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Resin vesicles in conifer seeds: morphology and allelopathic effects

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Manuscripts

1   **Resin vesicles in conifer seeds: morphology and allelopathic effects**

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Abstract—The seed coat of fir (*Abies*), hemlock (*Tsuga*), and cedar (*Thuja*) species contain terpenoid resin vesicles. Although information is limited about the morphology and allelopathy of these vesicles, their damage during seed processing can negatively impact germination success. We examined resin vesicle morphology of western redcedar (*Thuja plicata*), eastern white-cedar (*T. occidentalis*), amabilis fir (*Abies amabilis*), balsam fir (*A. balsamea*), grand fir (*A. grandis*), and subalpine fir (*A. lasiocarpa*) seeds by ¹H magnetic resonance imaging to characterize resin vesicle volume, shape, and number. Western redcedar genotypes with known differences in the quantity of foliar monoterpenes also had parallel differences in the resin vesicle volume of corresponding seeds. Germination assays with the cedar and fir species, eastern hemlock (*Tsuga canadensis*), mountain hemlock (*T. mertensiana*), and western hemlock (*T. heterophylla*) confirmed that resin vesicle damage prior to stratification (moist chilling) significantly reduced germination success for most species. Extracts of these resin vesicles from the *Abies* and *Thuja* species strongly inhibited the germination of *Arabidopsis* Col-0 seeds but inhibited the germination of only a small percentage of *Arabidopsis* abscisic acid-insensitive mutant *abi3-6* seeds. Resin extracts from *Thuja* species were 10 times more effective than those from *Abies* species in inhibiting *Arabidopsis* Col-0 germination.

Key Words—Magnetic Resonance Imaging (MRI), Germination, Conifer, Seeds, Terpenoids, Allelopathy.

Introduction

Most species of conifers have cones that contain, and sometimes ooze, oleoresin (Farjon 2008). These are thought to have important roles in the defense against cone and seed pests and pathogens. In addition, several conifer species, including those from the genera *Abies*, *Thuja*, and *Tsuga*, have resin vesicle structures in the seed coat containing liquid oleoresin. These vesicles appear as dark brown patches on the seed coat and vary by species in location and number (Figure 1). Resin vesicles are lined with epithelial cells and develop in the outer layers of the integument (Kshatriya et al. 2018; Owens and Molder 1977; Singh and Owens 1981, 1982). Often, only a single layer of cells separates the resin vesicles from the inner surface of the seed coat. Although information about conifer resin vesicles is limited (reviewed by Kolotelo 2005), several biological reasons for these resin vesicles have been postulated (Bonner and Karrfalt 2008), including protection from seed and cone pests such as herbivores (Lobo 2014; Rubino et al. 2012), and pathogens, allelopathy (Dässler and Zentsch 1959), inhibition of germination or promotion of dormancy, and protecting the embryo and megagametophyte from excessive drying (Gunia and Simak 1970).

The resin vesicles are thought to contain liquid terpenoid compounds of which monoterpenes predominate, although sesquiterpenes, diterpenes, and oxygenated terpenes are also present. Terpenoid compounds are part of a conifer's chemical defenses against pests, herbivores, and pathogens (Keeling and Bohlmann 2006). Until recently, the only detailed studies to quantify the contents have been done in European silver fir (*Abies alba* Mill.) (Čermák 1987; Čermák and Penka 1978; Penka et al. 1977; Wajs et al. 2010) and Korean fir (*Abies koreana*) (Wajs-Bonikowska et al. 2013). Čermák (1987) found that

monoterpenes contributed 90% of the components, and that limonene was present in the greatest quantity (approx. 60%-90% of monoterpene hydrocarbons), followed by α -pinene (5%-30%), 3-carene (3%-4%) and β -pinene (1%-2%). Interestingly, when quantifying the composition of individual resin vesicles on seeds from the same and different cones, Čermák (1987) also found significant differences in the composition of the resin depending upon the location of the resin vesicle around the seed, and that these differences were consistent between seeds. Recently, a more comprehensive survey of the terpenoid contents of conifer resin vesicles was published (Kshatriya et al. 2018). That study quantified the monoterpenoids and diterpenoids in the same species of conifers as were used in the present study.

There is no information on the heritability of seed resin vesicle morphology. However, the oleoresin and resin vesicles in other conifer tissues can have a strong genetic component (Hall et al. 2011; Robert et al. 2010). Western redcedar (*Thuja plicata*) has seed resin vesicles, and the abundance of monoterpenes in foliage has been shown to have a genetic component and which is correlated with inhibition to deer browsing (Foster et al. 2013; Russell and Yanchuk 2012; Vourc'h et al. 2002). The availability of seeds from these genotypes with known foliar monoterpenes provided an opportunity to examine whether there is a relationship between the seed resin vesicles and foliar monoterpenes.

