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# Seed Germination and Cryopreservation of Wild Lime (*Zanthoxylum fagara*)

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## Summary

Wild lime *Zanthoxylum fagara* (L.) Sarg., is a small tree having attractive evergreen foliage and fragrant, yellow flowers. Native to Florida and Texas, this drought tolerant species is sought after for use in butterfly gardens in warm climates or conservatories. Yet, its nursery availability and ornamental use remain limited, as propagation protocols are largely unknown. To determine if seed propagation is a reliable and efficient means of producing wild lime, a series of studies were conducted in incubators to evaluate the effects of seed origin, temperature, dormancy, and cryopreservation on germination. Initial x-ray analysis and seed viability tetrazolium (TZ) tests determined the majority of seeds were filled (90-98%) and moderately viable (86-87%), regardless of the location they were collected from. However, seeds collected from northcentral Florida (Gainesville) and south Florida (Miami) responded differently to temperature treatments deployed to mimic spring (29/19 °C), summer (33/24 °C), fall (27/15 °C) and winter (22/11 °C) conditions. After 8 weeks, northcentral FL seeds had 10.7-41.1% germination, with seeds in the coolest temperature (winter) having the lowest germination, whereas south FL seed germination ranged from 30.2-71.2% across temperatures with the lowest germi-

nation occurring in the warmest temperature (summer). Additionally, seeds were found to imbibe regularly (do not require scarification) and tolerate cryopreservation procedures for long-term storage but possess physiological dormancy that must be overcome prior to germination.

## INTRODUCTION

Wild lime (*Zanthoxylum fagara* (L.) Sarg.,) exhibits a suite of characteristics that may warrant its widened use for ornamental and ecologically friendly landscaping (**Fig. 1A-H)**. Native to Florida and Texas in the U.S., this small-sized tree belongs to the citrus family (*Rutaceae*) and is a host plant to several swallowtail butterfly species (FNPS, 2022). Wild lime is adaptable to different landscape conditions tolerating partial shade to sun, moderate salt spray, periods of drought, and cold hardiness in zones 8b-11. In the spring, this species boasts fragrant, yellow flowers occurring in the leaf axils (**Fig. 1C**) of separate male and female plants that are proceeded by one-seeded, long-stalked follicles (**Fig. 1D-G**) attractive to birds.

