A MULTIDISCIPLINARY APPROACH TO RESTORATION OF BUTTERNUT (*JUGLANS CINEREA*)

by

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For the village that it took to get me here

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ABSTRACT

Anthropogenically driven global change is disrupting ecosystems and habitats of many plant species, straining the ability of native species to survive and reproduce. The overarching goal of this research was to holistically work towards restoration of a threatened tree species by connecting research from different disciplines. In order to do so, the threatened butternut tree (*Juglans cinerea*) and its hybrids were used as a case study. Hybridization can incorporate stress tolerance in plants and could be a potential restoration tool. Evidence in some wild butternut populations indicates that naturalized hybrids of butternut with Japanese walnut (*Juglans ailantifolia*) may be more tolerant to butternut canker disease (BCD) than butternut, but this has not been formally tested. Thus, chapter 2 examined potential BCD tolerance within and between unadmixed and hybrid butternut inoculated with two BCD fungal isolates. Differences in canker growth were observed by fungal isolate, which could help to explain some differences in BCD severity found among butternut populations. Smaller and fewer cankers and greater genetic gains were detected in hybrid families, demonstrating that hybrids warrant further evaluation as a possible breeding tool for developing BCD-resistant butternut trees.

However, even with increased disease tolerance, hybrids must possess similar ecophysiological tolerances to their native progenitor to be an effective replacement. Butternut is extremely cold hardy, but Japanese walnuts are native to a warmer ecosystem, indicating potential disparities in extreme temperature tolerances between the two species and their hybrids. Thus, samples from mature trees were subjected to cold and heat treatments to compare relative extreme temperature tolerances within butternut and between butternut, Japanese walnut, and their hybrids. Within butternut, trees from colder areas exhibited less cold damage than those from warmer areas. Differences in heat damage among provenances occurred but did not follow a clear trend. Butternut exhibited greatest cold tolerance, Japanese walnut exhibited greatest heat tolerance, and hybrids were intermediate. Thus, the utility of hybrids for restoration could be limited at the extremes of the species' distributions.

A second, but different type of freeze test was conducted for chapter 4 using seedlings to gain a more nuanced understanding of cold tolerance within butternut and between butternut and its hybrids. No survival or damage differences were detected in butternut provenances, although seedlings from the coldest provenances experienced more delayed budbreak at the two warmest

treatments than those from warmer provenances. Interspecific differences were not observed in dieback but were detected in survival and budbreak. The hybrids had greater survival than butternut from warmer provenances at the lowest temperature treatment (-38 °C), but given that temperatures that low are extremely unlikely to occur in those provenances, it is not anticipated to give the hybrids an advantage if planted in those areas. However, the hybrids' earlier budbreak could limit the success of restoration with these hybrids in the coldest extents of butternut's range.

If hybrids, as well as genetically modified (GM) trees, are successfully developed for effective disease tolerance and to serve as an ecologically suitable replacement, success of restoration using hybrids will ultimately depend on those directly responsible for replanting efforts. A survey was administered to land managers in 46 organizations in Indiana to gauge perceptions of hybrid and GM trees, as well as current use of hybrid trees. Land managers had stronger concern for ecological, rather than economic, issues. Agreement was highest for using hybrid and GM trees for "conservation and restoration of at-risk species", "timber production", and "non-timber products (fruit, syrup, etc.)". However, perceptions varied by characteristics, such as concern type, age, and the type of land they managed. Ecological concern and the type of land being managed most strongly predicted current hybrid use. Overall, results indicate the majority of land managers in Indiana would likely be agreeable to recommendations towards using hybrids. However, most nonetheless had strong ecological concerns about their suitability as a native replacement. It is important to note, though, that consistent with the results of previous studies, great variation was seen within the performance and characteristics of the butternut hybrids in chapters 2-4. Thus, it may be possible with careful selection and breeding to harness this variation to develop disease tolerant and ecologically similar hybrids acceptable to land managers.

CHAPTER 1. INTRODUCTION

1.1 Plant species in crisis

Anthropogenically driven global change is disrupting ecosystems and habitats of many plant species, straining the ability of native species to survive and reproduce (Parker and Gilbert, 2004; Niu et al., 2014). Climate change, specifically, can cause stress directly through abiotic changes in the local environment (Anderegg et al., 2013; Augspurger, 2013) or indirectly, such as through the creation of optimal conditions for native pathogens and other pests (Dukes et al., 2009; Sturrock, 2012). Activities such as globalization and mass trade of plant material can inadvertently transports new pests and pathogens into novel environments, leading to invasion, which in turn, is often facilitated by climate change (Diez et al., 2012; Early et al., 2016). To survive, trees must respond with phenotypic plasticity (Franks et al., 2013; Zhu et al., 2018), adaptation (Davis and Shaw, 2001; Franks et al., 2013), or migration (Davis and Shaw, 2001; Fei et al., 2017). If this is not possible, and without intervention, species may face extinction (Thomas et al., 2004; Feeley et al., 2012). As evidence of this, 522 plant species have been classified as extinct (extinct, extinct in the wild, possibly extinct, or possibly extinct in the wild) within the last 200 years by the International Union for Conservation of Nature (IUCN), which the organization states “is likely to be a severe underestimate” (IUCN, 2020). Further, over 40% of the nearly 44,000 species of plants currently able to be evaluated by the IUCN are listed as threatened as of 2020. For full context, the IUCN estimates that there are currently nearly 423,000 plant species described.

1.2 Hybridization as a plant restoration tool

Hybridization, which consists of the mating of individuals of two distinct species or populations (Allendorf et al., 2013), is currently being considered as a possible restoration tool for incorporating novel traits to aid in plant species survival (Hamilton and Miller, 2015). Potential traits could include environmental stress tolerances in light of a changing climate (Hamilton and Miller, 2015) or resistance to introduced invasive diseases and pests (Sniezko and Koch, 2017). For example, hybrids of the endangered American chestnut, *Castanea dentata* (Marsh.) Borkh., have been created through careful crossing with the Chinese chestnut, *Castanea mollissima* Blume, followed by several generations of backcrossing to American chestnut (Steiner et al., 2017). These hybrids have exhibited tolerance to chestnut blight, *Cryphonectria parasitica* (Murrill) Barr. and are currently being evaluated in forest field trials (Clark et al., 2019). In another, more recent example, endangered native North American ash species (*Fraxinus* L.) are being crossed with Asian ash species in pursuit of hybrids resistant to emerald ash borer, *Agrilus plannipennis* Fairmaire (Koch et al., 2012).

While these examples illustrate that there are not just theoretical, but realized benefits to the use of hybrids, there are also many considerations that must be addressed before this tool can be most effectively - and safely - used. Jacobs et al. (2013) proposes a framework for American chestnut involving technological, ecological, and societal strategies that should be considered during the restoration process Figure 1.1). Technological approaches include breeding, hybridization, and genetic modification for incorporation of disease resistance (Jacobs et al., 2013). This resistance will ultimately need to be extensively tested for not just presence, but strength and durability, particularly in the field conditions in which these hybrids would ultimately be planted (Sniezko and Koch, 2017). Ecological considerations focus on activities such as overcoming potential ecological barriers and preventing unintended negative ecological implications (Jacobs et al., 2013). If the hybrids are to act as a substitute for the unadmixed species, they must be able to survive, reproduce, and fill the same ecological niche (Allendorf et al., 2013; Jackiw et al., 2015). Further, it will be essential to determine early on whether there is any potential for invasiveness (Muhlfeld et al., 2014), genetic swamping (loss of local adaptations by genetic dominance from another species; Allendorf et al. 2013), and outbreeding depression (reduced fitness or genetic incompatibilities; Allendorf et al. 2013) - all of which could detrimentally affect the target species and its ecosystem. Societal considerations, often overlooked yet essential, include cultural and economic values and government policy and regulation (Jacobs et al., 2013). Whether or not these types of trees are ultimately supported by science as both technologically effective and ecologically appropriate, the success of restoration efforts using hybrid trees will be strongly dependent on perceptions and acceptance to their use (Hall, 2007; Jacobs et al., 2013; Martín et al., 2019). Concentrating on just one or two of these areas (typically just technology or ecology), will decrease the probability of restoring the species. Shared, collaborative goals will allow for areas to overlap, and when all three overlap and are equally emphasized, restoration of the species is strongest and most likely to succeed (Figure 1.1b; Jacobs et al., 2013). This holistic approach takes advantage of tools from technology, ecology, and society and holds potential for the restoration of species beyond the American chestnut, such as the butternut, *Juglans cinerea* L.

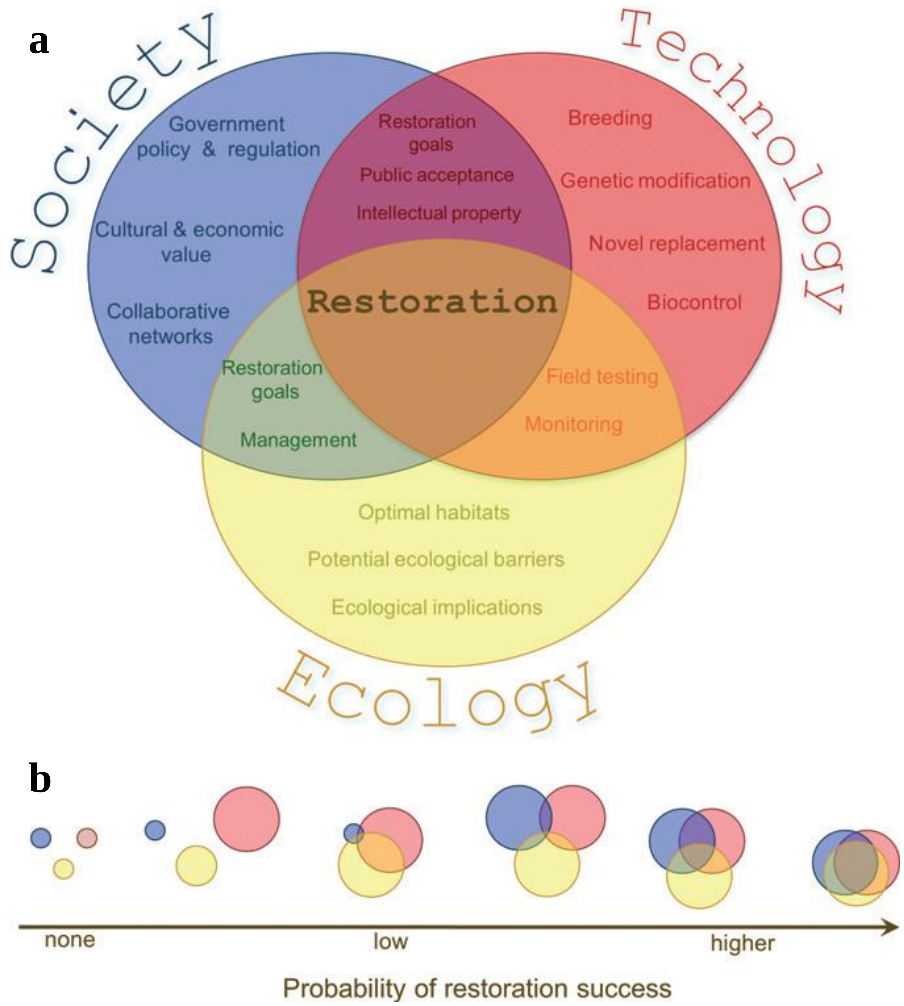

Figure 1.1. Conceptual framework for restoration of American chestnut (*Castanea dentata*) developed by Jacobs et al. (2013), consisting of three spheres: society (blue), technology (red), and ecology (yellow). (a) Examples of the different methods and tools from each area are listed, including those that overlap between spheres. (b) Ultimately, restoration efforts have the greatest probability of succeeding when all three areas are emphasized equally and overlap. Reprinted from Jacobs et al. (2013).

1.3 The case of the butternut tree

The butternut, also known as white walnut or oilnut, is a small- to medium-size hardwood tree native to the mixed hardwood forests of eastern North America (Rink, 1990). The species grows as far north as southern Ontario and Quebec, with small populations growing as far south as northern Mississippi, Alabama, and Georgia (Rink, 1990) (Figure 1.2a). The butternut's range is similar to that of black walnut (*Juglans nigra* L.) (Figure 1.2b), but extends further north into Canada and not as far south (Rink, 1990; Williams, 1990). Butternut is one of the most cold hardy *Juglans* species in the world, occurring in areas that can reach an average extreme minimum temperature as low as -40 °C (USDA hardiness zone 3; Dirr, 2009), and can grow at higher altitudes than black walnut (Rink, 1990; Williams, 1990). This relatively short-lived (around 75 years of age), shade intolerant species is found in deep, rich loamy areas along streambanks, as well as dry, rocky limestone sites (Rink, 1990; Ostry et al., 1994). The tree tends to occur sporadically as an individual or in small groups within forests and rarely occurs in pure stands (Rink, 1990). Other species commonly associated with butternut include black walnut, black cherry (*Prunus serotina* Ehrh.), beech (*Fagus grandifolia* Ehrh.), oak (*Quercus* L. spp.), hickories (*Carya* Nutt. spp.), elm (*Ulmus* L. spp.), maples (*Acer* L. spp.), basswood (*Tilia* L. spp.), tuliptree (*Liriondendron tulipifera* L.), Canadian hemlock [*Tsuga canadensis* (L.) Carr.], birch (*Betula* L. spp.), and white ash (*Fraxinus americana* L.) (Rink, 1990).

