

**Freeze-thaw effects on the biology of seeds and seedlings from exotic  
and native plants**

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## ABSTRACT

During the cold-moist conditions of winter, stratification occurs that alleviates diaspore dormancy in many species enabling spring germination. Winters in southeastern United States frequently have warm spells, lasting a few days, interrupted by cold (freezing) periods. In this study, the effects of a freeze during stratification and following a warm spell on diaspores and seedlings of locally common exotic and native plants were determined. In the laboratory, diaspores were exposed to freezing temperatures, following stratification and stratification + warm spell, that simulated a cold front passage and then they were incubated at warm temperatures. Freezing at both  $-10^{\circ}\text{C}$  and overnight reduced viability and germination in some of the species. Only seeds of two exotic *Lonicera* species germinated during the simulated warm spell, and these seedlings survived down to  $-5^{\circ}\text{C}$  but none survived overnight at  $-10^{\circ}\text{C}$ . In a common garden experiment, *Celtis*, *L. japonica* and *L. maackii* had cotyledons before a freeze; diaspores of other species only had roots. Seedlings of *L. japonica* and *L. maackii* had moderate survivorship, whereas those of *Celtis* had low survivorship. In the field, diaspores were sown inside and outside (control) of open top chambers (OTC). Viability of four species did not differ between the OTCs and control, probably due to the failure of the OTCs to warm above ambient conditions. However, viability of the native, but not exotic, species was greatly reduced with a warm spell interruption during winter. Thus, freezing may differentially affect seed viability and germination and seedling survivorship of exotic vs. native species and potentially influence competitive interactions between them.

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## CHAPTER I

### INTRODUCTION

In the eastern United States, and around the world, climate change effects on species and how they will cope with this environmental impact have gained attention (Guisan and Thuiller 2005). Species may show various responses from adaptation to migration and extinction (Davis and Shaw 2001). Besides species level responses, interactions among species also might change. For example, any shifts in the timing of germination could influence seedling establishment and survivorship of one species and alter the competitive ability and outcome of other species.

Among the many threats to forests (Hansen 2001), climate change impacts on the interactions between exotic and native species are a major concern. Exotic species are a threat to native biodiversity through competition, by reducing resources, and altering ecosystem processes (e.g. nutrient cycles) (Whitney 1994, Mack 2000). With climate change, exotics have been shown to have advantages over native species (Sandel and Dangremond 2011) due to their traits (e.g. seed size, flowering phenology, and growth). These advantages benefit exotic species by giving them a competitive advantage over native species, particularly during the growing season. However, during winter physiological processes take place that underpin life history traits of species (Campbell et al. 2005). For example, freeze (cold) tolerance is an important aspect governing geographic ranges of species (Kreyling et al. 2015). Although freeze tolerance has been relatively well studied in juvenile and mature native deciduous trees (Vitasse et al. 2014),

freeze tolerance of seeds and seedlings (i.e. plants with cotyledons) is less well known for both native and exotic species (Hawkins et al. 2003).

A common feature of winter in middle Tennessee, and in most parts of southeastern United States, is mild conditions and warm spells that last a few days. Following these warm periods, a cold front approaches which is usually associated with rainfall and then temperatures drop below freezing with soils being frozen. Soils may unthaw during the day and refreeze at night. Air temperatures gradually warm over a few days back to the conditions that soils remain unfrozen during the day and night. Under laboratory conditions, Flanigan (2012) found that seeds of exotic woody plants were less dormant, germinated at lower (winter) temperatures, and germinated during simulated warm spells. In the field, seeds of the exotics germinate before those of the native species and recently germinated seeds of the exotics have been observed during winter (Walck et al., unpubl. data). Given that milder winters and increased warm spells are predicted in the future with climate change, the chances of seedling establishment of exotics should increase. Although the laboratory and field studies show that seeds of exotics can germinate, the fate of the seedlings remain unknown under current and future climate conditions. If seedlings of the exotics can establish and survive during cold periods, then this would give them a pre-emptive competitive advantage compared to the native species.

In this study, the freezing tolerance of seeds and seedlings of native and exotic species of locally common trees, shrubs, and vines were determined in the laboratory. The relevance of the laboratory results was tested in the field using a common garden experiment and open top chambers that function as miniature greenhouses and simulate



warming with climate change (De Frenne 2015). My overall prediction is that seeds and seedlings of exotic species are more freeze tolerant than native species. I reason that since seeds of exotics have less dormancy and can germinate at lower temperatures and with less cold stratification, their seedlings would have evolved to tolerate freezing episodes that may occur in late winter.

## CHAPTER II

### MATERIALS AND METHOD

**Plant material:** Mature drupes or berries, berry-like cones (*Juniperus*), or bulbils (*Dioscorea*) of native and exotic species were collected between October and December 2014 and 2015 from middle Tennessee and used in both laboratory and field studies (Table 1). *Dioscorea* only produces bulbils in its non-native range of North America (Walck et al. 2010). After each collection, fruits and berry-like cones were macerated in water overnight and the pulp/cone were removed by hand. The seeds [i.e. true seeds or stones (endocarp + true seed)] and bulbils (hereafter, diaspores) were air-dried for 2-3 days, and then stored in an open container on a laboratory shelf (ca. 21°C) in dry conditions for less than a month before experiments began.

**Experiment 1 – Freeze/thaw cycling:** Diaspores of 8 species were used in this experiment (Table 1). The experiment was designed to simulate a mid-winter and late-winter freeze following cold stratification or following cold stratification + warm spell. Seeds were placed in 6 cm (diameter) x 1.5 cm (depth) plastic Petri dishes and bulbils were placed into 11 (diameter) x 4 (depth) cm polyethylene containers (with clear lids) filled with swimming pool filter sand. The sand was moistened with distilled water to saturation before diaspores were added to the dishes. Twenty-five diaspores were placed in each dish or container and three replicates were used for each treatment. To reduce moisture loss, dishes were sealed with plastic wrap (and lids were maintained on

containers); sand in each dish or container was moistened with distilled water as required throughout the experiment.