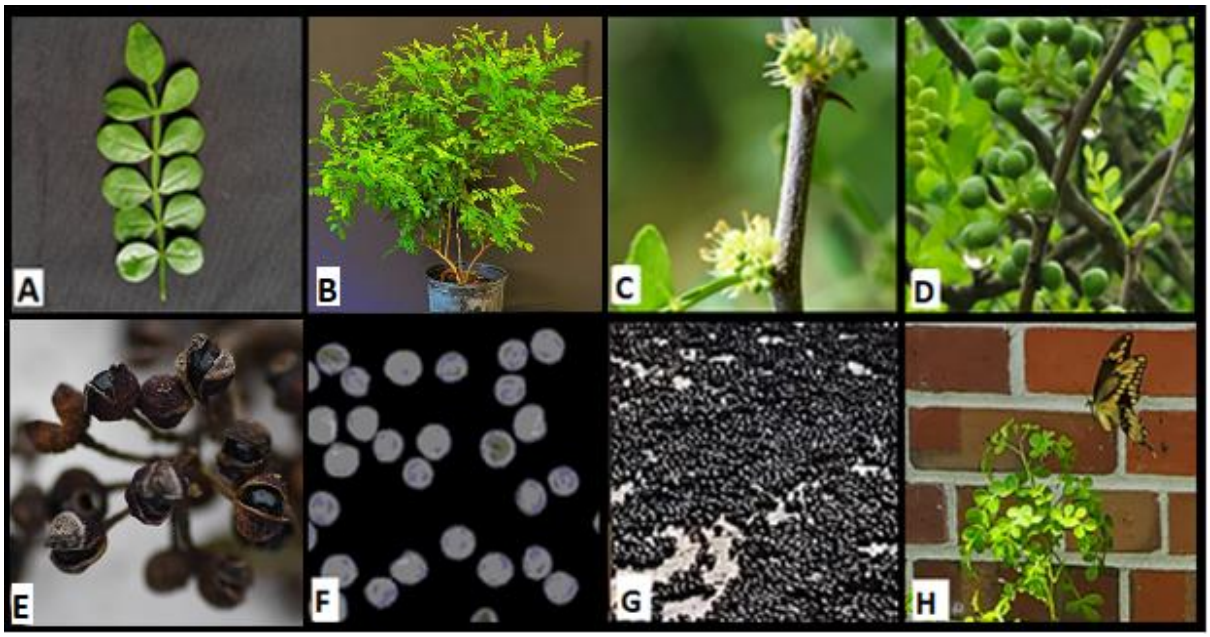


**Figure 1**. Ornamental and reproductive traits of wild lime featuring: (A) glossy, odd pinnately compound leaf with winged rachis, (B) woody growth habit, (C) inflorescences in yellow axillary panicles and stems with short, recurved stipular spines, (D) immature, glandulous fruit, (E) mature seeds in dehiscent capsules, (F) x-ray at 2000x showing embryo presence or absence, (G) globose, black seeds, and (H) swallowtail butterfly visiting host plant.

Consistent with poor germination of other closely related *Zanthoxylum* species (USDA Forestry Services, 2008), the germination of wild lime is irregular and sporadic. Seeds are likely to possess physiological dormancy (Datt et al., 2017; Patade et al., 2020), with unknown ability to tolerate

storage. Physiological dormancy is described as an inhibiting mechanism in the embryo that prevents radicle emergence (Baskin and Baskin, 2014). Practices to alleviate physiological dormancy include moist-chilling stratification, after-ripening, or application of gibberellic acid to replace a chilling requirement (Davies et al., 2018). Physical dormancy in comparison, is a condition when seeds fail to germinate because they are impermeable to water. To relieve this type of dormancy, scarification practices must be employed to mechanically or chemically abrade the seed coverings to allow for water uptake (imbibition) (Davies et al., 2018). In addition to dormancy, seed longevity (storability) is yet another factor that may influence germination response over time. Seeds that can tolerate drying may be ideal candidates for cryopreservation, a long-known technique used to maintain germplasm at ultra-low temperatures (Vendrame and Faria, 2011). With appropriate protocol development, cryopreservation could be a potential means of preserving seeds of wild lime for long periods of time and ultimately broadening its year-round availability for nursery production.

The overall goal of this study was to develop efficient and reliable methods to sexually propagate wild lime and subsequently increase its availability and use. A series of three studies was conducted with specific objectives to determine if: 1) germination differs by seed origin and temperature, 2) dormancy can be overcome by seed treatments and 3) seeds can be cryopreserved for long term storage.

## MATERIALS AND METHODS

**Effects of origin and temperature on seed germination.** On 27 Sept. 2021 and 31 Aug. 2021 wild lime seeds were harvested from two locations, the first from a cultivated population in northcentral Florida (Gainesville, FL) and the second from a natural population in south Florida (Miami, FL). Seeds were stored in paper bags at room temperature (22-25 °C) for 2-3 weeks prior to experimentation. A subsample of seeds from each location was examined using an Ultra Focus x-ray system with embryo fill calculated using Faxitron Vision software (US Forest Service National Seed Laboratory, Dry Branch, GA). Two replicates of 100 seeds were then cut laterally and stained overnight at 37 °C in a 1.0% TZ (2,3,5-triphenyl-2H-tetrazolium chloride) solution in accordance with the Association of Official Seeds Analysts (AOSA) rules for TZ testing (Iseldy, 2016). Seeds were considered viable when firm embryos stained evenly red under 10× magnification. From each location, an additional four replicates of 50 seed (experimental unit) were subjected to germination tests conducted in Percival I30VL light and temperature-controlled incubators set to mimic spring (29/19 °C), summer (33/24 °C), fall (27/15 °C) and winter (22/11 °C) alternating temperatures in Florida with a 12 hr photoperiod. Seeds were surface sterilized with a 10% bleach solution (0.75% a.i. NaClO) for 10 min., triple rinsed, and soaked overnight in sterile deionized (DI) water prior to placing in 10.9 x 10.9 cm transparent polystyrene germination boxes lined with two sheets of moistened white blotter paper beneath one sheet of unbleached crepe germination paper. Germination was checked three times a week and recorded as the first sign of radical emergence for a period of 6 months.