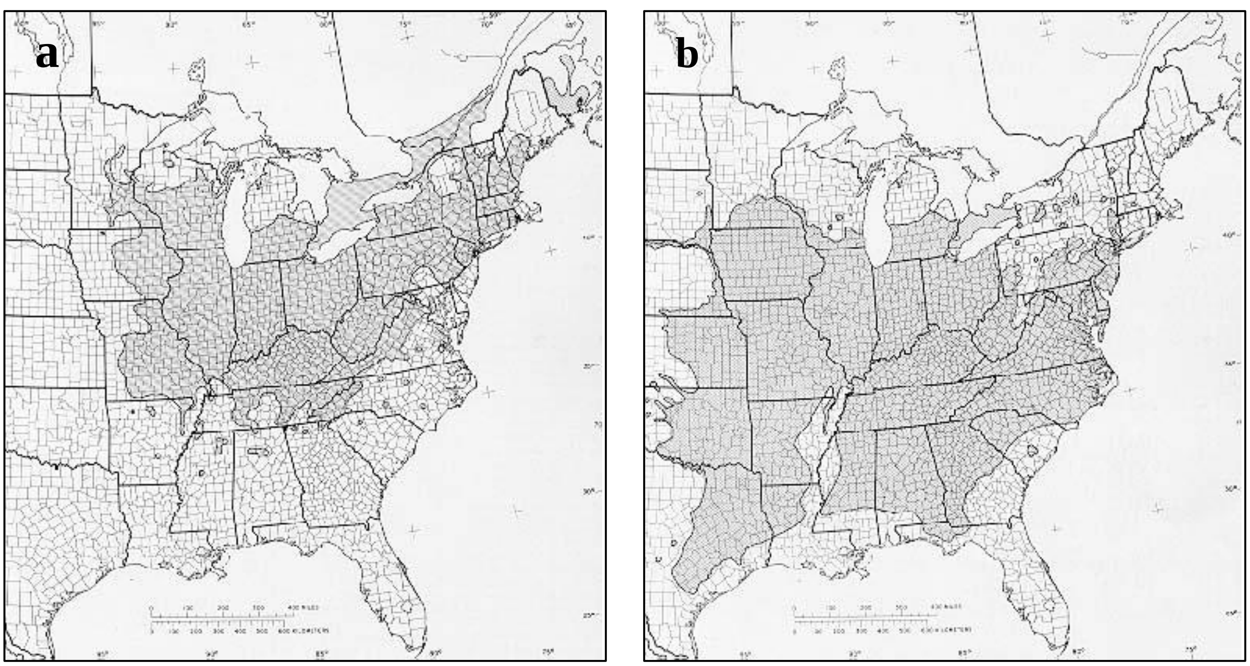

Figure 1.2. Species range map of (a) butternut (*Juglans cinerea*) and (b) black walnut (*Juglans nigra*). Reprinted from Rink (1990) and Williams (1990), consecutively.

Butternut has exceptionally long, alternately arranged, pinnately compound leaves (7-17 leaflets) and large, oblong nuts with a sticky, bright green hull surrounding the sharp shell that encases the kernel (Schultz, 2003; Farlee et al., 2010). The edible, sweet, and oily kernel, which inspired two of its common names, was valued by Native Americans as an energy-rich and long-lasting food source, as well as for many other cultural and medicinal purposes (Moerman, 1998; Abrams and Nowacki, 2008). Medicinally, butternut has shown a broader spectrum of antimicrobial activity than thirteen other North American hardwood species (Omar et al., 2000). Butternut seeds also serve as important food sourced for squirrels and other wildlife, particularly in the northern part of its range, where black walnut and many other masting species with large, energy-rich seeds are not present (Schultz, 2003; Farrar, 2017). Wood from the butternut is easily worked and economically valuable for furniture, paneling, and carving (Forest Products Laboratory, 2010).

1.3.1 Butternut canker disease

Butternut populations have declined dramatically due to butternut canker disease (BCD), caused by the fungus *Ophiognomonia clavigignenti-juglandacearum* (*Ocj*) (Nair, Kostichka, & Kuntz) Broders & Boland (Broders and Boland, 2011). The exact origin of the fungus is unknown; however, Broders et al. (2015) speculate that *Ocj* was most likely introduced on an imported exotic plant species, such as Japanese walnut (*Juglans ailantifolia* Carr.). The disease was first reported in 1967 in a woodlot in southwestern Wisconsin (Relund, 1971) and quickly spread throughout butternut's entire range (Ostry and Woeste, 2004; Broders et al., 2015). Natural openings in the tree, such as leaf scars, buds, branch crotches (Figure 1.3a), and bark fissures (Figure 1.3b) are common points of *Ocj* entry and establishment (Tisserat and Kuntz, 1983c, 1984). The disease manifests on limbs and boles as vertically oriented, elliptical to rhombus-shaped, sunken perennial cankers often with white margins and ink-black centers, eventually becoming partially covered by shredded bark (Figure 1.3b; Tisserat and Kuntz, 1983, 1984). Over time, the cankers multiply and coalesce, ultimately girdling and killing the affected tree (Tisserat and Kuntz, 1983c, 1984).

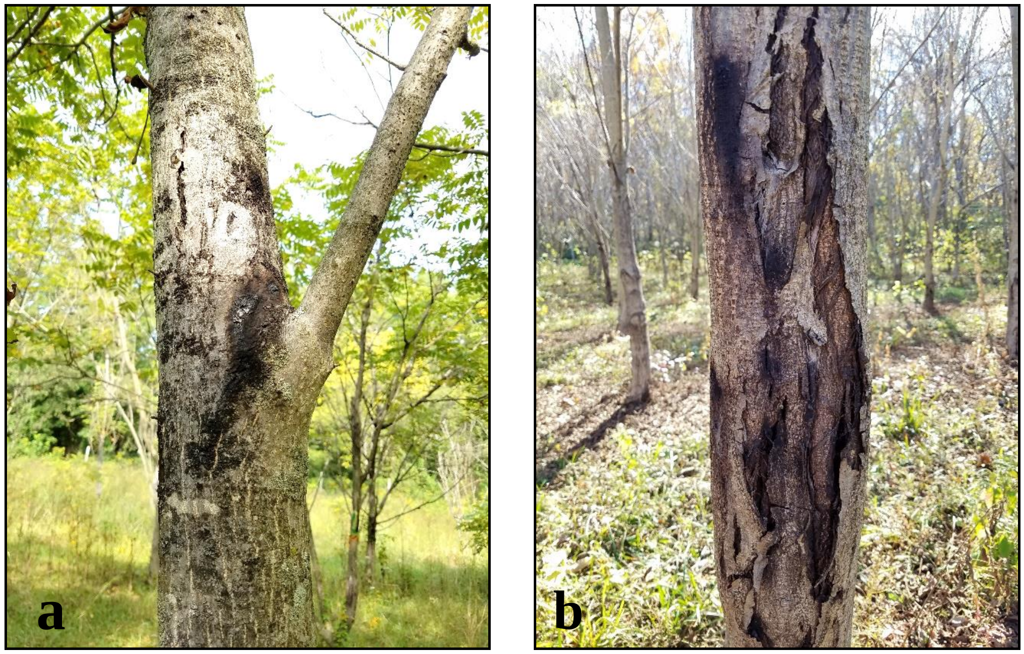

Figure 1.3. Butternut canker disease (*Ophiognomonia clavigignenti-juglandacearum*) of butternut (*Juglans cinerea*) manifesting in (a) a branch crotch and (b) bark fissures. Photo credit: Andrea Brennan, 2016.

BCD can be dispersed over long distances (up to 40 m away from diseased trees) by rainwater stemflow and raindrop impaction (Tisserat and Kuntz, 1983a, 1983b; Broders et al., 2015). While butternut is the only species reported to be killed by BCD, several other species have produced cankers through artificial inoculation with *Ocj*, including other *Juglans* species and hybrids (Orchard et al., 1982; Ostry, 1997; Ostry and Moore, 2007), and several species of *Carya*, also in the walnut family, Juglandaceae (Ostry, 1997; Ostry and Moore, 2007). *Ocj* has also been recovered 3-5 mo. post-inoculation from oak, chestnut, hazelnut (*Corylus* spp.), and cherry species after artificial inoculation, although no canker growth was evident (Ostry, 1997; Ostry and Moore, 2007). This indicates that, while these species may not be severely affected by BCD, other species growing in butternut's natural range could carry and harbor the fungus, allowing further spread of the disease (Ostry, 1997; Ostry and Moore, 2007).

Ocj has spread rapidly across butternut's entire range (Figure 1.4) and nearly eliminated natural regeneration of the tree species (Boraks and Broders, 2014). Across its range in the U.S., the number of butternut trees has decreased by 58% since the 1980s (Morin et al., 2017). Butternut was recently assigned to the A2.1 class (high current severity) of the Project CAPTURE (Conservation Assessment and Prioritization of Forest Trees Under Risk of Extirpation) framework, indicating the extreme sensitivity of butternut to BCD and the intensity of BCD's threat (Potter et al., 2019). The species is now considered endangered by the IUCN (Stritch and Barstow, 2019) and is listed under Canada's Species At Risk Act (SARA; Environment Canada, 2010). In the U.S., butternut has a conservation status of either critically imperiled (S1), imperiled (S2), or vulnerable (S3) in 21 states (NatureServe, 2017).

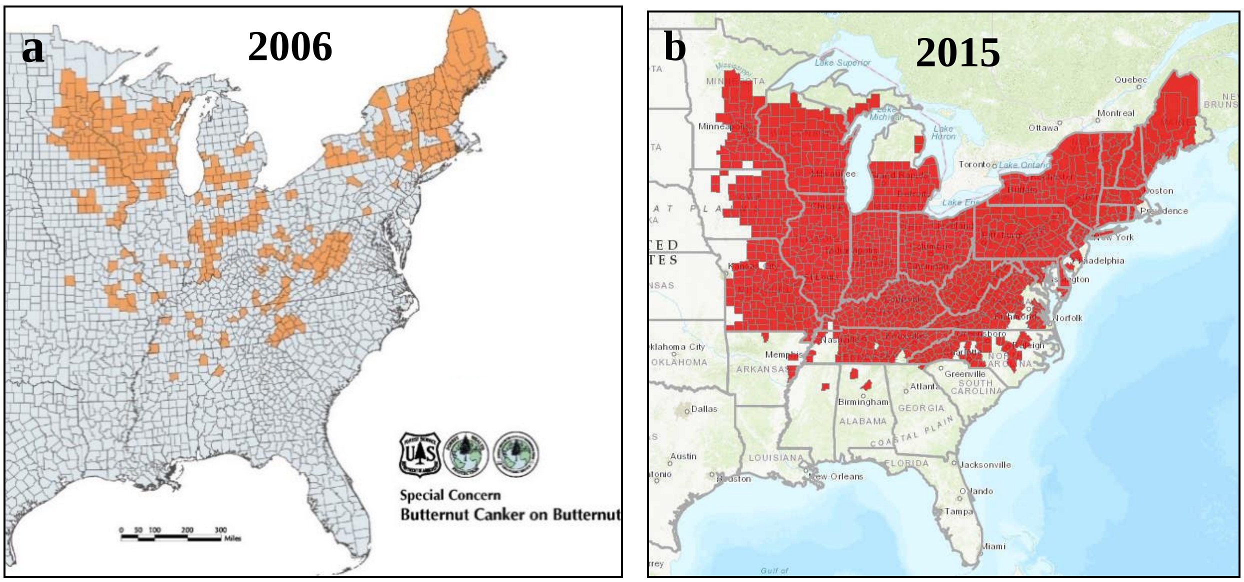

Figure 1.4. Disease risk maps of butternut canker disease (*Ophiognomonia clavigignenti-juglandacearum*) on butternut (*Juglans cinerea*) in the U.S, with county level observations of the disease on butternut in (a) 2006 in orange and (b) 2015 in red. Reprinted from USFS (2017).

1.3.2 Hybridization of butternut

Breeding for BCD resistance is considered a vital method for butternut conservation and restoration (Schultz, 2003; Environment Canada, 2010). However, similar to the case of several other forest tree species, such as American chestnut (Jacobs et al., 2013) or American elm (*Ulmus americana* L.; Martín et al., 2019), no effective disease resistance has been found in butternut populations to date. Healthy butternut individuals have been observed in the wild, surrounded by others that are severely cankered and declining due to BCD (Ostry and Woeste, 2004; Ostry and Moore, 2008). However, when clones and offspring from these healthy individuals have been selected, grown, and inoculated with the *Ocj* fungus under controlled conditions, the canker response is not consistent with the putative resistance observed in the wild, and varies greatly (Ostry and Woeste, 2004; Ostry and Moore, 2008). This has led to the current discussion of breeding butternut with a different, but BCD-resistant species in order to impart disease tolerance genes and aid in restoration of the species (Michler et al., 2006; Boraks and Broders, 2014).

Butternut is able to hybridize with two other walnut species: Persian walnut *(Juglans regia* L.) and Japanese walnut. The Persian walnut has shown high susceptibility to BCD and so is not an ideal choice for selected hybridization with butternut, but Japanese walnut has shown tolerance (Orchard et al., 1982). The latter species is estimated to have been released into the United States around 1860, particularly the popular heartnut variety, *J. ailantifolia* var. *cordiformis* (Crane et al., 1937). Japanese walnut readily hybridizes with butternut, and naturally occurring populations of their hybrids (*Juglans* × *bixbyi* Rehd.), sometimes referred to as buarts or buartnuts, have been observed throughout the United States (Hoban et al., 2009; Parks et al., 2013; Boraks and Broders, 2014). In a study of wild populations of butternut and naturally occurring hybrids in several states in the northeastern U.S., Boraks and Broders (2014) reported that the hybrids were significantly less affected by BCD (greater average vigor, less crown dieback, and fewer cankers) than unadmixed butternut. However, there are few controlled studies that not only directly compare BCD tolerance of the hybrids to unadmixed butternut, but other aspects of their potential use as a restoration tool, such as their ecophysiology, and societal perceptions to using them.

1.4 Summary of dissertation objectives

In light of rapid and severe global change, today's challenges are too massive, complex, and dynamic to simply specialize in one area without consideration of the multitude of others that might also have a stake in tackling the same challenges. The overarching goal for my dissertation research was to holistically consider restoration of a threatened tree species and link research from broadly different fields together in solving the challenge. I wanted to gain an understanding of and contribute to some of the disciplines that must work together in order to maximize restoration success. Hybridization, as well as related biotechnologies such as genetic modification, are emerging tools for tree restoration and hold great promise, but are not without valid concern. Thus, in my work, I explored hybridization for restoration of butternut as a case study, across biotechnological, ecophysiological, and societal disciplines.

Chapter 2 focuses on biotechnology through evaluation of breeding for BCD tolerance in butternut and its hybrids with Japanese walnut. While potential BCD tolerance was observed in naturalized hybrids occurring in wild butternut populations (Boraks and Broders, 2014), there have been no studies of BCD tolerance in controlled conditions to formally test whether the hybrids hold increased tolerance to their native progenitor. The objectives of this study were to examine potential BCD tolerance within and between butternut and its hybrids to determine if there is a difference in canker growth between isolates of *Ocj*. In order to meet these objectives, data from a pre-existing, multi-year BCD tolerance field study at Purdue University was analyzed and interpreted. The study was conducted with trees from multiple families of both butternut types inoculated with two different isolates of *Ocj*. Both artificially inoculated and naturally occurring infection were monitored.