Temperature- and light-controlled incubators and a temperature-controlled freezer were used in this experiment. The incubators were set to a constant temperature of 1 and 5°C and another to 12/12 h daily alternating temperature regime of 20/10°C. The 1°C was used to pre-condition the seeds prior to or directly after freezing and the 5°C was used for cold stratification. The alternating temperature represented the warm spell treatment and simulated a typical warm spell in middle Tennessee during winter. The daily photoperiod in the incubators was 12 h. Cool white 20 W fluorescent tubes, which produced a photosynthetic photon flux density (400–700 nm) at seed level of ca. 40  $\mu\text{mol m}^{-2} \text{s}^{-1}$ , were used as the light source. The freezer was set to 0, -5, and -10°C, with the adjustments between temperatures taking about 15 min; no lights were in the freezer.

Protrusion of a root (> 2 mm) from the diaspore was the criterion for the germination. Diaspores were checked for germination at 2-week intervals. At the end of the germination tests, the viability of the un-germinated seeds was determined using the pinch test: the seeds were pinched with forceps under a dissecting microscope and white, firm embryos were considered viable and brown, soft embryos were non-viable. A tetrazolium test confirmed that the white embryos were viable and the brown ones were not.

A diagram of the freeze/thaw experiment is shown in Figure 1. Diaspores received a simulated mid-winter freeze after a 6-week stratification period at 5°C or after a 6-week stratification period + 1 week at 20/10°C (warm spell). Diaspores were then acclimated at 1°C and sequentially moved from 0°C to -5°C to -10°C where they stayed

for 2 hours each. Then they were placed at  $-10^{\circ}\text{C}$ , where they stayed overnight, and then warming them by the same increments they were frozen by the following day. A set of seeds was removed following each freezing temperature and placed at  $20/10^{\circ}\text{C}$  for 2 weeks. Then, viability and germination were checked and percentages were calculated. For viability testing, percentage was based on the total number of seeds tested and germination based on the number of viable seeds. These percentages were compared to the control (no freezing).

**Experiment 2 – Late winter/early spring freezes in a common garden:** Seedlings of seven species (Table 1) were planted into moist Fafard Pro Mix soil in 8-cell trays (1 seedling/cell). They were acclimated by placing them at  $5^{\circ}\text{C}$  and then placing them outside for 3 days prior to planting. During a warm spell in February 2015, trays containing seedlings of each species were planted (with the top of the tray equal to the soil surface) randomly (3 trays/treatment) into an urban field into a 6 x 7 design with 0.25 m between each tray on 3 March 2015. The trays were randomly assigned two treatments: no leaf litter cover or 1-cm leaf (mostly oak) litter cover. Seedlings were followed weekly for 8 weeks during which time freezes occurred (on 1-2 March, 5-9 March, 28-29 March 2015) up until the final freeze on 5 April 2015; leaf litter was gently lifted just enough to check seedlings and then litter was replaced at weekly intervals. The last date of data collection was on 25 April 2015. Seedlings chosen for this experiment either had (1) exposed roots or (2) exposed roots and cotyledons. Plants received watering from rain.

**OTC construction and field placement:** OTCs were constructed of Solexx PRO 5 mm twin wall polycarbonate greenhouse (ca. 126 cm x 251 cm) panels (Solexx/Adaptive Plastics, Inc., Salem, OR), which has an R-value of 1.54. The panels were cut with a razor (Fig. 2) and assembled using 6.5 mm pan head self-tapping screws and liquid nails to anchor the panels to aluminum window flashing and wooden garden stakes (Fig. 3). The aluminum flashing (ca. 3.2 cm x 4.4 cm x 76.2 cm) was flattened so that there was no flange and cut to the same length as the side of the OTCs (34.29 cm). The wooden garden stakes were cut to the same length as the aluminum flashing so that the length of all sides of the OTCs were equal.

Ten OTCs were constructed: five used to test phenology and five used to test seed viability. The OTCs were oriented so that one side was in a north-south orientation. Associated with each of these OTCs was a control plot located 1 m (from each of OTC to each of control plot) on the north side from each OTC. The OTCs (and controls) were placed in a predominantly flat *Juniperus virginiana* woodland in Rutherford County, Tennessee; the sites for the OTCs in the woodland were selected based on appropriateness of the site (e.g. enough open space for the OTC and control, topography being mostly flat). The closest that any OTCs was ca. 15 m. The phenology and viability experiments were randomly allocated to a OTC. Inside of each OTC and control was plastic trays (27 x 54 cm) filled with limestone-derived topsoil common in middle Tennessee (Fig. 3). All of the seed trays were covered with window screens to reduce the impact of predation. Temperature and soil moisture were monitored inside and outside of three OTCs using HOBO micro station with temperature and soil moisture smart sensors.

Data were recorded hourly over 16 weeks when the phenology and viability studies were conducted.

For experiment 3 and 4, we used four species that contrasted in their seed ecologies (Table 1). The species were *Euonymus* (exotic, seeds are dormant and susceptible to freezes), *L. maackii* (exotic, seeds mostly non-dormant and readily germinate), *Celtis* (native, seeds are dormant and germinate late), and *Juniperus* (native, seeds moderately dormant and germinate early).

**Experiment 3 – Tracking phenology in OTCs:** Four hundred seeds (100 from each study species) were sown by hand on soil in each tray on 9 December 2015; no seeds were sown 1 cm around each edge of the tray. Each of the plots were checked weekly for 16 weeks, and if seedlings were present, they were counted and marked with aluminum wire to follow survivorship.

**Experiment 4 – Tracking seed viability in OTCs:** Twenty-five seeds each were placed into nylon mesh bags (10 x 10 cm) and randomly placed on top of the soil in each tray. To simulate a winter warm spell, three bags from each tray were removed on 22 February 2016 and given a 3-day warm spell at 20/10°C and then be returned to the field. The other bags remained in the field.

**Statistical analyses:** Analyses of variances (ANOVAs) were used to examine treatment effects. Germination percentages were arcsin-square root transformed for analyses to correct for non-normality of the data.

Table 1: Study species used in experiments.