Damage to the resin vesicles during commercial seed processing can have negative effects on germination success, and thus careful handling of cones and seeds is needed

when processing resin vesicle-containing species (Kitzmilller et al. 1975; Kolotelo et al. 2001). In addition, exposure of undamaged adjacent seeds to the resin from damaged vesicles of other seeds can reduce their germination success, and this effect is dependent upon whether resin exposure occurs prior to seed stratification or not (Gunia and Simak 1970; Kitzmilller et al. 1973). These observations suggest that exposure to the compounds in the resin vesicle affects germination.

Dormancy is maintained in seeds in part by the terpenoid plant hormone abscisic acid (ABA), with ABA flux and the responsiveness of seed tissues to the hormone being important (Kermode 2005). *Arabidopsis* seeds are efficient subjects for testing the allelopathic effects of plant secondary metabolites such as terpenoids on germination (Araniti et al. 2013; Araniti et al. 2017; Zhao et al. 2009), especially with the availability of mutants. For example, homozygous plants of the *Arabidopsis abi3-6* mutant produce non-dormant green seeds that are highly insensitive to exogenous ABA, and severely desiccation intolerant due to an internal deletion of the *ABI3* (*ABSCISIC ACID INSENSITIVE3*) gene (Nambara et al. 1994). We were interested to see whether the extracts of conifer resin vesicles would inhibit the germination of these mutant seeds in a manner similar to that observed for the Columbia Col-0 ecotype.

Magnetic resonance imaging (MRI) is a non-destructive imaging technique using the phenomenon of nuclear magnetic resonance (NMR). MRI is widely used in medical imaging, but is applicable in many other areas, including the imaging of plants and their seeds (Borisjuk et al. 2012, 2013; Terskikh and Kermode 2011; Terskikh et al. 2011).

Typically, ^1H resonances originating from the protons in water and oil, are imaged. Research instruments that can be used to image seeds can achieve resolutions of 10's of μm . Prior studies using MRI and NMR on conifer seeds include the examining the process of imbibition and oil content in western white pine seeds (Terskikh et al. 2005) and the deterioration of western redcedar seeds (Terskikh et al. 2008).

The present work provides details about the morphology of seed resin vesicles in several conifer species and examined some of their potential biological roles. This study had four objectives: (1) to use MRI to describe the morphology of resin vesicles of seeds of several conifer species; (2) to determine whether the abundance of seed resin vesicles has a genetic component by examining western redcedar seeds from parents with known genotypes for foliar monoterpenes; (3) to quantify the effect of resin vesicle-damage (release of constituent vesicle terpenoids) on germination success and (4) to determine whether resin vesicle contents have allelopathic effects on germination, using *Arabidopsis* seeds as proxies for seeds sympatric to conifers that might be exposed to resin vesicle constituents in natural, mixed species, seed beds.

Materials and methods

Seed Sources Dry seeds of amabilis fir (*Abies amabilis*), balsam fir (*A. balsamea*) grand fir (*A. grandis*), subalpine fir (*A. lasiocarpa*), yellow cedar (*Callitropsis nootkatensis*), eastern white-cedar (*T. occidentalis*), western redcedar (*Thuja plicata*), eastern hemlock (*Tsuga canadensis*), western hemlock (*T. heterophylla*), and mountain hemlock (*T. mertensiana*) were used. Seeds were already mechanically de-winged except for yellow

cedar, western redcedar and eastern white-cedar. Seeds from the maternal trees of crosses of high- and low-foliage monoterpene western redcedar were obtained as part of an on-going study (J. Russell, unpublished). The maternal tree genotypes were previously assessed for foliar monoterpenes as follows: 1368, not detectable; 1508, trace levels; 1371, low levels; 1372, high levels, and (J. Russell, unpublished observations). Additional information for the conifer seeds is provided in Supporting Table 1.

Arabidopsis thaliana Columbia (Col-0) ecotype and *abi3-6* mutant (Nambara et al. 1994) seeds were used to test the allelopathic effects of resin vesicle constituents. Dr. Eiji Nambara (U. of Toronto) provided heterozygous *abi3-6* F2 seeds. Homozygous *A. thaliana abi3-6* mutant plants (showing a reduced trichome phenotype) were recovered from seed originating from heterozygous plants. Mutant *abi3-6* seeds were harvested from the homozygous plants prior to seed desiccation (siliques were still yellow-green) and were used immediately.

¹H Magnetic Resonance Imaging A random selection of at least ten seeds with intact resin vesicles from each species was chosen for imaging. Seeds were weighed, and their dimensions measured under a dissecting microscope with a digital caliper. ¹H magnetic resonance imaging was completed on a Bruker AVANCE III 400 MHz NMR Spectrometer at Simon Fraser University with either a 2.5- or 5-mm diameter horizontal solenoid coil probe, with TopSpin (version 2.0PV) and ParaVision (version 5.1) software. Images were processed in the Fiji (Schindelin et al. 2012) distribution of ImageJ (version 2.0.0-rc/1.51d) (Schindelin et al. 2015). Total resin vesicle volume and the number of resin vesicles and cotyledons were determined from the images.

