doi: 10.17221/351/2014-HORTSCI

Effect of warm and cold stratification and ethanol treatment on germination of *Corylopsis* seeds

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Abstract

Kim J.H., Lee A.K., Suh J.K. (2016): **Effect of warm and cold stratification and ethanol treatment on germination of *Corylopsis* seeds**. Hort. Sci. (Prague), 43: 84–91.

Seed germination experiments with *Corylopsis coreana*, *C. sinensis* var. *calvescens*, and *Corylopsis gotoana* were conducted. *Corylopsis coreana* seeds were treated at 5, 7.5, 10, 12.5 and 20°C to study the upper temperature limit to break dormancy. Seeds of *C. sinensis* var. *calvescens* were treated 1 month with warm stratification (WS) at 10, 15, 20, and 25°C followed by cold stratification (CS) at 5°C for 0, 1, 2, and 3 months to understand the requirements of WS and CS to effectively break dormancy. Germination of *Corylopsis* seeds at 15–20°C without CS suggests that dormancy is considered shallow and the upper temperature limit to break is 12.5°C. Immersing seeds in water or ethanol induced seed germination which may result from leaching of inhibitors from the seed. Observation of seed coats following treatment with water and ethanol by means of low temperature scanning electron microscopy (LT-SEM) revealed elongated and rectangular/hexagonal shaped cells in *C. coreana* and irregular jigsaw puzzle-piece shapes in *C. gotoana*. In conclusion, dormancy in *Corylopsis* is considered shallow and the optimum CS is suggested to be 7.5°C, while the optimum WS is ranging from 12.5 to 20°C.

Keywords: *Corylopsis coreana*; *Corylopsis gotoana*; *Corylopsis sinensis* var. *calvescens*; optimal temperature; scanning electron microscopy; seed dormancy

Corylopsis Siebold & Zucc. is a genus of nearly 30 species of woody shrubs in the witch-hazel family (Hamamelidaceae). Shrubs within the genus, commonly known as winter hazels, are used as landscape plants due to their showy yellow flowers and early season flowering characteristics. *Corylopsis* has been propagated using *in vitro* techniques (Moon 2002), however, acclimatization was difficult. Although some forms are propagated by rooting of softwood cuttings, seeds have been used for mass propagation.

Dormancy in *Corylopsis* seeds is not well investigated. It is not known whether dormancy is induced by physical dormancy imposed by the seed coat, by physiological dormancy which imposed internally involving the embryo, or by both physical and physiological dormancy (Baskin, Baskin 2004; Hilhost 2008). To release seeds from dormancy imposed by the seed coat, scarification or abrasion of the seed coat is practiced with many types of seeds. In seeds without hard seed coat such as *Jasminus fruticans* L. stratification at warm and cold storage temperatures effectively break dormancy and induces germination (Pipinis et al. 2009). Scarification alone failed to induce germination of *Koelreutria paniculata* Laxm., which has a hard seed coat (Rehman, Park 2000). Scarification of seeds of *Styrax japonicus* L., which also have a

doi: 10.17221/351/2014-HORTSCI

hard seed coat, resulted in germination rates not higher than 50% (Kwon 1995), while warm stratification (WS) and cold stratification (CS) increased the germination rate to more than 80% (Roh et al. 2004). To effectively release seeds of many woody genera from physiological dormancy, seeds were subjected to WS followed by CS (1–5°C) (Dirr 1990; Batlla, Benech-Arnold 2003).

Some metabolites, such as phenolics (Siddiqui, Khan 2010) or flavonoids (Wada et al. 2011), may be leached out as a result of ethanol or hot water treatment (Cavanagh 1980). Ethanol at 0.2 to 0.5M in the dark or after exposure to 5 min of red light promoted germination of seed of fall panicum (*Panicum dichotomiflorum* Michx.) (Taylorson, Hendricks 1979). Plant growth regulators also affect seed germination. Generally, seed germination was promoted by a decrease in abscisic acid (ABA) concentration and an increase in gibberellin concentration (Hillhost 2008), although gibberellic acid (GA_3) treatment was not effective when seeds received a sufficient cold treatment (Roh et al. 2004).

