Pericarp-mediated chemical dormancy controls the fruit germination of the invasive hoary cress (*Lepidium draba*), but not of hairy whitetop (*Lepidium appelianum*)

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Abstract

This study provides a comparative analysis of the dormancy and germination mechanisms of the indehiscent fruits of hoary cress (*Lepidium draba* L.) and hairy whitetop (*Lepidium appelianum* Al-Shehbaz), two invasive weeds of the Brassicaceae. Germination assays comparing isolated seeds (manually removed from the fruits) and intact indehiscent fruits showed that the isolated seeds are nondormant and provided full germination for both species. In contrast to this, the species differed in the germination properties of their indehiscent fruits, in that *L. appelianum* fruits were nondormant, while the *L. draba* fruit coat (pericarp) conferred a coat-imposed dormancy. The pericarp of *L. draba* fresh fruit was water permeable, and neither mechanical scarification nor surface sterilization affected germination, supporting the concept that pericarp-mediated dormancy was not due to water impermeability or mechanical constraint. Washing of *L. draba* fruits with water, afterripening (dry storage), and treatment with gibberellin (GA) stimulated the germination of this species, all of which are indicative of physiological dormancy. Analyses of endogenous abscisic acid (ABA) and GA levels combined with treatment experiments with wash water from fresh and afterripened *L. draba* pericarps and with ABA dose–response quantification of germination revealed that ABA is a key component of a pericarp-mediated chemical dormancy in this species. Consistent with this, pericarp ABA levels decreased during afterripening and upon fruit washing, and isolated fresh or afterripened seeds did not differ in their ABA sensitivities. The possible roles of the ABA-mediated pericarp dormancy for the germination ecophysiology and weed management of these species are discussed.

Introduction

The noxious and invasive weeds hoary cress [*Lepidium draba* L.; also known as *Cardaria draba* (L.) Desv. or heart-podded hoary cress] and hairy whitetop (*Lepidium appelianum* Al-Shehbaz; also known as globe-podded hoary cress) belong to the Brassicaceae (Francis and Warwick 2008). These closely related species rank 8th out of the 45 most frequently listed noxious weeds of agricultural land, pastures, and riparian and waste areas in the western United States and Canada (Supplementary Figure S1; Mulligan 2002; Mulligan and Findlay 1974; Skinner et al. 2000). Both *L. draba* and *L. appelianum* are native to Eurasia and have high competitiveness and invasiveness in their native, expanded, and introduced ranges (Francis and Warwick 2008; Hinz et al. 2012). *Lepidium draba* and *L. appelianum* occur on a variety of soil types, including saline soils where moisture is in at least moderate supply (Darbyshire 2003; Hooks et al. 2018). They grow in regions with abundant irrigation (Francis and Warwick 2008), wet and dry grasslands, scrublands, and arid regions with alkaline soils (Mulligan 2002; Mulligan and Findlay 1974). The reproductive biology of *L. draba* is, at least in part, responsible for its wide distribution, but very little is known about the underpinning mechanisms. What is known about it is only based on studying the germination and seedling growth of "isolated" seeds, that is, seeds manually extracted from their fruits (Hooks et al. 2018; Rezvani and Zaefarian 2016; Rezaee et al. 2018). However, a key feature of *L. draba* and *L. appelianum* is that they produce indehiscent fruits that do not open to release seeds (Mühlhausen et al. 2013;
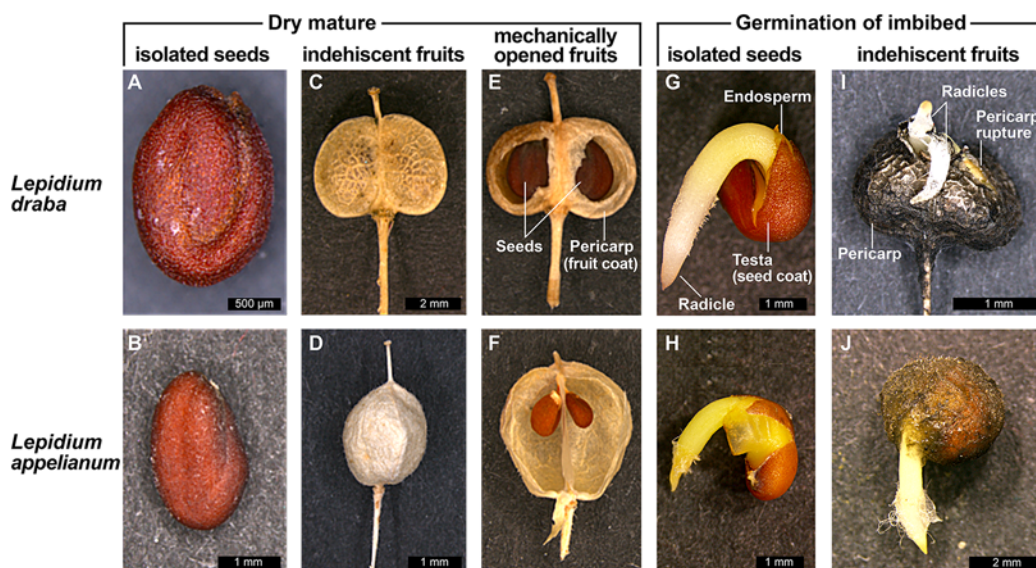
Figure 1. Seed and fruit structure and germination of *Lepidium draba* and *Lepidium appelianum*. Seeds tightly adhere to the fruit wall in *L. draba* but not in *L. appelianum*. (A) *Lepidium draba* seed (oval); (B) *L. appelianum* seed (oval and flattened); (C) *L. draba* fruit (heart-podded); (D) *L. appelianum* fruit (globe-podded); (E) *L. draba* manually opened fruits, seeds are tightly adhered to the pericarp (fruit wall); and (F) *L. appelianum* manually opened fruits, seeds are loosely adhered to the fruit wall. Radicle emergence through the ruptured testa and endosperm marks the completion of germination of imbibed seeds of *L. draba* (G) and *L. appelianum* (H). (I) Pericarp rupture and radicle emergence as visible events marking the completion of *L. draba* fruit germination. (J) Pericarp rupture and radicle emergence following the seed germination within the *L. appellianum* fruits. A Leica M165 FC Fluorescence Classic Stereomicroscope (Wetzlar, Germany) was used to take pictures of seeds and fruits.

Mummenhoff et al. 2009). This means that *L. draba* and *L. appelianum* seeds are dispersed encased in their fruit coat (pericarp) as indehiscent fruits (Figure 1C and D). Neither the dormancy and germination mechanisms of these indehiscent fruits nor the possible role of the pericarp in the control of fruit germination timing have been studied.

Molecular phylogenetic studies within the genus *Lepidium* revealed that indehiscent fruits (not releasing seeds) evolved independently several times from dehiscent fruits, that is, fruits that open at maturity to release seeds (Mühlhausen et al. 2013; Mummenhoff et al. 2009). These very closely related study species with indehiscent fruits, *L. draba* and *L. appelianum*, are therefore embedded in an abundant number of *Lepidium* species with dehiscent fruits. Indehiscent fruits may have evolved for several reasons: escape in time and space (Eriksson 2008; Hu et al. 2010), protection of seeds against predation (Mamut et al. 2014; Ohadi et al. 2011), or high soil-surface temperatures (Mamut et al. 2014; Moreira and Pausas 2012). Alternatively, fruits may control germination timing via pericarp-imposed dormancy to ensure that seedling establishment occurs in the right season (Hu et al. 2010; Ohadi et al. 2011; Mamut et al. 2014; Sperber et al. 2017).

