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Research Note

Ethanol exposure can inhibit red spruce (*Picea rubens*) seed germination

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Abstract

Flotation of seeds in solvents is a common means of separating unfilled and filled seeds. While a few protocols for processing red spruce (*Picea rubens*) seeds recommend ethanol flotation, delayed and reduced germination have been reported. We conducted an ethanol bioassay on seeds previously stored at -20°C to quantify the concentration required to separate red spruce seeds and the effects on germination. We used seeds from Canada (CAN) that had been exposed to ethanol during processing, and seeds from the United States (USA) that had not been exposed to ethanol during processing. Seeds were exposed to 10 ethanol concentrations (10-100%) and deionised water was used as a control. The effective concentration of ethanol for 50% (EC_{50}) of the seeds to sink ranged by source from 70.9 to 90.7%, with all seeds sinking in 100% ethanol. The use of less than 100% ethanol is not adequate for seed separation, as some filled seeds could float and be mistakenly categorised as unfilled. The mean EC_{50} of ethanol that inhibits germination was significantly higher for USA sources (52.7%), than for CAN sources (40.8%; $P < 0.05$). Ethanol concentrations that inhibited germination coincided with delays in germination. The mechanism of phytotoxity was not determined; however, damage during extraction, desiccation and storage at -20°C are potential sources. We recommend separating red spruce seeds by physical means rather than ethanol flotation to avoid potential negative impacts on germination.

Keywords: bioassay, ethanol, phytotoxicity, *Picea rubens*, red spruce, seed germination

Experimental and discussion

Seed flotation in organic solvents (e.g. ethanol, ether, pentane, hexane) that are less dense than water is a common means of separating unfilled seeds from a seed lot (Baldwin, 1932; McLemore, 1965; Brown, 1967; Lebrun, 1967; Barnett, 1971; Simak, 1973; Simpson and

Daigle, 2003). The literature provides numerous examples evaluating separation efficacy and safety of particular solvents, however these reports are typically species specific (e.g. Bonner and Karrfalt, 2008). Ethanol floatation was reported to reduce storage longevity of red spruce (*Picea rubens* Sarg.; Baldwin, 1932), spruce-pine (*Pinus glabra* Walt.; Barnett, 1970) and slash pine (*P. elliotti* Engelm.; Barnett, 1971), and to reduce and/or delay germination for red spruce (Baldwin, 1932), Japanese larch (*Larix japonica* Hort. Ex. Carriere) and related F1 hybrids (Kon and Kita, 2014), tamarack (*L. laricina* (Du Roi) K. Koch) (Eavy and Houseweart, 1987) and true firs (*Abies* spp.) (Edwards, 1980). In contrast, seeds of Crimean pine (*Pinus nigra* Arn. ssp. *pallasiana* (Lamb.) Holmboe; Avsar, 2010), white birch (*Betula papyrifera* Marsh.; Simpson and Daigle, 2003), Himalayan maple (*Acer caesium* Wall. Ex. Brandis) and elm (*Ulmus wallichiana* Planchon) (Phartyal *et al.*, 2002) were unharmed by short periods of ethanol exposure.

Published protocols for separating unfilled red spruce (Mosseler *et al.*, 2000) and black spruce (*Picea mariana* (Mill.) B.S.P.) seeds from a seed lot (Stoehr and Farmer, 1986) specifically recommend flotation in ethanol (C_2H_5OH). For example, Natural Resources Canada operates the National Tree Seed Centre (NTSC) in Fredericton, New Brunswick, and for decades, researchers there have used ethanol flotation to process red spruce seeds without any apparent damage or toxicity. Standard processing includes soaking the seeds in ethanol for 15 seconds or less so that unfilled seeds can be removed, rinsing filled seeds with cold tap water for 15-30 seconds, followed by drying (personal communication, Dr. Dale Simpson). Dry seeds are stored at -20°C and negative effects on seed longevity have not been reported.

