± SD		
		Germinated	Dormant	Dead
50°C/1 h	S	31.67 ± 8.50[d]	**15.00 ± 9.17[b]**	1.67 ± 1.15[a]
	C	**40.67 ± 3.06[bef]**	6.00 ± 1.73[cdef]	2.00 ± 2.00[a]
50°C/3 h	S	34.33 ± 4.16[de]	12.67 ± 4.51[bg]	2.00 ± 0.00[a]
	C	38.00 ± 5.29[def]	7.33 ± 2.31[efg]	3.33 ± 2.31[acd]
50°C/24 h	S	34.67 ± 3.79[de]	**13.33 ± 4.62[b]**	1.00 ± 1.00[a]
	C	**41.67 ± 1.53[bf]**	7.00 ± 2.00[def]	1.00 ± 0.00[a]
55°C/1 h	S	38.33 ± 4.73[befg]	10.67 ± 3.06[bfg]	1.00 ± 1.73[a]
	C	41.00 ± 3.61[bef]	5.33 ± 4.93[acdef]	2.33 ± 0.58[ac]
55°C/3 h	S	41.97 ± 3.79[bf]	4.33 ± 2.52[acde]	3.67 ± 2.08[acde]
	C	41.67 ± 3.21[bf]	0.33 ± 0.58[a]	7.33 ± 1.53[de]
55°C/24 h	S	**40.33 ± 2.89[bef]**	2.67 ± 1.53[acde]	6.33 ± 4.04[cde]
	C	32.00 ± 7.81[d]	1.33 ± 1.53[ac]	**15.33 ± 6.81[f]**
60°C/1 h	S	39.67 ± 2.52[def]	6.33 ± 3.06[cdef]	2.33 ± 1.53[ac]
	C	37.67 ± 3.21[def]	3.00 ± 1.00[acde]	**7.67 ± 2.89[e]**
60°C/3 h	S	**45.33 ± 2.52[bg]**	1.67 ± 2.08[acd]	2.67 ± 2.08[a]
	C	34.33 ± 2.89[de]	0.00 ± 0.00[a]	14.33 ± 3.51[ac]
60°C/24 h	S	**10.00 ± 3.00[c]**	0.00 ± 0.00[a]	39.00 ± 2.00[b]
	C	2.67 ± 2.08[a]	0.33 ± 0.58[a]	**45.33 ± 2.08[g]**

Analyses were applied to the data at final census. Seeds were cold stratified; burial treatment in the soil (*S*) and storing in an incubator (control; *C*), during winter before experiments. Means that share the same letter did not differ significantly within each column (*for germinated*, *for dormant* and *for dead*) ($p > 0.05$). Bold values indicate significant differences between S and C within the same treatment. All 3 replications with 50 seeds per replicate

[a] Type of cold stratification before experiment

dormancy, whereas those from Trees B had no physical dormancy (Fig. 1). In Tree I, a few seeds absorbed water during winter (Figs. 2, 3) and then germinated in an incubator (Fig. 4), indicating that most seeds from Tree I showed 'weak physical dormancy' indicating that the seeds would eventually germinate without any treatments as time goes by (Figs. 1, 4).

Endogenous development of water impermeability of the seed coat is often related to seed maturation (Koyama 1998; Baskin et al. 2004). For example, impermeable seed coats develop in *Pisum elatius* during seed dehydration (Marbach and Mayer 1974). Because the *R. pseudoacacia* seeds used in the present study were collected in October, when the pods and chalaza were already brown and dry, the

seeds were mature at the time of collection. Furthermore, seeds stored in dry conditions in an incubator during winter and fresh seeds showed similar germination curves (Figs. 1, 6), suggesting that dry condition after seed collection has little influence on the induction of physical dormancy in *R. pseudoacacia* seeds.