See Supporting Information for complete description.

Effect of Resin Vesicle Damage on the Germination of Conifer Seeds Stratification and germination were conducted in 35 x 10 mm disposable Petri dishes. Whatman 1 filter paper (30 mm dia., GE Healthcare Life Sciences) was placed in each dish over top of a square of germination paper (Versa-Pak Germination Paper, Seedburo Equipment Co., Des Plaines, IL USA) and moistened with distilled water. Imbibition, stratification, and germination conditions were conducted following Kolotelo (1997), using germination test codes G64 for amabilis fir and subalpine fir, G32 for grand fir, D1 for western redcedar, and G31 for western and mountain hemlock; and the National Tree Seed Centre's Germination Guidelines (Nov. 2015) for balsam fir and eastern white-cedar, except cold stratification temperatures were 4°C instead of 2°C for all species. Imbibition was performed at room temperature in 50 mL Falcon tubes with distilled water, which was replaced several times each day. After 24 or 48 h of imbibition, depending upon the test code, seeds were randomly divided into control seeds and those to be damaged. Each treatment had 8 dishes (biological replicates) containing 16 seeds, except for western redcedar, which had 6 replicates. Damaged and undamaged seeds were placed in separate dishes. To damage the resin vesicles, the outer surface of all visible resin vesicles on each seed were damaged with the tip of a forceps before placing the seeds onto the filter paper in the Petri dishes. Stratification was completed in the Petri dishes, sealed with Parafilm, and held in ZipLoc bags. For those species requiring re-drying part way through stratification, seeds were removed from the dishes and allowed to dry at room temperature to approximately 30% moisture content before returning to the dishes

with only a fresh and dry filter paper for the remainder of the stratification. Once stratification was complete, filter paper and germination paper were replaced with fresh ones moistened with 4 mL of distilled water and then the dishes were placed in germination conditions with 8 h of light and 16 h of dark (as specified in Kolotelo (1997) and the National Tree Seed Centre's Germination Guidelines (Nov. 2015)). Germination was observed daily, and a seed was considered germinated if the radicle length exceeded 50% of the seed length. This chosen length was a compromise between scoring germination as radicle emergence typically used by academic seed biologists and scoring germination as a radicle length of 4x seed length used by tree seed centres. On day 12-14 of the germination test, germinated seeds were removed (to aid in counting the remaining seeds) and additional water added to the germination paper if needed. Observations continued until the germination test day for each species was reached. The percentage of seeds that had germinated by the test day were calculated for each dish and two-tailed t -tests and Cohen's effect size (Cohen 1988) were completed on these percentages.

Extraction of Resin Vesicles from Conifer Seeds The outside surface of every resin vesicle on each seed was punctured under a stereo microscope with the tip of a scalpel and the seeds immediately placed into methanol (HPLC grade, Caledon Laboratories Ltd, Georgetown, ON, Canada). After all seeds were placed into the extraction solvent (enough to cover the seeds), the tube was vortexed and then the liquid removed. This was repeated 2 more times and then the three extracts were combined, and the total volume measured. The extract was stored at -20°C until use. The initial weights of the seeds were compared to their weights after extraction, after the solvent had been allowed

to evaporate at room temperature for at least 24 h, to calculate the mass of extractable material from the resin vesicles. Yellow-cedar seeds, which have a deep dormancy but have no resin vesicles to damage, were also extracted as a negative control and tested to confirm that the methanol did not extract inhibitory compounds from other parts of the seeds. Unlike the other seeds, no loss in weight could be measured for the yellow-cedar seeds after extraction.

Inhibition of Germination of Arabidopsis Seeds with Conifer Resin Vesicle Extracts

A dilution series in methanol (125 or 250 μ L) of the conifer resin vesicle extract was added onto a 30-mm diam. Whatman 1 filter paper (GE Healthcare Life Sciences) inside a disposable 35 x 10 mm Petri dish. After allowing the solvent to evaporate for 15 min, 250 μ L of 1/2 strength Murashige & Skoog medium (Modified Basal Medium with Gamborg Vitamins, PhytoTechnology Laboratories) containing a suspension of approximately 50-100 *Arabidopsis* seeds were added to the filter paper. The *Arabidopsis* Col-0 seeds were sterilized with 70% ethanol, washed 3x with distilled water and then 2x with 1/2 MS medium immediately prior to use. The *abi3-6* mutant seeds were placed directly into 1/2 strength Murashige & Skoog medium without sterilization. Dishes were sealed with Parafilm and then placed in the dark at 4°C for 4 days. After this stratification step, the dishes were placed in germination conditions (16 h light, 8 h dark, constant 22°C) and germination success scored daily by the emergence of the radicle from the endosperm and a radicle length at least half the seed length. The effective concentration of resin vesicle extract to inhibit 50% of the seeds from germinating (EC50) at each observation time point between 30 and 130 h was calculated in R (version 3.4.0, R Core

Team 2017) using the *drc* (version 3.0-1, binomial model LL2.3(), Ritz and Streibig 2005) and *MASS* packages (version 7.3-47, Venables and Ripley 2002). The EC50 response with time was then modeled with the *metafor* package (version 2.0-0, Viechtbauer 2010) to predict the EC50 value at 72 h (EC50_{72h}) and to compare EC50_{72h} values between resin vesicle extracts from the different species by the Tukey method (adapted from procedure described in Jensen et al. 2017).

