Germination Trials for Asian and North American Ash Species

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Abstract

North American ash trees (genus *Fraxinus*) have been affected by the emerald ash borer (*Agrilus planipennis* Fairmaire; EAB), an aggressive, invasive insect native to southeastern Asia; native Asian ash species are comparatively resistant to this phloem-feeding insect. Little research exists on optimal growing conditions for germinating seeds from various ash species, aside from those with the widest natural ranges. The objective for this research was to evaluate the effects of temperature, photoperiod, and seed scarification on germination of nine ash species. Germination rates and percentages were evaluated under three test conditions (controlled environment, agar-based solid medium, and potting mix). Chinese and green ash seeds germinated the best, while black, blue, common, and Manchurian ash seeds had the lowest germination rates across all three tests. Optimized germination techniques for ash will enable research into the EAB resistance mechanism of some ash species. In addition, North American nurseries may be able to use the information presented here to more efficiently grow Asian or other ash species that may be resistant to EAB.

Introduction

Ash trees (genus *Fraxinus*) are important in North America. These trees are fundamental aesthetic elements of nearly every city and suburban landscape, have high stumpage value, and are an important commercial lumber and pulp species for the furniture and paper- and tool-making industries. In addition, ash trees serve as significant Native American cultural resources and play an integral part in the ecology of North America (Cappaert and others 2005). The ash-tree industry in North America is threatened by a recently introduced invasive species: the emerald ash borer (*Agrilus planipennis* Fairmaire; EAB; MacFarlane and Meyer 2005; Poland and McCullough 2006). EAB larvae feed on phloem of ash trees, ultimately killing the tree by restricting vascular transport (figure 1). Because of the introduction of the EAB in North America, most nurseries in the United States, even those in the West, where EAB has yet to arrive, have stopped growing *Fraxinus* species. As a result, ash seedlings are increasingly unavailable to researchers interested in studying ash (especially in the context of EAB), so they must grow their own (figure 2). Little information has been published on optimal conditions for germination of Asian and European ash species. What limited research that has been done has focused on seed cutting or embryo-rescue treatments (both of which are time consuming) in the most common North American ash species, such as green (*Fraxinus pennsylvanica* Marshall), white (*F. americana* L.), black (*F. nigra* Marshall), and the European common ash (*F. excelsior* L.) (Steinbauer 1937; Villiers and Wareing 1964, 1965; McBride and Dickson 1972; Bonner 1975; Marshall 1981; Stinemetz and Roberts 1984;

Figure 1. Emerald ash borer larvae galleries under the bark of a mature white ash tree in Fort Wayne, IN, May 2009. (Photo by Darla French)

Figure 2. Three-year-old white ash trees that the authors grew from seed and maintained in the greenhouse. (Photo by Darla French)

Vandewalle 1985; Piotto 1994; Preece and others 1995; Piotto and Piccini 1998; Ashley 2002; Raquin and others 2002; Ashley and Preece 2004, 2009).

The objective for this research was to evaluate varying environmental conditions and seed treatments on germination of nine ash species.

Materials and Methods

Seed Source and Preparation

Nine ash species were included in this study (table 1). All seeds were stored in sealed containers over a desiccant at 40 °F (4 °C) in the dark until testing (up to 12 months). Seeds of all species were germinated under three different test conditions: potting mix; a controlled environment; and an agar-based, solid medium.

Before the germination tests, the pericarps were removed from the seeds, except for some of those used in the potting-mix test, in which pericarps were removed for two treatments and left intact for the third. To remove pericarps, seeds were soaked in tap water at room temperature for several hours to soften tissue and facilitate removal. Before the tests on an agar-based, solid medium and potting mix, seeds were imbibed for 20 minutes using 0.3M NaOH, followed by surface sterilization at two concentrations of calcium hypochlorite [$Ca(ClO)_2$] solution, each with 0.01 percent Tween® 20 (Sigma-Aldrich, St. Louis, MO) according to a protocol described by Raquin and others (2002). At the end of sterilization, seeds became white and translucent, and embryos were clearly visible. Seeds damaged by insects and those without embryos or showing necrosis were discarded. Seeds were then subjected to the tests described below. In all tests, seeds were considered germinated when both a radicle hook and cotyledons were apparent (figure 3).