Lepidium species with dehiscent fruits are known to have either physiologically dormant (PD) or nondormant (ND) seeds (Baskin and Baskin 2014; Finch-Savage and Leubner-Metzger 2006; Willis et al. 2014). Garden cress (*Lepidium sativum* L.) is an example of a species that produces ND seeds, and its endosperm acts as a constraint to radicle protrusion (Linkies et al. 2009; Müller et al. 2006; Steinbrecher and Leubner-Metzger 2017). ND seeds have the capacity to germinate over the widest range of normal physical environmental conditions. Warty peppercress (*Lepidium papillosum* F. Muell.) and mouse-ear cress [*Arabidopsis thaliana* (L.) Heynh.] are Brassicaceae species that produce PD seeds (Graeber et al. 2010, 2013). This form of dormancy provides seasonal cueing to ensure that germination occurs only upon specific environmental triggers (Baskin and Baskin 2004, 2014; Finch-Savage and

Leubner-Metzger 2006; Willis et al. 2014). Rupture of the testa (seed coat) and rupture of the endosperm are two sequential events during the germination of *L. sativum* and *A. thaliana* (Graeber et al. 2014; Steinbrecher and Leubner-Metzger 2017). Abscisic acid (ABA) specifically inhibits the endosperm rupture of these two Brassicaceae species (Graeber et al. 2014; Linkies et al. 2009; Müller et al. 2006; Steinbrecher and Leubner-Metzger 2017; Voegele et al. 2011). The seed dormancy–specific gene *Delay of Germination1* (*DOG1*) is widespread, including within the genus *Lepidium*, and together with ABA controls the dormant state and seed response toward environmental conditions (Graeber et al. 2010, 2013, 2014). While ABA inhibits germination and maintains PD, the antagonistically acting gibberellins (GA) release PD and promote germination of ND and PD seeds (Finch-Savage and Leubner-Metzger 2006). Rezvani and Zaefarian (2016) demonstrated that GA treatment replaces the light required for the germination of *L. draba* "isolated" (i.e., manually removed from fruits) seeds, but nothing is known about the hormonal and pericarp-associated mechanisms underpinning the germination of *L. draba* and *L. appelianum* indehiscent fruits.

The pericarp (fruit coat) can control germination and seedling establishment timing by inhibiting or delaying water uptake (Cousens et al. 2010; Sperber et al. 2017); via germination-inhibiting chemicals, including ABA (Baskin and Baskin 2014; Mamut et al. 2014; Sari et al. 2006); or by other means of inhibiting radicle protrusion, including pericarp-imposed mechanical dormancy (Cousens et al. 2010; Lu et al. 2015; Neya et al. 2008; Sperber et al. 2017; Steinbrecher and Leubner-Metzger 2017). Within the Brassicaceae, the known cases of pericarp-imposed dormancy are not due to complete water impermeability of fruit and seed coats, and the encased seeds are either ND or PD. In lesser swinecress (*Lepidium didymum* L.) fruits, for example, the ND seeds are encased by a hard pericarp that confers a mechanical constraint to full water uptake required for the completion of germination by

radicle protrusion (Sperber et al. 2017). In the case of *L. didymum*, the tight encasement of the seeds by the pericarp, which prevails even after the pericarp-mediated dormancy has been released, does not allow the seeds to germinate within the fruits. It is therefore the fruit itself that eventually completes germination, with visible radicle emergence through all the layers covering the seed (endosperm, testa) and the fruit (pericarp). Similar cases in which the germination of PD or ND seeds and their radicle emergence from the dispersed indehiscent fruits are restrained by the water-permeable pericarp have been described (Cousens et al. 2010; Lu et al. 2015, 2017; Zhou et al. 2015). There are, however, cases in which the seed or fruit coats confer complete water impermeability (Baskin and Baskin 2003; Gama-Arachchige et al. 2013; Smykal et al. 2014; Steinbrecher and Leubner-Metzger 2018). This—and only this—is then called physical dormancy, which has water-impermeable seeds and/or fruit coats as its hallmark (Baskin and Baskin 2003, 2014; Steinbrecher and Leubner-Metzger 2017, 2018).

The properties, possible roles, and mechanisms of the pericarp in the germination of indehiscent fruits of *L. draba* and *L. appelianum* have not been studied. Because noxious and invasive weeds are a major concern for agriculture and biodiversity, knowing the ecophysiological mechanisms of the indehiscent fruits of *L. draba* and *L. appelianum* is important to inform effective management strategies.

Materials and Methods

Seed Sources

Two *L. draba* accessions from two continents, KM 1296 (from Logan's Market, Malheur, OR, USA) and KM 1568 (from a vineyard near Hayesdorf, Austria) were used for this work. Freshly harvested mature fruits of *L. draba* (KM 1296 and KM 1568) and *L. appelianum* (KM 1754; obtained from J Gaskin, USDA, Fremont County, Wyoming, USA) were collected from plants cultivated in the Botanical Garden, Osnabrueck University, Germany, in 2014 to 2015. In addition, fresh mature fruits were harvested for both *L. draba* accessions in 2015 to 2016, and further for KM 1568 in 2016 to 2017. After drying at room temperature for 10 d, fruits with encased seeds were sealed in aluminum bags, vacuumized, and stored at −20 C for up to 3 wk to retain their fresh mature status until experiments were initiated, following a protocol described by Baskin and Baskin (2014). Initial tests with freshly harvested material revealed that the 3-wk storage did not affect the maximum germination of the fruits, demonstrating the fresh mature state was preserved during the storage at −20 C.

Germination Assays, Afterripening Storage, and Treatments

Germination was quantified using "isolated seeds" (seeds manually removed from the fruits by mechanically opening the pericarp; Figure 1) and indehiscent fruits (seeds enclosed within pericarps). Three technical replicates, each containing 25 fresh isolated seeds and 25 fruits as biological replicates were assigned to germination assays as follows. Germination assays were carried out under a 12/12 h light regime (white light at ~100 µmol m^{-2} s^{-1}) at optimum temperature (25/15 C, 12/12 h) for the species studied. Isolated seeds and fruits (seeds within pericarp) were incubated for 28 d, and visible protrusion of the radicle was recorded as the completion of germination (Baskin and Baskin 2014; Mamut et al. 2014; Tang et al. 2010; Zhou et al. 2015). To determine whether

dormancy is released during dry afterripening storage, isolated seeds and fruits were stored in laboratory conditions (25 ± 2 C, 51% relative humidity) for 0 (fresh as the control), 4, 8, 12, and 16 wk. To investigate whether cold stratification releases dormancy, isolated seeds and fruits were incubated in the imbibed state in darkness at 4 C for 0 (fresh as the control), 4, 8, 12, and 16 wk. To investigate how gibberellic acid (GA$_3$; CAS: 77-06-5, A4586, AppliChem GmbH, Darmstadt, Germany) or (±)-abscisic acid (ABA; CAS: 14375-45-2, A1049, Sigma-Aldrich, USA) affects germination, isolated seeds and fruits were incubated without (distilled water as the control) or with a defined hormone concentration dissolved in dimethyl sulfoxide (ca. 0.01% v/v) added.

Water Uptake, Mechanical Constraint, and Chemical Inhibitor Experiments

To investigate whether the pericarp is water permeable or not, water imbibition was compared between fresh isolated seeds and fruits. Three replicates of 20 fresh isolated seeds and three replicates of 10 intact fruits were compared. Each replicate was weighed using an electronic balance and placed on filter paper moistened with distilled water in petri dishes. Before being weighed, seeds were blotted with paper towels to remove excess moisture at 0, 1, 3, 6, 9, 12, and 24 h, and at 2-d intervals thereafter until the final constant mass was achieved, following a methodology described by Mamut et al. (2014). Percentage of increase in mass was calculated as $[(W_i − W_d)/W_d] \times 100$, where W_i is mass of imbibed seeds within pericarp or fresh isolated seeds and W_d is mass of dry seeds within pericarps or of fresh isolated seeds (Baskin and Baskin 2014; Liu et al. 2015; Mamut et al. 2014). To test whether the pericarp mechanically constrains germination or not, the following germination tests were compared: (1) The pericarp was completely removed without damaging the seeds (Lu et al. 2015; Mira et al. 2015). (2) The whole dispersal unit (fruit) was tested as a control (Hu et al. 2010; Liu et al. 2015). (3) The pericarp was mechanically scarified with a razor blade without damaging the seeds to test whether it prevents the protrusion of the radicle or not (Mira et al. 2015). This scarification removed a small piece of pericarp layer in the region where the radicle end of the seed is localized. (4) Surface sterilization of fruits was used as another comparison (Sperber et al. 2017). To test whether the pericarp confers a chemical dormancy to constrain germination or not, the following germination tests were compared: (1) The pericarp was completely removed without damaging the seeds (Lu et al. 2015; Mira et al. 2015). (2) The whole dispersal unit (seeds within pericarp) was tested as a control (Hu et al. 2010; Liu et al. 2015). (3) Scarified fruits with the mechanical constraint completely removed were analyzed to determine whether soluble chemicals would inhibit germination by leach out from the pericarp (Baskin and Baskin 2014; Mamut et al. 2014). (4) Seeds within the pericarp were washed for 24 h to show whether chemical inhibitors are removed by washing with a large volume of water or not (Hu et al. 2010). Moreover, seeds within the pericarp washed in 1 L of de-ionized water for 0 h (nonwashed as the control) were compared with seeds within pericarp washed for 6, 12, 18, and 24 h and fresh isolated seeds (nonwashed) to show the effect of pericarp-mediated chemical inhibitors on germination. Furthermore, fresh and afterripened pericarps 300 mg were washed with 3 ml of distilled water using a shaker at 100 rpm for 6 h, and wash water from fresh pericarp, afterripened pericarp, or previously washed fresh pericarp (rewashed) was applied for germination tests.