**Effects of seed scarification and pre-hormone treatment on imbibition and germination.** Using the seeds from northcentral FL a seed imbibition study was conducted using four replicates of 25 seeds subjected to one of three treatments. The first subsample of seeds was mechanically scarified using coarse grit sandpaper and then soaked overnight in DI water. The second set of seeds was also mechanically scarified but soaked in 500 mg/L gibberellic acid ($GA_3$) for 24 hr. The third set of seeds served as the control and was only soaked overnight in DI water for 24 hr. Dry weight ($W_0$) of each replicate was recorded prior to soaking and then wet weight ($W_1$) was recorded in 12-hr increments until the experiment ended after 336 hr (2 wk). Increase in fresh weight was calculated using the following equation: $[(W_1 - W_0)/ W_0] \times 100$.

A second study was conducted using four replications of 50 seeds that were either scarified with coarse grit sandpaper or treated with $GA_3$ and kinetin at two different rates (200 mg/L $GA_3$ + 100 mg/L kinetin or 400 mg/L $GA_3$ + 200 mg/L kinetin). Seeds were soaked overnight prior to placement in germination boxes arranged in incubators set at 29/19 °C with a 12-hr photoperiod. Germination was observed three times a week and recorded for 6 months.

**Effects of cryopreservation on seed germination.** Seeds from the northcentral FL origin (7 wk post collection) were placed in a desiccator to determine initial moisture content. Four replications of 50 seeds (northcentral FL origin) were subjected to five different cryopreservation treatments and included a non-treated control. The control was not immersed in a plant vitrification solution 2 (PVS2) nor liquid nitrogen (LN). Cryopreservation treatments consisted of seed 1) immersed in PVS2 but not LN, and 2) immersed in LN but not PVS2, 3) immersed in PVS2 followed by LN, 4) pre-cooled with ice prior to immersion in PVS2 and then LN, and 5) pre-cooled with ice, immersed in a solution of PVS2 plus 1.0% phloroglucinol (PG) followed by LN. Both the control and the PVS2 only (treatment 1) were held at room temperature for 72 hours. After 72 hr in LN (treatments 2-5) cryotubes were removed and rapidly reheated in a water bath held at 39.7 °C. Seeds were rinsed and soaked overnight prior to placing in incubators set to 29/19° C. Germinates were recorded weekly for 6 months and presented at 1-month intervals.

*Statistical analysis*. Germination data were analyzed using Generalized Non-Linear Models procedures as implemented in SAS PROC NLMIXED (SAS/STAT 14.1; SAS Institute, Cary, NC) through a 3-parameter logistic growth model

$$Proportion\ germinated = \frac{c}{(1 + Exp(-a \cdot (DAS - b)))}$$

where a = growth rate, b = inflection point, c = asymptotic final germination, and DAS = Days after the start of the experiment. Monthly means were predicted from the fitted curve and treatments and or parameters compared using pairwise t-tests.