Results

¹H Magnetic Resonance Imaging of Conifer Seed Resin Vesicles Figure 1 shows a diagrammatic view of a conifer seed with resin vesicles. We successfully used MRI to image the resin vesicles of conifer seeds and estimate the total resin vesicle volume of each seed. A typical one-dimensional ¹H NMR spectrum from a seed is shown in Supporting Figure 1. A summary of the observations is shown in Table 1 and in Figures 2-3. Orthogonal views of 3-dimensional reconstructions of the seeds are shown in Figure 4. We would occasionally observe an MRI image with a frilly edge on the outside surface of resin vesicles coinciding with a vesicle that appeared to be undamaged but not full (wrinkled). Whether this was due to gradual evaporation of the contents during storage or insufficient filling during vesicle development is not known (some seedlots have been in storage at -20°C for many years, see Supporting Table 1). Imaging the hemlock seeds could not be undertaken because the resin vesicle constituents were too viscous (western hemlock) or had crystalized completely (mountain and eastern hemlock), and the ¹H MRI signal requires imaging mobile protons (i.e. liquid materials, because of T1 and T2 relaxation time considerations). The mountain hemlock seeds were

the oldest seeds used in this study, having been in frozen storage since collected and processed in 1982 (Supporting Table 1).

Morphology of Resin Vesicles The morphology of the resin vesicles around the seed coat differed between species, and although there was variation within a species, some trends were observed. There were differences in the location, shape, and distance of resin vesicles from the megagametophyte of the seed (Table 2). In the case of grand fir, the resin vesicles appeared to impinge on and distort the shape of the megagametophyte (Figure 2C). No obvious differences in the number of resin vesicles on the adaxial versus abaxial sides of seeds were observed.

Although only one seedlot was examined for each species, the resin vesicle volume and number varied widely both within a seedlot and between species. Resin vesicle volume generally increased with increased seed weight (Figure 5A), but the number of resin vesicles per seed did not increase with seed weight (Figure 5B). In fact, the lightest seeds, from eastern white-cedar, had the largest number of resin vesicles per seed.

Resin Vesicles of Western Redcedar Seeds Derived from Genotypes with Different Levels of Foliar Monoterpenes Western redcedar seeds originating from controlled crosses of genotypes with different levels of foliar monoterpenes were imaged. Although these seeds were part of a crossing experiment, these seeds were from the same maternal trees, not seeds from the offspring of these crosses. Thus, as the seed coat is maternally derived tissue, we should have expected no effect of the pollen source on these seed

coats. An effect of pollen source might be observable in the seeds of the offspring of these crosses, but these seeds were not yet available. We found significant differences between maternal sources in both the number and total volume of resin vesicles (Tables 3 and 4). The volume of the resin vesicles increased with the increasing quantity of foliar monoterpenes found in these genotypes (J. Russell, unpublished observations), suggesting that there may be a linked genetic component between these two traits.

Effect of Damage to Resin Vesicles on Germination Success of Conifer Seeds

The response to resin vesicle damage varied depending upon the species. Amabilis-, grand-, and subalpine-firs all showed a significant reduction in germination success with resin vesicle damage, but there was no difference for balsam fir (Figure 6A-D). Western redcedar showed a slight, but non-significant, reduction in germination success (Fig. 11E), but eastern white-cedar showed a significant reduction in germination success with damage (Fig. 11F). Western and mountain hemlock did not show any difference, but damage to eastern hemlock resin vesicles significantly reduced germination success (Fig. 11G-I). The germination success rates of the undamaged seeds in this study were similar to the information available from the seed providers.

Effect of Conifer Resin Vesicle Extracts on Germination Success of Arabidopsis Seeds

Resin vesicle extracts from all species examined had a dose-dependent effect on the germination success of *Arabidopsis* Col-0 seeds (Figure 7A-F), contrary to extracts from yellow-cedar seeds, which lack resin vesicles (Figure 7G). The EC₅₀_{72h} values (concentration of extract resulting in inhibition of 50% of the seeds at 72 h) ranged from

2.1 to 7.7 seed equivalents per mL of growth medium (Table 5). The weight of resin vesicle components as a portion of the total seed weight ranged from 7 to 21%. Accounting for the differences in seed size, and the amount of material extracted from the resin vesicles of the different species, the $EC_{50_{72h}}$ value in units of mg of extractables per mL of growth medium ranged from 0.63 to 9.8 mg/mL (Table 5). This emphasized that, although the eastern white-cedar and western redcedar were some of the smallest seeds extracted, the extractable material in these seeds was approximately ten times more effective at inhibiting germination than the extractable material from the fir seeds. The $EC_{50_{72h}}$ values (in mg/mL) from the different fir extracts were not significantly different from each other. The $EC_{50_{72h}}$ values from eastern white-cedar and western redcedar extracts were not significantly different. However, extracts from both cedar species were significantly different from the fir species tested. When extracts from grand fir or western redcedar seeds were tested with seeds from the *Arabidopsis abi3-6* mutant, which are insensitive to ABA, only a slight inhibition of germination success was observed at the highest doses (Figure 7H-I).