Quantification of Endogenous ABA and GA Metabolites

The ratio between ABA (induces dormancy) and bioactive GA (induces germination) controls seed germination (Baskin and Baskin 2014; Finch-Savage and Leubner-Metzger 2006; Née et al. 2017). Fresh and afterripened isolated seeds, fresh and afterripened pericarp tissues, fresh pericarp tissues washed for 0 h (nonwashed as the control) were compared with fresh pericarp tissues washed 6, 12, 18, and 24 h to study the levels of endogenous ABA and GA metabolites. For ABA metabolite analysis, plant tissue (approximately 20 mg of each sample's dry weight) were homogenized and extracted for 1 h in 1 ml ice-cold methanol/water/acetic acid (10/89/1, v/v/v). Internal standard mixtures, containing 20 pmol each of $(-)$-$7',7',7'$-$[^2H_3]$-phaseic acid; $(-)$-$7',7',7'$-$[^2H_3]$-dihydrophaseic acid; $(-)$-$8',8',8'$-$[^2H_3]$-neophaseic acid; $(+)$-$4,5,8',8'$-$[^2H_5]$-ABAGE; $(-)$-$5,8',8',8'$-$[^2H_4]$-$7'$-OH-ABA, and $(+)$-$3',5',5',7',7',7'$-$[^2H_6]$-ABA were added to each of the samples. The homogenates were centrifuged ($36,670 \times g$, 10 min, 4 C) after extraction, and the pellets were then re-extracted in 0.5 ml extraction solvent for 30 min. The combined extracts were purified by solid-phase extraction on Oasis® HLB cartridges (60 mg, 3 ml; Waters, Milford, MA, USA), then evaporated to dryness in a Speed-Vac (UniEquip, Planegg, Germany), and finally analyzed by UPLC-ESI $(\pm)$-MS/MS (ultra-performance liquid chromatography-electrospray ionization tandem mass spectrometry) (Turečková et al. 2009). For GA metabolite analysis, sample preparation and analysis were performed according to the method described in Urbanová et al. (2013) with some modifications. Briefly, 5-mg freeze-dried seed samples were ground to a fine consistency using 3-mm zirconium oxide beads (Retsch, Haan, Germany) and an MM 301 vibration mill at a frequency of 30 Hz for 3 min (Retsch) with 1 ml of ice-cold 80% acetonitrile containing 5% formic acid as extraction solution. The samples were then extracted overnight at 4 C using a benchtop laboratory rotator (Stuart SB3, Bibby Scientific, Staffordshire, UK) after adding 17 internal gibberellin standards ($[^2H_2]GA_1$, $[^2H_2]GA_3$, $[^2H_2]GA_4$, $[^2H_2]GA_5$, $[^2H_2]GA_6$, $[^2H_2]GA_7$, $[^2H_2]GA_8$, $[^2H_2]GA_9$, $[^2H_2]GA_{15}$, $[^2H_2]GA_{19}$, $[^2H_2]GA_{20}$, $[^2H_2]GA_{24}$, $[^2H_2]GA_{29}$, $[^2H_2]GA_{34}$, $[^2H_2]GA_{44}$, $[^2H_2]GA_{51}$, and $[^2H_2]GA_{53}$; purchased from Lewis Mander, Canberra, Australia). The homogenates were centrifuged at 36 670 $\times g$ (10 min, 4 C, using a Beckman Coulter Avanti™ 30, Indianapolis, IN, USA) and 4 C for 10 min and the corresponding supernatants were further purified using reversed-phase and mixed-mode SPE cartridges (Waters) and analyzed by ultra-high-performance chromatography–tandem mass spectrometry (Micromass, Manchester, UK). GA metabolites were detected using the multiple-reaction monitoring mode for the transition of the ion $[M–H]^-$ to the appropriate product ion. Masslynx v. 4.1 software (Waters) was used to analyze the data, and the standard isotope dilution method (Rittenberg and Foster 1940) was used to quantify the GA levels.

Data Analysis

One-way ANOVA was conducted to analyze the association between treatments for the water imbibition, the various germination assays, and the levels of ABA and GA metabolites. Data were subjected to one-way ANOVA, with post hoc comparisons made by a Tukey's honest significant difference test. The rejection threshold for all analyses was P < 0.05. SigmaPlot v. 13 (Systat Software, San Jose, CA, USA) and PRISM v. 7.0a (GraphPad, San Diego, CA, USA) were used to generate graphs of the results.

Results and Discussion

Comparative Germination Analysis of Isolated Seeds and Indehiscent Fruits

The dispersal units of the related invasive, noxious, and weedy Brassicaceae species *L. draba* and *L. appelianum* are indehiscent fruits (Figure 1). The aim of the present study was to comparatively investigate the roles of the fruit coat (pericarp) in the dormancy mechanisms and germination biology of these species. To achieve this, the germination of isolated seeds (i.e., seeds manually removed from fruits by opening the pericarp; Figure 1A and B) was compared with germination of indehiscent fruits (Figure 1C and D). Visible emergence of the radicle through all covering layers (endosperm, testa, pericarp) was used as the criterion to score the completion of germination of seed or fruit populations. For isolated seeds, germination was accompanied by testa and endosperm rupture (Figure 1G and H) as described for the seeds of *L. sativum* (Müller et al. 2006). For the indehiscent fruits of *L. draba*, the tight encasement of the seeds by the pericarp did not allow the seeds to germinate within the fruits. Instead the fruit itself eventually completed germination with visible radicle emergence through all the covering layers of the seed (endosperm, testa) and fruit (pericarp) and visible pericarp rupture (Figure 1I). The pericarp rupture and visible radicle protrusion next to the tip of the *L. draba* fruit is therefore mechanically very similar to the fruit germination of *L. didymum* (Sperber et al. 2017). For *L. appelianum* fruits, the seeds germinated within the fruits and subsequent embryo expansion eventually led to radicle emergence through the pericarp (Figure 1J).

The objectives of the first set of experiments were to identify the seed dormancy class (Baskin and Baskin 2004; Finch-Savage and Leubner-Metzger 2006; Willis et al. 2014) and to reveal the role of the pericarp. Therefore, the germination of freshly harvested mature fruit and isolated seed populations of *L. draba* and *L. appelianum* were compared with their germination responses in the afterripened state (Figure 2; Supplementary Figure S2) and their responses to cold stratification (Figure 2) and to GA treatment (Figure 3). Fresh isolated seed populations germinated at a high percentage (ca. 90%) in both species. The high percentage of germination of isolated seeds was not appreciably affected by dry afterripening storage (Figure 2A; $F(4, 20) = 0.834$, P = 0.433), cold stratification (Figure 2B; $F(4, 20) = 1.371$, P = 0.274), and treatment of isolated seeds with GA$_3$ (Figure 3A; $F(4, 10) = 0.06$, P = 0.992). It is well established that these treatments release physiological dormancy (Baskin and Baskin 2014; Finch-Savage and Leubner-Metzger 2006; Willis et al. 2014). The isolated seeds of both the U.S. (KM 1269) and the Austrian (KM 1568) *L. draba* accessions germinated to a high percentage (ca. 90%) independent of the harvest year (2014 to 2015, 2015 to 2016, or 2016 to 2017) and state (fresh vs. afterripened) (Supplementary Figure S2 and corresponding statistics in Supplementary Table S1). Because both freshly harvested and afterripened isolated seed populations of *L. draba* and *L. appelianum* germinated at a high percentage and were not affected by these treatments, we conclude that these seeds are physiologically ND at maturity.