When seeds of *Corylopsis coreana* Uyeki and *C. sinensis* var. *calvescens* Rehder & E. H. Wilson were germinated, a few *C. coreana* seeds had germinated after 2 months at 10°C without CS at 5°C, although CS was required for more than 90% of seeds to germinate (Roh et al. 2008). Further, variable response among *Corylopsis taxa to* seed treatment (Roh et al. 2008) may suggest differences in anatomy of the seed coat (Haughn, Chaudhury 2005), or leaching of certain metabolites involved in seed germination, when *C. sinensis* var. *calvescens* was soaked in water for 16 hours.

Therefore, these studies were conducted to investigate the following: germination of *C. coreana* seed as influenced by WS at different temperatures and CS; the effect of CS duration on germination of seed of *C. sinensis* var. *calvescens*; the effect of ethanol treatment on germination of seed of *Corylopsis gotoana* and *C. coreana*; and on changes in the shape of cells in the seed coat.

MATERIAL AND METHODS

Plant materials and seed germination treatments. Plants were grown outdoors at Beltsville, USA and seeds were collected from *Corylopsis coreana* Uyeki, *C. gotoana* Makino, and from *C. sinensis* var. *calvescens* Rehder & E.H. Wilson (NA57391). Seeds were harvested as specified in each experiment and only those seeds that sank after immersion in water for 15 min to remove empty seeds were used in these experiments.

Seeds were sown in pots and covered with about 0.5 cm deep as specified in each experiment, which were filled with Metro Mix 200 or Pro-Mix BX germination medium (Premier Horticulture Inc., Quakertown, USA) and received various temperature treatments. Cold stratification (CS) was provided at 5°C in a refrigerator. Seeds were considered germinated when the hypocotyl emerged from the medium, and recorded as specified in each experiment.

Germination of *C. coreana* as influenced by temperature (Experiment No. 1). One hundred seeds were sown in 10 cm pots filled with germination medium, and pots were placed in incubators maintained at 5, 7.5, 10, 12.5, and 20°C for one month in 2011, and then transferred to an air-conditioned greenhouse maintained at 18.5°C/18°C (day/night). After an additional month, all pots with non-germinated seeds were transferred to CS for 3 months, and then were returned to the greenhouse to record the number of germinated seeds. The number of seeds germinated one month after the end of temperature treatment was counted and the final number of seeds germinated was counted one month following removal from CS. The number of days for the first seeds to germinate was counted from the time of the seeds were sown. Each treatment was replicated 3 times, with 100 seeds per replication.

Effect of temperature treatments prior to CS and duration of CS on seed germination of *C. sinensis* var. *calvescens* (NA57391) (Experiment No. 2). One hundred seeds sown on February 2, 2011 were placed in incubators maintained at 10, 15, 20, and 25°C for one month of temperature treatment, followed by 0, 1, 2, and 3 months of CS. After CS, pots were moved to an air conditioned greenhouse maintained at 18.5°C/18°C (day/night), and the number of seedlings germinated was recorded daily. The number of days to germination was counted on completion of CS. Each treatment was replicated 3 times, with 100 seeds per replication.

Effect of ethanol treatment and cold stratification on germination and seed coat cell shape in *C. coreana* and *C. gotoana* as determined by low temperature-scanning electron microscopy (LT-SEM) (Experiment No. 3). One hundred *C. coreana* seeds harvested on October 12, 2012 were stored dry at 5°C until May 27, 2013. On that date, seeds

doi: 10.17221/351/2014-HORTSCI

either remained dry (dry) or were treated with 30 ml of distilled water (0% ethanol (EtOH)) for 75, 150 or 300 min, 25% ethanol (EtOH) for 30, 60, or 120 min, 50% EtOH for 15, 30 or 60 min, or 75% EtOH for 10, 20 or 40 min, on a shaker at 50 rpm. Each treatment was replicated 3 times, with 100 seeds per replication. Liquid-treated seeds were then dried at 20°C overnight in darkness and 100 seeds were then sown in 15 cm pots filled with the medium, and stored at 20°C for 7 weeks. Once the number of seeds remained unchanged for 45 days, pots were moved to CS for 2 months, and then moved back to the 20°C temperature regime for germination. The number of seeds germinated was counted weekly for 6 weeks. The total number of seeds germinated was obtained by combining the number of seeds germinated before and after CS.