The potential phytotoxicity of ethanol flotation to red spruce seeds was observed by our group during an initial effort to propagate red spruce seedlings from 48 half-sib families collected in Vermont, USA. Seeds were collected from natural stands in August-September 2015, and left to air-dry before processing in November 2015. The processing included flotation in ethanol (approximately 15 seconds), followed by rinsing with tap water and drying (based on Mosseler *et al.* (2000) and Major *et al.* (2005)). Germination rates varied by family and ranged from 0 to 71% (mean of 20%) which was much lower than expected (e.g. 77% mean germination in 30 days (Baldwin, 1934)). After storage at -20°C for approximately three months, additional germination tests were conducted. Germination from the original 48 families was largely absent, while one family collected and processed separately had a germination rate of > 75%. This single seed lot had not been soaked in ethanol; providing our first indication that ethanol may have contributed to poor germination.

To improve our understanding of the potential toxicity of ethanol to red spruce seeds we asked two questions: 1) What concentration of ethanol is needed to make filled red spruce seeds sink? 2) What concentration of ethanol inhibits or delays red spruce germination? To answer these questions we performed an ethanol bioassay on two seed lots that were not previously treated with ethanol and another two seed lots that were exposed to ethanol during processing.

Two red spruce seed sources from Mount Mitchell, North Carolina, USA (USA) were collected in 2016 and processed at the USDA Forest Service, National Seed Laboratory in Dry Branch, Georgia: MT-10, single tree (35.75354 °N, -82.24500 °W; 1525 m a.s.l.)

and MT-103, bulk collection (35.75685 °N, -82.25188 °W; 1700 m a.s.l.). The unfilled seeds were removed with a blower and filled seeds were dried and stored at -20°C prior to experimentation. Two sources from New Brunswick, Canada (CAN) were provided from the collections of the NTSC: #20001215, single tree, Perth-Andover (46.73333 °N, -67.6500 °W) and #20001226, bulk collection, Coy Brook (46.26667 °N, -65.5500 °W). CAN seeds were processed and stored in a similar fashion, except the lots were soaked in ethanol for 15 seconds or less to remove unfilled seeds, rinsed with cold tap water for 15-30 seconds, dried and stored at -20°C.

To determine the minimum ethanol concentration required to sink filled red spruce seeds, ethanol (200 proof, anhydrous, ACS/USP grade) concentrations from 10-100% in 10% increments were prepared; deionised water was used as a control. Twenty filled seeds from each source were dipped in each ethanol concentration and the control for 30 seconds. All seeds were immediately rinsed with deionised water for 30 seconds, and then soaked in deionised water for 24 hours to imbibe for germination. Twenty seeds from each source and ethanol concentration combination were drained and placed on moist blotter paper in Petri dishes. Seeds were incubated in an Achieva precision tabletop light/dark germinator (Seedburo Equipment Co., Des Plaines, USA) set to 20°C in darkness for 16 hours followed by 30°C in light for eight hours (AOSA, 2016). Germination was observed every 2-3 days, and totaled when the emerging radicle was 2-3 mm in length. USA and CAN sources were incubated in the same germinator with identical settings for 34 days, however germination of each source was assessed in separate trials. While a 30 second exposure time was used for the main experiment, we also exposed CAN sources to 100% ethanol for 15 seconds to determine if germination rate was affected by a shorter exposure duration.

A four parameter logistic regression equation was used to model the effect of ethanol concentration on both the sinking of filled seeds and on seed germination after one 30 second exposure (equation 1):

$$Y_{Eth} = Y_{Min} + \frac{Y_{max} - Y_{min}}{1 + \left(\frac{Eth}{EC_{50}}\right)^{-b}} \quad (1)$$

where Y_{Eth} is the number of seeds (out of 20) that sank (or germinated) at a given ethanol concentration (Eth), EC_{50} is the effective concentration where 50% of the seeds are affected between Y_{min} and Y_{max}, and b is the slope of the curve. Curve fitting and parameter estimation was performed using SigmaPlot 12.5 software (Systat Software, Inc., San Jose, USA). Differences in ethanol EC_{50} for seed flotation and germination success were analysed by country of origin and processing (CAN, sources 1215 and 1226; USA, sources MT-10 and MT-103) using ANOVA (SAS 9.4, SAS Institute, Cary, NC). Both variables were found to be normally distributed with the Shapiro-Wilk and Kolmogorov-Smirnov tests meeting the assumptions of the analysis.