<b>Species</b>	<b>Experiments</b>
<i>Dioscorea polystachya</i> (Chinese yam)	1, 2
<i>Euonymus fortunei</i> (winter creeper)	1, 3, 4
<i>Ligustrum sinense</i> (Chinese privet)	1
<i>Lonicera japonica</i> (Japanese honeysuckle)	1, 2
<i>L. maackii</i> (Amur honeysuckle)	1, 2, 3, 4
<i>Celtis laevigata</i> (sugarberry)	1, 2, 3, 4
<i>Cocculus carolinus</i> (coralberry)	2
<i>Juniperus virginiana</i> (eastern redcedar)	1, 3, 4
<i>Parthenocissus quinquefolia</i> (Virginia creeper)	1, 2
<i>Vitis vulpina</i> (frost grape)	2

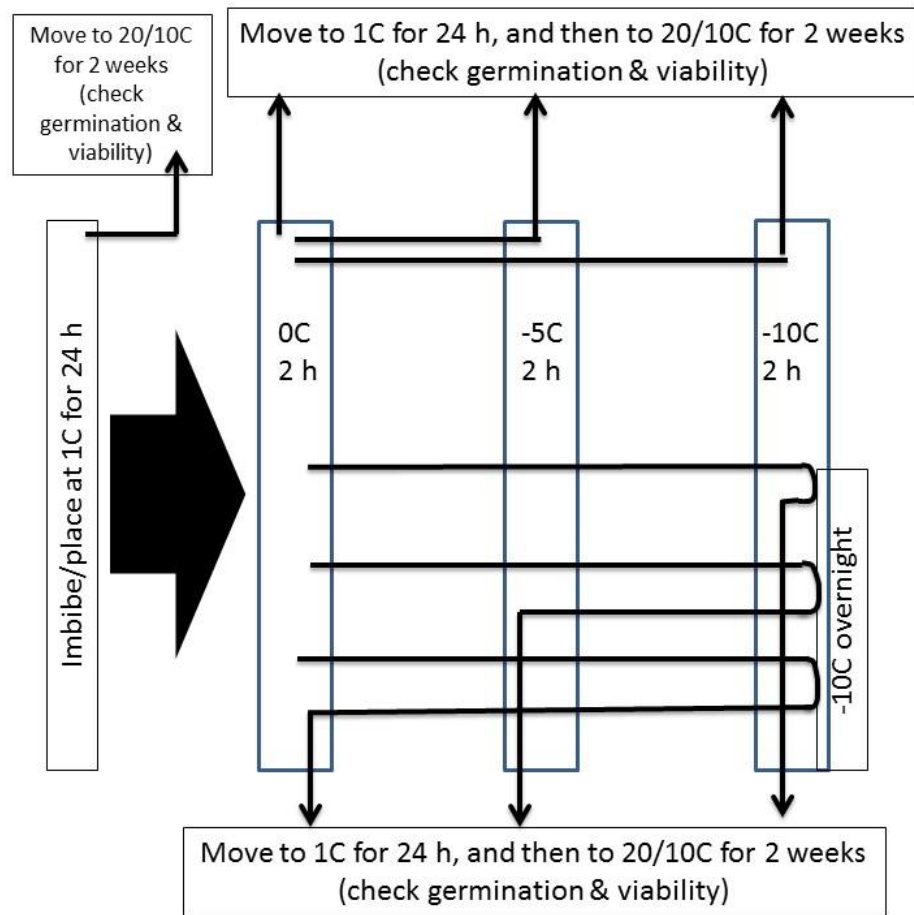


Figure 1. Diagram of freeze/thaw experiment.



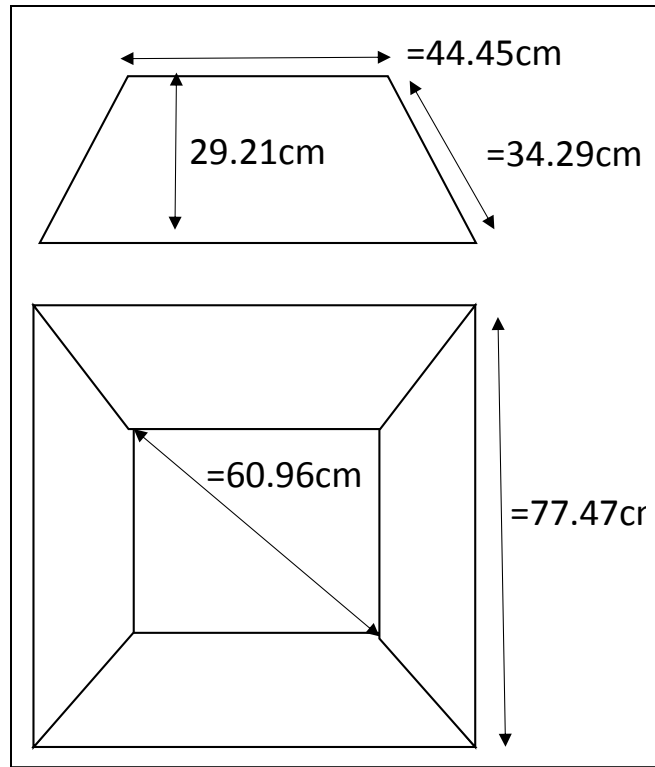


Figure 2. Schematic of open top chambers showing dimensions of side panels (top) and bottom panel (bottom).



Figure 3. OTC with tray placed inside and covered by screen.

## CHAPTER III

### RESULTS

**Experiment 1 – Freeze/thaw cycling:** For diaspores that were frozen after 6 weeks of cold stratification, viability decreased for the exotics *Dioscorea*, *Euonymus*, and both *Lonicera* species and for the native *Juniperus* particularly when frozen overnight (Fig. 4). For other species, viability either remained about the same through the freezing episodes, as in the natives *Celtis* and *Parthenocissus*, or it increased, as in the exotic *Ligustrum*. Germination decreased for all exotic species across the freezing treatments. In contrast, germination increased and then decreased following the overnight freeze for the native *Celtis* and *Juniperus* or it was unaffected by freezing for the native *Parthenocissus* (Fig. 5).