## RESULTS AND DISCUSSION

**Seed viability and germination.** Seeds from both origins (northcentral and south FL) had similarly high embryo fill (90-98%) and pre-germination viability (86-87%) revealing that both seed populations were of similar quality ($P$ =0.5425) at the start of experimental treatments (**Table 1**). When seeds were placed in incubators set to mimic seasons in Florida (29/19, 33/24,

27/15 and 22/11 °C), a significant origin × season interaction occurred ($P \leq 0.0001$) revealing that seeds from each origin did not respond similarly to temperature. As such, seeds from northcentral FL reached 10.7-41.1% germination among all temperatures with winter showing the lowest germination, and nonsignificant differences observed between all other seasons (**Table 1**).

**Table 1**. Initial embryo fill was calculated using x-ray software to non-destructively determine presence or absence of embryos in seed, viability was based on 24-hr tetrazolium (0.1%) staining of laterally cut seeds was cut laterally of a subsample of wild lime seeds collected from northcentral and south Florida prior to germination test. Least squares germination means from a generalized linear mixed model analysis 35, 45 and 55 Days after the start of the experiment conducted in incubators set to mimic summer (33/24 °C), spring (29/19 °C), fall (27/15 °C) and winter (22/11 °C) seasons.

| | Days after start (DAS) | | |
|---|---|---|---|
| Origin/Season | 35 | 45 | 55 |
| North Central FL | | | |
| Spring | 23.9 (16.9, 32.8) a[z] | 28.2 (19.2, 39.3) a | 28.9 (20.9, 38.4) a |
| Summer | 26.0 (18.0, 35.9) a | 37.6 (26.5, 50.2) a | 32.9 (23.8, 43.5) a |
| Fall | 24.0 (16.7, 33.3) a | 34.1 (23.8, 46.2) a | 41.1 (31.4, 51.6) a |
| Winter | 7.6 (4.5, 12.7) b | 8.9 (4.9, 15.7) b | 10.7 (6.5, 17.0) b |
| | Embryo fill = 90% | Initial Viability = 86% | |
| South FL | | | |
| Spring | 56.0 (50.7, 61.2) b | 61.3 (56.6, 65.7) b | 62.0 (56.7, 67.0) b |
| Summer | 21.3 (17.1, 26.2) c | 26.5 (22.3, 31.1) c | 30.2 (25.1, 35.9) c |
| Fall | 67.5 (61.4, 73.0) a | 70.3 (65.0, 75.0) a | 71.2 (65.4, 76.4) a |
| Winter | 52.4 (47.2, 57.6) b | 56.2 (51.6, 60.7) b | 59.3 (54.1, 64.2) b |
| | Embryo fill = 98% | Initial Viability = 87% | |

| Source | P > F |
|---|---|
| Origin | ≤ 0.0001 |
| Season | ≤ 0.0001 |
| Origin*Season | ≤ 0.0001 |
| DAS | ≤ 0.0001 |
| Origin*DAS | 0.3891 |
| Season*DAS | 0.692 |
| Origin*Season*DAS | 0.2954 |

[z] Means within origin and time (DAS) followed by the same letter are not statistically different at $\alpha = 0.05$.

Whereas seeds from south FL reached similar germination percentages between 59.3-71.2% at 29/19, 27/15 and 22/11 °C. While germination in the fall temperature (27/15°C) was significantly higher than spring and winter (29/19 and 22/11 °C), they were on average 2.0-2.4 times greater than the 33/24 °C (summer) treatment (**Table 1**). This suggests that seeds possess some level of dormancy that cannot be overcome passively by these fixed temperatures in an 8-wk study. Further, seeds collected from warmer climates of south FL preferred cooler temperatures of spring, winter and fall for optimal germination.

Results from the imbibition study showed that sandpaper scarified seeds with or without $GA_3$ imbibed similarly to non-scarified seeds ($P$=0.6574) with fresh mass increases ranging from 11.7 to 13.8% among treatments (Data not presented). This suggests seeds of wild lime can absorb water normally and do not have physical dormancy. Further, when seeds were subjected to scarification or hormonal pretreatments, after one month germination was 4.9 times greater when seeds were treated with 400 mg/L $GA_3$ and 200 mg/L kinetin compared to seeds treated with sandpaper (**Table 2**).