Discussion

Our imaging of the resin vesicles in several conifers provided a standardized set of observations to guide further studies and showed variation between seeds within the same seedlot and between species. The variation within a species across its geographic range was not examined. This work complements the histological examination of these resin vesicle-containing conifer seeds recently described (Kshatriya et al. 2018). The percentage of seed dry weight composed of resin vesicle constituents we measured (7-

21%) was consistent with those found for these species by other methods (Kshatriya et al. 2018), and with the 20% reported in the European silver fir (*Abies alba* Mill.) (Čermák 1987). However, up to 60% was reported in noble fir (*A. procera*) (Kandya and Ogino 1986).

There was a positive relationship between foliar monoterpene content and seed resin vesicle volume in western redcedar seeds from genotypes that exhibit high and low monoterpene content within their foliar tissues. Terpenoids are products of terpene synthase and cytochrome P450 enzymes in the tree (Keeling and Bohlmann 2006). These enzymes are part of large gene families and many enzymes are necessary to produce the complex mixture of terpenoids present in foliar and resin vesicle tissues. We would expect natural variation across the population in the expression and kinetic abilities of these enzymes, as has been shown in Sitka spruce (*Picea sitchensis*) (Hall et al. 2011). These enzymes are also being identified in western redcedar (Gesell et al. 2015). However, the genotypes examined in this study that completely lacked seed resin vesicles in association with their lack of foliar monoterpenes are likely to differ in the larger control mechanisms of terpenoid defense pathways rather than in variation of specific terpene synthases. The presence of terpenoid compounds in western redcedar foliage is important for defense against various herbivores (Kimball et al. 2012; Vourc'h et al. 2001; Vourc'h et al. 2003) and the genetics of this trait are currently being investigated (Foster et al. 2013; Vourc'h et al. 2002). There is moderate to high heritability of foliar monoterpene quantities in western redcedar (Russell and Yanchuk 2012), but the heritability for seed resin vesicle volumes is not known. Assessing the resin vesicles on

the seeds produced from plants germinated from the seeds used in this study would be useful for this. The resin vesicles found in a seed may be linked to the foliar phenotype shown by the maternal parent. The visual inspection of seeds under a microscope may rapidly identify high/low resin vesicle seeds in bulk collections or collections without prior phenotyping of the maternal foliar monoterpenes. These selected seeds can then be used for subsequent germination and foliar monoterpene testing.

We found that resin vesicle damage immediately after imbibition had a significant effect on germination success. Damage occurring at this step is only one situation where a seed may get damaged. In commercial processing, damage could occur during cone processing, seed processing, storage, imbibition, or stratification (Kolotelo et al. 2001). Damage during commercial seed processing prior to storage provides the opportunity during storage for the volatile constituents to evaporate and also the diterpenoids to polymerize. Notably, we found the contents of the hemlock resin vesicles were viscous or crystalized, even in the absence of visible damage.

Consistent with the suggested allelopathic role of conifer resin vesicles (Bonner and Karrfalt 2008), extracts from the resin vesicles inhibited the germination success of *Arabidopsis* seeds. Although *Arabidopsis* is not a known sympatric competitor to conifer seedlings, it provided a standardized seed for *in vitro* testing. Natural competitor species to conifer seedlings, and other conifer species, will need to be tested in future studies. Significantly higher potency of *Thuja* versus *Abies* resin vesicle extracts was observed. In *Thuja plicata*, α -thujone and β -thujone are major foliage constituents (Foster et al.

2013), whereas they are absent or found at lower levels in *Abies* spp. (e.g. Smirnov 1972; Zavarin et al. 1977). Kshatriya et al. (2018) have recently identified and quantified the monoterpenes and diterpenoids found in these and the other resin vesicle-containing conifer seeds studied here. Additional studies are needed to establish whether these ketones confer this difference in inhibitory potency, and to quantify the inhibitory potency of each resin vesicle constituent. The conifer resin vesicle extracts did not significantly inhibit the germination success of *Arabidopsis abi3-6* mutant seeds, which are insensitive to ABA because they do not synthesize the ABI3 protein, a transcription factor of the ABA signal transduction pathway. Although the data are not sufficient to prove that resin vesicle constituents act via the abscisic acid signaling pathway, they suggest that this pathway might be involved. Additional studies and other *Arabidopsis* mutants from other parts of the ABA pathway (affecting ABA biosynthesis or ABA signal transduction) would need to be tested to corroborate these results. The role conifer seed resin vesicles play in the allelopathy and other aspects of chemical ecology in the forest is very limited. In the commercial seed processing setting, resin vesicle leakage from damaged seeds is likely to affect the germination success of adjacent seeds coming in contact with the resin, as has been shown in *Abies alba* (Gunia and Simak 1970).