Chapters 3 and 4 involve ecophysiology and examined the extreme temperature tolerances of butternut and its hybrids with Japanese walnut. In order for hybrid butternut to be an ecological equivalent to its native progenitor species, it must be able to survive and reproduce in the conditions that naturally occur in butternut's native range. Temperature and water availability are important in determining species distributions (Berry and Bjorkman, 1980; Woodward and Williams, 1987). Crystal and Jacobs (2014) evaluated the moisture tolerances of hybrid butternut in relation to both its progenitors and found that hybrids had less tolerance to drought than butternut, the drought-tolerant parent, and less flood tolerance than Japanese walnut, the flood-tolerant parent. However, the temperature tolerances of these species have not been compared. While butternut grows in areas where the minimum average temperature can reach -40 °C (Dirr, 2009), in the coldest parts of Japanese walnut's distribution in Hokkaido, Japan (GBIF Secretariat, 2019), it rarely drops below -20 °C (Japan Meteorological Agency, 2012). Butternut and Japanese walnut both experience average high summer temperatures of around 30 ºC in the hottest parts of their distributions, however Japanese walnut lives in an overall warmer ecosystem than butternut. Given this potential disparity in temperature tolerances, the objective of chapters 2 and 3 was to understand how hybrid butternut responds to cold and hot temperature extremes compared to its progenitors. Specifically, chapter 3 is a relative comparison of cold and heat tolerances of butternut, Japanese walnut, and two types of hybrids: F1 hybrids (butternut × Japanese walnut) and backcross hybrids (F1 × butternut). Excised plant material from mature field trees were exposed to cold and heat treatments in the laboratory to provide a comparative assessment of the species' temperature tolerances. Since extreme cold tolerance is such a key trait of butternut and its niche in the northern part of its range, an additional cold experiment was conducted with butternut and F1 hybrid seedlings for chapter 4. The seedlings were germinated and grown in common conditions in the greenhouse and exposed to cold treatments in the laboratory.

Chapter 5 examines perceptions to the use of biotechnology, specifically, hybrid and genetically modified (GM) trees in general, rather than for butternut specifically. Even if extensive scientific evaluations have shown a particular hybrid or GM tree as effective and safe for use in restoration, if the biotechnology is not perceived positively, there would likely be resistance to its use, as seen with GM agronomic crops (Sedjo, 2010). Evaluations of perceptions to hybrid and GM trees, prior to their launch and implementation, would aid in understanding and proactively addressing potential concerns so that the restoration process is more effective. A specific population that would be critical in any implementation of hybrid and GM trees is land managers – those responsible for the management and planting of trees. Thus, the overall objective of chapter 5 was to gauge land manager perceptions to the use of hybrid and GM trees, as well as current use of hybrid trees. An online survey, informed by preliminary interviews, was disseminated to land managers in numerous organizations from a broad set of disciplines (forestry, horticulture, botany, ecology, etc.) asking about concerns, perceived advantages, and potential uses for hybrid and GM trees, as well as current use of hybrid and at-risk tree species.

The final chapter synthesizes the findings of the BCD tolerance screening, ecophysiological experiments, and land manager perceptions study and how they contribute to butternut restoration. Informed by these findings, recommendations for future work are identified, in order to further efforts in restoration of butternut, as well as at-risk tree species in general.

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CHAPTER 2. HYBRID BREEDING FOR RESTORATION OF THREATENED FOREST TREES: EVIDENCE FOR INCORPORATING DISEASE TOLERANCE IN *JUGLANS CINEREA*

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2.1 Abstract

Hybridization is a potential tool for incorporating stress tolerance in plants, particularly to pests and diseases, in support of restoration and conservation efforts. Butternut (*Juglans cinerea*) is a species for which hybridization has only recently begun being explored. This North American hardwood tree is threatened due to *Ophiognomonia clavigignenti-juglandacearum*, the causal fungus of butternut canker disease (BCD), first observed in 1967. Observational evidence in some wild *J. cinerea* populations indicates that naturalized hybrids of *J. cinerea* with Japanese walnut (*Juglans ailantifolia*) may be more tolerant to BCD than non-admixed *J. cinerea*, but this has not been formally tested in a controlled trial. We aimed to examine potential BCD tolerance within and between *J. cinerea* and *J. cinerea* × *J. ailantifolia* hybrids and to determine if there is a difference in canker growth between BCD fungal isolates. Five-year-old *J. cinerea* and hybrid trees were inoculated with two *Ocj* fungal isolates collected from natural infections found in two different sites in Indiana, USA and a blank control (agar only). Measurements of both artificially induced and naturally occurring cankers were taken at 8-, 12-, 20-, 24-, and 32-months post-inoculation. Differences in canker presence/absence and size were observed by fungal isolate, which could help explain some of the differences in BCD severity seen between *J. cinerea* populations. Smaller and fewer cankers and greater genetic gains were seen in hybrid families, demonstrating that hybrids warrant further evaluation as a possible breeding tool for developing BCD-resistant *J. cinerea* trees.

2.2 Introduction

Native and non-native diseases and pests are increasingly threatening ecosystems, especially forests, across the globe (Ennos, 2015; Early et al., 2016). This is driven in large part by anthropogenically driven activities, such as globalization and mass trade of plant material that inadvertently transports new pests and pathogens into novel environments (Early et al., 2016). Climate change compounds the problem by providing ideal environments for pests and pathogens (Dukes et al., 2009) and weakening host species, making the host species more vulnerable to attack (Diez et al., 2012). Some species are not able to acclimate or adapt to these increased threats and are facing extinction (Thomas et al., 2004; Bellard et al., 2016).

Hybridization is currently under consideration as a possible tool to incorporate stress tolerance in support of restoration and conservation efforts (Hamilton and Miller, 2015). There are concerns that hybrids could be detrimental to both the target species and its ecosystem through potential invasion (Muhlfeld et al., 2014), outbreeding depression (genetic incompatibilities or reduced fitness; Allendorf et al. 2013), and genetic swamping (loss of local adaptations by genetic dominance from another species; Allendorf et al. 2013). However, desirable traits, such as disease and pest resistance conferred through hybridization, may be one of few remaining tools to save some species (Sniezko and Koch, 2017). Perhaps the most notable example of using hybridization to support an endangered species is the American chestnut (*Castanea dentata* (Marsh.) Borkh.), which has been crossed with the Chinese chestnut (*Castanea mollissima* Blume) in pursuit of resistance to chestnut blight [*Cryphonectria parasitica* (Murrill) Barr.] (Steiner et al., 2017; Clark et al., 2019; TACF, 2020). The American Chestnut Foundation (TACF), one of the leading organizations in this effort, has been breeding and backcrossing *C. dentata* hybrids for three generations over 30 years and is currently trialing hybrids with increased resistance in several restoration sites in the eastern US (TACF, 2020).

Another, lesser-known example where hybridization is being considered to save an endangered plant species, is butternut (*Juglans cinerea* L.), a North American hardwood tree species (Michler et al., 2006). While *J. cinerea* shares a native range in the eastern United States similar to black walnut (*Juglans nigra* L.), *J. cinerea* does not extend as far south and is one of few deciduous tree species in the far northern areas of the United States and southern Canada (Rink, 1990; Farrar, 2017). As a masting species, the tree is ecologically important for providing large, energy-rich nuts for both wildlife and humans (Schultz, 2003), but also holds economic importance through high quality wood products (Forest Products Laboratory, 2010). Culturally, *J. cinerea* has been used by Native Americans for a wide variety of purposes, including for medicine, food, dyes, and canoe construction (Moerman, 1998). Medicinally, *J. cinerea* has been documented to have a broader spectrum of antimicrobial activity compared to many other North American hardwood species (Omar et al. 2000).

Unfortunately, *J. cinerea* populations are now in severe decline due to butternut canker disease (BCD), caused by the fungus *Ophiognomonia clavigignenti-juglandacearum* (*Ocj*) (Nair, Kostichka, & Kuntz) Broders & Boland (Broders and Boland, 2011). The disease, first reported in Wisconsin in 1967 (Relund, 1971), manifests as vertically oriented, elliptical cankers that develop on limbs and boles, often causing the surrounding outer bark to peel (Tisserat and Kuntz 1984). Over time, the cankers multiply and coalesce, ultimately girdling and killing affected trees (Tisserat and Kuntz 1984). The reduction in *J. cinerea* populations by BCD has nearly eliminated natural regeneration (Boraks and Broders, 2014), to the point that it is now considered endangered by the International Union for Conservation of Nature (Stritch and Barstow, 2019). *Juglans cinerea* is also listed under Canada's Species At Risk Act (Environment Canada, 2010) and in the United States, the species has a conservation status of either critically imperiled (S1), imperiled (S2), or vulnerable (S3) in 21 states (NatureServe, 2017).

Despite the sporadic occurrence of healthy *J. cinerea* trees in the wild, no durable resistance to BCD has been found in populations of *J. cinerea* to date, with all showing susceptibility upon further testing. For example, when Ostry and Moore (2008) inoculated grafted clones from twelve canker-free source trees with *Ocj*, all individuals displayed susceptibility to the disease. This has led to the concept of using hybridization to incorporate disease resistance into the species (Michler et al., 2006; McKenna et al., 2011; Boraks and Broders, 2014). *Juglans cinerea* does not hybridize with *J. nigra*, the only other *Juglans* conspecific in the eastern deciduous forest (Rink, 1990). However, *J. cinerea* does hybridize with the Japanese walnut (*Juglans ailantifolia* Carr.; Rink 1990). A study of wild populations of both non-admixed *J. cinerea* and its naturalized hybrids with *J. ailantifolia* found possible tolerance in hybrids compared to its native progenitor, with *J. cinerea* exhibiting an average of 4.5 cankers per tree versus an average of 2.5 for its hybrids (Boraks and Broders, 2014). However, there have been no controlled evaluations to formally test whether the hybrids hold increased BCD tolerance to *J. cinerea*.

The objectives of this study were to examine potential BCD tolerance within and between non-admixed *J. cinerea* ("*J. cinerea*") and *J. cinerea* × *J. ailantifolia* hybrids ("hybrids," unless otherwise noted) and to determine if there is a difference in canker growth between isolates of *Ocj*. Our hypotheses were as follows: 1) hybrids will have greater tolerance to BCD than *J. cinerea*; 2) some *J. cinerea* and hybrid families will show greater tolerance to BCD than other families; and 3) there will be a difference in canker infection by different *Ocj* isolates. To test these hypotheses, a multi-year field study was conducted using *J. cinerea* and hybrid trees inoculated with two different isolates of *Ocj*.

2.3 Materials and methods

2.3.1 Plant material

In the fall of 2002, seeds were collected from presumed *J. cinerea* trees in an open-pollinated clone bank in Rosemount, MN, USA originating from putatively resistant surviving trees in the wild (family accessions 709-750; Table 2.1 and S1). Seeds were also collected from six wild presumed *J. cinerea* trees in northern Indiana, USA (family accessions 702-708). The seeds were stratified in a cooler at 2.8 °C through winter and germinated in a greenhouse in April 2003. The sprouted seeds were planted in a lowland field of Purdue University's Martell Forest (West Lafayette, IN, USA 40.4313991, -87.0389821) in May 2003. Approximately ten seedlings were planted per family (half-sib progenies sharing the same maternal parent) as two five-tree plots in a randomized row-block design with a spacing of 3.7 m between rows and 1.8 m within rows.

An initial visual screening of the seeds was conducted to exclude F1 hybrids at planting. Our original goal was to include only *J. cinerea* families, and in particular, those from healthy wild trees that we considered as putatively resistant parents. However, by the third growing season in 2005, early genetic identification methods were being developed (Aradhya et al., 2006; Zhao and Woeste, 2011) and many *J. cinerea* × *J. ailantifolia* hybrids among our *J. cinerea* germplasm collection had been detected which allowed us to examine these "complex" hybrids for phenotypic differences in leaf size, twig color, and terminal and lateral bud characteristics to distinguish these from *J. cinerea*. For the families in the present study, the phenotypic traits of seedlings were rated in the fall of 2005 by two independent observers as 2 = *J. cinerea*, 1 = *J. cinerea* and hybrid mix, or 0 = hybrid, using the methods that ultimately became the basis for those of Woeste et al. (2009). We recognize that phenotypic assessment is imperfect, but Hoban and Romero-Severson (2011) found that nut growers only using their own personal experience, and no key, were able to correctly identify their *J. cinerea* or hybrid trees 85% of the time. Therefore, we have high confidence that phenotypic methods used by expert foresters with long experience with these species should be able to make successful species designations in most cases. However, we also performed DNA tests on a subset of individuals from all families in 2009 using chloroplast markers (Aradhya et al., 2006; Zhao and Woeste, 2011), as well as ITS region, mitochondrial, and nuclear markers (Zhao and Woeste, 2011), which confirmed the initial phenotypic *J. cinerea* or hybrid genotype of each family. Further, a second subsample of 39 *J. cinerea* and hybrid trees from those included in the current study were also genetically analyzed in 2019 using the nuclear markers of Hoban et al. (2008) and chloroplast markers of McCleary et al. (2009). For the 31 samples that successfully amplified, the results of this genetic analysis subsample matched with the initial identification designations. From these analyses, we determined that seven of the Rosemount families and all six wild-collected Indiana families were *J. cinerea* × *J. ailantifolia* hybrids. Ultimately, 203 *J. cinerea* trees from 23 different families and 106 hybrid trees from 13 different families were included in the study.

2.3.2 Inoculations

Two different fungal isolates of *Ocj* were used for the inoculations. Both were collected from natural, spontaneous infections found in Indiana, the first from one of our seedlings in a breeding block at Martell Forest in West Lafayette (IN-1375-4A, "isolate 1") and the second from the Hoosier National Forest in southern Indiana (IN-1378-3, "isolate 2"). These were chosen in order to use isolates representative of the state in which the study was being conducted, and these specific isolates had already been collected and isolated by Michael Ostry and Melanie Moore (USDA Forest Service, Northern Research Station, St. Paul, MN) and thus were readily available. Samples for initiating cultures were collected from cankered branches in early August 2008 and grown on malt agar in darkness at 20°C. Inoculum was prepared from sporulating cultures after two months. Inoculations were applied to the trees at 5 years old in 2008, from late September to early October, when trees have been shown to be most susceptible to infection from *Ocj* (Ostry and Moore, 2008). The inoculation application method was similar to that developed by Anagnostakis (1992) for screening chestnut trees (*Castanea* spp.) for tolerance to chestnut blight. Holes (6-mm diameter) were drilled into the main trunk at approximately breast height, through the bark and slightly into the sapwood. A 6-mm diameter plug of inoculum (agar with *Ocj*) was then inserted into each hole, with fungal hyphae facing inwards, towards the cambium. A single layer of masking tape was then wrapped around each inoculation wound. Each hole was spaced 20 cm apart, running in a vertical line down the trunk. Each tree received five inoculation points in the following order: the first, top-most (apical) hole was plugged with a blank control (agar only); the second and third holes with *Ocj* isolate 1; and the fourth and fifth holes with *Ocj* isolate 2.