Table 1. Ash (*Fraxinus*) species and seed sources used in this study. Seeds for all tests were obtained in 2009 and 2010 from Sheffield's Seed Co., Inc. (Locke, NY), or Lawyer Nursery (Plains, MT). Lot numbers available upon request.

Species	Scientific name	Seed source
Black ash[a]	*F. nigra* Marshall	Ontario, Canada
Blue ash[a]	*F. quadrangulata* Michx.	Indiana, United States
Chinese ash[a]	*F. chinensis* Roxb.	Beijing, China
European common ash[a]	*F. excelsior* L.	Poland
Flowering ash	*F. ornus* L.	Hungary
Green ash[a, b]	*F. pennsylvanica* Marshall	South Dakota, United States[a] Pennsylvania, United States[b]
Manchurian ash[a, b]	*F. mandschurica* Rupr.	Beijing, China
Pumpkin ash[a]	*F. profunda* Bush	Louisiana, United States
White ash[a, b]	*F. americana* L.	North Dakota, United States

[a] Seed from Sheffield's Seed Co., Inc. (Locke, NY).

[b] Seed from Lawyer Nursery (Plains, MT).

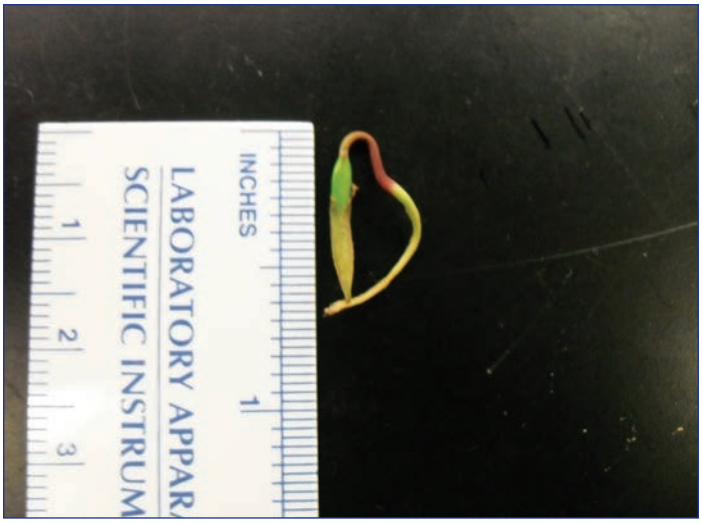

Figure 3. Example of a germinated Chinese ash seedling exhibiting a radicle hook and the presence of cotyledons. (Photo by Darla French)

Controlled-Environment Test

For the controlled-environment test, incubators were set at four constant temperature treatments: 50, 59, 68, and 77 °F (10, 15, 20, and 25 °C, respectively) and a fifth treatment consisted of alternating 59 °F in the dark and 77 °F in the light (15 and 25 °C, respectively). At each temperature treatment, 8-h and 16-h photoperiods were used for a total of 10 treatments. There were three replicates for each temperature/photoperiod treatment. One hundred intact seeds per treatment replicate per species were placed on blotter paper in clear 6-oz (177-ml), 3.5-in (89.0-mm) diameter plastic jars (Parkway Plastics, Inc., Piscataway, NJ) and were wetted with ~0.2 oz (~5.0 ml) tap water (figure 4). Seeds were neither imbibed nor sterilized before the test. Containers were left in the incubators for 30 days, with germination recorded every 5 days. Rewetting of blotter paper with tap water was performed periodically during the 30 days.