Another 10 seeds were treated either with distilled water or 75% EtOH for 40 min, and washed twice with 25 mL of distilled water, after which the seed coats were imaged using LT-SEM as described previously (Roh et al. 2012). Images of the control seeds treated with distilled water and of seeds treated with 70% EtOH were compared.

Data analysis. Data were subjected to analysis of variance (ANOVA) using Statistical Analysis System software ver. 9.0 (2002; Cary, USA). Experiments during greenhouse phase after temperature and EtOH treatment in the laboratory or incubators were completely randomized. In Experiment No. 1, temperature was the independent variable with three replications per treatment. Temperatures and durations of CS (Experiment No. 2), and *Corylopsis* species (*C. coreana* and *C. gotoana*) and EtOH concentration and duration were included in the model for analysis, and data were reanalysed by species to understand the effect of ethanol concentrations and treatment durations once it was determined that there was a significant effect by species. The number of days to germination was counted from sowing date after temperature or EtOH treatment. The final germination percentage and the number of days to reach to the final germination was recorded when seeds did not germinate for 30–45 days. Means were compared by the Tukey-Kramer's test (*honestly significant difference* (HSD)) or Duncan's multiple range test as indicated in each table.

RESULTS

Germination of *C. coreana* as influenced by temperature and 5°C cold stratification treatment (Experiment No. 1)

The first seeds to germinate were observed at day 28 (prior to CS) in the 20°C storage temperature treatment. This was significantly earlier than the 46 to 50 days required for the first seeds to germinate at the other temperature regimes: 5, 7.5, 10, and 12.5°C (Table 1). At the time seeds were moved to CS, the germination percentages were significantly lower for the 12.5°C (14%) and 20°C (9%) temperature regimes in comparison with the lower temperature treatments. After CS, the final germination percentages were 66, 82 and 67% at 5, 7.5, and 10°C, respectively, which was significantly higher than at 12.5 and 20°C.

Table 1. Effect of storage temperature and cold stratification on seed germination of *Corylopsis coreana* (Experiment No. 1)

Storage temperature (°C) for 1 month after sowing	No. of days for the first seed to germinate[1]	Germination (%)	
		2 month after sowing before cold stratification[2]	6 month after sowing after cold stratification
5	50[a3]	71[a]	66[b]
7.5	46[a]	60[a]	82[a]
10	46[a]	51[a]	67[b]
12.5	49[a]	14[b]	49[c]
20	28[b]	9[b]	45[c]
Level of significance[4]	**	**	**

[1]number of days to germination was counted from March 18; [2]seeds were cold stratified (CS) at 5°C for 3 months; [3]means with different letters within each column are significantly different by Duncan's multiple range test, $P \leq 0.01$; [4] significant $P \leq 0.05$ (*) or $P \leq 0.01$ (**)

doi: 10.17221/351/2014-HORTSCI

Effect of temperature treatments prior to cold stratification and duration of CS on seed germination of *C. sinensis* var. *calvescens* (NA57391) (Experiment No. 2)

Without CS only 3 seeds (0.3%) of the control germinated at 53 days, 1.3% of seeds germinated at 84 days after sowing in the greenhouse (GH) maintained at 18.5°C/18°C (day/night) (GH; control) (Table 2). Without CS (0 month), only 3.7, 0.7, and 1.0% of seeds germinated; at pre-sowing temperature of 10°C (10°C/0 month), 15°C or 20°C (20°C/0 month) seeds germinated in 22, 35, and 42 days, respectively. Seeds treated at 25°C (25°C/0 month) did not germinate for 78 days when data collection was terminated. Without CS, the highest final germination (26% in 42 days) was observed with seeds treated at 10°C (10°C/0 month). No seeds germinated thereafter.

When seeds received 1 month of CS following pre-sowing temperature treatment (Table 2), the first seeds germinated in 15 days, regardless of pre-sowing temperatures, and the final germination ranged from 60 to 79% in less than 21 days. Percentages were not significantly different among pre-sowing temperature treatments. When seeds received 2 or 3 months of CS (Table 2), no significant differences in the days to the first seeds germination and the final germination were observed among pre-sowing

Table 2. Effect of temperature and the duration of cold treatment on seed germination of *Corylopsis sinesis* var. *calvescens* (NA57391) (Experiment No. 2)