For diaspores that were frozen after 6 weeks of cold stratification + 1 week of a warm spell, up to 30 and 84% of seeds from the exotics *L. japonica* and *L. maackii* germinated during the warm spell, respectively. Seedlings of these two species survived if frozen down to -5°C but none survived below -5°C and frozen overnight (treatment,  $P < 0.0001$ ; Fig. 6). For the remaining species that did not germinate during the warm spell, viability decreased for the exotics *Dioscorea* and *Euonymus* and for the native *Celtis* across the freeze treatments (Fig. 7). For the natives *Juniperus* and *Parthenocissus*, viability was unaffected. Germination decreased for all exotic and native species across the freezing treatments (Fig. 8).

**Experiment 2 – Late winter/early spring freezes in a common garden:** For seedlings with only a root, survivorship differed among species ( $p < 0.0001$ ) but litter had no effect ( $p = 0.057$ ) among species (interaction,  $p = 0.187$ ). Survivorship for the native *Vitis* was much reduced with litter compared to that of one exotic and two other native species which did not differ appreciably between litter present and absent (Fig. 9). Survivorship was 50% or higher for most species, except *Vitis* which was below 20%. For seedlings with a root and cotyledon, survivorship differed among species ( $p < 0.0001$ ) but litter had no effect ( $p = 0.643$ ) among species (interaction,  $p = 0.150$ ). No seedlings of the native *Celtis* survived but moderate percentages of the exotic *Lonicera* survived (Fig. 10).

**Experiments 3 & 4 – Tracking phenology and seed viability in OTCs:** Although mean temperatures inside our OTCs did not differ from ambient (control) temperatures, maximum temperatures in the OTCs were higher than the ambient particularly when sunlight filtered through the redcedar canopy (data not shown). On the other hand, soil water content was higher inside than outside the OTCs. Neither the start nor peak of germination differed between seeds sown inside vs. outside the OTC (data not shown). Seed viability of the four species did not differ between the OTCs and control (Figs. 11, 12). However, viability of the native, but not exotic ( $p \geq 0.171$ ), species was greatly reduced with a warm spell interruption during winter ( $p < 0.0001$ ).

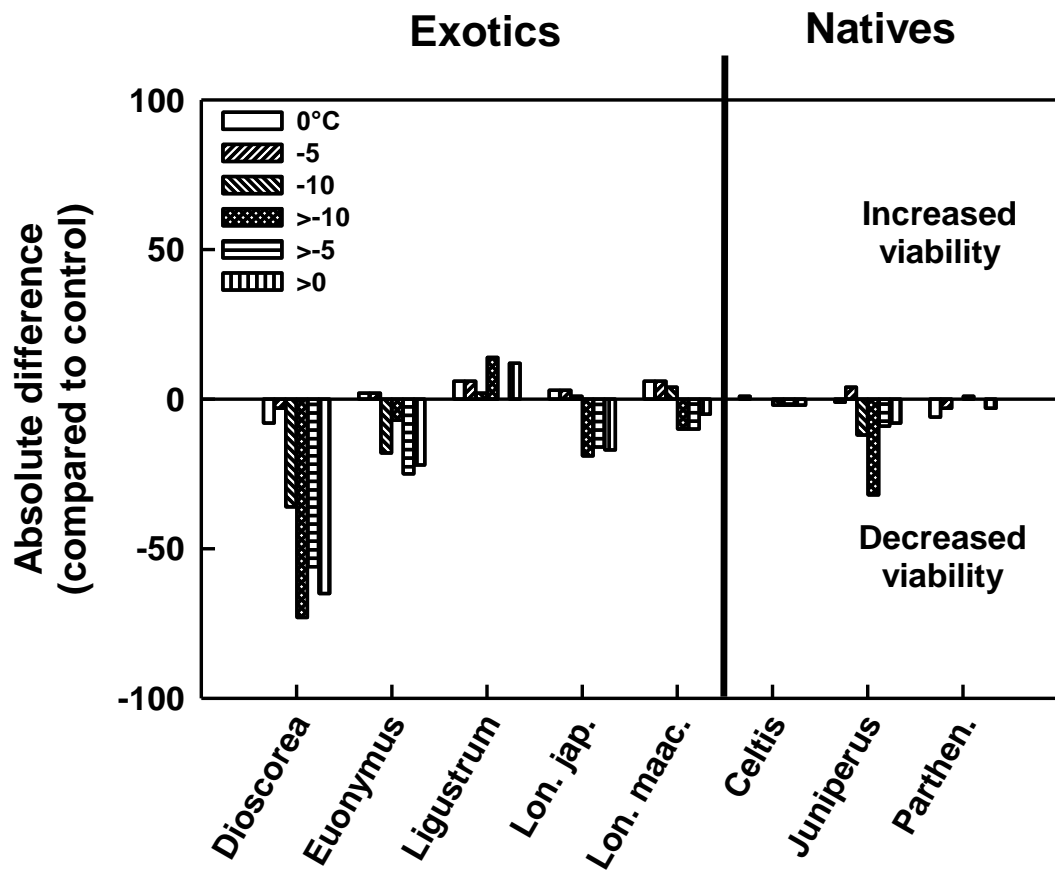


Figure 4. Viability for diaspores of exotic and native species subjected to 6 weeks of cold stratification at 5°C followed by freezing starting at 0°C down to -10°C and then frozen overnight (>) and brought from -10°C to 0°C. Following all freezing treatments (including the control), diaspores were incubated at 20/10°C in light for 2 weeks.