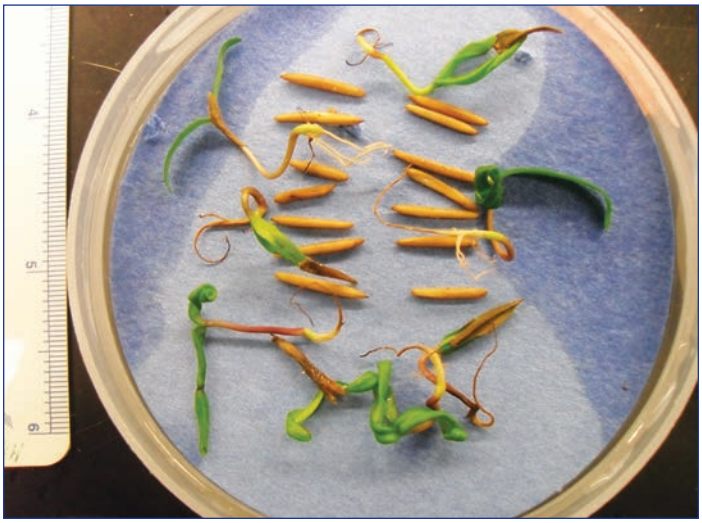

Figure 4. Chinese ash seeds and seedlings 20 days after sowing in the controlled-environment experiment. (Photo by Darla French)

Agar-Based, Solid-Medium Test

For the agar-based, solid-medium test, imbibed, sterilized seeds were plated on Murashige and Skoog (MS) media (Murashige and Skoog 1962) containing 3 percent sucrose. Seeds were either left intact, or were cut to remove 0.2 in (0.5 cm) from cotyledonary ends of seeds as described by Ashley and Preece (2004). Three replications were made of at least 100 seeds per species for each of the two treatments. Plated seeds were left at room temperature under 40W Sylvania Gro-Lux lamps for 60 days (16-h photoperiod), with germination recorded every 5 days (figure 5).

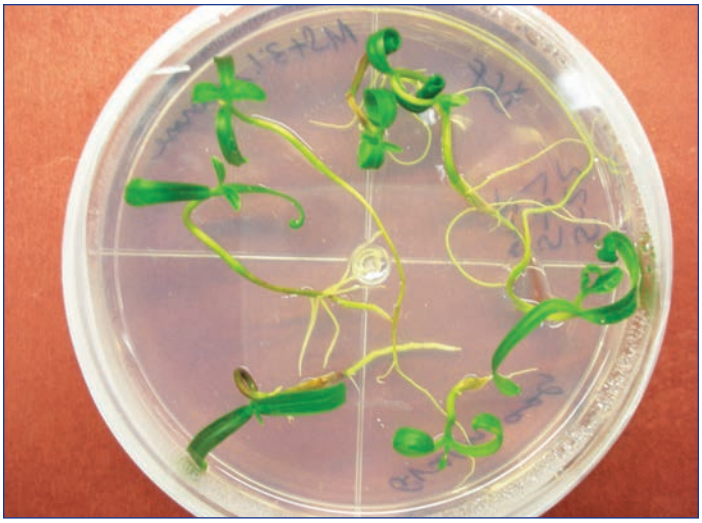

Figure 5. Green ash seedlings 30 days after sowing on agar-based solid medium. (Photo by Darla French)

Potting-Mix Test

For the potting-mix tests, sterilized seeds were planted in standard 36-cell seed trays (overall dimensions: 11 by 21 in [28 by 54 cm]; volume of each cell: ~3 in^3 [49 cm^3]) in Premier® Pro-Mix® PGX Grower Mix (Premier Horticulture, Ltd., Quakertown, PA), a high-porosity, peat-based germination and growing medium optimized for seedling production in plug systems (figure 6). Seed were sown at a depth of 0.2 to 0.4 in (0.5 to 1.0 cm) as recommended by the supplier. As with those tested in the agar-based, solid medium, seeds were either left intact, or they were cut as described by Ashley and Preece (2004). In a third treatment, seeds without the pericarp removed were sown into the potting mix. Three replications were made of at least 100 seeds per species for each treatment. Seeds were left under a combination of 600W high-intensity discharge lamps; high-pressure sodium lamps (PARsource Lighting Solutions, Petaluma, CA); and 60W cool white, energy-efficient fluorescent lamps (4,100 K [color temperature], 6,150 initial lumens; Grainger, Inc.. Lake Forest, IL) for 60 days (16-h photoperiod), with germination recorded every 5 days. Seed trays were watered regularly to maintain adequate moisture in the potting mix.