Pre-sowing temperature[1] (°C)	Storage at 5°C (month)	50% final germination		Germination (%)	
		No. of days to[2]	germination % at day[3]	final germination	final after 5°C
GH	GH	53	0.3	84	1.3
10	0	22	3.7	42	26
15	0	35	0.7	47	1.3
20	0	42	1	48	1.3
25	0	0[4]	0[4]	0[4]	0[4]
10	1	15	2.7	19	79
15	1	15	1	21	77
20	1	15	0.3	21	65
25	1	15	1.7	21	60
10	2	8	0.7	19	91
15	2	8	1	21	91
20	2	8	0.3	21	92
25	2	9	0.3	21	90
10	3	11	0.3	20	89
15	3	12	6.7	20	90
20	3	11	0.7	19	93
25	3	11	0.7	20	90
Level of significance[5]					
Pre-sowing temperature (PST) (°C)		ns	ns	*	ns
Months at 5°C		**	ns	**	*
PST × months at 5°C		**	ns	ns	ns
HSD[6] $P \leq 0.01$		13.0	7.2	7.2	13.5

[1]seeds were stored for 1 month in the greenhouse (GH) at 10, 15, 20, and 25°C followed by germination in the greenhouse (GH-GH) or cold stratified at 5°C for 0, 1, 2 or 3 months; [2]number of days reaching to 50% level of the final germination was rounded up; [3]germination percentage at days when germination reached to 50%; [4]seeds did not germinate for 84 days; [4]number of days cannot be calculated due to a low final germination percentage; [5]non-significant (ns) or significant at $P \leq 0.05$ (*) or $P \leq 0.01$ (**); HSD – Honestly significant difference (Tukey-Kramer's test)

doi: 10.17221/351/2014-HORTSCI

temperature treatments, and seeds germinated in 9–12 days, reaching final germination rates of over 89% in 19–20 days. Interaction of the first seeds germination between pre-sowing temperature and duration of CS was observed.

Effect of ethanol treatment and cold stratification on germination and seed coat images of *C. coreana* and *C. gotoana* (Experiment No. 3)

The germination percentage was higher in *C. gotoana* both before CS (>19.3% at 0–7 weeks) and the final germination percentage after 2 months of CS 22.0% (Cs-Fi) than in *C. coreana* >12.3% at 0–7 weeks and >13.3% at Cs-Fi (Table 3). Germination of *C. coreana* seeds that were not treated with ethanol and not soaked in water reached 2.7% and 18.7% in 3 weeks (0–3 weeks) and 7 weeks (0–7 weeks), respectively, before receiving 2 months of CS (Table 3) as compared to 9.3% and 38.7% in *C. gotoana*. The final germination percentage of *C. coreana* seeds after CS (Cs-Fi) was 21.7%, which was not different from germination prior to 2 months of CS (0–7 weeks). However, germination was 38.7% in *C. gotoana* in 7 weeks (0–7 wk) before 2 months of CS, which is higher than that of *C. coreana* and the final germination was further increased to 58.7% in 6 weeks (Cs-Fi) which was also significantly higher than that of *C. coreana*.

Immersing *C. coreana* seeds for 150 min prior to 2 months of CS and 75 min after 2 months of CS increased germination percentage significantly; however, this effect was not observed in *C. gotoana* (Table 3). When *C. coreana* seeds were treated with

Table 3. Effect of ethanol concentration and seed soaking duration on seed germination of *Corylopsis coreana* and *C. gotoana* (Experiment No. 3)