2.3.3 *Evaluation*

Survival was recorded each time canker growth was measured. Cankers resulting from the inoculations were evaluated at 8, 12, 20, 24, and 32 months after the inoculations were applied. The maximum vertical lengths (l) and horizontal widths (w) of each canker were recorded. The canker length and width were used to calculate the area (A) of the inoculated canker, using the formula for an ellipse (oval):

$$A = \frac{l}{2} \times \frac{w}{2} \times \pi \qquad (\text{cm}^2) \qquad (1)$$

Cankers occurring from natural *Ocj* infection (outside of inoculation areas) began appearing four years after planting in 2006, which was confirmed by isolation of the fungus from several samples of the naturally formed cankers in August 2008. Evaluations of the natural cankers were conducted concurrently with the artificially induced cankers at 8, 20, and 32 months following the inoculations. Natural cankers were rated for cumulative incidence and size using an ordinal scale. Incidence was rated from 0 to 3, where 0 = no natural cankers; 1 = 1 or 2 cankers; 2 = 3 to 5 cankers; and 3 = 6 or more cankers (McKenna et al., 2011). Size was based on the average size of the natural cankers (length × width), rated from 0 to 3, where 0 (none to very small) = less than ~30 × 10 mm; 1 (small) = ~30-59 × 10-19 mm; 2 (medium) = ~60-99 × 20-24 mm; and 3 (large) = ~100 × 25 mm or greater sized cankers (McKenna et al., 2011).

2.3.4 *Data analysis*

All data was analyzed in R v. 3.5.3 (R Core Team, 2019). There was insufficient mortality by the conclusion of the study to conduct a valid statistical analysis of survival, so only survival percentages are reported. The control inoculations did not produce cankers and were not included in the statistical analyses. Canker growth for the remaining inoculations was analyzed at the species/hybrid level using a two-part model to account for the high level of zero growth instances in the early time points of the study. Both parts of the model were conducted using R package ‘lme4’ (Bates et al., 2015). The first part used a linear mixed model to analyze the percent of individuals in each family with canker growth present over time. The second part evaluated canker area over time with linear mixed models only for inoculations where growth was present, using natural-log-transformed data to meet the assumption of normality of errors. For both parts, species/hybrid, fungal isolate, time, and block within the plot (three-level categorical variable) were considered fixed effects and family was considered a random effect. Since the second part of the model evaluated at the individual level, individual tree was also included as random and nested within family. To facilitate breeding selection and evaluate variation at the family level, Best Linear Unbiased Predictors (BLUPs; Isik et al. 2017) were generated from a linear mixed model, as in the second part of the inoculated canker model. However, only a subset of the data was used to analyze canker area for inoculations where growth was present at the last time point (32-months post-inoculation), thus, time was not included in the analysis of this data subset. The BLUPs (random effects) for each family were taken from the model and estimates of accuracy were calculated for each BLUP based on its standard error (SE) and the family variance (S) as: (Mrode, 2014):

$$Accuracy = \sqrt{1 - (\frac{SE^2}{S})} \tag{2}$$

Accuracy estimates are the correlation between true and predicted breeding values (Mrode, 2014) and are used in plant and animal breeding to evaluate confidence in predictions in lieu of the SE (Isik et al., 2017). The BLUPs were converted to breeding values (BV) by multiplying by two and adding the 32-month canker area population grand mean (μ). The BV was then converted to a percent gain relative to the population mean:

$$Genetic\ gain = \frac{\mu - BV}{\mu} \times 100 \quad (\%) \tag{3}$$

A positive genetic gain indicates a family with artificial canker sizes smaller than the population mean, while a negative genetic gain indicates a family with canker sizes greater than the population mean. The families were finally ranked in order of greatest to smallest gains to assist in family breeding selection. The incidence and size of naturally formed cankers was analyzed using cumulative link mixed models (also called ordinal regression or proportional odds models) with R package ‘ordinal’ (Christensen, 2019). Species/hybrid, fungal isolate, time, and plot block were set as fixed effects. Individual tree nested within family were set as random effects.

2.4 Results

2.4.1 Survival

By the conclusion of the study (32-months post-inoculation), there was 96% and 92% survival for *J. cinerea* and hybrid trees, respectively.

2.4.2 Artificially induced infection

The percent of individuals with canker growth present at the inoculation site strongly increased over time ($\chi^2 = 186.87$, $p < 0.0001$; Figure 2.1A). There was no difference in the presence of canker growth following inoculation between *J. cinerea* and hybrid trees ($\chi^2 = 0.14$, $p = 0.713$). However, there was a strong difference by fungal isolate ($\chi^2 = 421.48$, $p < 0.0001$), with much greater presence of canker growth resulting from inoculations with isolate 1 than isolate 2. There was a moderate interaction between species and time ($\chi^2 = 5.74$, $p = 0.017$), but there was no interaction evident between species/hybrid and fungal isolate ($\chi^2 = 2.94$, $p = 0.086$); fungal isolate and time ($\chi^2 = 0.13$, $p = 0.720$); or species/hybrid, fungal isolate, and time ($\chi^2 = 0.20$, $p = 0.657$).

The size of cankers resulting from the inoculations strongly increased over time ($\chi^2 = 1418.95$, $p < 0.0001$; Figure 2.1B). Canker growth on the hybrids was smaller than on *J. cinerea* and by the final timepoint, the average inoculated canker area (non-zero) on hybrid trees was 41.9 (±3.4) cm^2 compared to 61.8 (±4.1) cm^2 on *J. cinerea* trees ($\chi^2 = 8.65$, $p = 0.003$). There was also a difference in fungal isolate, with an average canker area of 48.3 (±2.9) cm^2 for isolate 1 versus 55.4 (±4.7) cm^2 for isolate 2 by the final timepoint ($\chi^2 = 5.34$, $p = 0.021$). A strong interaction was present between species/hybrid and time ($\chi^2 = 19.78$, $p < 0.0001$), with canker growth increasing more rapidly in *J. cinerea* than the hybrids. However, there was no interaction evident between species/hybrid and fungal isolate ($\chi^2 = 0.31$, $p = 0.580$); fungal isolate and time ($\chi^2 = 0.54$, $p = 0.463$); or species/hybrid, fungal isolate, and time ($\chi^2 = 0.97$, $p = 0.325$).

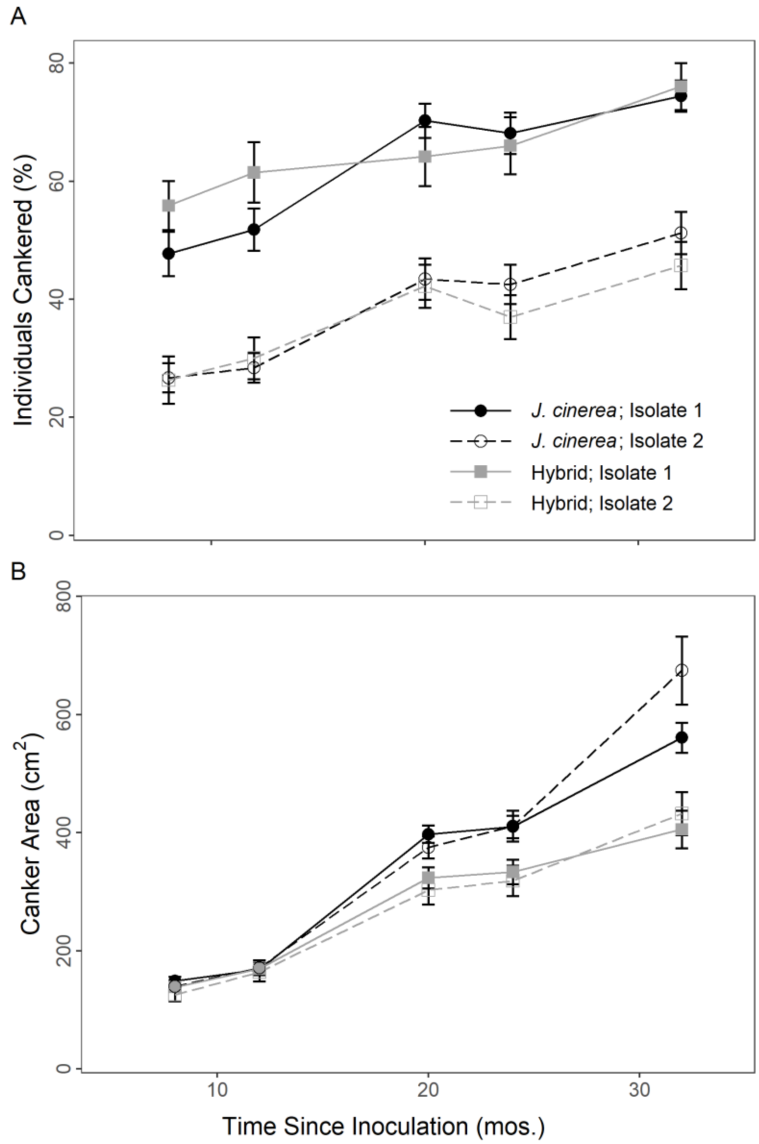

Figure 2.1. (A) Percent of individuals cankered and (B) canker area over time on *Juglans cinerea* and its hybrids with *Juglans ailantifolia* following inoculation with two different isolates of *Ophiognomonia clavigignenti-juglandacearum*, the causal fungus of butternut canker disease. Isolate significantly affected both percent of individuals cankered ($p < 0.0001$) and canker area ($p = 0.021$). Species/hybrid affected canker area ($p = 0.003$), but not percent of individuals cankered ($p = 0.713$).

By the conclusion of the study at 32-months post-inoculation, genetic gains based on canker size ranged from -12 to 14% (Table 2.1). There was distinct separation of families by genetic gains based on canker size. In the top-ranking quarter (5 to 14% gains), seven of nine families were hybrids, while in the bottom quarter (-12 to -5% gains), eight of nine of families were *J. cinerea*.

Table 2.1. Best Linear Unbiased Predictors (BLUPs), accuracy estimates, breeding values (BVs), and genetic gains of families of *Juglans cinerea* and its hybrids with *Juglans ailantifolia* based on canker size (area). Cankers were measured 32-months following inoculation with *Ophiognomonia clavigignenti-juglandacearum*, the causal fungus of butternut canker disease. A positive genetic gain indicates a family with canker sizes smaller than the population mean, while a negative genetic gain indicates a family with canker sizes greater than the population mean.

Family	Species/Hybrid	BLUP	Accuracy	BV	Gain (%)
707	Hybrid	-0.57	0.75	7.10	14
706	Hybrid	-0.46	0.67	7.32	11
711	Hybrid	-0.43	0.84	7.37	11
750	Hybrid	-0.37	0.81	7.49	9
704	Hybrid	-0.35	0.83	7.53	9
702	Hybrid	-0.34	0.81	7.57	8
748	Hybrid	-0.28	0.78	7.69	7
736	*J. cinerea*	-0.20	0.85	7.84	5
712	*J. cinerea*	-0.19	0.80	7.86	5
710	Hybrid	-0.12	0.83	8.00	3
713	*J. cinerea*	-0.09	0.76	8.06	2
717	*J. cinerea*	-0.08	0.81	8.07	2
730	*J. cinerea*	-0.08	0.85	8.07	2
709	*J. cinerea*	-0.08	0.86	8.08	2
731	Hybrid	-0.05	0.83	8.13	1
738	*J. cinerea*	-0.03	0.80	8.18	1
734	Hybrid	0.01	0.80	8.26	0
714	*J. cinerea*	0.03	0.84	8.29	-1
742	*J. cinerea*	0.06	0.80	8.36	-1
708	Hybrid	0.07	0.80	8.38	-2
728	*J. cinerea*	0.08	0.81	8.39	-2
722	*J. cinerea*	0.09	0.86	8.41	-2
732	Hybrid	0.09	0.81	8.42	-2
727	*J. cinerea*	0.12	0.83	8.48	-3
715	*J. cinerea*	0.13	0.76	8.50	-3
723	*J. cinerea*	0.19	0.84	8.61	-5
747	*J. cinerea*	0.20	0.86	8.63	-5
726	*J. cinerea*	0.20	0.80	8.63	-5
743	*J. cinerea*	0.21	0.84	8.65	-5
733	*J. cinerea*	0.22	0.85	8.67	-5
744	*J. cinerea*	0.26	0.82	8.75	-6
741	*J. cinerea*	0.27	0.81	8.78	-7
718	*J. cinerea*	0.33	0.87	8.90	-8
746	*J. cinerea*	0.33	0.85	8.90	-8
735	Hybrid	0.36	0.86	8.95	-9
716	*J. cinerea*	0.49	0.81	9.22	-12

Family variance = 0.098. Population mean = 8.237 (log transformed from mm^2).

2.4.3 Naturally occurring infection

Incidence of naturally occurring cankers increased strongly over time ($\chi^2 = 404.76$, $p < 0.0001$; Figure 2.2). Species/hybrid was also an important predictor of natural canker incidence, with *J. cinerea* having a greater incidence of natural cankers than the hybrids at all timepoints ($\chi^2 = 24.53$, $p < 0.0001$). As an example, by the final timepoint, 12% and 21% of *J. cinerea* had natural cankers in classes 0 (lowest incidence) and 3 (greatest incidence), respectively, compared to 42% and 5% in hybrids (Figure 2.2). There was no evidence of an interaction between species/hybrid and time for natural canker incidence ($\chi^2 = 2.67$, $p = 0.263$).

The size of naturally occurring cankers increased greatly over time ($\chi^2 = 264.82$, $p < 0.0001$; Figure 2.2). *Juglans cinerea* had larger natural cankers than the hybrids at all timepoints ($\chi^2 = 23.95$, $p < 0.0001$). At the final timepoint, 12% and 11% of *J. cinerea* had cankers in size classes 0 (smallest) and 3 (largest), respectively, versus 42% and 1% of hybrids (Figure 2.2). No evidence of an interaction between species/hybrid and time was found for the size of natural cankers ($\chi^2 = 2.62$, $p = 0.270$).