Response of seeds to cold stratification

Seeds from Tree B showed no physical dormancy (Figs. 1, 2, 3). Most seeds in replicate 2 died in the soil (Fig. 3). Seeds from Tree B absorbed water during the winter (Figs. 2a, 3). The seed coat of the buried seeds from Tree B

Table 3 Multiple comparisons for heat shock experiments for Tree K

Thermal regime	Type[a]	Mean ± SD		
		Germinated	Dormant	Dead
50°C/1 h	S	3.67 ± 3.06[adf]	45.67 ± 2.31[b]	0.00 ± 0.00[a]
	C	3.00 ± 1.00[ac]	45.67 ± 1.53[b]	0.33 ± 0.58[a]
50°C/3 h	S	3.00 ± 1.00[ac]	45.33 ± 1.53[b]	0.67 ± 0.58[a]
	C	2.33 ± 2.31[a]	46.67 ± 1.53[b]	0.00 ± 0.00[a]
50°C/24 h	S	2.33 ± 1.53[a]	47.00 ± 1.73[b]	0.00 ± 0.00[a]
	C	2.33 ± 0.58[a]	47.33 ± 0.58[b]	0.00 ± 0.00[a]
55°C/1 h	S	3.33 ± 1.53[ac]	45.00 ± 1.00[bh]	0.67 ± 0.58[a]
	C	3.00 ± 1.00[ac]	44.00 ± 1.00[bghi]	0.67 ± 0.58[a]
55°C/3 h	S	7.00 ± 2.00[def]	41.33 ± 3.79[fgh]	1.33 ± 2.31[acd]
	C	7.67 ± 3.51[eg]	39.00 ± 1.00[ef]	1.67 ± 1.53[acd]
55°C/24 h	S	6.00 ± 1.00[cfg]	**41.33 ± 1.15[fgh]**	2.67 ± 1.53[acd]
	C	9.00 ± 1.73[eg]	34.67 ± 3.51[cd]	**5.67 ± 1.15[b]**
60°C/1 h	S	7.33 ± 2.08[eg]	38.33 ± 1.15[df]	**4.00 ± 2.65[bd]**
	C	7.00 ± 1.73[deg]	40.67 ± 0.58[fg]	1.00 ± 1.00[ac]
60°C/3 h	S	12.67 ± 4.73[h]	**36.00 ± 3.61[dei]**	0.67 ± 1.15[a]
	C	14.33 ± 2.08[h]	32.00 ± 1.00[c]	2.67 ± 2.08[acd]
60°C/24 h	S	21.00 ± 0.00[b]	23.67 ± 3.51[a]	3.67 ± 3.51[bc]
	C	20.67 ± 1.53[b]	23.33 ± 4.73[a]	4.00 ± 3.00[bd]

Details are the same as in Table 2

[a] Type of cold stratification before experiment

**Table 4** Multiple comparisons for heat shock experiments for Tree B

Thermal regime	Type[a]	Mean ± SD		
		Germinated	Dormant	Dead
50°C/1 h	S	6.67 ± 5.51[a]	0.00 ± 0.00[a]	27.00 ± 20.88[abcdef]
	C	**42.33 ± 7.02[b]**	0.00 ± 0.00[a]	5.00 ± 5.57[ac]
50°C/3 h	S	24.00 ± 20.52[cefgh]	0.00 ± 0.00[a]	6.67 ± 6.43[acd]
	C	26.33 ± 18.34[bdf]	0.00 ± 0.00[a]	11.00 ± 8.72[acde]
50°C/24 h	S	12.33 ± 16.17[ade]	0.00 ± 0.00[a]	22.00 ± 21.93[acdef]
	C	**37.00 ± 6.56[bh]**	0.00 ± 0.00[a]	6.67 ± 2.52[acd]
55°C/1 h	S	30.33 ± 24.42[bg]	0.33 ± 0.58[a]	3.67 ± 6.35[a]
	C	41.33 ± 3.51[b]	0.33 ± 0.58[a]	4.67 ± 3.06[ac]
55°C/3 h	S	5.00 ± 2.00[a]	0.00 ± 0.00[a]	42.00 ± 3.61[bf]
	C	9.00 ± 1.00[ac]	0.00 ± 0.00[a]	30.33 ± 8.33[bdef]
55°C/24 h	S	1.33 ± 1.53[a]	0.00 ± 0.00[a]	31.00 ± 26.85[bef]
	C	5.00 ± 0.00[a]	0.00 ± 0.00[a]	41.00 ± 3.61[bf]
60°C/1 h	S	3.67 ± 0.58[a]	0.00 ± 0.00[a]	30.67 ± 26.56[bdef]
	C	10.67 ± 2.52[ade]	0.00 ± 0.00[a]	36.33 ± 2.52[bf]
60°C/3 h	S	4.67 ± 3.79[a]	0.00 ± 0.00[a]	28.33 ± 23.25[bcdef]
	C	7.33 ± 2.08[ae]	0.33 ± 0.58[a]	37.00 ± 8.54[bf]
60°C/24 h	S	0.00 ± 0.00[a]	0.00 ± 0.00[a]	34.67 ± 23.97[bef]
	C	0.67 ± 1.15[a]	0.00 ± 0.00[a]	48.67 ± 1.53[b]