All four seed sources had germination rates ranging from 75 to 100% in the absence of ethanol. The mean EC_{50} of ethanol affecting the flotation of filled seeds was 84.3% for USA sources and 69.3% CAN sources, though the difference was not statistically significant ($F = 5.02$, $P = 0.15$) (figure 1A). The range of response exhibited by the four

sources indicates that using less than 100% ethanol is not adequate, as some filled seeds could float and be mistakenly categorised as unfilled. The mean EC_{50} of ethanol that inhibited red spruce germination was higher for USA sources (52.7%) than CAN sources (40.8%) (F = 21.93, $P = 0.043$) (figure 1B). Thus, ethanol concentrations that enabled filled seeds to sink also inhibited germination. The germination of CAN sources after a shorter (15 second) exposure to 100% ethanol was 5%.

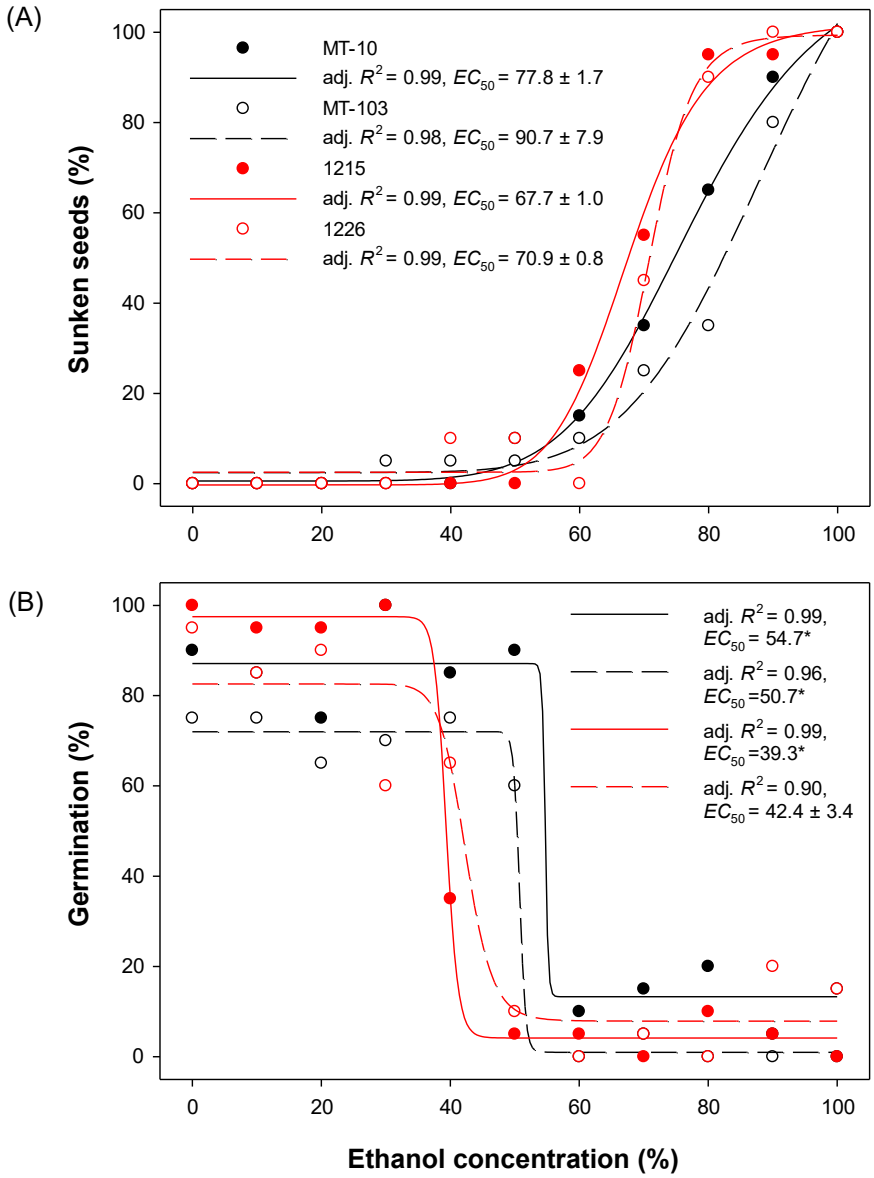

Figure 1. Four parameter logistic regression of ethanol concentration on the percentage of filled seeds that sank (**A**) and the percentage of seeds that germinated (**B**) after 30 seconds exposure. USA seed sources are in black and CAN sources are in red. Twenty seeds were used for each ethanol concentration × seed lot. Standard error of EC_{50} is presented except where noted with an asterisk, the model requires a minimum of two observations on the slope of the function to calculate standard error.

Cumulative germination of USA sources displayed a similar pattern, in which seeds exposed to ethanol concentrations < 50% germinated without delay in comparison with the control, while seeds exposed to ≥ 50% ethanol concentrations exhibited delayed germination or did not germinate at all (figure 2). CAN sources were more sensitive to ethanol and experienced germination delays at concentrations of 30% (1226) and 40% (1215) ethanol.