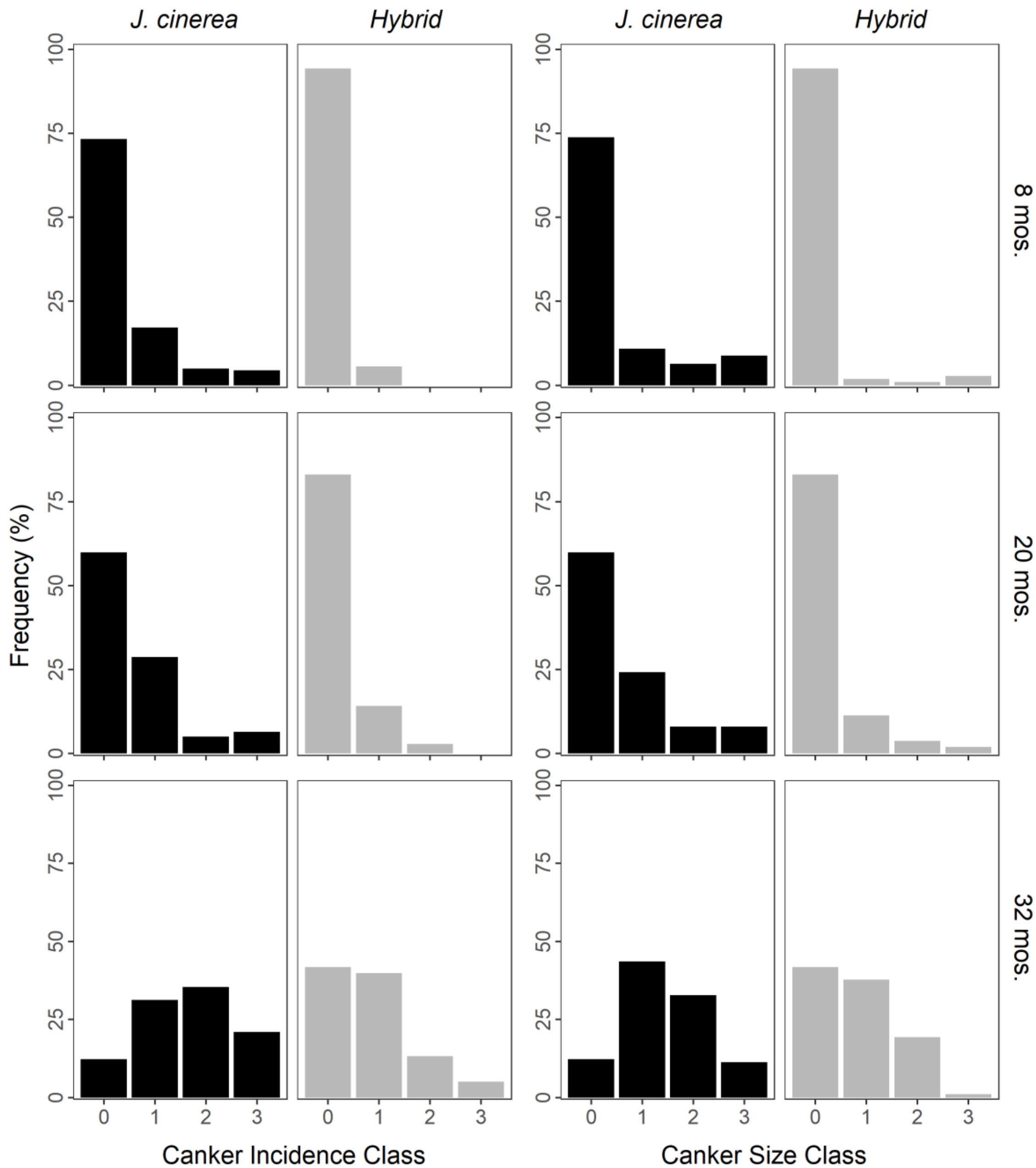

Figure 2.2. Frequency of trees of *Juglans cinerea* and its hybrids with *Juglans ailantifolia* with naturally occurring cankers by different incidence and size classes over time since the initiation of the study. Cankers were formed by *Ophiognomonia clavigignenti-juglandacearum*, the causal fungus of butternut canker disease. Incidence was rated from class 0 (no natural cankers) up to 3 (6 or more cankers). Size was based on the average size of the natural cankers (length × width), rated from class 0 (none to very small; less than ~30 × 10 mm) up to 3 (large; ~100 × 25 mm or greater). *Juglans cinerea* and hybrids were significantly different for both natural canker incidence and size at all timepoints ($p < 0.0001$ for all).

2.5 Discussion

2.5.1 Effect of fungal isolate

Supporting our hypothesis, the two *Ocj* isolates used for the inoculations in our study resulted in different levels of canker occurrence and size (Figure 2.1), which is consistent with studies by Ostry and Moore (2008) and Broders et al. (2012, 2015). In the current study, although the specific fungal isolate used in inoculations played a role in canker size, isolate played a much larger role in predicting the presence/absence of canker growth. This could indicate stronger variability in the ability of different *Ocj* isolates to initiate host infection. With differing levels of aggressiveness, the specific isolates present within a certain location may contribute, in part, to help explain why some areas experience more severe and sudden BCD outbreaks than others (Broders et al., 2015; Morin et al., 2017). However, it is likely that habitat and environment also play a strong role in determining occurrence of infection in these situations as well (Boraks and Broders, 2014; Labonte et al., 2015; Morin et al., 2017).

2.5.2 Tolerance of Juglans cinerea *and its hybrids*

Although there was no significant difference in inoculated canker absence/presence between *J. cinerea* and its hybrids, the hybrids did have smaller cankers (averaging nearly 1/3 smaller by the end of the study) that grew slower than those on the progenitor species (Figure 2.1). Further, hybrid families had the greatest genetic gains in terms of canker size by 32-months post-inoculation (Table 2.1). When considering naturally occurring infection, the hybrids also had both fewer and smaller cankers than *J. cinerea* (Figure 2.2). Thus, our hypotheses that hybrids would show greater tolerance to BCD than *J. cinerea* was mostly supported. This trend was also seen in a study of populations of wild *J. cinerea* and naturalized hybrids in the northeastern US, where the hybrids were found to be much less affected by the disease and had fewer cankers, less dieback, and greater vigor than trees of *J. cinerea* (Boraks and Broders, 2014). It should be noted, however, that while hybrids in the current study were more tolerant on average than *J. cinerea*, some hybrids performed worse than average and some *J. cinerea* performed better than average (Table 2.1).

Black and Neely (1978) reported that *J. cinerea* × *J. ailantifolia* hybrids also had greater tolerance than *J. cinerea* to another *Ophiognomonia* fungal species, anthracnose (*Ophiognomonia leptostyla* (Fr.) Sognov). These results in *J. cinerea* can be compared to hybrids and other diseases in *Juglans*. In the aforementioned study, hybrids of *J. nigra* with four other *Juglans* species consistently showed greater anthracnose tolerance than their highly susceptible *J. nigra* parent (Black and Neely, 1978). Conversely, in another study, hybrids of Persian walnut (*Juglans regia* L.) and iron walnut (*Juglans sigllata* Dode) showed similar or even greater susceptibility to walnut bacterial blight (*Xanthomonas arboricola* pv. *juglandis* Pierce) than both their progenitors (Jiang et al., 2019). Heightened susceptibility to crown gall disease (*Agrobacterium tumefaciens* Smith & Townsend) has also been documented in hybrids of northern California black walnut [*Juglans hindsii* (Jeps.) Jeps. ex R.E. Sm.] and *J. regia* (McKenna and Epstein, 2003). Thus, disease tolerance in *Juglans* hybrids that is greater than one or both of the parents is not guaranteed and depends on the specific host-pathogen interaction for each disease. Further, in relation to pest resistance, *J. ailantifolia* and its hybrids with both *J. cinerea* and *J. nigra* have expressed greater susceptibility to butternut curculio (*Conotrachelus juglandis* LeConte) than the two native North American progenitors (Wilson & Corneil 1978). This illustrates that in attempting to obtain BCD resistance in *J. cinerea*, it will be critical that increased susceptibility to native pests not also be inadvertently incorporated.

Interspecific hybrids have also been developed in other genera with the goal of incorporating disease resistance, or tolerance, into a susceptible and endangered native species. As discussed previously, *C. dentata* × *C. mollissima* hybrids backcrossed to *C. dentata* have been developed with increased resistance to chestnut blight compared to their susceptible *C. dentata* progenitor (Steiner et al., 2017; Clark et al., 2019; TACF, 2020). After strong selection for *C. dentata*-specific traits and blight resistance, second (B_2) and third (B_3) backcross hybrid lines developed at TACF's Meadowview Research Farm (Meadowview, VA, USA) were found to have average blight areas (B_2) or blight ratings (B_3), significantly different and intermediate to their American and Chinese chestnut progenitors, but not different from those of the F_1 generation (Steiner et al., 2017). However, Clark et al. (2019) reported that blight resistance in *C. dentata*, *C. mollissima*, B_1, B_2, and B_3 Meadowview backcross hybrids ultimately varied when planted across different sites in the first natural forest field trials testing this resistance. While the *Castanea* hybrids held yearly resistance rankings that were intermediate to that of their progenitors in two of the sites (NC and VA), there was no significant difference between any of the progenitors or hybrids in a third site (TN). Given such genotype × environment variation, it is essential that future work test *J. cinerea* and hybrid families in a common garden plots across multiple sites in order to assess the durability of possible BCD resistance. Efforts have also been pursued to develop Dutch elm disease (*Ophopstoma* spp.) resistant hybrids for restoring the endangered American elm (*Ulmus americana* L.) and several other *Ulmus* spp. affected by the disease (Brunet and Guries, 2016; Griffin et al., 2017; Martín et al., 2019). While progress has been made with promising hybrids and a few *U. americana* varieties (Brunet and Guries, 2016; Griffin et al., 2017; Martín et al., 2019), it has been slowed by incompatibility and ploidy barriers (Ager and Guries, 1982). These issues do not appear to be an issue with *J. cinerea* × *J. ailantifolia* hybrids given the large number of naturalized hybrids present in the landscape (Hoban et al., 2009).

Consistent with our second hypothesis, both *J. cinerea* and hybrid families separated out by genetic gains on 32-month canker size, with some families showing greater tolerance than others, indicating a possible genetic basis to disease tolerance (Isik et al., 2017). While hybrids tended to rank highest in genetic gains, some *J. cinerea* families, such as 736 and 712, had modest gains as well. However, the finding of a potential genetic basis to BCD tolerance in the current research must be compared to a heritability study of a wild population of *J. cinerea* in Wisconsin. LaBonte et al. (2015) primarily concluded that genetic differences explained little of the variance in mortality, and that environmental and site differences were stronger predictors. It was also reported that while genetics were not correlated with survival, there were low, but significant correlations between genetics and canker-related traits, including canker number, which is consistent with the present study. The population assessed by LaBonte et al. (2015) only contained non-admixed *J. cinerea* trees, which are believed to have originated from a small number of mother trees, limiting the genetic diversity. The present study, in contrast, included seeds propagated from long-term surviving selections collected from across a wide geographic range and inter-pollinated together in a grafted orchard, expanding the genetic diversity of our test families. Additionally, our study did not include environmental and site factors as in LaBonte et al. (2015), so a comparison with the current study's results on heritability of BCD tolerance in hybrids is not entirely possible.

Survival, as assessed by LaBonte et al. (2015), is likely a better measure in ultimately identifying the most BCD tolerant trees than the canker-related traits we evaluated in just under three years. However, the high survival (over 90%) for both *J. cinerea* and hybrid trees by the conclusion of the present study suggests that more than 32 months are required to understand the full potential of tolerance differences between the species and hybrids once *Ocj* infection begins.

Further, the research of LaBonte (2015), as well as Clark et al. (2019) with *C. dentata* (discussed earlier), both underscore the need for BCD tolerance screenings on multiple different sites to understand possible genotype × environment interactions. Sambaraju et al. (2018) reported that multiple factors, notably weather, influence *Ocj* epidemiology. It is likely that the successful restoration of *J. cinerea* will not be accomplished solely through the integration of genetic BCD resistance, but in combination with appropriate site selection and silvicultural practices (Jacobs et al. 2013).

Ultimately, beyond any increased disease tolerance or resistance that hybrids may hold compared to their progenitor species, it is essential to also consider how well such hybrids fill both the economic and ecological niches of the progenitor species they are intended to replace, including reproductive potential, physiology, invasiveness, and wood quality. These qualities have been evaluated to a moderate extent in *J. cinerea*, *J. ailantifolia*, and their hybrids. Crystal and Jacobs (2014) reported that the hybrids exhibited both intermediate drought and flood tolerance relative to their *J. cinerea* (more drought tolerant) and *J. ailantifolia* (more flood tolerant) progenitors. Phenotypically, Crystal et al. (2016) projected that most hybrids will tend more towards their *J. ailantifolia* progenitor, although some hybrids did occupy the same space as their *J. cinerea* progenitor. The concerns of dissimilar hybrid and *J. cinerea* phenotypes, along with the intermediate environmental tolerances of the hybrids, could limit their ability to act as a suitable replacement for *J. cinerea*, potentially changing the distribution of the species. However, in a phenotypical study of *C. dentata* hybrids and their progenitors, which are at a much more advanced breeding stage than *J. cinerea* hybrids, 96% of hybrid trees in the third backcross generation were distinctly different from their *C. mollissima* progenitor, and closely resembled *C. dentata* (Diskin et al. 2006). Thus, using *C. dentata* as an example threatened hardwood species for restoration (Jacobs et al., 2013), it may be possible to develop hybrids that are similar to *J. cinerea*, at least phenotypically, with careful selection and breeding.

2.6 Conclusion

Differences in canker occurrence and size by *Ocj* isolates were observed in this study, which may explain some of the differences in BCD severity reported among different *J. cinerea* populations. Hybrid families had smaller and fewer cankers and greater genetic gains compared to *J. cinerea* families, demonstrating that hybrids could be a possible breeding tool for developing

BCD-resistant *J. cinerea* trees. Further, the genetic gain separation of families by canker size indicates potential heritability of BCD tolerance (under the timeframe of the current study). This is promising for the development of resistance breeding programs using hybrids, but possibly *J. cinerea* as well. Hybridization in *J. cinerea* is one of just a few examples in plants where hybrids are being considered not only for preserving a species' economic value (timber and nut production), but also for ecological (restoration and conservation) and cultural purposes (ethnobotanical and medicinal). Thus, this study provides further evidence that hybrids represent a potentially effective tool for incorporating disease resistance to aid in restoration of threatened tree species.

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CHAPTER 3. CAN COLD AND HEAT TOLERANCES SUGGEST ADAPTIVE LIMITATIONS FOR *JUGLANS CINEREA* RESTORATION USING HYBRIDS?