Details are the same as in Table 2

[a] Type of cold stratification before experiment
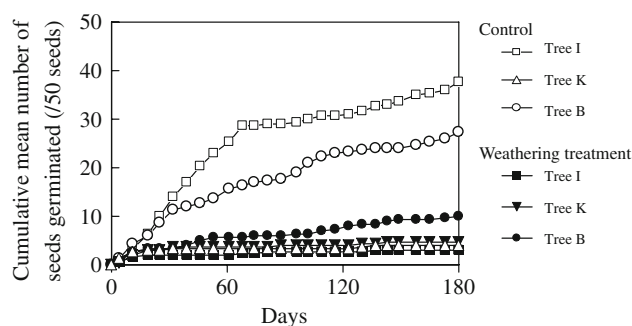
Fig. 6 Seed germination curves after weathering treatment; weathering treatment (*closed symbol*), control (*open symbol*)

Table 5 Multiple comparison by one-way ANOVA with LSD test for the final number of germinated seeds in weathering experiment (180 days)

Type[a]	Mean ± SD		
	Tree I	Tree K	Tree B
Out	**3.00 ± 1.00**[b]	4.67 ± 1.15[be]	**10.00 ± 6.55**[ce]
In	37.67 ± 1.53[a]	4.00 ± 1.00[b]	27.33 ± 0.58[d]

Out and *In* indicate weathering treatment outdoors and dry storage in an incubator (3.8°C) during winter, respectively. Means that share the same letter did not differ significantly ($P > 0.05$). Bold values indicate significant differences between Out and In within the same seed source. All 3 replications with 50 seeds per replicate

[a] Type of cold stratification before experiment

was stripped off, exposing a pale-yellowish cotyledon, which should strongly affect seed sensitivity to microbial and fungal attacks. This result implies that it is not adaptive for fresh seeds from Tree B to remain in the soil. In turn, germination curves in Tree I did not differ markedly between Fig. 1 and Fig. 4a. Besides, most seeds from Tree K did not germinate after cold stratification. These results imply that cold stratification during winter had little influence on the break of seed dormancy in *R. pseudoacacia*.

Response of seed germination to diurnal thermal conditions

In this section, we discuss the seed germination for control responses in both the diurnal thermal regime experiment and the heat shock experiment. The difference between the burial treatment and control will be discussed in the next section.

Weak physical dormancy was observed in most seeds from Tree I. Tree I seeds germinated in response to a wide range of heat shock treatments (Fig. 5, Table 2). Additionally, the final number of germinated seeds did not differ

significantly among diurnal thermal regime experiments (Table 1), implying that weak physical dormancy in Tree I seeds could be broken by moderate temperatures. The number of dead seeds increased significantly with increasing temperature and treatment time (Table 2), suggesting that seeds from Tree I are less tolerant of high temperatures. Because all dead seeds absorbed water before decay, high mortality in high heat shock treatment (60°C) may be caused by denaturation at high temperatures.

Because the seeds from Tree I did not absorb water during winter (Figs. 2, 3) and were unlikely to be attacked by microbes or fungi in the soil (Fig. 3), these seeds can form a soil seed bank. We are still investigating whether seeds from Tree I remain dormant in the soil for years. In another experiment, we found that seeds from another tree did not absorb water in the soil until the end of the second winter, although the seeds germinated after first wintering in a laboratory (Yamada and Masaka, unpublished data). Furthermore, some seeds collected from natural soil seed banks germinated in the laboratory without any treatment (Masaka and Yamada, unpublished data). These facts imply that factors other than the temperature may also influence the break of seed dormancy.