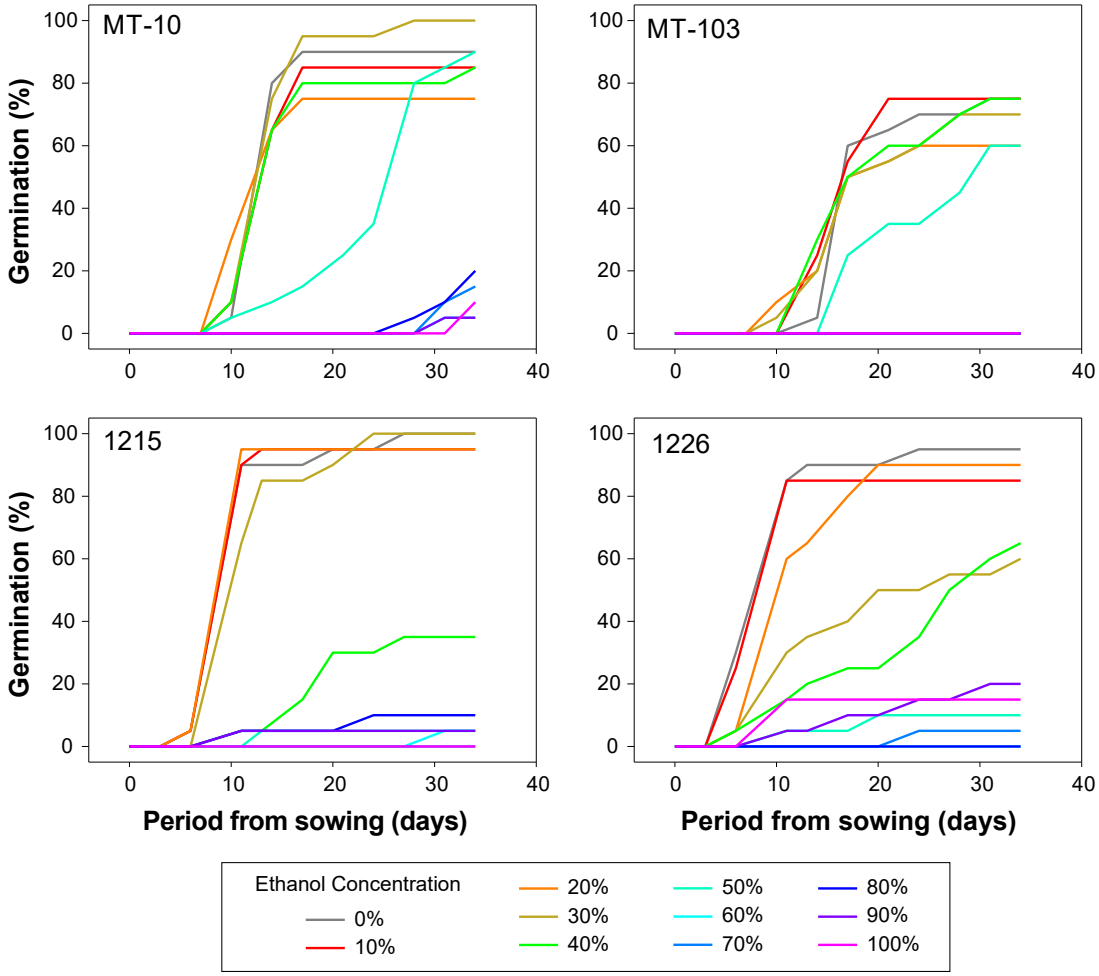

Figure 2. Cumulative germination of 20 red spruce seeds from USA (MT-10, MT-103) and CAN (1215, 1226) sources treated with a 30 second exposure to deionised water or ethanol concentrations

Ethanol flotation prior to long-term storage at -20°C is part of the NTSC standard protocol to process red spruce seeds and does not appear to be deleterious to seed health or longevity of the CAN sources, as they maintained germination rates of 95% or greater after 17 years in storage in the present study. Yet, the results clearly demonstrate that the same seeds are susceptible to ethanol toxicity when subject to additional exposure at concentrations as low as 30%. One potential explanation is that once seeds are dried

and placed into cold storage, they become more susceptible to ethanol toxicity. Namely, changes in the permeability or physical damage of the seed coat may occur in storage that could increase the likelihood that seeds imbibe ethanol and experience toxic effects. In this regard, our bioassay has limitations as it only used seeds stored at -20°C and does not directly replicate the protocols used by Mosseler *et al.* (2000), (Major *et al.*, 2005) and the NTSC. The significant difference between USA and CAN EC_{50} of ethanol is notable, but the seed lots have numerous differences that could contribute to this phenomenon including crop year, latitude, climate, duration of storage and prior ethanol exposure. Drying followed by storage at -20°C does not account for situations in which newly collected seeds, cleaned and processed with ethanol at the NTSC, maintain vigour in storage while our initial attempts with Vermont seeds processed with a similar technique gave poor results.