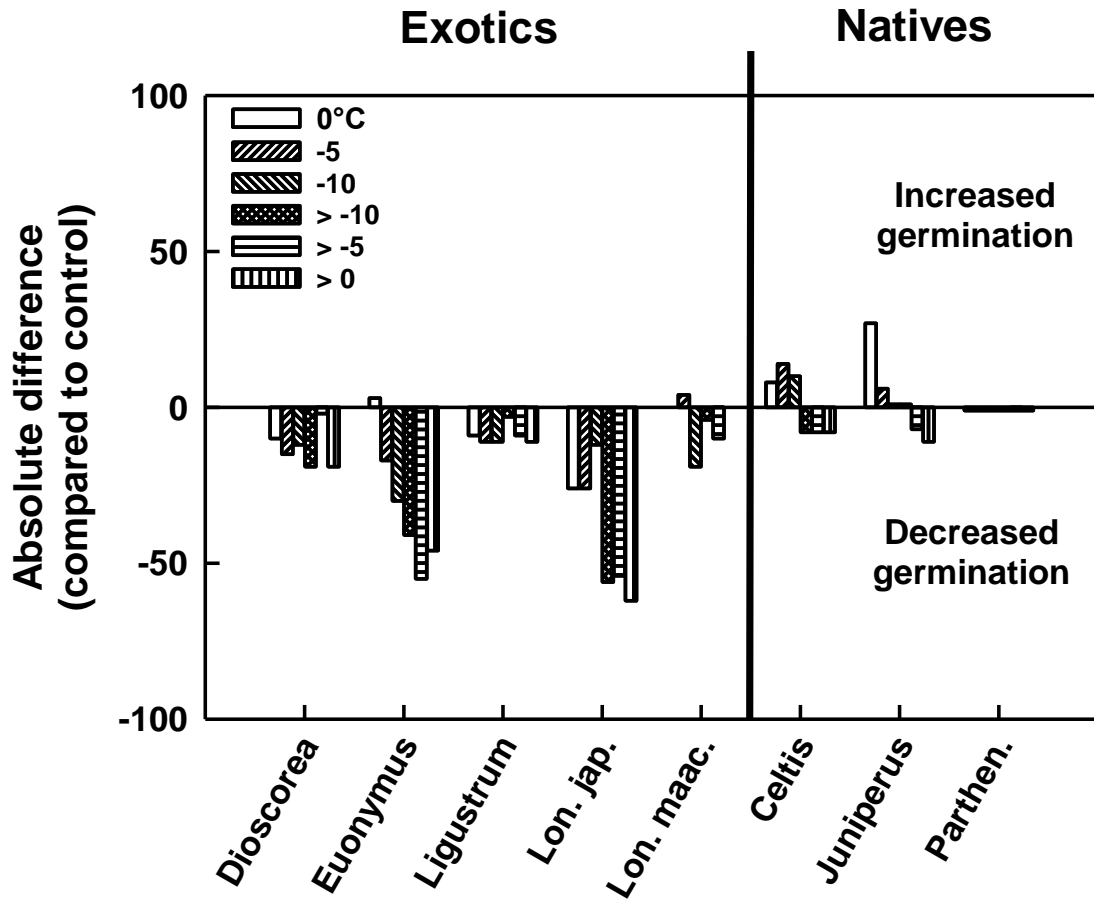


Figure 5. Germination for diaspores of exotic and native species subjected to 6 weeks of cold stratification at 5°C followed by freezing starting at 0°C down to -10°C and then frozen overnight (>) and brought from -10°C to 0°C. Following all freezing treatments (including the control), diaspores were incubated at 20/10°C in light for 2 weeks.

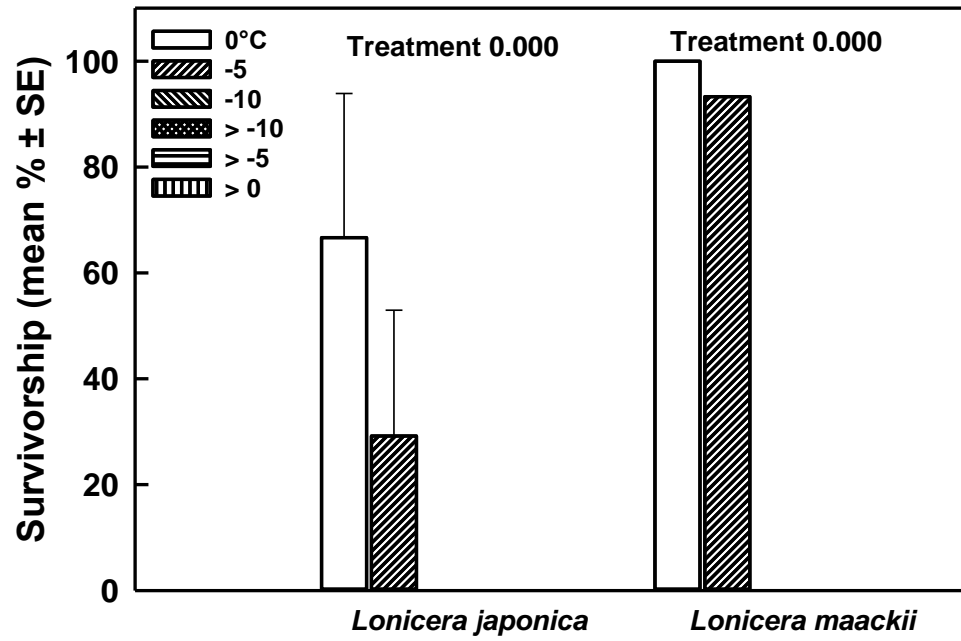


Figure 6. Survivorship of seedlings that germinated during warm spell and frozen (see Fig. 1).