Analysis of endogenous GA metabolite levels in fresh and afterripened seed and pericarp tissues in *L. draba* revealed the presence of the bioactive gibberellins GA$_1$, GA$_3$, GA$_4$, and GA$_7$ (Figure 3B), as well as their precursors and inactive metabolites (unpublished data). The dry seed and pericarp contained nanogram quantities of these GA metabolites, with GA$_1$ being the dominant bioactive GA. No striking differences were evident between fresh and
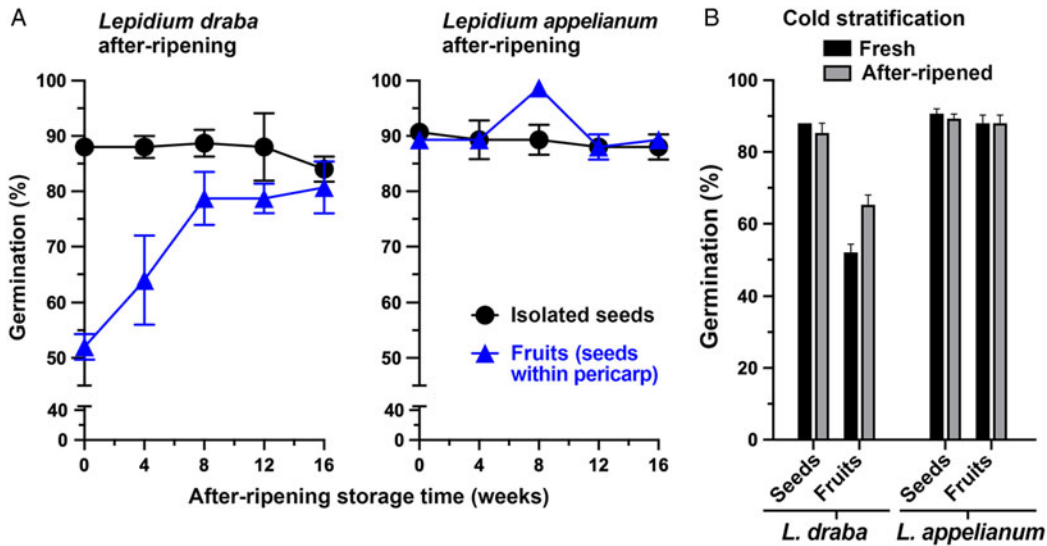
Figure 2. The effect of afterripening and cold stratification on the germination of *Lepidium draba* and *Lepidium appelianum* isolated seeds and indehiscent fruits (seeds within pericarp). (A) The effect of afterripening (dry) storage at room temperature and humidity. (B) The effect of cold stratification in the imbibed state under dark conditions in a refrigerator (4 C). Mean values ± SE ($N = 3 \times 25$) of accessions KM 1296 and KM 1754 (2014 to 2015 harvest) at optimal germination assay conditions (12/12-h light regime at 25/15 C day/night for 28 d) are presented.
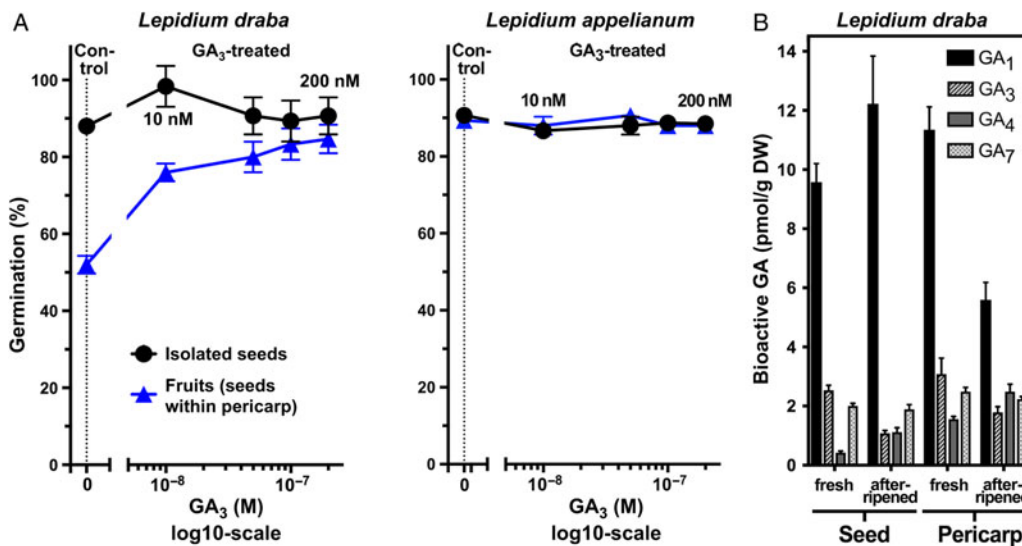

Figure 3. The effect of gibberellic acid (GA_3) treatment on the germination of *Lepidium draba* and *Lepidium appelianum* fresh and afterripened seeds and fruits and the levels of endogenous bioactive gibberellins (GA). (A) Dose response for the effects of exogenous GA_3 on germination responses of fresh isolated seeds and fruits (seeds within pericarp). Mean values ± SE ($N = 3 \times 25$) of accessions KM 1296 and KM 1754 (2014 to 2015 harvest) at optimal germination assay conditions (12/12-h light regime at 25/15 C day/night for 28 d) are presented. (B) Endogenous levels of bioactive gibberellins (GA_1, GA_3, GA_4, and GA_7) in fresh and afterripened seeds and pericarps of *L. draba*. $N = 4 \times 20$ mg (dry weight, DW) of seed/pericarp are presented.

afterripened seeds (Figure 3B). The finding that fresh and afterripened seeds do not appreciably differ in the levels of bioactive GAs further supports the conclusion that *L. draba* seeds are ND.

Our study species, *L. draba* and *L. appelianum*, are highly invasive weeds throughout the Middle East, Asia, Australia, New Zealand, Canada, and the western United States (Chipping and Bossard 2000; Gaskin 2006; Gaskin et al. 2005; McInnis et al. 2003) and are dispersed in disturbed areas, including roadsides. Analysis of their global distribution using the Global Biodiversity Information Facility (https://www.gbif.org/) database suggests that their main occurrence in the western part of North America is expanding toward the eastern regions for both

species (Supplementary Figure S1). Bani-Aameur and Sipple-Michmerhuizen (2001) and Presotto et al. (2014) reported that the lack of dormancy in weedy species increases the survival ability of the species, because germination and early seedling growth are the most critical factors for species establishment. Therefore, higher germination and seedling recruitment have been recognized as being among the major factors promoting naturalization success of invasive species (Fernández-Pascual et al. 2013; Mandák 2003; Udo et al. 2016; Walck et al. 2011). Rezvani and Zaefarian (2016) found that isolated seeds of *L. draba* are light requiring and that GA_3 treatment can replace the light requirement to trigger germination. Similar to *L. draba* and *L. appelianum* (Figures 1–3), other

Brassicaceae species, including *Noccaea papillosa* (Boiss.) F. K. Mey (Kirmizi 2017), *L. sativum* (Graeber et al. 2014), and *L. didymum* (Sperber et al. 2017), produce ND seeds. In contrast to this, other Brassicaceae weedy species, for example, *Coincya rupestris* subsp. *leptocarpa* (Gonz. Albo) Leadlay, *Coincya rupestris* subsp. *rupestris* Porta & Rigo ex Rouy (Copete et al. 2005), clasping pepperweed (*Lepidium perfoliatum* L.) (Tang et al. 2010), *L. papillosum* (Graeber et al. 2013), *A. thaliana* (Baskin and Baskin 2014), *Chorispora sibirica* (L.) de Candolle, Syrian mustard [*Euclidium syriacum* (L.) W. T. Alton], *Goldbachia laevigata* (Marschall von Bieberstein) de Candole, *Spirorhynchus sabulosus* Karelin & Kirilov, *Sterigmostemum fuhaiense* H. L. Yang, *Tauscheria lasiocarpa* Fischer ex de Candolle (Lu et al. 2015), and *Isatis violascens* Bunge (Zhou et al. 2015) produce PD seeds (Baskin and Baskin 2014).