3.1 Abstract

Hybridization could incorporate traits to native species for surviving detrimental global change. However, to serve as an effective substitute for restoration, hybrids must survive and reproduce in the same distribution as the target species. An ecophysiological evaluation of hybrids versus progenitor species could contribute to predicting potential hybrid distribution and utilization areas. *Juglans cinerea* L. is an endangered, exceptionally cold hardy North American tree species that can hybridize with the non-native *Juglans ailantifolia* Carr. Preliminary evidence indicates their hybrids could hold resistance to *Ophiognomonia clavigignenti-juglandacearum*, the fungal disease threatening *J. cinerea*. Consequently, hybrids are being evaluated as a possible conservation tool. This study aims to compare relative cold and heat tolerances within *J. cinerea* provenances (USDA plant hardiness zones 4, colder, to 7, warmer) and between *J. cinerea*, *J. ailantifolia*, and their hybrids (F1 hybrids - *J. cinerea* × *J. ailantifolia*, and backcross hybrids - F1 × *J. cinerea*). For the cold test, current-year twigs were subjected to five freeze treatments (-38 to 5 °C). Resulting damage was estimated using electrolyte leakage. In the heat test, leaflets were subjected to six hot water bath treatments (30 to 54 °C) and damage was estimated as change in chlorophyll fluorescence. Cold tolerance differed more than heat tolerance among *J. cinerea* provenances and across species. Within *J. cinerea,* trees from colder areas exhibited less cold damage than those from warmer areas. Differences in heat damage between hardiness zones occurred but did not follow a clear trend. Consequently, at the intraspecific level cold and heat tolerance were not correlated. However, at the interspecific level cold and heat tolerance were negatively correlated. *J. cinerea* exhibited greatest cold tolerance, *J. ailantifolia* exhibited greatest heat tolerance, and hybrids were intermediate. Thus, the utility of hybrids for restoration could be limited at the ecophysiological extremes of species' distributions.

3.2 Introduction

Global change is causing the habitats of many tree species to become unsuitable for survival and reproduction (Parker and Gilbert 2004, Niu et al. 2014). This can be direct, through abiotic changes in the local environment, climate change, such as increased extreme events like frost or heat and drought (Anderegg et al. 2013, Augspurger 2013) or indirect, such as through facilitation of increasingly optimal conditions for native pests (Dukes et al. 2009) or the arrival of non-native invasive species, including pests and diseases (Early et al. 2016). While native species might not be able to deal with these new and/or increased stressors, other related species might have higher tolerance. Hybridization, which consists of the mating of individuals of two distinct species or populations (Allendorf et al. 2013), could incorporate desirable traits to native species. For instance, hybrids could hold desirable traits to aid in species survival, such as disease and pest resistance (Sniezko and Koch 2017) or environmental stress tolerances for a changing climate (Hamilton and Miller 2015). Consequently, hybrids are currently being evaluated as a possible conservation tool (Allendorf et al. 2013, Hamilton and Miller 2015, Jackiw et al. 2015). However, there are many concerns towards using hybrids, including potential invasiveness (Muhlfeld et al. 2014), genetic swamping (Allendorf et al. 2013), and outbreeding depression (Allendorf et al. 2013), which could detrimentally affect the target species and its ecosystem. To serve as an effective substitute for conservation, hybrids must fill the same ecological niche as the native species by being able to survive and reproduce in the same distribution as the target species, while also being ecologically equivalent (Allendorf et al. 2013, Jackiw et al. 2015, Crystal et al. 2016).

Temperature and water availability are important in determining species distributions (Berry and Bjorkman 1980, Woodward and Williams 1987). Cold (e.g. Wisniewski et al. 2003, Fernández-Pérez et al. 2018) and drought (Reddy et al. 2004, Crimmins et al. 2011) have been studied extensively. Fewer studies have evaluated heat tolerances (e.g. Wahid et al. 2007, O'Sullivan et al. 2017), despite increasing relevance to climate change (Teskey et al. 2015). Even fewer studies have compared both heat and cold tolerances together, which allows for better delineation of the temperature spectrum that contributes to species distribution limits (Burr et al. 1993; Cunningham and Read 2006). Further, evaluation of hybrid ecophysiology compared to their progenitor species could contribute to predicting hybrid distributions and potential areas of utilization. However, this aspect has not been widely studied, particularly for tree species (Himrane et al. 2004; Crystal and Jacobs 2014; Pinchot et al. 2017).

The butternut (*Juglans cinerea* L.) is a North American tree species for which hybrids are currently being considered to support conservation efforts (Michler et al. 2006, Broders et al. 2015). In its natural range, *J. cinerea* can experience temperatures as low as -40 °C (USDA plant hardiness zone 3; Rink 1990; Dirr 2009), and is one of only a few deciduous tree species that can grow in the far northern areas of the United States and southern Canada (Farrar 2017). This tree produces masts of exceptionally large, energy-rich nuts that are an excellent food source for both wildlife and humans (Schultz 2003), while also holding economic value through, veneer-quality wood (Forest Products Laboratory 2010). Unfortunately, butternut canker disease, caused by the fungus *Ophiognomonia clavigignenti-juglandacearum* (Nair, Kostichka, & Kuntz) Broders & Boland, has caused rapid declines in *J. cinerea* populations across its entire range since its discovery in 1967 (Broders et al. 2015). Consequently, the species is now considered "endangered" by the International Union for Conservation of Nature (Stritch and Barstow 2019) and newer tools to preserve *J. cinerea*, and the ecological services it provides, are needed.

Breeding for butternut canker disease resistance is considered an important method for *J. cinerea* conservation (Schultz 2003, Environment Canada 2010). However, similar to the case of the American chestnut (*Castanea dentata* (Marsh.) Borkh.; Jacobs et al. 2013), no effective resistance has been found in *J. cinerea* to date, leading to discussion of hybrid breeding (Michler et al. 2006, Boraks and Broders 2014). While black walnut (*Juglans nigra* L.), the only other *Juglans* species co-occurring with *J. cinerea,* is able to resist butternut canker disease, it does not hybridize with *J. cinerea* (Williams 1990). However, Japanese walnut (*Juglans ailantifolia* Carr.), an exotic species, can hybridize with *J. cinerea,* and there is evidence that their hybrids are more tolerant to the disease than *J. cinerea* (Brennan et al. 2020 – Chapter 2; Boraks and Broders 2014). Even with increased disease tolerance, the hybrids must possess closely similar ecophysiological tolerances as *J. cinerea* to be an effective replacement. While *J. cinerea* grows in areas where the minimum average temperature can reach -40 °C (Dirr 2009), in the coldest parts of *J. ailantifolia*'s distribution in Hokkaido, Japan (GBIF Secretariat 2019), it rarely drops below -20 °C (Japan Meteorological Agency 2012). While *J. cinerea* and *J. ailantifolia* both experience average high summer temperatures of around 30 ºC in the hottest parts of their distributions, *J. ailantifolia* lives in an overall warmer ecosystem than *J. cinerea*. Given this potential disparity in temperature tolerances, it is imperative to understand how hybrids respond to temperature extremes relative to their progenitor species. Additionally, a deeper inspection of temperature tolerances by provenance is also needed for *J. cinerea*, which would allow for potential range-wide variation in adaptations to be uncovered. This, in turn, would aid restoration efforts through the selection and use of properly adapted trees (Bischoff et al. 2008).

The objective of this study was to compare relative cold and heat tolerances within *J. cinerea* from different USDA plant hardiness zone provenances and between *J. cinerea*, *J. ailantifolia*, and their hybrids. It was hypothesized for *J. cinerea* that the lower the hardiness zone of the provenance (and thus colder the area where the plant material originated), the greater the cold tolerance, with the reverse hypothesis for heat tolerance. Between species, it was hypothesized that *J. cinerea* would have the greatest cold tolerance, *J. ailantifolia* would have the greatest heat tolerance, and the hybrids would be intermediate to the progenitor species. To test these hypotheses, two experiments were conducted using samples from the same set of mature trees.

3.3 Materials and methods

3.3.1 Plant material

We selected 35 trees of *J. cinerea*, 13 trees of *J. ailantifolia*, 12 trees of F1 hybrids (*J. cinerea* × *J. ailantifolia*), and 12 trees of backcross hybrids (F1 × *J. cinerea*; hereon Backcross-*Jc*). Within *J. cinerea* were 5-12 trees (based on availability) from provenances corresponding to USDA plant hardiness zones 4-7 (USDA 2012). Hardiness zone 3 and 8 correspond to the coldest and warmest distribution limit of the species, respectively (Rink 1990, USDA 2012). However, only material from zones 4-7, which represents the majority of the *J. cinerea* population, was able to be obtained for the study. Note that two *J. ailantifolia* trees used in the heat test died and therefore, were excluded from the cold test. All plant material used was collected from seven- or eight-year-old seed-grown trees grown in a *Juglans* species common garden located in two nearby plots of the Hardwood Tree Improvement and Regeneration Center in Purdue University's Martell Forest (West Lafayette, IN, USA, 40.4313991, -87.0389821; Table B.1). Samples from all trees were genetically analyzed to confirm species identity using nuclear satellite marker methods (Hoban et al. 2008).

3.3.2 Cold tolerance test

Current year twigs were collected from each tree between 17:00-19:00 solar time in November 2018 (late fall). Minimum average daily temperature for the three weeks prior to collection was 2 °C. Under these conditions, trees were assumed to be cold acclimated, but unlikely to have experienced frost damage. Twigs were collected from the top-west side of the tree, the most exposed tree crown area that avoided effects from neighbor trees. After harvesting, twigs were moved immediately to the laboratory inside plastic bags with wet paper. Samples were stored overnight at 2.3 °C in a refrigerator to allow for full hydration. Cold tests were carried out the following morning. Two different cold hardiness assays were conducted on November 5th and 12th. On each date, half of the trees per species were analyzed.

The cold test was conducted following the methodology described in Haase (2011). Terminal and basal ends of the twigs were discarded, and the twigs were then cut into 2-cm segments. The segments were placed into 20 mL copolymer polypropylene vials (RPI Corp., Mount Prospect, IL) and filled with 13 mL of deionized water. A twig segment from each tree was tested at each of the four target temperatures: -14, -22, -30, and -38 °C. Additionally, one set of samples from each tree was placed in a refrigerator at 2.3 °C as a control. Four samples of each tree were placed in a programmable freezer (40-12, ScienTemp, Adrian, MI). Beginning with an initial temperature of 2 °C, the temperature was decreased at 5 °C hour^{-1}. At -2 °C all samples were shaken for approximately 5 s to promote ice nucleation. The temperature was held constant for one hour once the target temperature was reached, after which time the vials designated for that test temperature were removed, and the temperature then continued to decrease to the next temperature. After removal, samples were placed in a refrigerator at 2.3 °C for 12 hours and for an additional 24 hours at room temperature in darkness to allow for complete thawing.

After thawing, electrolyte leakage (EL) was measured using a conductivity meter (SevenEasy, Mettler Toledo, Columbus, OH). Maximum conductivity was measured after subjecting the samples to autoclave (Medallion, Amsco/Steris, Washington, MO) for 30 minutes at 121 °C and 100 kPa above atmospheric pressure and 12 hours at room temperature for complete electrolyte release. Damage was calculated with the equation:

$$\mathrm{EL} = \frac{c_\mathrm{i}}{c_\mathrm{f}} \times 100 \qquad (\%) \qquad (1)$$

where "c_i" is the initial conductivity and "c_f" is the maximum conductivity of each sample.

3.3.3 Heat tolerance test

Leaf samples were collected from mid-June to early July 2018 (late spring to early summer). The maximum average daily temperature was 29 °C for the three weeks prior to harvesting. Therefore, leaves were assumed to be heat acclimated, but unlikely to have experienced heat damage. Selected leaves were fully mature, without initial visual damage, and were collected from the top-west side of the tree, the most exposed tree crown area and which avoids the effect of neighbor trees. Leaves were measured for field maximum photochemical efficiency of PSII [$(F_V/F_M)_{field}$] using a fluorimeter (Handy PEA, Hansatech Instruments, Norfolk, UK). Immediately after harvesting, each leaf was wrapped at the base with a moist paper towel and placed in a plastic bag and transported to the laboratory in an insulated cooler. The leaf samples were kept in the laboratory overnight in darkness at room temperature. Heat tests were carried out during the following two days. Five heat hardiness assays were conducted between June 12th and July 11th. In each assay, roughly half the trees per each species were analyzed at two temperatures (35-37 trees per temperature and assay).

Heat treatments were applied using the hot water bath method (Marias et al. 2016). Apical and bottom leaflets were discarded. Selected leaflets were measured for initial maximum photochemical efficiency of PSII [$(F_V/F_M)_{pre}$] and assigned randomly to one of the target temperatures: 30 (control), 42, 45, 48, 51, or 54 °C. Then, the leaflets were wrapped with a wet paper, aluminum foil and sealed inside a heat durable and watertight plastic bag to prevent the sample from touching the hot water as is standard in heat tolerance tests (Kreeb 1990). Leaflets were then subjected to a specific temperature by immersing the samples in a water bath (89032-220, VWR, Radnor, PA) at the target temperature for 30 minutes. The temperature of two randomly selected leaflets were continuously monitored with a thermocouple (1312-EN-01, Professional Instruments, Hong Kong). Following the water baths, leaflet wraps were eliminated, and leaflets were placed in 20-ml plastic vials with 3 ml of deionized water and kept at room temperature and darkness for 24 hours. Maximum photochemical efficiency of PSII was measured 24 h later [$(F_V/F_M)_{post}$]. In all instances, the maximum photochemical efficiency of PSII of seedlings under in the field and before the test, remained stable (see Table B.2), confirming that leaflet responses to temperature treatments could be attributed to heat stress. Heat damage was calculated with the equation:

$$F_V/F_M \text{ reduction} = \frac{(F_V/F_M)_{pre} - (F_V/F_M)_{post}}{(F_V/F_M)_{pre}} \times 100 \quad (\%) \qquad (2)$$

3.3.4 Statistical analysis

All data analyses were conducted in R v. 3.4.3 (R Core Team 2019). Hierarchal nonlinear regression was used to analyze the cold differences in cold tolerances between species (species model) and hardiness zone provenances (*J. cinerea* model) with R packages 'nlstools' (Baty et al. 2015), 'lme4' (Bates et al. 2015), and 'arm' (Gelman and Hill 2007). A four-parameter logistic model (Pinheiro and Bates 2000) nested for each species was used:

$$\text{EL} = a + \frac{b-a}{1+e^{\left(\frac{c-T}{d}\right)}} \quad (\%) \qquad (3)$$

where a is the horizontal asymptote on the left side (equivalent to maximum cold damage), b is the horizontal asymptote on the right side (equivalent to minimum cold damage), c is the inflection point of the linear section of the curve (equivalent to the lethal temperature for 50% (LT_{50})), d is the inverse value of the slope (equivalent to the inverse rate of cold damage by temperature), and T is the temperature.

The data from the heat test did not conform to a logistic curve, thus, linear mixed models were used with individual tree nested in species (species model) or hardiness zone (*J. cinerea* model) as a random effect using R package 'nlme' (Pinheiro et al. 2019). Post hoc tests using Tukey's HSD were conducted using R package 'emmeans' (Lenth et al. 2019). Significance level was established at $\alpha = 0.05$. F_V/F_M reduction data was logit-transformed to meet the assumption of normally distributed errors. The variance in the response variable increased as the temperature increased, so the variance was fixed within each treatment temperature to improve model fit.