**Table 2**. Mean germination percentage and rate (T50FG) of mechanically scarified (seeds were placed between coarse grit sandpaper sheets and gently rubbed until visibly abraded), hormonally pretreated [seeds were soaked for 24 hr in gibberellic acid ($GA_3$) and kinetin at two rates] and a non-treated control for wild lime seeds placed in incubators set at 29/19 °C with a 12-hr photoperiod for 6 months.

| Month after treatment | Sandpaper scarification | 200 mg/L GA3 + 100 mg/L kinetin | 400 mg/L GA3 + 200 mg/L kinetin | Control (non-treated) |
|---|---|---|---|---|
| | Germination percent (%) | | | |
| 1 | 5.5 (4.6, 6.4) d[z] | 13.5 (11.5, 15.6) b | 26.7 (24.1, 29.3) a | 10.1 (8.6, 11.5) c |
| 2 | 24.3 (22.0, 26.6) b | 31.1 (29.6, 32.5) a | 30.4 (29.2, 31.5) a | 23.2 (21.2, 25.1) b |
| 3 | 34.1 (32.7, 35.4) a | 32.8 (31.4, 34.2) a | 30.4 (29.2, 31.6) a | 26.7 (25.4, 28.0) b |
| 4 | 35.2 (33.6, 36.9) a | 32.9 (31.4, 34.4) a | 30.4 (29.2, 31.6) a | 27.1 (25.5, 28.7) b |
| 5 | 35.3 (33.6, 37.1) a | 32.9 (31.4, 34.4) a | 30.4 (29.2, 31.6) a | 27.1 (25.5, 28.8) b |
| 6 | 35.3 (33.6, 37.1) a | 32.9 (31.4, 34.4) a | 30.4 (29.2, 31.6) a | 27.2 (25.5, 28.8) b |
| | Number of days until 50% of final germination (T50FG) was reached | | | |
| | 51 (47, 55) a | 34 (30, 37) b | 21 (18, 23) c | 38 (32, 43) b |

[z] Means within a row followed by the same letter are not statistically different at $\alpha = 0.05$.

However, this effect diminished as germination time increased. After 6 months, germination of non-treated control seeds was the lowest at 27.2%, while germination amongst seed treatments was non-significant and ranged from 30.4-35.3% (treated with sandpaper or GA + kinetin). More pronounced was the effect of treatments on germination rate. Seeds treated with the higher 400 mg/L $GA_3$ + 200 mg/L kinetin solution took the least amount of time (21 d) to reach 50% of their final germination

compared to 34 d for seeds that were treated with half the concentration (200 mg/L $GA_3$ + 100 mg/L kinetin) and 51 days for seeds that were scarified. Gibberellins have long been known to stimulate germination by increasing hydrolytic enzyme activity in seeds and embryo growth potential (Baskin and Baskin, 2014). The positive effect of $GA_3$ and kinetin on germination percentage and speed is consistent with Datt et al. (2017) who found that seeds from the closely related Timroo (*Zanthoxylum armatum*) responded better to this combination of hormones than any other pre-treatment.

**Seed cryopreservation.** Initial percent moisture of seeds was 6.7%, considered acceptable for cryopreservation (Davies et al., 2018). Seeds that germinated after precooling on ice before submersion into solutions of PVS2, or PVS2 and 1% Phloroglucinol, then immersed into LN were statistically significant from the control (**Fig. 2**), revealing that seeds can be cryopreserved successfully using either method. This also reveals that seeds are tolerant of maturation drying and therefore considered orthodox, a benefit to commercial propagation. It is plausible that collection time and storage length influenced seed deterioration of wild lime.