Distinct Roles of the Pericarp in Lepidium draba and Lepidium appelianum Fruit Germination

Figure 2 shows that while seeds of both species are physiologically ND and germinate readily, the two species differ in the germination responses of their indehiscent fruits. In populations of *L. appelianum* fruits, the seeds germinated within the fruits and subsequent radicle expansion led to emergence through the pericarp (Figure 1J) of ca. 90% already in the fresh mature state (Figure 2A). In contrast to this, populations of *L. draba* fresh fruits exhibited pericarp rupture and visible radicle emergence (Figure 1I), with only ca. 50% of the fruits completing germination (Figure 2A). Interestingly, afterripening for 16 wk resulted in ca. 90% fruits germinating in *L. draba* with visible pericarp rupture (Figure 2A). This finding for *L. draba* was evident for both the U.S. (KM 1269) and the Austrian (KM 1568) accessions and consistent over the harvest years (2014 to 2015, 2015 to 2016, 2016 to 2017) (Supplementary Figure S2 and corresponding statistics in Supplementary Table S1). As with afterripening, treatment with GA3 also increased the maximum fruit germination percentage of *L. draba* from ca. 50% to ca. 90% (Figure 3A). In contrast to this, cold stratification had no appreciable effect on the maximum germination percentage of *L. draba* (Figure 2B). When the germination responses of fruits and isolated seeds (Figures 2 and 3; Supplementary Figure S2) are compared, it is clear that in *L. appelianum* the pericarp has no effect on the maximum germination percentage of the population, but in *L. draba* it inhibited the germination of about half of the population. We conclude that while both species have ND seeds, the roles of the pericarps differ. In *L. appelianum*, the pericarp does not affect the germination capacity, while the indehiscent fruits of *L. draba* have pericarp-imposed dormancy. Interestingly, this pericarp-imposed dormancy of the *L. draba* indehiscent fruits can be released by afterripening and by GA3 treatment (Figures 2 and 3).

To further investigate the finding that the pericarp confers coat dormancy in *L. draba*, while it does not affect the germination in *L. appelianum*, we compared the patterns of water uptake of fruits and seeds. Figure 4 shows that the water uptake patterns of isolated seeds of both species were very similar, with three typical phases: imbibition (phase 1), plateau (phase 2), and completion of germination by radicle emergence and subsequent growth (phase 3) (Finch-Savage and Leubner-Metzger 2006). In Figure 4A a comparison of fresh mature fruits of *L. draba* with fresh mature isolated seeds shows that the pericarp slowed down the water uptake during imbibition (phase 2) and delayed the onset and rate of phase 3 water uptake, which in seeds is associated with radicle emergence
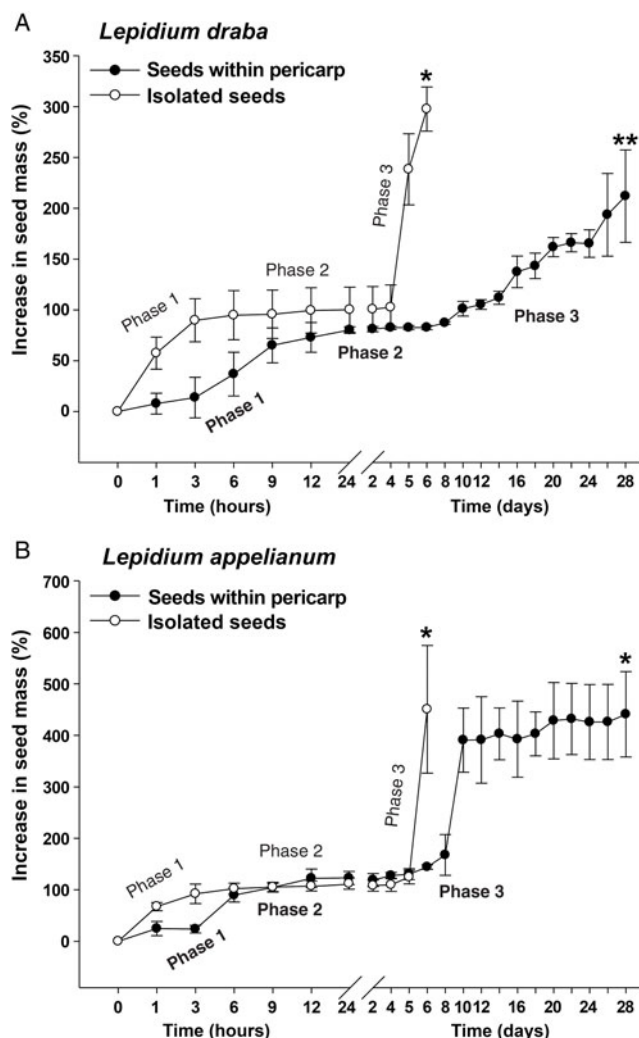

Figure 4. The effects of the pericarp (fruit coat) on the water uptake of (A) *Lepidium draba* and (B) *Lepidium appelianum* seeds. A single asterisk refers to the time of full (>90%) completion of germination of fresh isolated seeds or fruits (seeds within pericarp), whereas a double asterisk refers to the maximum germination (52%) due to the pericarp-mediated dormancy of *L. draba* (see Figure 2A). Isolated seeds and fruits exhibit a typical three-phase pattern of water uptake by seeds: phase 1 (imbibition) is followed by the plateau phase 2 (metabolic activation), and upon endosperm rupture, the radicle emergence is associated with phase 3 (water uptake indicative for the completion of germination). $N = 3 \times 20$ (fresh seeds) of accessions KM 1296 and KM 1754 (2014 to 2015 harvest); $N = 3 \times 10$ for each time point measured (fresh seeds within pericarp).