3.4 Results

3.4.1 Cold tolerance

Electrolyte leakage greater than that of the control began at the -14 °C treatment (Figure 3.1). After this point, the species and hybrids rapidly began to differentiate, with the greatest separation occurring at -38 °C, the lowest temperature tested. Among the four parameters defining the shape of the logistic function, the largest difference between cold damage among *Juglans* species was observed for parameter a (Table 3.1), where differences are indicated by the absence of overlapping SE. Parameter *a* explained 19% of the variance of the model while the remaining parameters, taken together, explain less than 5% of the variance (0.9, 2.6, and 0% of the variance for b, c and d parameters respectively). Parameter *a*, which defines the asymptotic maximum damage reached at the lowest temperature tested, indicated that *J. cinerea* sustained the lowest maximum damage, while *J. ailantifolia* suffered the greatest. The F1 hybrids reached maximum damage at levels intermediate to those of their progenitors and were statistically different from them. Damage sustained by the backcross-*Jc* hybrids was closer to that of *J. cinerea* than to the F1 hybrids, and no differences were observed between the backcross-*Jc* hybrids and the *J. cinerea* progenitor. Variation was also observed in the species model for parameter *c* (equivalent to the LT_{50}). Fitted values of parameter *c* revealed that the LT_{50} occurred in the same manner as that of parameter *a* (maximum damage), with maximal values predicted for *J. ailantifolia* and minimal values for *J. cinerea*, respectively, while intermediate values were noted for both the backcross-*Jc* and F1 hybrids, which did not differ from each other. Little to no differences were seen in parameters *b* (minimum EL) or *d* (inverse rate of cold damage by temperature) between *Juglans* species. Among *J. cinerea* hardiness zones, only parameter *a* explains sufficient variance to generate statistical differences (5.2% of variance explained for parameter *a* and less than 0.05% for the remaining parameters). Trees with provenances in hardiness zones 4 and 5 sustained lower maximum damage than those from zones 6 and 7.

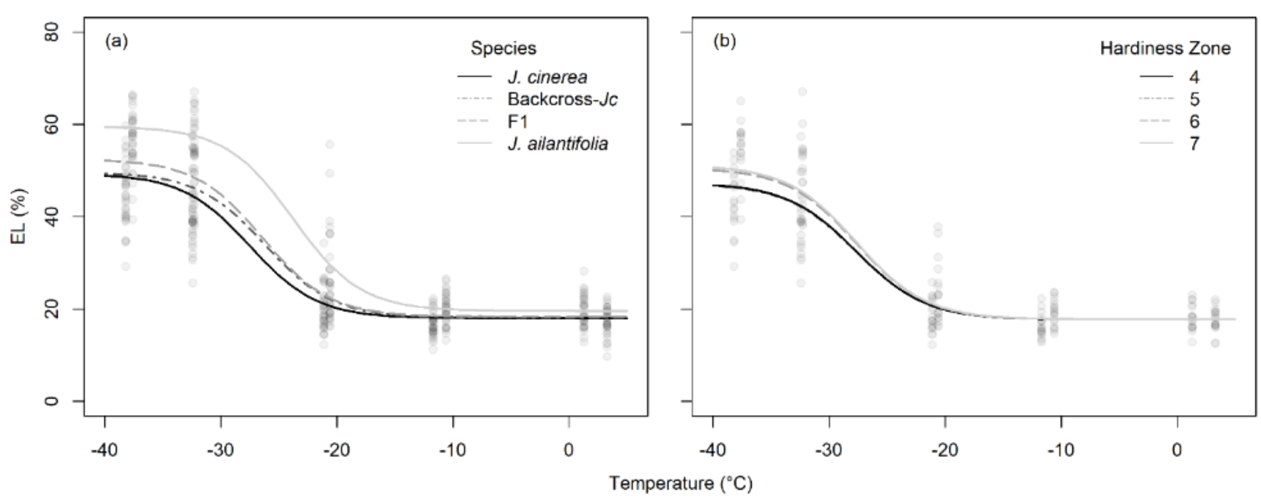

Figure 3.1. Electrolyte leakage (EL) measured on excised twig segments of (a) *Juglans* species and their hybrids and (b) USDA plant hardiness zone provenances within *J. cinerea* following five low temperature treatments relative to maximum EL. Each point represents the EL (%) of an individual sample and lines represent a logistic function fit for each species and hardiness zone.

Table 3.1. Estimated parameters (± SE) of a fitted logistic function with four parameters for electrolyte leakage (EL) and temperature across *Juglans cinerea* provenances (USDA plant hardiness zones) and across *Juglans* species and their hybrids. Parameter *a* indicates the maximum EL (%), *b* indicates the minimum EL (%), *c* indicates the LT_{50} (°C), and *d* indicates the inverse rate of cold damage by temperature. Upper case letters indicate differences among species, while lower case letters indicate differences among *J. cinerea* provenances. The global model utilized all possible data sets simultaneously to arrive at a single model that best fit all species and hybrids.

	Parameter			
Model	*a*	*b*	*c*	*d*
Global	52.76 ± 2.43	18.55 ± 0.78	-26.02 ± 1.04	2.91 ± 0.37
Juglans cinerea	49.37 ± 0.33 A	18.03 ± 0.07 A	-27.51 ± 0.12 A	2.91 ± 0.00
Hardiness zone 4	47.35 ± 0.36 a	17.78 ± 0.02	-27.68 ± 0.03	2.99 ± 0.00
Hardiness zone 5	47.20 ± 0.46 a	17.77 ± 0.02	-27.69 ± 0.04	2.99 ± 0.00
Hardiness zone 6	50.62 ± 0.29 b	17.77 ± 0.01	-27.65 ± 0.02	2.99 ± 0.00
Hardiness zone 7	51.20 ± 0.32 b	17.78 ± 0.01	-27.64 ± 0.03	2.99 ± 0.00
Hybrids	-	-	-	-
Backcross-*Jc*	49.63 ± 0.56 AB	18.14 ± 0.12 A	-26.18 ± 0.21 B	2.91 ± 0.00
F1	52.49 ± 0.56 B	18.35 ± 0.12 A	-26.40 ± 0.21 B	2.91 ± 0.00
Juglans ailantifolia	59.60 ± 0.59 C	19.59 ± 0.13 B	-23.78 ± 0.22 C	2.91 ± 0.00

3.4.2 Heat tolerance

There was no reduction in F_V/F_M below 42 °C (Figure 3.2). Above 42 °C, the higher the temperature, the greater the reduction in F_V/F_M in all the species (χ^2 = 2257.84, P < 0.001). However, there were no differences in F_V/F_M reduction between 45 and 48 °C. All *Juglans* species had the same response to temperature, as indicated by an absence of any significant species × temperature interaction (χ^2 = 10.75, P = 0.18). Between species, *Juglans cinerea* experienced greater reduction in F_V/F_M than *J. ailantifolia*. Both the F1 and backcross-*Jc* hybrid types fell between the parent species in terms of heat damage, with no statistical differences from their progenitor species or from each other. Likewise, *J. cinerea* seed sources had the same response to temperature, and no species × temperature interaction was evident (χ^2 = 2.91, P = 0.41). In the provenance model, both hardiness zone (χ^2 = 12.38, P = 0.006) and temperature (χ^2 = 1867.38, P < 0.001) were significant variables in determining reduction in F_V/F_M following the heat treatments. Trees from hardiness zone 5 experienced the greatest reduction in F_V/F_M while those from zones 4 and 7 the lowest F_V/F_M reduction, and trees from hardiness zone 6 experienced intermediate F_V/F_M reduction.

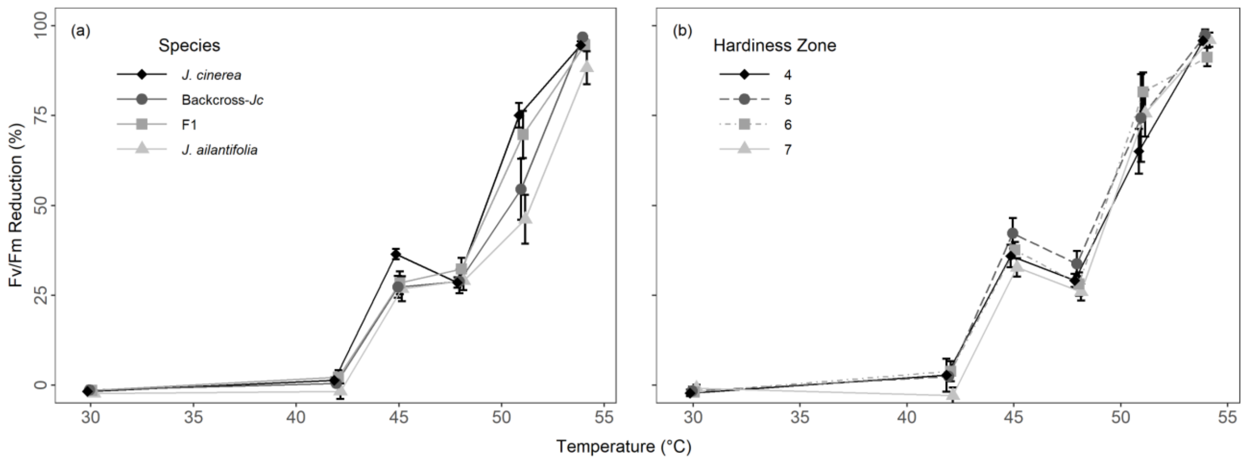

Figure 3.2. Reduction in F_V/F_M measured on excised leaflets of (a) different *Juglans* species and their hybrids and (b) USDA plant hardiness zone provenances within *J. cinerea* following six high temperature treatments relative to pre-treatment levels. Data are means and SE.

3.5 Discussion

3.5.1 Cold tolerance

Consistent with our hypotheses, provenances of *J. cinerea* from colder areas (lower hardiness zone numbers) exhibited greater cold tolerance than those from warmer areas when assessed by electrolyte leakage in current-year twigs. This result was similar to that reported for the Persian walnut (*Juglans regia* L.), where individuals from colder provenances sustained less damage following cold treatments (Guàrdia et al. 2013) and performed better on colder sites (Hemery et al. 2005) than individuals from warmer provenances. Similar results have also been reported in other species, such as white ash (*Fraxinus americana* L.; Alexander et al. 1984) and maritime pine (*Pinus pinaster* Aiton; Corcuera et al. 2011). Differences in cold tolerance among *J. cinerea* provenances reported here were quite small. The biggest difference was in the maximum damage at the lowest temperature tested (parameter *a*), which differed by only 4.00% among provenances, and the variance in LT_{50} (parameter *c*) was not significant (Table 3.1). Specifically, provenance differences were between the two lowest temperature hardiness zones (4 and 5) versus the two highest temperature hardiness zones (6 and 7), rather than between each hardiness zone individually. Thus, the provenance of *J. cinerea* planting material can be considered for cold tolerance at a broader scale than a single hardiness zone, like in groups of zones.

The variation in cold tolerance between *Juglans* species was greater than that seen within provenances of *J. cinerea*. The main differences were in the LT_{50} and maximum damage reached at the lowest temperature. Thus, the results suggest that there were likely earlier activated and more effective cold tolerance mechanisms in the more cold hardy species in the study. Consistent with the study's hypotheses, *J. cinerea*, the species native to the coldest areas, exhibited the greatest cold tolerance while *J. ailantifolia* exhibited the least. Also, as hypothesized, the hybrids exhibited cold tolerance levels intermediate to their parent species. Further, although the difference in LT_{50} for either of the hybrid types relative to *J. cinerea* was statistically significant, the magnitude of the difference was small (approximately 1 °C), while the difference with *J. ailantifolia* was higher (2-3ºC). This indicates that cold tolerance is a heritable and dominant trait, because the response of both hybrid types was closer to that of the most cold tolerant parent, *J. cinerea*. These results align closely with those reported by Ebrahimi et al. (2020) in a cold hardiness evaluation of seven *Juglans* species and their hybrids, including *J. cinerea* crossed with *J. ailantifolia* and *J. regia*. When less cold tolerant species were crossed with more cold tolerant species, the resulting hybrids had intermediate levels of cold tolerance, often closest to the cold hardiest progenitor. The dominance of cold tolerance in hybrids has also been observed in species from genera outside of *Juglans.* A naturally occurring hybrid, Barnes' aspen (*Populus* × *smithii* B. Bovin), a cross between bigtooth aspen (*Populus grandidentata* Michx.) and quaking aspen (*Populus tremuloides* Michx.), shared stem freezing tolerance closest to the most cold hardy parent, *P. grandidentata* (Deacon et al. 2019). Further, a study of hybrids of interior Douglas-fir (*Pseudotsuga menziesii* (Mirb.) Franco var. *glauca*) and coastal Douglas-fir (*Pseudotsuga menziesii* (Mirb.) Franco var. *menziesii*) also reported that hybrids were intermediate to their parents in cold hardiness, but most closely resembled the most cold tolerant progenitor, *P. menziesii* var. *glauca* (Rehfeldt 1977).

3.5.2 Heat tolerance

Contrary to the hypotheses, the hardiness zone where the material originated was not a good predictor of heat tolerance, as it was with cold tolerance. While there were differences in heat tolerance of *J. cinerea* by hardiness zone, there was no clear trend linking seed source and heat tolerance. Specifically, provenances from intermediate hardiness zones (5 and 6) showed higher heat tolerance than those from coldest and warmest hardiness zones (4 and 7, respectively). This could be because USDA plant hardiness zones are designated based on the average annual extreme minimum temperature (USDA 2012). While, more broadly, species native to colder places tend to have lower heat tolerances than those from warmer areas (Cunningham and Read 2006, O'Sullivan et al. 2017), the relationship between cold and heat tolerances seems to be more complex than simply being inverse of each other. Knight and Ackerly (2002), for example, reported that heat tolerance was not necessarily greater for species with warm-climate distributions when measured in a common environment. Similarly, at the intraspecific level, Marias et al. (2016) found no difference in thermotolerance between seedlings from different populations within ponderosa pine (*Pinus ponderosa* Lawson & C. Lawson) and *P. menziessii*, and suggests that this could be due to seedling growth and acclimation in the same environment.

While an inverse relationship between heat- and cold tolerance was not seen between provenances of *J. cinerea* in this study, it was observed between *Juglans* species. In accord with the research hypotheses, *J. ailantifolia* exhibited the greatest heat tolerance, *J. cinerea* had the least, and the hybrids were intermediate. Several studies have also reported intrageneric differences in heat tolerance of tree species, such as within *Quercus* L. (Hamerlynck and Knapp 1994) and *Picea* Mill. (Zhang et al. 2018). The LT_{50} for high temperature was above 49 ºC in all species included in the current study. This is a similar value to that of other temperate species, as well as species in other types of ecosystems, such as tropical (Cunningham and Read 2006) or desert ecosystems (Curtis et al. 2014). Additionally, the biggest differences in heat tolerance among species were found above 48 ºC, although differences were small. Both facts together suggest that high temperatures have a limited effect on species distribution.