Ethanol (EtOH) concentration (%)	Seed soaking duration (min)	Germination (%)[1]							
		weeks			Cs-Fi	weeks			Cs-Fi
		0–3	0–7	Cs-1		0–3	0–7	Cs-1	
		C. coreana				*C. gotoana*			
dry	0	2.7^{b2}	18.7^{bc}	0.7^{b}	21.7^{c}	9.3^{abz}	38.7^{ab}	3.3^{ab3}	58.7^{a}
	75	3.3^{b}	28.0^{b}	1.3^{b}	36.3^{b}	11.7^{a}	40.3^{a}	2.3^{ab}	54.7^{a}
0	150	4.0^{b}	18.0^{bc}	0.0^{b}	21.3^{c}	4.3^{ab}	24.3^{abc}	1.7^{b}	45.7^{a}
	300	1.3^{b}	12.3^{c}	0.0^{b}	17.0^{c}	10.7^{ab}	2.7^{a}	4.0^{ab}	66.3^{a}
	30	11.3^{a}	38.0^{a}	7.7^{a}	57.7^{a}	2.0^{ab}	19.3^{c}	0.7^{b}	25.3^{b}
25	60	2.7^{b}	15.3^{c}	0.3^{b}	17.3^{c}	2.3^{ab}	21.0^{bc}	0.3^{b}	22.0^{b}
	120	3.0^{b}	19.0^{bc}	0.7^{b}	20.0^{c}	5.0^{ab}	26.0^{abc}	3.3^{ab}	46.3^{a}
	15	2.0^{b}	16.3^{c}	2.0^{b}	20.0^{c}	5.0^{ab}	34.0^{abc}	2.3^{ab}	48.7^{a}
50	30	3.3^{b}	19.3^{bc}	0.3^{b}	20.7^{c}	7.7^{ab}	39.7^{ab}	4.7^{ab}	63.7^{a}
	60	3.0^{b}	20.3^{bc}	0.3^{b}	22.0^{c}	8.7^{ab}	35.7^{abc}	5.3^{ab}	63.7^{a}
	10	2.0^{b}	17.7^{bc}	0.3^{b}	20.3^{c}	1.3^{b}	25.3^{abc}	4.3^{ab}	49.0^{a}
75	20	1.7^{b}	10.7^{c}	1.3^{b}	13.3^{c}	11.7^{a}	43.3^{a}	9.0^{a}	67.3^{a}
	40	1.7^{b}	12.3^{c}	0.7^{b}	13.3^{c}	5.3^{ab}	28.3^{abc}	4.0^{ab}	48.7^{a}
Level of significance[4]									
Ethanol concentration (A)		***	**	*	***	**	***	*	***
Treatment duration (B)		*	***	**	***	ns	ns	ns	*
A × B		***	***	*	***	*	*	ns	**

[1]germination percentage was recorded 3 and 7 weeks prior to cold stratification (CS) for 2 months at 5°C and 1 week after CS (CS-1 week) and final germination percentage recorded in 7 weeks (CS-Fi); [2]means with different letters within each column are significantly different by Duncan's multiple range test, $P \leq 0.01$; [3]means with different letters within each column are significantly different by Duncan's multiple range test, $P \leq 0.05$; [4]non-significant (ns) or significant at $P \leq 0.05$ (*), 0.01 (**), or 0.001 (***)