In summary, the morphology of resin vesicles on conifer seeds varied by species. The importance of these vesicles in conifer ecology is not fully understood. However, we have shown that they have a significant allelopathic effect on heterospecific seeds, and that they also exhibit negative effects on their own germination success if they are damaged after imbibition.

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Table 1: Seed and resin vesicle physical attributes

Common Name	Species	Seed weight (mg)	Seed length (mm)	Seed width* (mm)	Cotyledon number	Resin vesicle number	Resin vesicle volume (mm ³)
Amabilis fir	<i>Abies amabilis</i>	28.9±8.3	8.5±1.1	3.7±0.4	4.8±0.4	5.2±1.3	3.5±1.4
Grand fir	<i>A. grandis</i>	21.8±4.7	7.3±0.7	3.5±0.3	5.9±0.3	7.3±1.5	4.1±0.8
Subalpine fir	<i>A. lasiocarpa</i>	16.6±3.5	6.0±0.6	3.1±0.3	4.4±0.5	5.2±2.1	2.1±1.3
Balsam fir	<i>A. balsamea</i>	10.0±1.1	5.2±0.4	2.7±0.3	4.1±0.3	5.2±0.9	1.5±0.3
Western redcedar	<i>Thuja plicata</i>	1.6±0.3	4.2±0.3	1.1±0.1	2.0±0.0	8.2±1.8	0.15±0.03
Eastern white-cedar	<i>T. occidentalis</i>	1.3±0.3	3.6±0.2	1.2±0.1	2.0±0.0	11.6±2.6	0.085±0.023

*Marginal to medial width.

All measurements are averages ±SD for seeds imaged ($N=10$, equal numbers of enantiomorphs where appropriate). Resin vesicle number and volume, and cotyledon number were determined from MRI images; other measurements were determined under stereomicroscope with a digital caliper. Seed measurements were for dry seed after commercial processing, which included removing the seed wing for fir species.

Table 2: Description of resin vesicle morphology

Common Name	Morphology of resin vesicles*
Amabilis fir	disperse, capped-m-c, asymmetrical, loose-intermediate, elongate
Grand fir	micropylar-disperse, capped-c, asymmetrical, v. tight (distortion of megagametophyte), ovate-elongate
Subalpine fir	disperse, capped-c, asymmetrical, intermediate-tight, elongate
Balsam fir	micropylar-disperse, semi-capped-c, asymmetrical, tight, elongate
Western redcedar	micropylar, symmetrical, intermediate-tight, v. elongate
Eastern white-cedar	micropylar, semi-symmetrical, loose, elongate

*Position around seed: *micropylar*, more resin vesicles were found at the micropylar end than at the chalazal end; *disperse*, found all over seed coat; *chalazal*, more resin vesicles were found at the chalazal end than at the micropylar end; *capped*, a resin vesicle surrounded the –m, micropylar end and/or the –c, chalazal end of the seed; *symmetrical*, resin vesicles usually appeared on the adaxial/abaxial and medial/marginal positions at the same time; *semi-symmetrical*, resin vesicles occasionally appeared on the adaxial/abaxial and medial/marginal positions at the same time; asymmetrical, no symmetry in resin vesicles between adaxial/abaxial and medial/marginal positions; Distance from resin vesicles to megagametophyte: *loose*, large gap; *intermediate*, small gap; *tight*, no gap;

Shape: *ovate*, almost round; *elongate*, extended length in chalazal-micropylar direction; v. *elongate*, elongated for nearly the full length of the seed.

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Table 3: Resin vesicle volumes in seeds of western redcedar of known genotype with high and low foliar monoterpene content

	1368	1371	1372	1508	Combined* (n)
1368	0	0	0	0	0 (12) ^a
1371	0.047±0.013	0.041±0.003	n.a.	n.a.	0.044±0.009 (6) ^{bc}
1372	0.049±0.026	0.052±0.028	0.11±0.03	n.a.	0.070±0.038 (9) ^c
1508	n.a.	n.a.	n.a.	0	0 (3) ^{ab}

Resin vesicle analysis was by ¹H MRI (average±SD mm³/seed, N=3 for each cross where available). Numbers on the left indicate maternal genotype and the numbers at the top indicate paternal genotype. The maternal tree genotypes were previously assessed for foliar monoterpenes as follows: 1368, not detectable; 1371, low levels; 1372, high levels, and 1508, trace levels (J. Russell, unpublished observations). Means followed by the same letter were not significantly different by multiple comparison test ($p>0.05$) after significant Kruskal-Wallis test with the R package *pgirmess* (version 1.6.7 , Giraudoux 2017).