Statistical Analyses

Statistical comparisons were made between treatments for each experiment using one-way analysis of variance (ANOVA) based on means of three replicates per test.

Figure 6. Green ash seedlings 20 days after sowing on potting mix medium. (Photo by Darla French)

Results

Controlled-Environment Test

ANOVA tests were used to analyze germination rates after 30 days in the controlled-environment experiment by species, temperature, and photoperiod (table 2). Chinese and green ash seeds had higher germination rates as temperature increased, regardless of photoperiod. In addition, both Chinese and green ash seeds had greater germination under the shorter photoperiod than seeds at the same temperature under the longer photoperiod. Pumpkin ash only germinated at 68 °F (20 °C) under a short photoperiod but did not germinate under any other conditions. Black, common, and Manchurian ash seeds failed to germinate under any condition, suggesting their stratification needs and/or required germination conditions were not met.

Agar-Based, Solid-Medium Test

For all species on the MS medium, germination rates tended to be more rapid and higher for seeds in the "cut-seed" treatment than those in the "intact-seed" treatment (table 3); however, flowering ash was the only species for which a statistically significant difference occurred between treatment groups ($p = 0.0108$).

Table 2. Germination percentages for five species of ash seeds germinated on wetted filter paper in incubators over 30 days under five temperature regimes and two photoperiods. Black, blue, common, and Manchurian ash seeds were also tested but did not germinate under any temperature/photoperiod regime. Species names followed by the same letter did not differ significantly in overall germination after 30 days at alpha = 0.05. No statistically significant differences were observed among temperature treatments. The 8-h photoperiod resulted in significantly higher overall germination ($p = 0.0051$) than the 16-h photoperiod. No statistically significant interactions occurred among species, temperature, or photoperiod.

Ash species (significance)	Days after sowing	10 °C		15 °C		20 °C		25 °C		15–25 °C	
		8 h	16 h	8 h	16 h	8 h	16 h	8 h	16 h	8 h	16 h
Chinese (A)	10	1	0	34	0	55	3	15	14	81	0
	20	1	0	37	2	59	19	26	28	93	2
	30	1	0	37	6	59	20	26	30	93	2
Flowering (B)	10	0	0	0	0	0	0	0	0	0	0
	20	0	0	0	2	0	4	0	0	37	0
	30	0	17	1	15	0	4	0	0	43	0
Green (A, B)	10	0	0	6	0	29	1	6	3	85	0
	20	0	0	24	1	34	8	6	7	89	0
	30	1	0	24	1	34	9	6	7	89	0
Pumpkin (B)	10	0	0	0	0	39	0	0	0	0	0
	20	0	0	0	0	40	0	0	0	0	0
	30	0	0	0	0	40	0	0	0	0	0
White (B)	10	0	0	0	0	0	0	0	0	0	0
	20	0	0	2	0	0	0	0	0	5	0
	30	9	0	3	0	0	0	0	0	5	0

Table 3. Percent germination for eight species of ash seeds on agar-based medium. Seeds were either cut or left intact. Blue ash seeds were also tested, but they did not germinate under either seed treatment. Within a species, means did not differ significantly among treatments with the exception of flowering ash.