and embryo postemergence growth. While all *L. draba* seeds completed the germination process within ca. 6 d, even after 28 d, the *L. draba* fruit population had only reached ca. 50%. The results in Figure 4A demonstrate that the *L. draba* pericarp is water permeable, that is, the fruits do not have physical dormancy, but the pericarp confers coat dormancy in association with slowing down the water uptake and the transition to phase 3 and the completion of fruit germination. In contrast to this, Figure 4B shows that when fruits and isolated seeds of *L. appelianum* were compared, the pericarp did not appreciably affect the water uptake patterns and permitted maximum germination. We conclude that the pericarp-imposed dormancy of *L. draba* fruits is caused by some mechanism that decreases the water uptake and inhibits the transition from phase 2 to phase 3 required for the completion of germination.
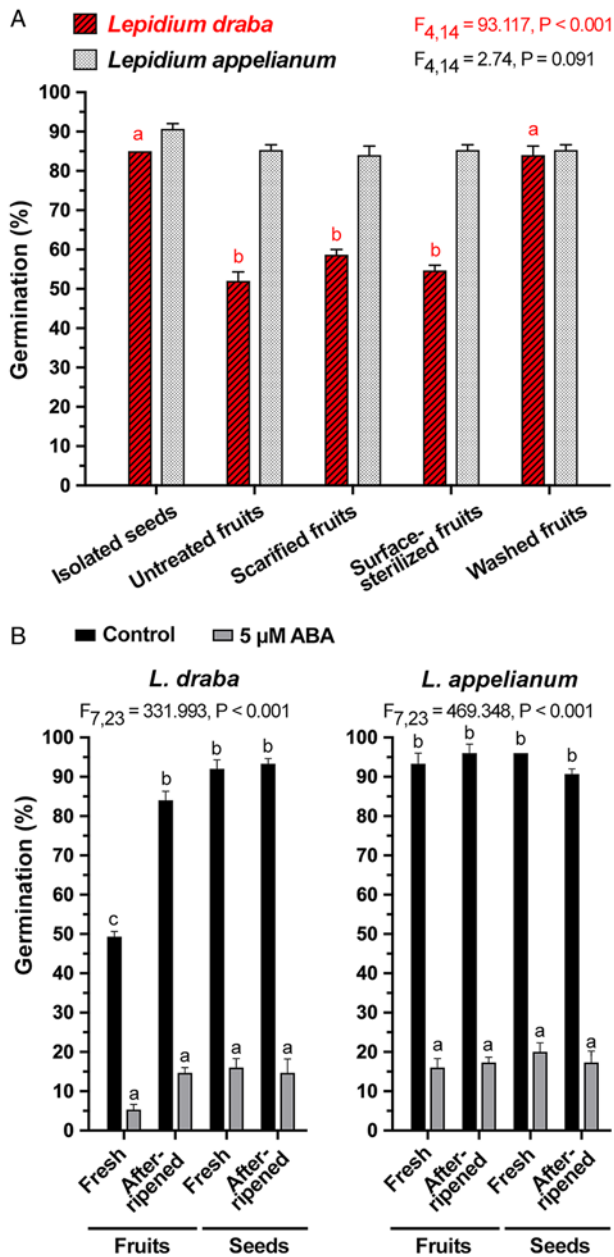
Figure 5. The effects of pericarp scarification, sterilization, washing, and abscisic acid (ABA) treatment on the germination of *Lepidium draba* and *Lepidium appelianum* freshly harvested mature fruits. (A) Germination of fresh isolated seeds, untreated fresh fruits (seeds enclosed within untreated pericarp), scarified fresh fruits (seeds enclosed within scarified pericarp, that is, mechanical constraint of pericarp removed by scarification with razor blade), surface-sterilized fresh fruits (seeds enclosed within surface-sterilized pericarp to eliminate possible microbial activity), and washed fresh fruits (fruits washed for 24 h to remove water-soluble chemical inhibitors) of *L. draba and L. appelianum*. (B) Germination of fresh and afterripened indehiscent fruits and isolated seeds without (control) and with addition of 5 μM ABA. Mean values ± SE ($N = 3 \times 25$) of accessions KM 1296 and KM 1754 (2014 to 2015 harvest) at optimal germination assay conditions (12/12-h light regime at 25/15 C day/night for 28 d) are presented. Different letters (a, b) designate significantly different mean values as determined by Tukey's pairwise multiple-comparison test ($P < 0.05$).

ABA-mediated Inhibition as Mechanism for the Pericarp-imposed Dormancy in Lepidium draba

To investigate the mechanisms through which the pericarp confers coat dormancy to seeds in *L. draba* fruits, we conducted scarification and leaching experiments. Figure 5A shows that neither pericarp scarification (i.e., removing a possible mechanical constraint by manually removing the part of the pericarp covering the seed's radicle end with razor blade) nor pericarp surface sterilization to eliminate possible microbial activity removed the pericarp-imposed dormancy. The germination percentages of mechanically scarified fruits (58%) and surface-sterilized fruits (54%) did not differ significantly from nonsterilized fruits (control, 52%) ($F(2, 120) = 3.8$, $P = 0.086$) of *L. draba*. The treatments did not affect the ca. 90% germination of subsequently isolated *L. draba* seeds, and the comparison with *L. appelianum* shows that neither the scarification nor the sterilization treatment had negative effects (Figure 5A). The finding that neither pericarp scarification nor surface sterilization affected the pericarp-mediated dormancy of *L. draba* strongly suggests that lack of germination is not mechanical in nature and that microbial activity is not involved in its release. It is therefore different from the recent finding in *L. didymum* that the pericarp-imposed dormancy is caused by a mechanical constraint and the microbial activity of common fungi is involved in the release of the pericarp-imposed dormancy (Sperber et al. 2017). Many other Brassicaceae species, including wild radish (*Raphanus raphanistrum* L.) (Cousens et al. 2010), *Diptychocarpus strictus* (Fischer ex Marschall von Bieberstein) Trautvetter (Lu et al. 2010), turnipweed [*Rapistrum rugosum* (L.) All.] (Ohadi et al. 2011), *Lachnoloma lehmannii* Bunge (Mamut et al. 2014), *C. sibirica*, *E. syriacum*, *G. laevigata*, *S. sabulosus*, *S. fuhaiense*, *T. lasiocarpa* (Lu et al. 2015), and *I. violascens* (Zhou et al. 2015) seem to have a coat dormancy with a mechanical component similar, at least in part, to the pericarp-imposed mechanical dormancy of *L. didymum* (Sperber et al. 2017). In contrast to this, in *L. appelianum*, the pericarp does not confer a coat dormancy at all.

In contrast to pericarp scarification, washing of fresh *L. draba* fruits removed the pericarp-imposed dormancy and caused ca. 90% germination, as is the case for the isolated ND seeds of *L. draba* (Figure 5A). For the ND *L. appelianum* fruits, germination occurs without the washing treatment (Figure 5A). This finding strongly suggests that chemical inhibitors present in the pericarp of fresh *L. draba* fruits cause the pericarp-imposed dormancy, while such inhibitors are lacking in *L. appelianum*. Washing may have caused leaching out of the chemical inhibitors from *L. draba* pericarps, thereby releasing the dormancy. To further quantify the effect of the inhibitors, we added wash water of *L. draba* pericarps to the germination media of fresh (Figure 6A) and afterripened (Figure 6C) *L. draba* seeds. The wash water from fresh *L. draba* pericarps caused a significant delay in the onset of seed germination and in the maximum germination percentages of fresh (Figure 6A) and afterripened (Figure 6C) seeds. In contrast to this, no inhibition of seed germination was obtained with wash water from afterripened or wash water from previously washed fresh pericarps (Figure 6A and C). It is therefore clear that only the fresh *L. draba* pericarp contains water-soluble germination inhibitors that leach out and thereby inhibit germination.