Baldwin (1932) studied the effect of red spruce seed flotation in 100% ethanol after one and two years of storage at 10°C. Seeds were exposed to ethanol for up to two minutes (no rinsing step specified) then placed in a germinator for 30 days. Interestingly, seeds exposed to ethanol after prolonged storage without prior ethanol exposure broke dormancy faster and had higher germination rates (> 70%) than untreated seed (55-70%). However, Baldwin (1932) found that seeds collected and treated with ethanol prior to being put into storage degraded, albeit at different rates. Specifically, after 14 months, only 10.5% of seeds germinated from collections made in 1927, but 78.7% of seeds germinated and were viable from seed collections made in 1928. These results were attributed to differences in crop years (e.g. weather), but differences in processing or storage conditions could have had a role in predisposing the 1927 crop to ethanol damage. Together, Baldwin's study and ours suggest that exposure to ethanol can reduce seed viability, although the magnitude of reduction in germination appears to vary among seed lots and possibly also storage conditions.

Seemingly small differences in handling, seed extraction, de-winging, seed moisture content and/or duration of the ethanol treatment may account for variable and reduced germination proportions among red spruce seed lots. Considering the results of Baldwin (1932) together with our findings, red spruce varies in its sensitivity to ethanol and the underlying mechanisms are not clearly understood. Our results demonstrate that filled red spruce seeds will only reliably sink in 100% ethanol, and that more dilute ethanol solutions result in ineffective separation. We have not identified the mechanism by which ethanol enters the seed from short exposures resulting in phytotoxicity, but the results clearly show that red spruce seeds previously stored at -20°C are sensitive to ethanol toxicity even for short exposure times. We recommend separating red spruce seeds by physical means (e.g. blowers) rather than ethanol flotation to avoid potential negative impacts on germination.

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