Days after sowing	Black		Chinese		Common		Flowering		Green		Manchurian		Pumpkin		White	
	Seed cut	Seed intact	Seed cut	Seed intact	Seed cut	Seed intact	Seed cut	Seed intact	Seed cut	Seed intact	Seed cut	Seed intact	Seed cut	Seed intact	Seed cut	Seed intact
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
5	0	0	4	2	0	0	0	0	0	0	0	0	0	0	0	0
10	1	0	12	48	0	6	0	0	42	8	0	0	0	0	0	2
15	3	0	21	48	0	6	0	0	49	8	0	0	0	0	0	2
20	5	0	24	52	0	6	0	0	58	21	1	0	0	0	2	2
25	5	5	26	56	1	6	11	4	62	35	3	0	2	0	3	2
30	5	5	26	56	1	6	18	5	65	35	9	0	6	0	3	2
35	8	5	26	56	5	6	24	5	66	35	11	0	11	0	8	2
40	8	5	27	56	5	6	37	8	66	36	12	1	12	0	8	2
45	8	5	44	56	10	9	54	16	66	37	13	1	19	0	9	2
50	9	5	49	56	10	9	55	17	66	37	18	1	20	0	12	2
55	11	5	57	56	10	9	55	17	66	52	22	1	20	0	12	2
60	11	5	57	56	10	9	55	18	66	52	27	1	20	0	12	2

Potting-Mix Test

When sown into potting mix, only one-half of the species germinated (table 4). Within those, no seeds in the "pericarp removed" treatment germinated. Of the other two treatments, more seeds germinated if they were left intact than if they were cut, though none of those differences were statistically significant, likely because of the high variability between replicates.

Discussion

Seeds require specific environmental conditions to emerge from dormancy. The requirements for germination are species-specific and include variables such as temperature, moisture, and photoperiod. Chinese and green ash seeds germinated the best across all three tests and all treatment levels; unless more optimal conditions are found for seeds of the remaining species of ash, future research will likely focus on these two species. While tetrazolium chloride tests were not completed to test seed viability, it can be inferred that the seeds used in this study were alive, because all species showed at least some level of germination in the agar-based, solid-medium test.

Under the conditions tested, flowering, pumpkin, and white ash seeds were moderately difficult to germinate. In the case of these three species, dormancy may involve chemical inhibitors (Villiers and Wareing 1964, 1965; McBride and Dickson 1972) that are not present in Chinese and green ash seeds. Ashley and Preece (2004) suggest that white ash seeds have a much deeper, more complex dormancy compared with Chinese and green ash seeds.

Table 4. Percentages of seeds from five species of ash germinated on a peat-based potting mix in a greenhouse. Seeds were either cut or left intact; intact seeds either had the pericarp intact or removed. Seeds with pericarps did not germinate. Between the other two treatments, the germination means were not statistically different for any species.

Days after sowing	Chinese			Flowering			Green			Pumpkin			White		
	Seed cut	Seed intact without pericarp	Seed intact with pericarp	Seed cut	Seed intact without pericarp	Seed intact with pericarp	Seed cut	Seed intact without pericarp	Seed intact with pericarp	Seed cut	Seed intact without pericarp	Seed intact with pericarp	Seed cut	Seed intact without pericarp	Seed intact with pericarp
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
5	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
10	3	6	0	0	0	0	6	3	0	0	0	0	1	0	0
15	11	14	0	0	0	0	11	7	0	0	0	0	1	0	0
20	17	16	0	0	0	0	13	15	0	0	0	0	1	1	0
25	18	19	0	0	0	0	16	25	0	0	1	0	1	1	0
30	18	20	0	0	1	0	17	31	0	0	11	0	2	1	0
35	18	20	0	0	1	0	17	32	0	0	11	0	2	1	0
40	18	20	0	0	1	0	17	32	0	0	11	0	2	1	0
45	18	21	0	0	1	0	17	32	0	0	12	0	2	1	0
50	19	21	0	0	1	0	17	33	0	0	15	0	2	1	0
55	27	29	0	0	1	0	17	33	0	0	15	0	2	1	0
60	36	41	0	0	1	0	17	33	0	0	15	0	2	1	0

Black, blue, common, and Manchurian ash seeds had the lowest germination rates across tests, which may indicate that their embryos were immature. Some evidence suggests that low germination of black and common ash may be because of immature embryos (Steinbauer 1937; Villiers and Wareing 1964; Vanstone and LaCroix 1975; Wagner 1996). Because Manchurian ash seeds appear to have similar seed and embryo morphology to those in black and common ash, their lower germination may also be related to physiologically immature embryos.