To investigate whether pericarp-released ABA is involved in mediating the pericarp-imposed dormancy of *L. draba*, we initially compared the germination responses to treatment with ABA of fresh and afterripened fruits and isolated seeds of both species. Treatment with 5 μM ABA inhibited the germination of fruits and seeds of both species in both physiological stages (Figure 5B). Fresh and afterripened seeds of both species seem to be equally sensitive to inhibition by ABA. Interestingly, seeds in fresh *L. draba* fruits were more sensitive to the inhibition compared with those in fresh *L. appelianum*
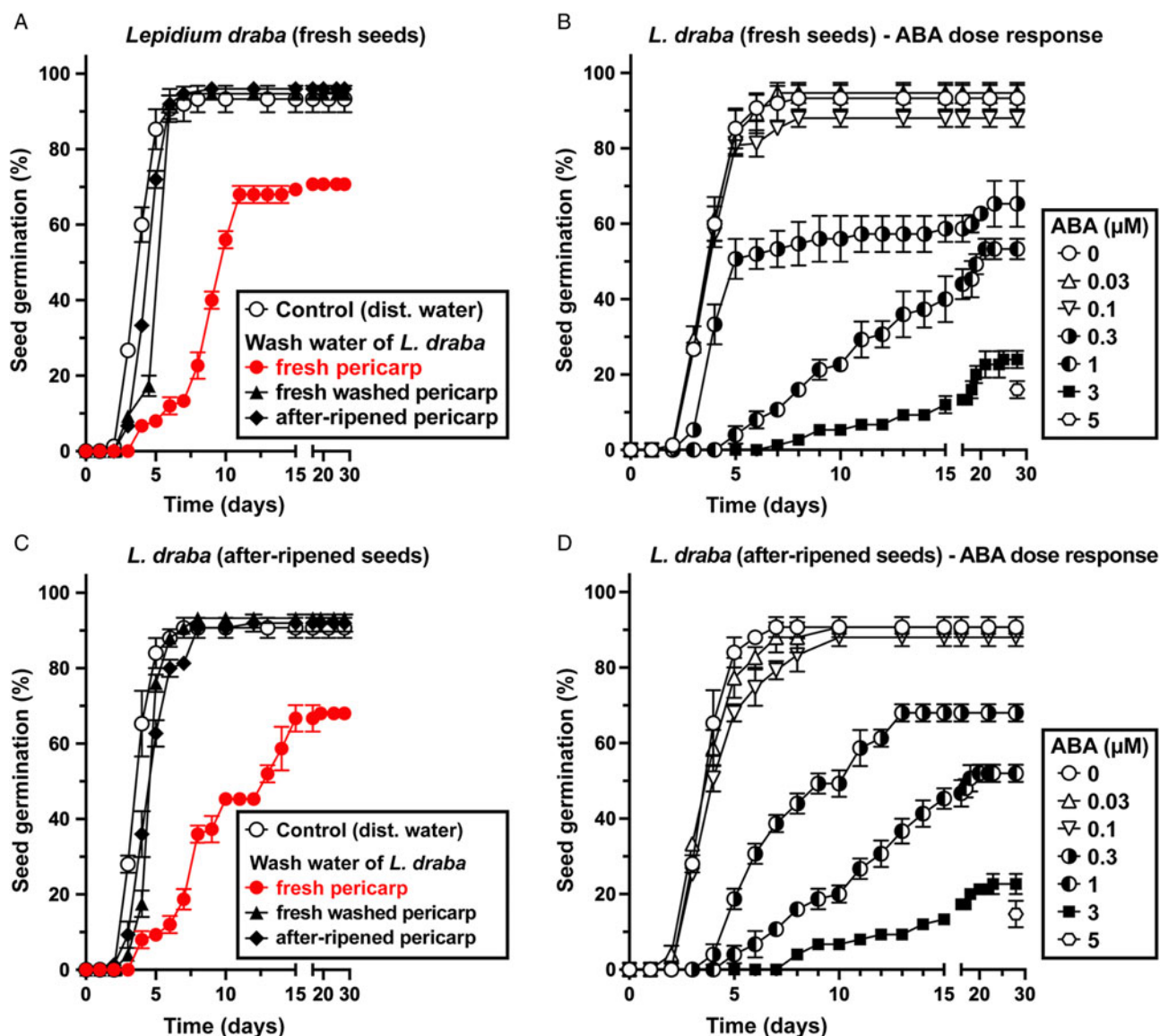
Figure 6. The effect of treatment with abscisic acid (ABA), wash water from fresh pericarp, wash water of washed fresh pericarp, and wash water of afterripened pericarp on the germination kinetics of *Lepidium draba* isolated seeds. (A) The effect of wash water from *L. draba* pericarp on the germination of *L. draba* fresh seeds. (B) Germination dose response of *L. draba* fresh seeds incubated with different ABA concentrations applied. (C) The effect of wash water from *L. draba* pericarp on the germination of *L. draba* afterripened seeds. (D) Germination dose response of *L. draba* afterripened seeds incubated with different ABA concentrations applied. Mean values ± SE ($N = 3 \times 25$) of accessions KM 1296 and KM 1754 (2014 to 2015 harvest) at optimal germination assay conditions (12/12-h light regime at 25/15 C day/night for 28 d) are presented. Pericarp tissues weighing 300 mg were washed with 3 ml of distilled water using a shaker at 100 rpm for 6 h to obtain the pericarp wash water applied in the germination assays.

fruits (Figure 5B). To precisely quantify the seed sensitivities, we conducted dose–response experiments for the ABA treatment with fresh (Figure 6B) and afterripened (Figure 6D) *L. draba* seeds. These results demonstrated that increasing ABA concentrations caused a similar delay in the onset of the completion of seed germination of fresh and afterripened *L. draba* seeds (Figure 6). Increasing ABA concentrations also caused a decrease in the maximum germination percentages reached after ca. 1 mo of incubation of the seed populations. This quantification demonstrated that the fresh and afterripened *L. draba* seeds had the same ABA sensitivity (Figure 7A). A comparison of these ABA sensitivity values with the results obtained with the wash water of fresh pericarp (Figure 7B) revealed that its inhibitory effect corresponds to a ca. 0.3 µM ABA concentration in the incubation medium (Figure 7).

Germination and dormancy are controlled by the hormonal balance between promoting GAs and inhibiting ABA (Finch-Savage and Leubner-Metzger 2006). To investigate whether ABA is involved in the pericarp-mediated dormancy of *L. draba*, we analyzed the endogenous hormone levels of fresh and afterripened *L. draba* seeds, pericarps, and fruits (Figure 8). In contrast to the low GA levels, fresh and afterripened dry seeds of *L. draba* contained equally high levels of ABA. Interestingly, the ABA content of *L. draba* fresh mature pericarp was more than 20-fold higher compared with the afterripened pericarp (Figure 8A). Remarkably, when we calculated this, the pericarp ABA content will upon pericarp washing lead to a ca. 0.1 µM ABA concentration in the wash water of fresh *L. draba* pericarps and to a ca. 0.005 µM ABA concentration in the wash water from afterripened pericarp.
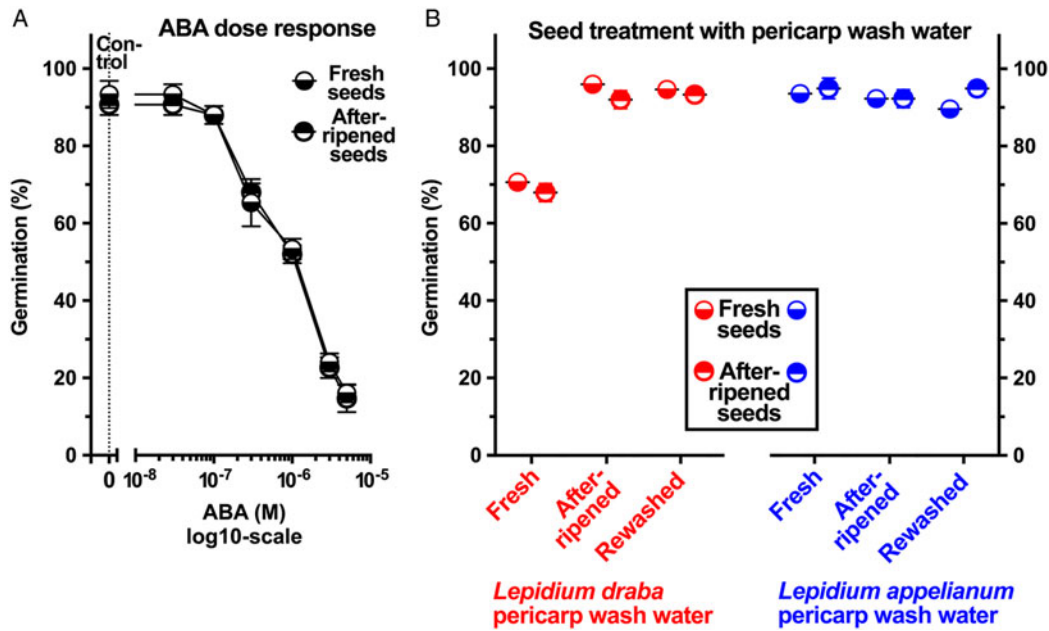
Figure 7. The effect of exogenous abscisic acid (ABA), wash water from *Lepidium draba* pericarp (fresh, fresh-washed, and afterripened) on the germination of *L. draba* fresh and afterripened isolated seeds. (A) Germination dose-response of *L. draba* seeds incubated with different ABA concentrations. (B) The effect of wash water from pericarp on the germination of *L. draba* seeds. Wash water of fresh *L. draba* pericarp inhibits at a level similar to 0.3 µM ABA. *Lepidium appelianum* pericarp does not contain ABA or other water-soluble compounds that may inhibit germination. Mean values ± SE ($N = 3 \times 25$) of accessions KM 1296 and KM 1754 (2014 to 2015 harvest) at optimal germination assay conditions (12/12-h light regime at 25/15 C day/night for 28 d) are presented. Pericarp tissues of 300 mg were washed with 3 ml of distilled water using a shaker at 100 rpm for 6 h to obtain the pericarp wash water applied in the germination assays.
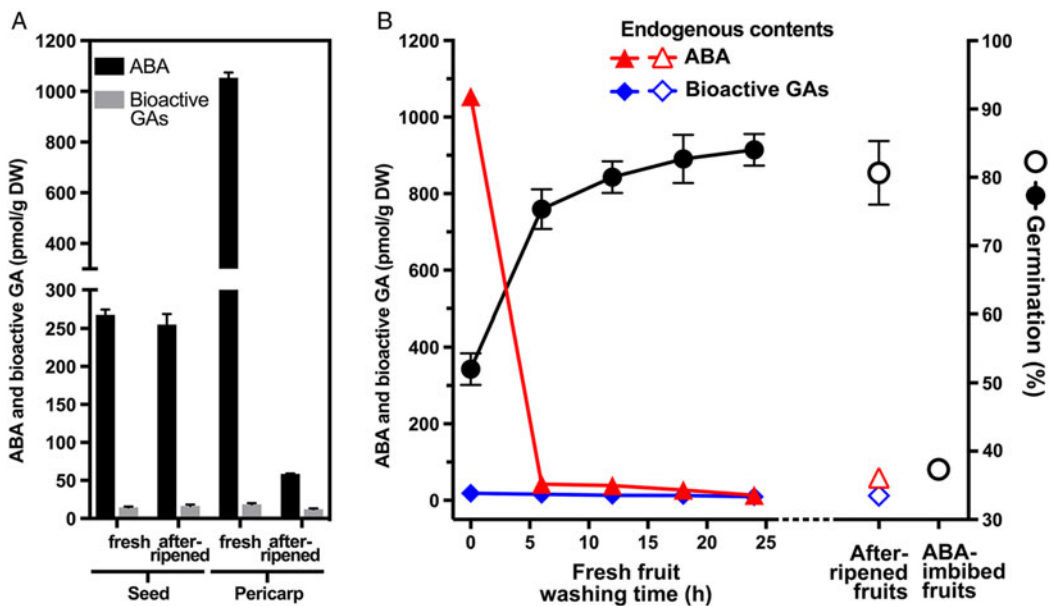