* Data for seeds originating from the same maternal genotype were combined because the seed coat originates only from maternal tissue. n.a., The crosses between these genotypes were not completed.

Table 4: Number of resin vesicles in seeds of western redcedar of known genotype with high and low foliar monoterpene content

	1368	1371	1372	1508	*Combined (n)
1368	0	0	0	0	0 (40) ^a
1371	7.0±1.1	7.7±1.3	n.a.	n.a.	7.3±1.2 (20) ^b
1372	5.9±1.4	5.2±1.3	5.6±0.5	n.a.	5.6±1.2 (30) ^b
1508	n.a.	n.a.	n.a.	0	0 (10) ^a

Resin vesicles counted by visual examination under a stereo microscope (average±SD, $N=10$). Numbers on the left indicate maternal genotype and the numbers at the top indicate paternal genotype. Means followed by the same letter were not significantly different by multiple comparison test ($p>0.05$) after significant Kruskal-Wallis test with the R package *pgirmess* (version 1.6.7, Giraudoux 2017).

* Data for seeds originating from the same maternal genotype were combined because the seed coat originates only from maternal tissue. n.a., The crosses between these genotypes were not completed.

Table 5: Summary of resin vesicle extract EC50_{72h} values for the inhibition of *Arabidopsis* (Col-0) germination

Common Name	Extractables (weight percent)	EC50 _{72h} (seed Eq/mL ± SE)	EC50 _{72h} (mg/mL ± SE)
Amabilis fir	10.9	2.5±0.5	8.3±1.8 ^{a*}
Grand fir	21.0	2.1±0.5	9.8±2.5 ^a
Subalpine fir	16.5	2.6±0.5	6.3±1.3 ^a
Balsam fir	13.3	6.4±0.6	8.1±0.8 ^a
Western redcedar	10.7	6.7±0.6	0.83±0.07 ^b
Eastern white-cedar	7.0	7.7±0.6	0.63±0.05 ^b

* Means followed by the same letter were not significantly different ($p>0.05$) by the Tukey method.

Figure Legends

Figure 1: Diagram of resin vesicle-containing conifer seeds.

Adapted from figures of European silver fir (*Abies alba*) seeds by Čermák (1987). (A) Adaxial view of ovuliferous scale with seeds. *CA*, cone axis; *OS*, ovuliferous scale; *Br*, bract; *S₁* and *S₂*, seeds; *W₁* and *W₂*, seed wings. Seed wings completely cover the adaxial side of the seeds. (B) Seed shown from abaxial side with resin vesicles appearing on marginal (*marg*) and medial (*med*) seed sides and both micropylar (*mic*) and chalazal (*cha*) ends. *m*, the median plane. Seed wing partially sheaths the marginal and medial surfaces of abaxial side of seed. (C) Cross-sectional view of seed (through plane shown in (A) and (B)). *m*, median plane, *e*, embryo, *mg*, megagametophyte. Resin vesicles appear on the adaxial (*ad*), abaxial (*ab*), medial (*med*), and marginal (*marg*) seed sides. The seed wing, *W* (dotted line), loosely surrounds the seed except where the seed attaches to the ovuliferous scale (not shown) on the abaxial side.

Figure 2: ¹H MRI of fir seeds showing resin vesicle morphology.

Representative images of fir seeds obtained with the 5-mm horizontal ¹H MRI probe with FOV 4.4 mm × 4.4 mm, 256 × 256 pixels, in-plane resolution 17 µm × 17 µm per pixel, TE 10.6 ms, TR 1000 ms, 64 scans averaged, and an acquisition time of 4 h 33 min. (A) Amabilis fir seed, slice thickness 370 µm. (B) Balsam fir seed, slice thickness 300 µm. (C) Grand fir seed, slice thickness 350 µm. (D) Subalpine fir seed, slice thickness 300 µm. Resin vesicles can be seen as bright objects surrounding the megagametophyte and embryo (in grey). Images are shown left to right, top to bottom, from micropylar end to chalazal end. The medial seed side is at the bottom of each image.

Figure 3: ^1H MRI of cedar seeds showing resin vesicle morphology.

Representative images of cedar seeds obtained with the 2.5-mm horizontal probe, FOV $1.5\text{ mm} \times 1.5\text{ mm}$, 64×64 pixels, in-plane resolution $23\text{ }\mu\text{m} \times 23\text{ }\mu\text{m}$ per pixel, 256 scans averaged, TE 7.6 ms, TR 1500 ms, and an acquisition time of 6 h 49 min. (A) Western redcedar seed, slice thickness $150\text{ }\mu\text{m}$. (B) Eastern white-cedar seed, slice thickness $160\text{ }\mu\text{m}$. Resin vesicles can be seen as bright objects surrounding the megagametophyte and embryo (in grey). Images are shown left to right, top to bottom, from micropylar end to chalazal end. The adaxial seed side is at the bottom of each image.