Embryo excision and culture have been used to overcome dormancy in many cases, such as when a chemical inhibitor is present, when the seed coat is impenetrable to the growing embryo, or when seeds require complex stratification to overcome dormancy (Arrillaga and others 1992). Embryo culture, however, is often a difficult, labor-intensive process that requires extreme care to avoid damaging the embryo, and our experience has shown that, for most species of ash, the percentage of embryos that survived the excision process is very low (data not shown). Therefore, manual embryo excision is not a recommended method for increasing ash seed germination rates. In this study, seed cutting was a quicker way to increase germination rates without excessively damaging embryos, although for species with immature embryos, including black (Vanstone and LaCroix 1975) and Manchurian ash, even seed cutting may not significantly improve germination, as indicated by the low germination rates for these species in this study.

Hormonal treatments are often used to overcome seed dormancy. In the case of common ash, Lewandowska and Szczotka (1992) showed that addition of kinetin or gibberellin before cold stratification stimulated the breaking of dormancy but lowered overall germination rates, as compared with a control group. Determining which hormones, at what rate, and in conjunction with what temperature or humidity, however, can be a time-consuming process. Still, it may be a worthwhile undertaking for seeds from some species, such as flowering, pumpkin, and white ash, which showed low overall germination rates, even with seed cutting.

The method of Ashley and Preece (2004) for removing the cotyledonary tip of the ash seed to promote germination is much more efficient than embryo culture or hormone treatments at overcoming dormancy for most species of ash. Nurseries and researchers may be able to use the information obtained here as a first step toward identifying the quickest and most optimal germination conditions for a variety of *Fraxinus* species. Based on our results, photoperiod seems to have a greater effect than temperature on the germination of Chinese, green, and pumpkin ash seeds. This environmental effect may be because of differing evolutionary adaptations for climate, or it may suggest a deeper interaction between photoperiod and temperature on germination rate, as is seen in eastern hemlock (*Tsuga canadensis* L.; Stearns and Olson 1958). Our results suggest that the ash seed pericarp, while protecting the seed from environmental conditions, also serves as a physical barrier to germination (possibly also as a chemical barrier; Thapliyal and Nautiyal 1989). Further, an intact seed coat (i.e., one that has not received any cutting treatment) may prevent endosperm degradation products from leaching out, as may happen with cut seeds placed in potting mix or soil. On solid media, perhaps nutrients from the endosperm are less likely to leach from the incision because the seed sits only on top of the media, rather than being embedded in moist potting mix, as was done here.

The slower, lower overall germination rates of black, blue, common, flowering, Manchurian, pumpkin, and white ash as compared with Chinese and green ash (table 3) may suggest that the denser, thicker seed coats and slightly different seed shape we observed for seeds of these species act as germination barriers. As suggested by Ashley and Preece (2004), the cutting treatment may serve a dual purpose in enhancing germination rates: first, by removing a physical barrier to the emergence of radicles and cotyledons and, second, by allowing for a more efficient gas exchange, thereby promoting the germinative process (Villiers and Wareing 1965).

We recommend that a better procedure for surface sterilization and imbibition of ash seeds be developed. Microbial contamination was a major limitation in these germination experiments. While the procedure for preparing seeds for plating or planting included two progressively more stringent steps for sterilization (Raquin and others 2002) during initial imbibition, mold and fungi were major problems in both the incubator and agar-based, solid-medium tests. This undesirable effect may be because of incomplete surface sterilization (Singh and others 1992). A more reliable procedure could be developed by including additional sterilization steps or reagents. Tetrazolium chloride tests should also be implemented to ensure that seedlot viability is as expected. In addition, humidity control could be incorporated into future trials to further evaluate optimum conditions for germinating ash seeds.

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