Figure 8. The effect of afterripening and washing on the abscisic acid (ABA) and gibberellin (GA) levels of *Lepidium draba* fruits. (A) Endogenous levels of ABA and bioactive GAs in fresh and afterripened dry seeds and pericarps. (B) ABA and bioactive GA levels during washing of fresh *L. draba* fruits, as compared with afterripened fruits, and with the resultant maximum germination responses presented. Mean values ± SE ($N = 3 \times 25$) of accessions KM 1296 and KM 1754 (2014 to 2015 harvest) at optimal germination assay conditions (12/12-h light regime at 25/15 C day/night for 28 d) are presented. $N = 4 \times 20$ mg (dry weight, DW) of seed/pericarp for ABA and bioactive GA analysis. For a detailed statistical analysis of the ABA contents and their catabolites, see Supplementary Table S2.

Compared with the ABA dose responses (Figure 7A), only the concentration from the fresh pericarp would inhibit germination, and this is exactly what we observed (Figure 7B). In agreement with the ABA in the pericarp playing a role in the chemical dormancy of *L. draba* fruits, the increased germination percentages of washed

fruits were associated with a decrease of the ABA content during the washing process (Figure 8B). Analysis of the ABA catabolites demonstrates that these also decrease during the washing process (Supplementary Table S2) and that the classical 8′-hydroxylation pathway via phaseic acid and dihydrophaseic acid plays a major

role in ABA degradation (Turečková et al. 2009). Further, the high germination proportion of afterripened fruits was associated with a low ABA content (Figure 8B), and afterripened fruits did not germinate when imbibed in the presence of ABA (Figure 8B). We therefore conclude that fruit washing and afterripening released chemical dormancy by eliminating ABA from the pericarp by leaching and/or degradation.

As for *L. draba*, but not *L. appelianum*, pericarp-mediated chemical dormancy is suggested to occur in crop and weed species of Brassicaceae, including *R. raphanistrum* (Cheam 1986; Mekenian and Willemsen 1975), *L. lehmannii* (Mamut et al. 2014), and *Leptaleum filifolium* (Willd.) DC. (Lu et al. 2017). Chemical inhibitors that leach out from the pericarp are thought to cause seed dormancy and can be removed by washing intact fruits with a large volume of water (Baskin and Baskin 2014; Hu et al. 2010; Liu et al. 2015; Mamut et al. 2014). In general, the pericarp can inhibit germination by (1) physical means, that is, inhibition of water imbibition (Cousens et al. 2010) and reduction of gas exchange (Adkins et al. 2002; Hu et al. 2009); (2) mechanical means, that is, mechanical resistance on the radicle protrusion is imposed by the pericarp (Baskin and Baskin 2014; Hu et al. 2010; Sperber et al. 2017); and/or (3) chemical inhibitors residing inside the fruit coat inhibit germination (this study; Mamut et al. 2014; Sari et al. 2006). In the Brassicaceae, physical dormancy has not been recorded to date (Baskin and Baskin 2014), and the possible dormancy types for fruits as dispersal units in this family are either nondormancy and/or pericarp-mediated mechanical (Cousens et al. 2010; Ohadi et al. 2011; Sperber et al. 2017) and chemical dormancy (this study; Cheam 1986; Mamut et al. 2014; Mekenian and Willemsen 1975).

Conclusions and Future Research

Weeds compete with crops for water and nutrients, causing significant yield reduction (Bijanzadeh et al. 2010; Olorunmaiye and Olorunmaiye 2009). Sequestration of these resources by weeds results in vigorous growth, increased seed production, and ease of weed population establishment, all of which have direct impact on crop agriculture and crop yield (Korres 2005). This is particularly true for *L. draba*, an invasive and agronomically problematic weed in western United States and Canada (Al-Shehbaz and O'Kane 2002; Gaskin 2006). Pericarp-mediated chemical dormancy in *L. draba* plays a critical role in the weediness of this species, because the germination biology is controlled by the dormancy mechanism. Dormancy has a crucial role in the survival of the species in determining the germination timing. Dormancy in weed seedbanks cycles throughout the season, with soil temperatures and moisture providing the key environmental factors to regulate dormancy mechanisms to time germination in variable field environments (Finch-Savage and Footitt 2017; Walck et al. 2011). Dormant weed seedbanks could be greatly depleted by stimulating early-season germination and then killing the young seedlings (Westwood et al. 2018). Based on our findings, 2 to 3 mo of afterripening or 1 d of fruit washing eliminated the ABA in the pericarp and thereby released the pericarp-mediated dormancy of *L. draba*. In the soil, this dormancy release would therefore occur slowly in dry conditions and quickly in very wet conditions. Because the pericarp is dead tissue, the removal of this ABA- and pericarp-mediated primary dormancy mechanism would be irreversible. Ultimately, corresponding weather conditions would affect the timing of weed germination and subsequent seedling emergence. The timing of these events constitutes major traits that determine the success of a weed in agricultural ecosystems (Cousens and Mortimer 1995), and understanding seed germination timing under natural conditions is therefore crucial to weed management (Karimmojeni et al. 2014). This point becomes particularly important in the case of our study species *L. draba*, which is listed as an "invasive species" (Al-Shehbaz and O'Kane 2002; Gaskin 2006). The success of future weed management strategies targeting this weed will depend on knowledge of its biological, phenological, and reproductive characteristics and population dynamics. An understanding of the weed establishment time within a particular cropping system enables timely implementation of control strategies (Karimmojeni et al. 2014).

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Data availability statement. All data presented or analyzed are included in this published article or are available from the corresponding authors.

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