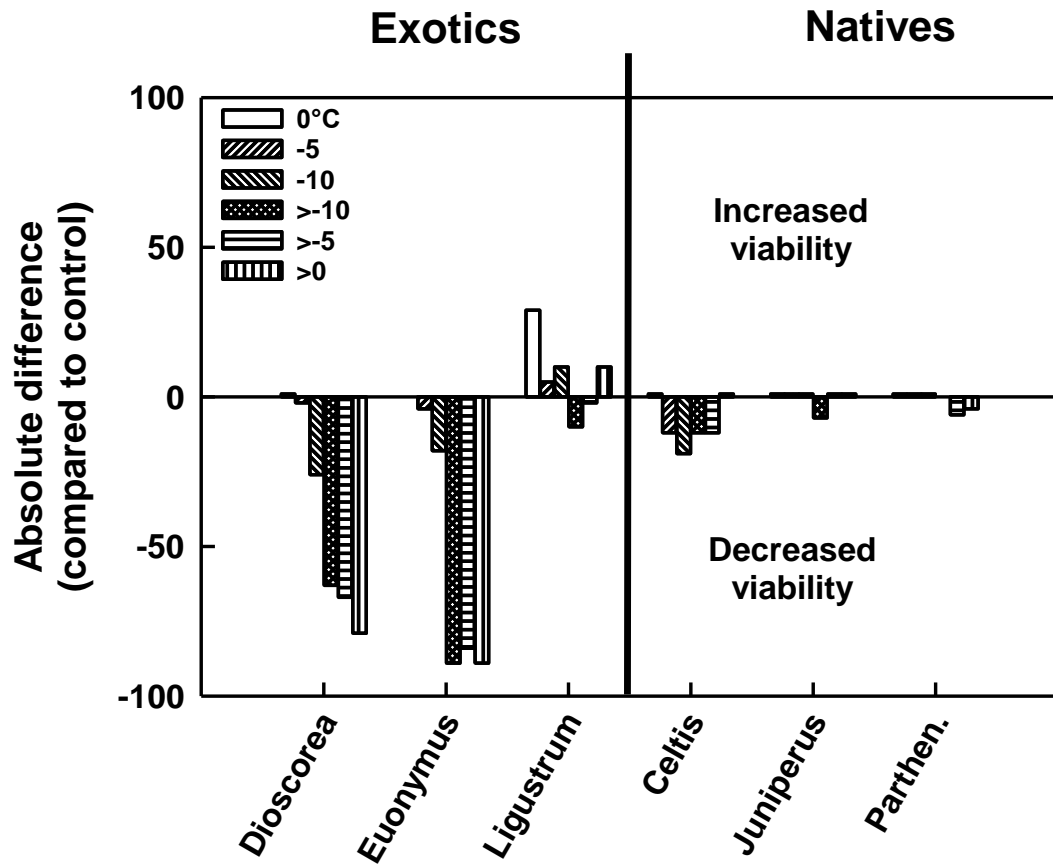


Figure 7. Viability for diaspores of exotic and native species subjected to 6 weeks of cold stratification at 5°C + 1 week warm spell at 20/10°C followed by freezing starting at 0°C down to -10°C and then frozen overnight (>) and brought from -10°C to 0°C. Following all freezing treatments (including the control), diaspores were incubated at 20/10°C in light for 2 weeks.



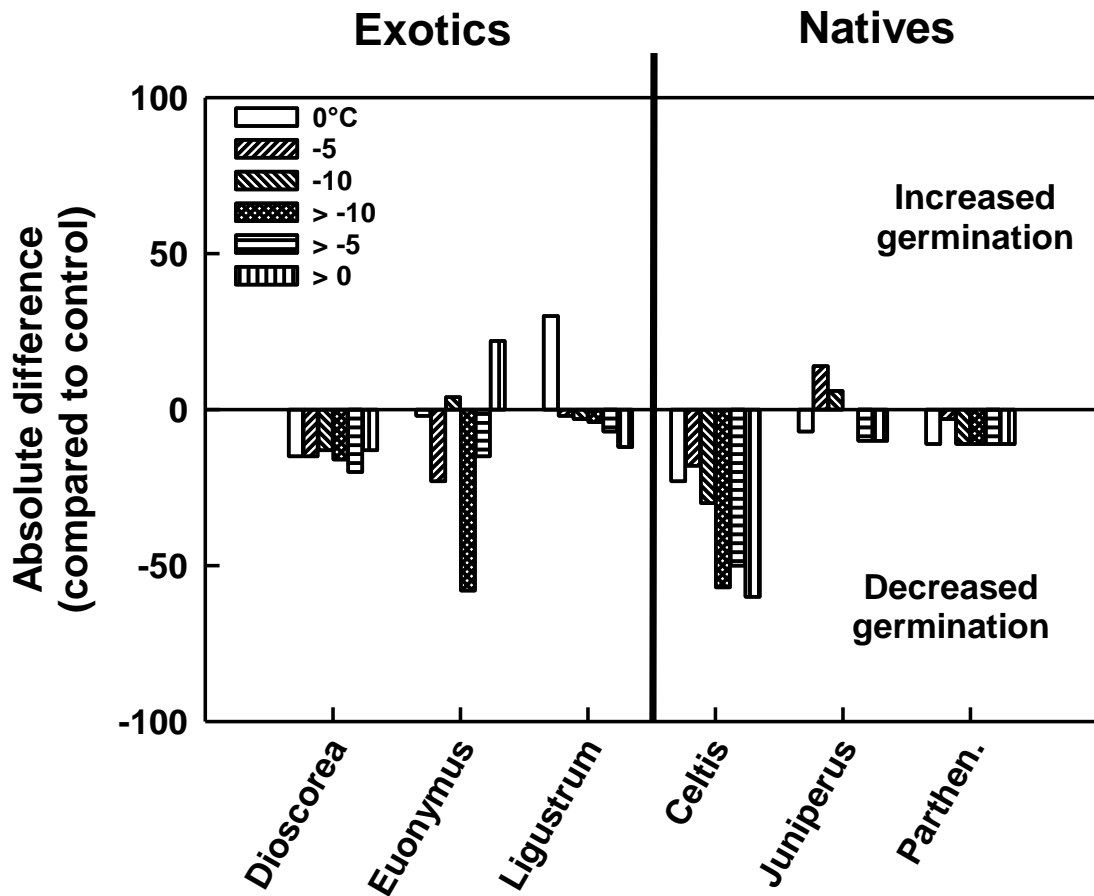


Figure 8. Germination for diaspores of exotic and native species subjected to 6 weeks of cold stratification at 5°C + 1 week warm spell at 20/10°C followed by freezing starting at 0°C down to -10°C and then frozen overnight (>) and brought from -10°C to 0°C. Following all freezing treatments (including the control), diaspores were incubated at 20/10°C in light for 2 weeks.