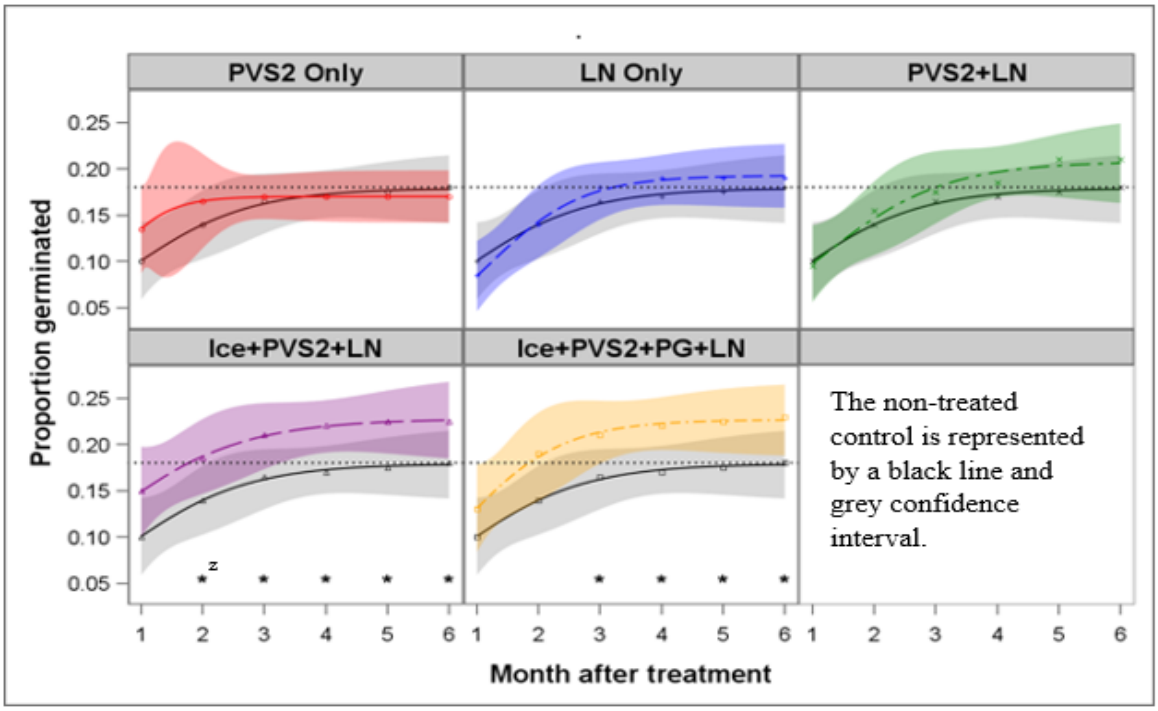


**Figure 2**. Mean germination over a 6-month period and approximate 95% prediction intervals estimated from a 3-parameter logistic growth mixed model as implemented in SAS® PROC NLMIXED (SAS/STAT 14.1; SAS Institute, Cary, NC). The experiment consisted of a non-treated control and five treatments. The control had no immersion in plant vitrification solution 2 (PVS2) nor liquid nitrogen (LN), and treated seeds were either immersed in PVS2 but not LN, immersed in LN but not PVS2, immersed in PVS2 then LN, pre-cooled on ice for one hour, immersed in PVS2 and then LN, and pre-cooled on ice for one hour, immersed in a solution of PVS2 plus 1.0% phloroglucinol (PG) then LN. The control and PVS2 only (treatment 1) were held at room temperature for 72 hrs. Rinsed seeds were then placed in germination boxes for 6 months at 29/19 °C. The dotted horizontal reference line indicated the maximum germination for the non-treated control.

## CONCLUSION

Results presented herein suggest that wild lime seeds are not physically dormant but do possess physiological dormancy. Seed source influences germination of this species and should be considered when propagating. Seeds are tolerant of desiccation and cryopreservation making them an ideal candidate for long term storage. Cold/warm stratification through move-along experiments where seeds are manually shifted through mimicked seasons during a single year are currently in progress to ascertain the depth of physiological dormancy and reach germination at levels high enough for commercial application. In addition, promising experiments are in progress to explore asexual propagation of this species by cuttings and tissue culture.

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