Figure 4: Orthogonal views of reconstructed 3-dimensional ^1H MRI images of representative seeds showing resin vesicle morphology.

(A) Amabilis fir; (B) balsam fir; (C) grand fir; (D) subalpine fir; (E) western redcedar; (F) eastern white-cedar. Regular ripples in the images are artifacts due to sampling slices and are not actual seed features. Images were created with the Volume Viewer plug-in for ImageJ. False color indicates relative signal intensity. See Figure 1 for abbreviations. These 3-dimensional reconstructions are shown as animated movies in Supporting material.

Figure 5: Relationship between seed weight and resin vesicle volume and number.

Resin vesicle volume was positively associated with increasing seed weight (Pearson $R^2=0.83$, $p<0.0001$, all species combined, $N=61$) (A). Inset shows the cedar seeds in an expanded range. The number of resin vesicles was weakly negatively associated with

increasing seed weight ($R^2=0.23$, $p<0.0001$, all species combined, $N=61$) (B) and resin vesicle volume ($R^2=0.12$, not shown, $p<0.007$, all species combined, $N=61$).

Figure 6: Effect of resin vesicle damage on germination success of the damaged seeds.

Comparison of germination success for seeds with damaged or undamaged resin vesicles in: (A) amabilis fir ($p=0.007$, Cohen's $d=1.6$); (B) balsam fir ($p=0.68$, Cohen's $d=0.2$); (C) grand fir ($p=0.03$, Cohen's $d=1.2$); (D) subalpine fir ($p=0.002$, Cohen's $d=1.9$); (E) western redcedar ($p=0.12$, Cohen's $d=1.0$); (F) eastern white-cedar ($p=0.001$, Cohen's $d=2.0$); (G) western hemlock ($p=0.56$, Cohen's $d=0.3$); (H) eastern hemlock ($p=0.003$, Cohen's $d=1.8$); (I) mountain hemlock ($p=0.79$, Cohen's $d=0.1$). Peak value (PV) indicates the point where the cumulative germination percent divided by the number of days was the maximum. Vertical green line indicates the test day for the appropriate germination test code for each species. An asterisk indicates a significant ($p\leq 0.05$) difference on the germination test day. p -Values (two-tailed t -test) and Cohen's effect size (d) are shown above at this test day. $N=6$ (for western redcedar) or 8 (all other species), 16 seeds per replicate.

Figure 7: Effect of resin vesicle extracts at various doses on the germination success of *Arabidopsis* Col-0 and *abi3-6* mutant seeds.

Comparison of germination success for *Arabidopsis* Col-0 seeds exposed to methanol extract of resin vesicles from: (A) amabilis fir; (B) balsam fir; (C) grand fir; (D) subalpine fir; (E) western redcedar; (F) eastern white-cedar; (G) yellow cedar; and with

Arabidopsis abi3-6 mutant seeds exposed to methanol extract of resin vesicles from: (H) grand fir; (I) western redcedar. The concentration of resin extracts that inhibited 50% of the Col-0 seeds from germinating after 72 h in germination conditions ($EC_{50_{72h}}$) calculated from this data are shown in Table 4. $N=3$ for each dose, with approximately 85 seeds per replicate.

Supplementary Information

cjfr-2018-0221suppla.pdf

Additional information about seed sources and MRI methods.

cjfr-2018-0221supplb.mp4

Animations of the 3-dimensional ^1H MRI reconstruction of the seeds shown in Figure 4.

A

B

A

B

Figure 6: Effect of resin vesicle damage on germination success of the damaged seeds. Comparison of germination success for seeds with damaged or undamaged resin vesicles in: (A) amabilis fir ($p=0.007$, Cohen's $d=1.6$); (B) balsam fir ($p=0.68$, Cohen's $d=0.2$); (C) grand fir ($p=0.03$, Cohen's $d=1.2$); (D) subalpine fir ($p=0.002$, Cohen's $d=1.9$); (E) western redcedar ($p=0.12$, Cohen's $d=1.0$); (F) eastern white-cedar ($p=0.001$, Cohen's $d=2.0$); (G) western hemlock ($p=0.56$, Cohen's $d=0.3$); (H) eastern hemlock ($p=0.003$, Cohen's $d=1.8$); (I) mountain hemlock ($p=0.79$, Cohen's $d=0.1$). Peak value (PV) indicates the point where the cumulative germination percent divided by the number of days was the maximum. Vertical green line indicates the test day for the appropriate germination test code for each species. An asterisk indicates a significant ($p \leq 0.05$) difference on the germination test day. p -Values (two-tailed t -test) and Cohen's effect size (d) are shown above at this test day. $N=6$ (for western redcedar) or 8 (all other species), 16 seeds per replicate.

82x156mm (300 x 300 DPI)

27x12mm (300 x 300 DPI)