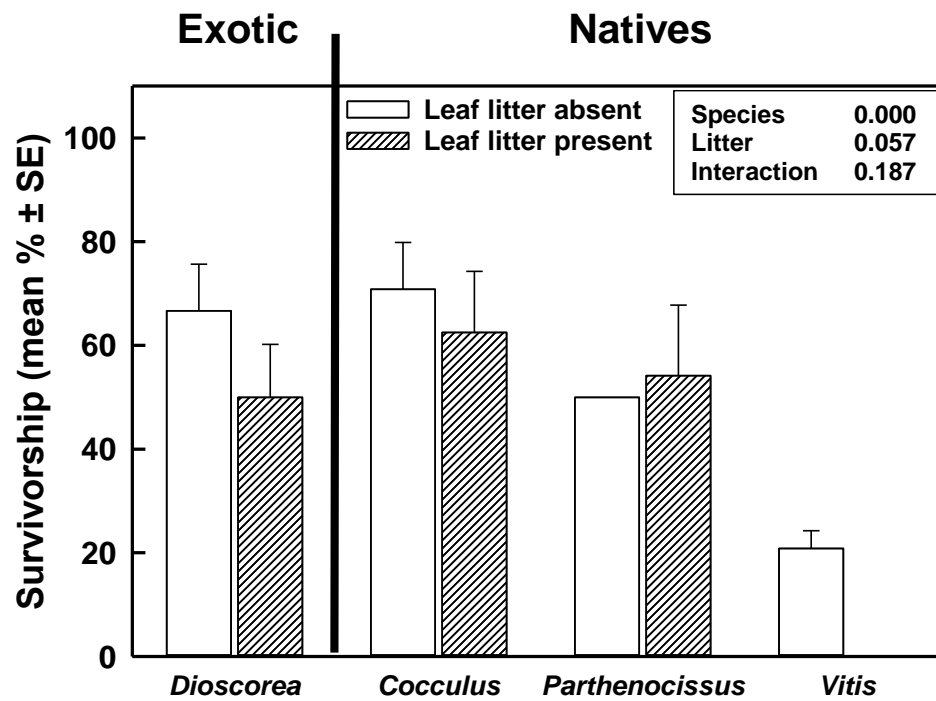


Figure 9. Survivorship of seedlings with exposed roots during late winter/early spring freezes.

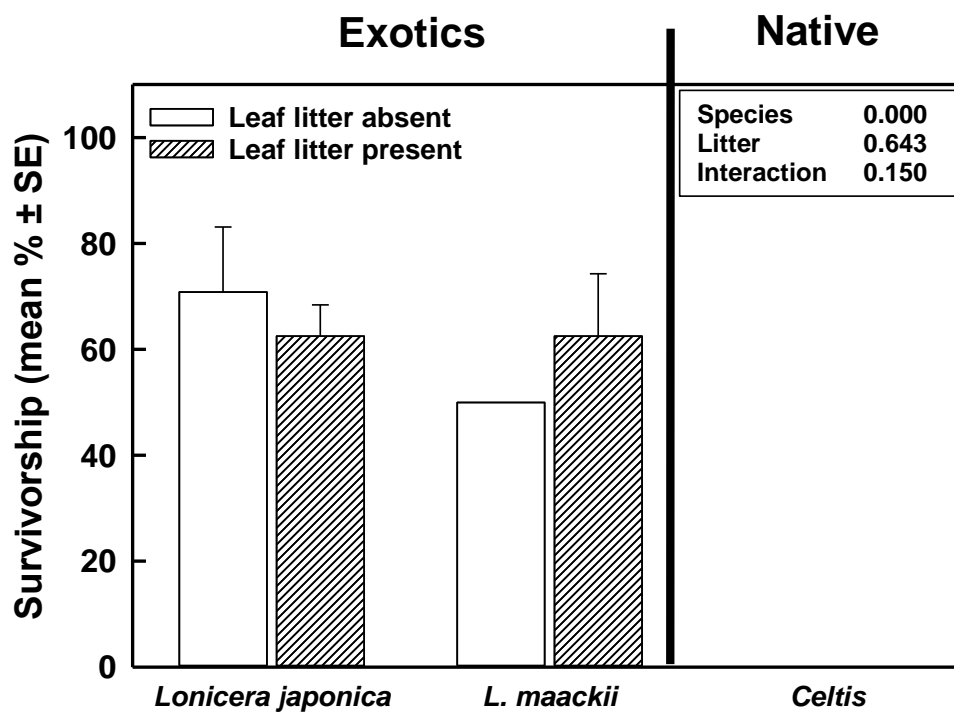


Figure 10. Survivorship of seedlings with exposed roots and cotyledons during late winter/early spring freezes.

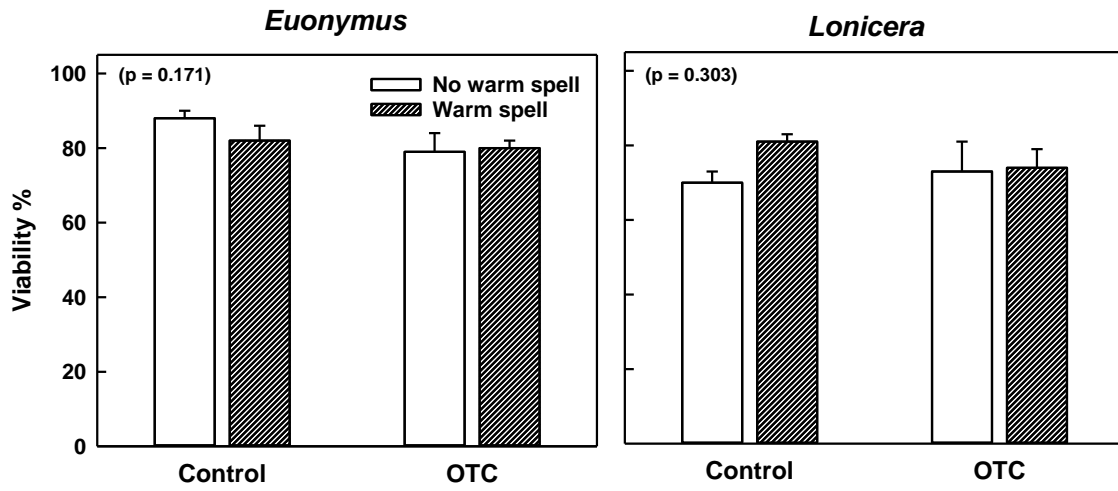


Figure 11. Mean  $\pm$  SE viability of seeds from two exotic species placed inside an open top chamber (OTC) and in a control with and without a warm spell. *Lonicera* refers to *L. maackii*.