doi: 10.17221/351/2014-HORTSCI

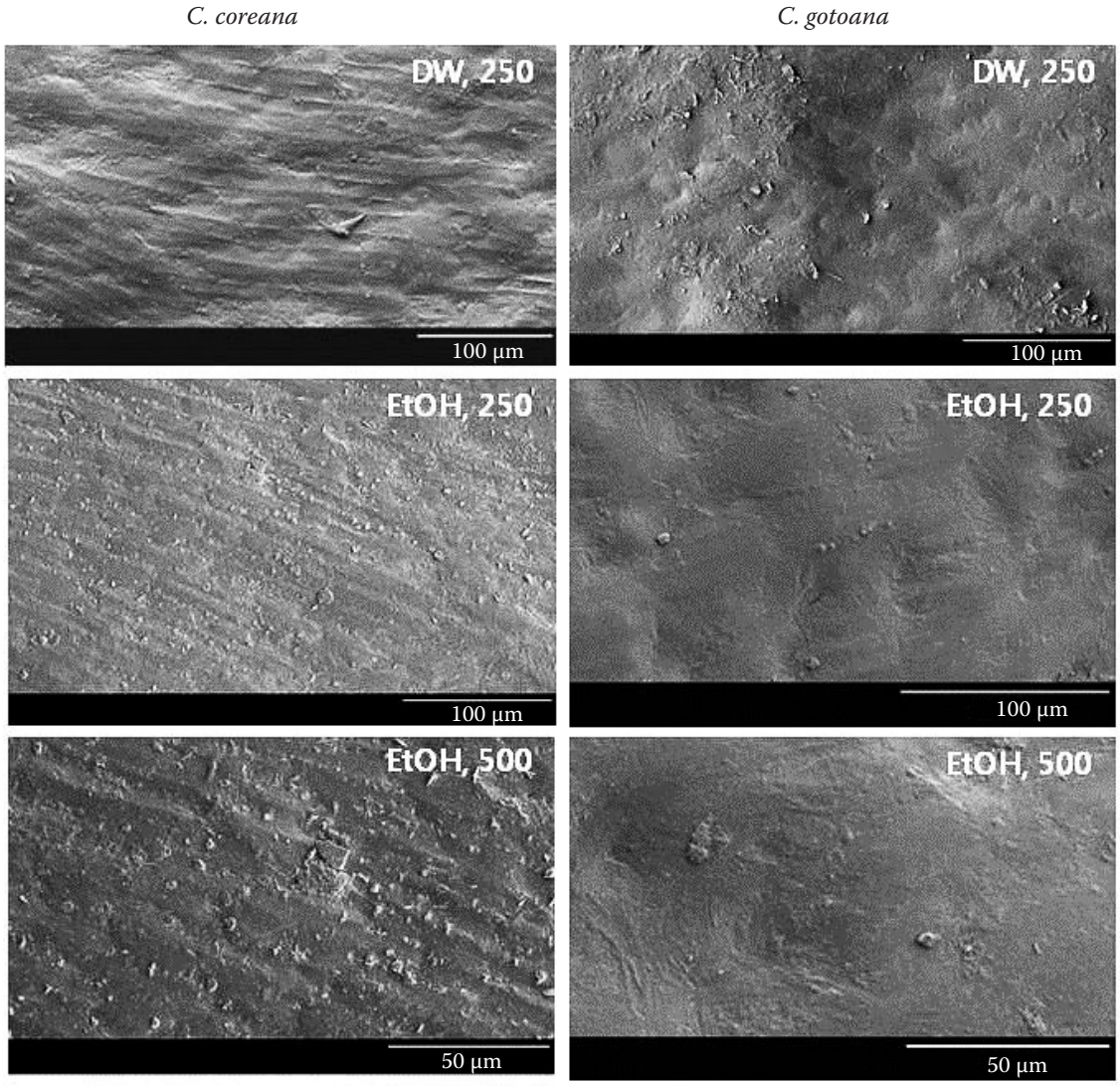

Fig. 1. Low temperature-scanning electron micro-photos of the seed coat surfaces of *C. coreana* and *C. gotoana* immersed in distilled water (DW) and 75% ethanol (EtOH) for 40 min at magnification 250× (bar – 100µm) and 500× (bar – 50 µm)

25% ethanol solution for 30 min, germination was significantly increased to 38% in 7 wk (0–7 wk), before CS, and 57.7% after 2 months of CS (Cs-Fi) (Table 3), as compared to germination percentages of all other ethanol treatments, regardless of the concentrations and durations.

The seed coats treated with distilled water (DW) showed intrinsic differences in cell shape; elongated and rectangular/hexagonal shaped cells were observed in *C. coreana* and irregular jigsaw puzzle-piece shapes in *C. gotoana* (Fig. 1). At a higher magnification (500×), individual cells were visible on the surface of *C. coreana*, while only the smooth surface of the seed coat was visible in *C. gotoana*. On the surface of *C. coreana* seeds, and especially on the surface of *C. coreana* seeds treated with 75% ethanol, aggregates or droplets of unidentified deposits were observed more than on the surface of *C. gotoana* seeds.

DISCUSSION

Seed germination is affected by many factors, such as maturity of seeds and release from dormancy by stratification. When stratification is necessary, optimum upper (CS) and lower (WS) temperatures to maximize germination with min. treatment duration must be determined. Dormancy is broken following WS and/or CS in many woody plants, including *Styrax japonicus* (Young, Young 1992; Roh et al. 2004, 2011). Without CS,

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low percentage of *C. coreana* seeds germinated at 18.5°C/18°C (day/night) (Roh et al. 2008).

Effective WS and CS on seed germination: level of dormancy and upper temperature limit for CS and/or lower temperature limit for WS

The ability of *Corylopsis* seeds to germinate at a constant WS (15 or 20°C) without CS suggests that dormancy is shallow, but the level of dormancy varies seed by seed. The upper temperature limit of CS to break dormancy for *C. coreana* seeds was 10°C, at which 14% of seeds germinated. Less than 5% of mature seeds of *Empetrum hermaphroditum* Hagerup exhibiting intermediate physiological dormancy germinated at temperatures of 15–25°C/6–15°C (day/night) in light and germination was increased with CS treatment; perhaps germination was influenced by fluctuating 6–15°C at night, with temperatures between WS (≥15°C) and CS (~0–10°C) (Baskin et al. 2002).