The physical dormancy observed in Tree K seeds implies that most seeds form a soil seed bank under natural conditions. In Tree K seeds, neither a diurnal thermal regime after cold stratification (Fig. 4, Table 1) nor weathering during winter affected the break of physical dormancy (Fig. 6, Table 3). The seeds responded to a long, high temperature treatment (Fig. 5, Table 3). This condition may occur in the soil during a fire. Thus, our results imply that seeds from Tree K may respond to fire under natural conditions; the original range of *R. pseudoacacia* includes the Appalachian Mountains (Huntley 1990), where fire plays an important role in forest dynamics (Elliott et al. 1997, 1999; Lafon et al. 2005) and *R. pseudoacacia* forms a soil seed bank (Lambers et al. 2005). Seeds may rarely contribute to regeneration in Japan or other humid regions because they are not fire-prone ecosystems.

The heat shock treatment was not as effective for seed germination in Tree B seeds as it was for seeds from Tree K and Tree I, because the seeds showed no physical dormancy. Many Tree B seeds died due to heat shock (Fig. 5, Table 4). There was no significant difference in the number of germinated seeds from Tree B among thermal regimes in the control (Table 1). As long as the seeds from Tree B could escape from microbial and fungal attacks, the seeds would be able to germinate immediately even in a cool spring.

Physical dormancy induced by cold, and the ecological significance of an aerial seed bank.

The number of germinated seeds from Trees I and B in the weathering treatment was significantly lower than that in the control (Fig. 6, Table 5); the number of germinated seeds did not differ significantly in Tree K seeds (Fig. 6, Table 5). These results indicate that seeds from Trees I and B acquire physical dormancy by weathering during winter (Fig. 6). Similarity in germination curve between fresh seeds (Fig. 1) and seeds stored in dry conditions in an incubator during winter (Fig. 6), especially in Tree I, imply that freezing rather than dry conditions influence the induction of physical dormancy in *R. pseudoacacia* seeds. This induction of physical dormancy is a surprising result because a freezing or chilling treatment generally enhances the break of seed dormancy. This result suggests that seed pods remaining in the crown function as an aerial seed bank. Aerial seed banks are often observed in fire-prone ecosystems (e.g., Harper 1977; Lamont et al. 2003). *R. pseudoacacia* may be able to produce various levels in dormancy with respect to weather conditions during the winter.

The ecological significance of the induction of physical dormancy by weathering seems to differ between Trees B and I. Seeds from Tree I first showed weak physical dormancy (Figs. 2, 3) and were unlikely to be attacked by microbes or fungi in the soil during winter (Fig. 3). Tree I may produce various levels in physical dormancy in response to the thermal environment experienced during winter. In contrast, seeds from Tree B were unable to overwinter in the soil. Therefore, the seed pods that remain in the crown escape the microbial and fungal attack experienced by seeds in the soil.

Induction of physical dormancy was also found in the burial treatment (Tables 1, 2). In Tree I seeds, there were significantly more dormant seeds in the burial treatment than in the control under all thermal regimes (Table 1) and in the 50°C heat shock treatments (Table 2). The difference between the burial treatment and the control can be explained by the difference in thermal environment between the soil surface (nearly 0°C under snow cover) and the incubator (3.8°C). These results also suggest that physical dormancy was induced by freezing. Significantly more seeds from Tree I germinated and died in the control than in the burial treatment (Table 2), also reflecting the difference in the number of physically dormant seeds. Thus, seeds in weak physical dormancy are more likely to die after long, high heat shock treatment than seeds in physical dormancy. Further study is necessary to confirm anatomical changes in the seed coat caused by freezing.

Induction of physical dormancy in Trees I and B is an adaptive character. Because *R. pseudoacacia* is a typical pioneer species, seedlings cannot survive under a forest canopy. The seeds have to remain dormant until the appearance of bare ground created by a disturbance. However, we did not study the demography of seed pods in the crown. Differences in the manner of seed shedding among trees need to be investigated in relation to seed dormancy variation.

In conclusion, seed dormancy of *R. pseudoacacia* was complex. We found that seed germination characters differed markedly among only three trees. It implies that the original geographic variation might be disrupted during introductions, and more varied patterns may be found in nature. This variability has enabled *R. pseudoacacia* to spread rapidly worldwide. Geographic variation in seed germination characters in the original habitat should be evaluated to understand the reproductive traits of *R. pseudoacacia*. The dynamics of the aerial and soil seed banks will be verified in future studies.

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