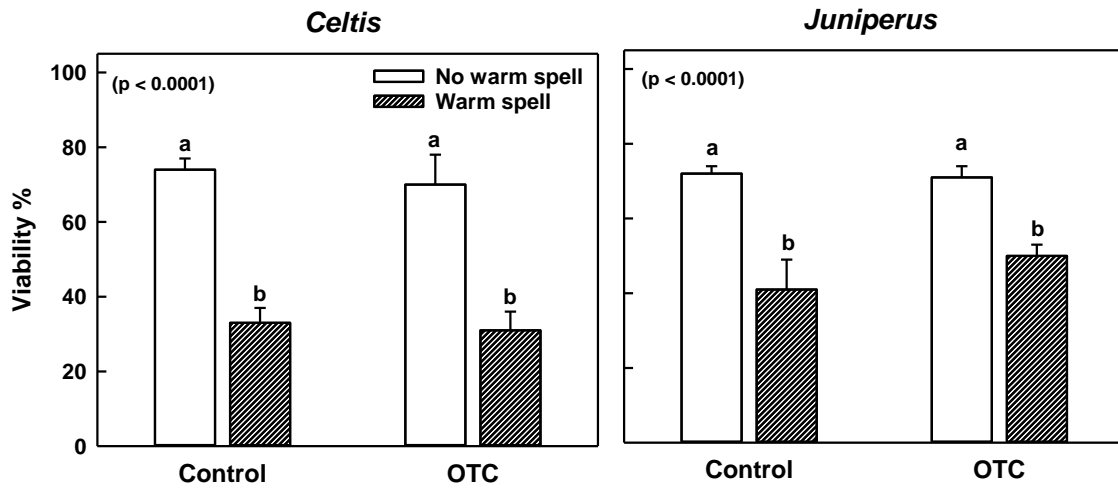


Figure 12. Mean  $\pm$  SE viability of seeds from two native species placed inside an open top chamber (OTC) and in a control with and without a warm spell.

## CHAPTER IV

### DISCUSSION

Responses to a freeze/thaw cycle differed among species as well as between warm spell vs. no warm spell treatments. Bulbils of the exotic *Dioscorea* were the most sensitive to a simulated mid-winter freeze compared to seeds of all the other species, showing a large reduction in viability, when a freeze interrupted cold stratification or followed a warm spell (Fig. 4). This result is not too surprising given the extremely thin epidermal layer surrounding the bulbil as compared to relatively thick endocarps surrounding seeds of the other species. In addition to *Dioscorea* bulbils, seeds of the exotic *Euonymus* showed highly reduced viability during a freeze following a warm spell (Fig. 7). The reason for the decreased viability of *Euonymus* seeds during a freeze following a warm spell as compared to a freeze interrupting cold stratification is that during the warm spell seeds of this species swelled and the seed coat/endocarp split exposing the embryo. During the freeze, the embryos of *Euonymus* were exposed to the low temperatures.

In addition to viability being affected by a freeze/thaw cycle, germination was mostly reduced during a freeze/thaw cycle across species. A freeze interrupting cold stratification most highly reduced germination of the exotics *Euonymus* and *Lonicera japonica* much more so than that of the other exotic or native species (Fig. 5). Although germination of *Euonymus* also was highly affected by a freeze following a warm spell, seeds of the native *Celtis* were affected the most (Fig. 8). The cause(s) for the decreased germination associated with freezing are unclear.

Seedling survivorship of exotic vs native species in the field during a late winter freeze was highly dependent on whether the shoot was exposed. In species where only the root was exposed, survivorship was low for the native *Vitis* but moderate for an exotic and two native species (Fig. 9). In species where both shoot and root were exposed, moderate survivorship occurred for two exotic *Lonicera* species but no seedlings of a native species survived (Fig. 10). This may indicate that seedlings of the two exotics are more freeze tolerant as compared to the native species, but further tests need to be conducted. On the other hand, seedlings of the exotic *Lonicera* species died when exposed they germinated in the laboratory during a mid-winter warm spell and then exposed to freezing temperatures (Fig. 6). Supporting this laboratory result, seedlings of *Lonicera maackii* observed in the field during a warm spell in mid-December died during a freeze in late winter (J. Walck, pers. obs.). Thus, seedlings of the exotic *Lonicera* species may germinate during warm spells. If a freeze occurs following the warm spell, these seedlings have a higher chance of surviving as compared to native species

In the field, viability did not differ between simulated warming (with OTCs) and control for the two native and two exotic species (Figs 11, 12). The lack of a difference might have been due to temperatures inside and outside the OTCs being similar. The OTCs were placed in a highly shaded redcedar woodland. The maximum temperature sometimes differed between the OTCs and controls when the sun directly penetrated through the canopy onto the OTCs. OTCs have been used mostly in open-canopy ecosystems (e.g. tundra) or in deciduous forests, where they would be exposed to more direct sunlight throughout the year (tundra) or during late autumn to early spring (deciduous forest) (DeFrenne et al. 2010). Thus, my experiment using OTCs in a shaded

habitat may show a limitation for use of this experimental method in climate change studies.

In the field OTC experiment, viability of seeds by early spring differed between those given an artificial warm spell during winter and those not subjected to one. Seed viability of the native species *Celtis* and *Juniperus* was reduced with a warm spell (Fig. 12), but that of the exotic species *Euonymus* and *L. maackii* was unaffected (Fig. 11). Since warm spells are a relatively common feature of winter weather in middle Tennessee, the overall implications of this reduced viability may be that more seeds of exotic species remain viable in the soil seed bank than those of native species.

A few ecological consequences may be drawn from my research. If seeds of exotic species, particularly both *Lonicera* species in Middle Tennessee, germinate earlier and not be affected by freeze events, especially in late winter, they would have an advantage for pre-emption of resources over native species. On the other hand, not all exotic species would show a similar response. Viability of the exotics *Dioscorea* and *Euonymus* were highly reduced with freezing. During mild winters, some exotic species may germinate and establish earlier than native species. Although during cold winters with warm spells, seeds of exotics might germinate seedlings would die during a freeze. However, later in winter, exotic seedlings show higher survivorship than native seedlings. Over time with winter warming, exotics may gain an increased advantage over native species at the seed and seedlings stages of the life cycle.



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