Some seeds germinated when harvested on August 2, and this suggests that dormancy may not be deep in seeds of *C. coreana, C. sinensis* var. *calvescens*, or *C. gotoana*. Dormancy may not be deep in early harvested seeds which are immature. Although WS for as long as 3–5 months has been suggested prior to CS (Dirr 1990), another study with just 1–2 months of WS resulted in a high germination percentage. Also, in *Styrax japonicus*, 1–2 months of WS was sufficient to obtain a high germination percentage (Roh et al. 2004), as opposed to 3–4 months of WS (Dirr 1990; Kwon 1995).

Based on these experiments, it is evident that seeds of both *Corylopsis* species are not in deep dormancy and able to germinate even at the wide temperature ranges of 12.5 and 20°C for WS, followed by 2 months of CS. To achieve more than 80% germination, it is recommended that *C. coreana* seeds be stratified at 7.5°C for 1 month, and other species tested at temperatures of 5–10°C for longer than 1 month.

Effect of ethanol treatment on germination of *C. gotoana*

Immersing *C. coreana* seeds for 30 min at 20% ethanol (EtOH) seemed to promote germination, as did immersing *C. gotoana* for 20 min at 75% EtOH. Further evaluation should be carried out to determine the effects of ethanol and water on seeds, employing ethanol concentrations in the 15–75% and immersion durations of 10–30 minutes. This approach may be reasonable since ethanol effects were observed in the 7 weeks prior to CS and the 1 week after CS treatments. It has been suggested that immersing seeds in water may cause leakage of ABA, thus lowering the ABA content as observed in seeds of *Arabidopsis thaliana* (L.) Heynh. (Ali-Rachedi et al. 2004). Reduced ABA content was also observed in developing embryos of *Triticum aestivum* L. incubated in water at 20°C at 30–40 days after pollination, when premature germination was observed (Suzuki et al. 2000). Ethanol at 0.2–0.5M promoted seed germination of fall panicum in dark (Taylorson, Hendricks 1979). Perhaps it is not the effect of ethanol but rather the effect of immersing seeds in water that leaches inhibitors, thus affecting seed germination.

With regard to seed coat morphological changes leading up to germination, mucilage in the apoplast of the epidermis is extruded when *Arabidopsis* seeds are immersed in water (Haughn, Chudhury 2005), while dissolved and empty-looking cells with distinguishable cell walls are observed in the seed coats of *Linum usitatissimum* L. (Barbary et al. 2009). It is not known whether changes in seed coat morphology such as the presence of aggregates or droplets of unidentified deposits are related to the differences in the seed germination patterns observed in *C. coreana* and *C. gotoana*. Mucilage produced by the seed coat may facilitate seed germination (Western 2012). Also, placing seeds in solutions such as ethanol/water extract, typically for 24 h, should be investigated (Liu, White 2012).

Currently, the changes in abscisic acid and gibberellins are being investigated and preliminary data indicate that ABA concentration is significantly decreased following WS, not CS. Soaking seeds in EtOH solution may cause leaching ABA from seeds, resulting in increased germination. In *Styrax japonicus*, ABA concentration was significantly decreased following WS (Horimoto et al. 2011).

In conclusion, dormancy of seeds in three *Corylopsis* taxa, *C. coreana*, *C. gotoana* and *C. sinensis* var. *calvescens* is considered shallow due to the fact that seeds germinate without CS. To achieve > 80% germination, it is recommended that seeds be stratified (WS) at 12.5–20°C, followed by CS at 7.5°C. Changes in morphology of the seed coat following EtOH treatment as well as a significant effect of treatment

doi: 10.17221/351/2014-HORTSCI

duration on germination further supports the idea that germination inhibitors are leached from seeds immersed in ethanol, which requires further experiments to investigate the effect of leachate on germination of seeds that do not show dormancy. Further, it is suggested that seed coat of *Corylopsis* is not hard.

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Received for publication October 16, 2014
Accepted after corrections May 7, 2015

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