An unexpected finding in the heat tests was the near constancy of F_V/F_M reduction between the 45 and 48 °C treatments, which was seen consistently across all species, hardiness zones, and treatment batches. Although a direct explanation of this result was not found in the existing literature, it is reasonable to speculate that one or more rapidly induced physiological mechanisms may have been activated and effective from around 45-48 °C. For example, a study of mustard seedlings (*Sinapis alba* L.) found that rapid rises in salicylic acid by more than 400% (compared to the control) occurred within 30 minutes of a 1-hour long heat treatment at 45 °C (Dat et al. 1998). Salicylic acid is a plant hormone and signaling molecule linked to the production of antioxidants and heat shock proteins (among other biochemical factors) and is thus known to provide thermal protection against heat shock (Larkindale and Knight 2002, Snyman and Cronjé 2008).

3.5.3 Implications of cold and heat tolerance for restoration

In this experiment, heat tolerance was not correlated, either negatively or positively, with cold tolerance across *J. cinerea* provenances. However, the most cold tolerant species, *J. cinerea*, was also the least heat tolerant species and the reverse pattern was found in *J. ailantifolia*, indicating that at the interspecific level, cold and heat tolerance were negatively correlated. Freezing versus high temperature stress can be contrasting, such as with cell membrane stability, where freezing stress causes the membrane to become more rigid, while high temperature stress causes it to become more fluid (Sung et al. 2003, Taiz et al. 2015). Yet, both temperature extremes can also lead to the same or similar effects, such as protein destabilization and oxidative stress (Sung et al. 2003, Taiz et al. 2015). Thus, some mechanisms of freezing tolerance, such as molecular chaperone proteins and antioxidants, also provide cross-protection against high temperatures, even though it may not actually be necessary for a plant or species to have both strong freezing and heat tolerance in its particular distribution (Sung et al. 2003, Taiz et al. 2015).

This study demonstrates differences in heat- and cold tolerances between provenances of *J. cinerea* and among *Juglans* species. Specifically, differences across species in cold tolerance were bigger than heat tolerance. Although both measures provide useful information on the nature of genetic adaptation to source environment, differences in cold tolerance indicate a much stronger link to the distribution of the species and provenances than did differences in heat tolerance. Additional work, particularly using additional cold and heat tolerance metrics, is needed to fully explain this outcome. Electrolyte leakage has long been used to evaluate cold tolerance (Lassoie and Hinckley 1991, Earnshaw 1993, Haase 2011), and chlorophyll fluorescence similarly has been a powerful tool to assess heat tolerance (Schreiber and Berry 1977, Bilger et al. 1984). Both approaches have been documented to correspond with temperature tolerances using other established metrics. However, in each case, these tests measure only one response on part of the plant. Consequently, the methodologies used do not allow us to determine the real temperature thresholds for the distribution of the species, though comparative analysis of species is still highly informative. Future evaluations that utilize the whole plant and employ multiple assays of stress indicators could provide a more realistic assessment that might better inform the relationships among cold tolerance, heat tolerance, and distribution.

Based on our results for *J. cinerea*, safe and effective matching of the provenance of a potential planting material to a particular planting site will be best accomplished by considering the low temperatures – rather than the high temperatures – experienced in that provenance. Differences in the cold and heat tolerances of the hybrids are small compared to *J. cinerea*. It should be acknowledged, however, that material from the very northern and southern extremes of the range (USDA zones 3 and 8) were not able to be included in the study. Nonetheless, the results still suggest that the hybrids might be planted successfully in the same distribution area as *J. cinerea*. However, special care should be taken when planting the hybrids in the colder, more northern extremes of the distribution due to the ecological importance of *J. cinerea* in those areas. Because the hybrids' heat tolerance did not differ from *J. cinerea*, the hybrids could likely tolerate the heat in the southern extremes of *J. cinerea*'s range, but would not likely expand the southern distribution. Additionally, it is essential that there was no loss in heat tolerance found in the hybrids, particularly with the rise in temperature projected under climate change for the southern portions of *J. cinerea*'s distribution (Ghannoum and Way 2011, Kunkel et al. 2013).

Additionally, hybrids have shown intermediate tolerances and traits to the progenitor species in other aspects. Specifically, *J. cinerea* × *J. ailantifolia* hybrids have less tolerance to drought than *J. cinerea*, the drought-tolerant parent, and less flood tolerance than *J. ailantifolia*, the flood-tolerant parent (Crystal and Jacobs 2014). In a discriminant analysis of the vegetative and adaptive traits for the same species, the hybrids were found to vary widely, with only some of them being able to occupy the same space as their *J. cinerea* progenitor (Crystal et al. 2016). Taken together, the results of ecological studies indicate that the hybrids may not be able to fully fill the niche of *J. cinerea*. Thus, it is ultimately recommended that if these hybrids are to be used for restoring *J. cinerea*, hybrid families should be carefully screened and evaluated for their ecophysiological tolerances and matched to the climate of the target planting areas, particularly for the northern and southern extremes. The ecophysiology of the hybrids is only one of many aspects that need to be considered if hybrids are to be used to restore a threatened species (Allendorf et al. 2013, Jackiw et al. 2015). A full recommendation on the use of hybrids for restoring *J. cinerea* cannot be made without also evaluating other hybrid characteristics, both ecological and economical, such as reproductive potential, invasiveness, growth rate, form, and wood quality compared to the native progenitor species (Allendorf et al. 2013, Woodcock et al. 2017).

3.6 Conclusion

Although cold tolerance of different sources of *J. cinerea* varied according to provenances, differences were relatively small, suggesting that provenances should be considered for *J. cinerea* at broader geographical scales. However, there was no pattern detectable in the heat tolerance across *J. cinerea* provenances. Consequently, heat and cold tolerance did not seem to correlate at the interspecific level. Furthermore, differences in cold and heat tolerance were larger in magnitude at the interspecific than intraspecific level. Additionally, heat tolerance was negatively correlated with cold tolerance at the interspecific level. *Juglans cinerea* was the most cold tolerant species, *J. ailantifolia* was the most heat tolerant species, and their hybrids expressed intermediate responses in comparison to both progenitors at both temperature extremes. In general, differences among species in cold tolerance were larger than for heat tolerance, indicating that low temperatures were better able to explain species distribution patterns. With respect to cold, the hybrids were closer to the most cold tolerant progenitor (*J. cinerea*), suggesting that cold tolerance is a heritable and dominant trait. Interestingly, results suggest the activation of heat tolerance mechanisms above 45 ºC, which was common to all species evaluated. Differences in cold- and heat tolerances of the hybrids were small compared to the *J. cinerea* parent in this study, indicating that hybrids could be used in some circumstances for restoration, when primarily considering their temperature tolerances. However, the utility of hybrids for restoration could be limited at the ecophysiological extremes of a species' distribution.

3.7 References

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CHAPTER 4. COLD HARDINESS TRAITS SUGGEST A LACK OF ECOLOGICAL SIMILARITY BETWEEN A PROGENITOR FOREST TREE SPECIES AND ITS HYBRIDS

4.1 Abstract

Many plant species are threatened with extinction by the effects of rapid global changes. Hybrids developed with novel traits, such as increased disease and pest tolerance, could support species growth in both natural and cultivated stands. For a hybrid to be an adequate substitute for its native progenitor, it must fulfill roughly the same niche ecology in the same distribution. In this regard, cold tolerance is an especially important trait, as low temperatures strongly define species' distributions, both latitudinally and altitudinally. The butternut (*Juglans cinerea* L.) is a threatened tree species native to the northeastern U.S. and southeastern Canada for which hybrids (*Juglans* × *bixbyi* Rehd.) are being considered. *Juglans cinerea* can survive in areas reaching as low as -40 °C, while Japanese walnut (*Juglans ailantifolia* Carr.), the other progenitor, typically only experiences temperatures down to -20 °C. This could indicate a cold tolerance disparity between *J.* × *bixbyi* and the species it is meant to replace, *J. cinerea*. A whole-plant freeze test was conducted at 7 (control), -10, -20, -30, and -38 °C using one-year-old seedlings of *J. cinerea* from USDA hardiness zones 4, 5, and 6 (coldest to warmest), and *J.* × *bixbyi*. Survival, dieback damage, and rate of spring budbreak were measured. No species or hardiness zone group exceeded the LT_{50} until the coldest treatment of -38 °C, demonstrating the extreme cold tolerance of both *J. cinerea* and *J.* × *bixbyi*. Budbreak phenology was most uniform at the -20 °C treatment, possibly indicating the importance of low, non-lethal temperatures for these species. No survival or damage differences were detected in provenances of *J. cinerea*, although seedlings from the coldest provenances (zone 4) experienced more delayed budbreak at the two warmest treatments than those from warmer provenances (zones 5 and 6). Interspecific differences were not observed in dieback but were detected in survival and budbreak. *Juglans* × *bixbyi* had greater survival than *J. cinerea* from warmer provenances at the -38 °C treatment, but given that temperatures that low are extremely unlikely to occur in those provenances, it is not anticipated to give the hybrids an advantage if planted there. However, the earlier budbreak of *J.* × *bixbyi* could cause it to be asynchronous to *J. cinerea*'s ecosystem and more vulnerable to spring frosts, calling into question the ability of *J.* × *bixbyi* to serve as an adequate substitute for *J. cinerea*.

4.2 Introduction

Many plant species are threatened by the effects of rapid global changes, such as climate change (Niu et al. 2014) and attacks from both native and nonnative pests and diseases (Dukes et al. 2009). Hybridization, or the crossing of different species (Allendorf et al. 2013), is increasingly being proposed as a potential tool to help species resist some of these stressors and support restoration and conservation efforts (Hamilton and Miller 2015, Jackiw et al. 2015). Hybrids developed with novel traits, such as increased tolerance to environmental stressors (Hamilton and Miller 2015) or pests and diseases (Sniezko and Koch 2017) could support species survival and growth in both natural and cultivated stands. One notable example is the functionally extinct American chestnut (*Castanea dentata* (Marsh.) Borkh.), which has been bred with the Chinese chestnut (*Castanea mollissima* Blume) in an intense backcrossing effort to obtain chestnut blight [*Cryphonectria parasitica* (Murrill) Barr.] resistant hybrids (Clark et al. 2019).

Use of hybrids is not without concern, though, with invasion (Muhlfeld et al. 2014), outbreeding depression (Allendorf et al. 2013), and genetic swamping (Allendorf et al. 2013) being cited as potential consequences of their use. Further, for a hybrid to be an adequate substitution for its native progenitor, it must fulfill the same niche ecology and be able to survive and reproduce in the same growing range (Allendorf et al. 2013, Jackiw et al. 2015). However, there is limited literature comparing ecophysiological tolerances of hybrid plants to their progenitors. The literature that is available in this area indicates that hybrids tend to hold intermediate tolerances compared to the parent species (Hamerlynck and Knapp 1994, Crystal and Jacobs 2014, Zhang et al. 2018, Deacon et al. 2019, Chapter 3), which could limit the utility of hybrids at the extremes of the target progenitor's distribution.

Cold temperatures strongly define plant species' distributions, both latitudinally and altitudinally (Berry and Bjorkman 1980, Woodward and Williams 1987). While the importance of cold survival and damage has clear implications, phenology is also important in understanding potential vulnerability to the increasing frequency of late spring frosts with climate change (Augspurger 2013, Muffler et al. 2016). Damage from late spring frosts can be particularly severe for species whose flowers emerge with their leaves in the spring: leaves can be recovered later in the growing season, but the loss of flowers due to a late spring frost destroys the plant's reproductive capacity for that year. Thresholds in the mechanisms by which individuals respond to environmental conditions, i.e. phenology in response to cold, can lead to ecosystem-level nonlinear responses, particularly when foundation species are already close to environmental tipping points (Williams et al. 2015). Recent evidence suggests that several important ecosystems may soon cross such thresholds (Williams et al. 2015), and thus, it is essential to understand the impact a hybrid could have on an ecosystem if used as a substitute for native species. Consequently, cold tolerance is a particularly important aspect of hybrids to consider for fulfilling a progenitor's niche.

One particularly cold hardy species for which hybrids are being considered is the butternut (*Juglans cinerea* L.), a tree native to the northeastern United States and southeastern Canada (Rink 1990). *Juglans cinerea* is one of few deciduous species that can survive in such northern latitudes and is also the only species in these areas that provides masts of large, energy rich seeds (Schultz 2003, Farrar 2017). With its valuable, rot-resistant, veneer-quality wood, *J. cinerea* is also important economically (Forest Products Laboratory 2010). Unfortunately, its populations have experienced steep declines due to the fungal butternut canker disease [*Ophiognomonia clavigignenti-juglandacearum* (Nair, Kostichka, & Kuntz) Broders & Boland] since it was first reported in 1967 (Morin et al. 2017). *Juglans cinerea* is now classified as "endangered" both by the International Union for Conservation of Nature (Stritch and Barstow 2019) and Canada's Species At Risk Act (Environment Canada 2010), and has various protective designations in 21 states in the United States (NatureServe 2017).

Breeding for butternut canker disease resistance has been considered an important tool in restoring and conserving *J. cinerea* (Michler et al. 2006, Environment Canada 2010). However, no effective and durable disease resistance has been discovered in *J. cinerea* as of yet, which has triggered some to propose the use of hybridization with another species (Michler et al. 2006, Boraks and Broders 2014). While *J. cinerea* is the only species in its range affected by the disease, the only other co-occurring *Juglans* species, black walnut (*Juglans nigra* L.), is incompatible (Rink 1990). However, there is a non-native species, Japanese walnut (*Juglans ailantifolia* Carr.), that can mate with *J. cinerea,* forming *Juglans* × *bixbyi* Rehd. (Rink 1990). An observational study of wild populations of *J. cinerea* with naturalized *J.* × *bixbyi* (Boraks and Broders 2014) and a controlled field study of the two species (Brennan et al. 2020 – Chapter 2), have both indicated increased tolerance to butternut canker disease in *J.* × *bixbyi* compared to *J. cinerea*.

Despite the reported disease tolerance in *J.* × *bixbyi*, these hybrids may not be able to occupy the same niche as *J. cinerea*, particularly in the northern extents of the latter's range, due to a disparity in the naturally occurring temperatures of the progenitors' distributions. *Juglans ailantifolia* rarely experiences temperatures below -20 °C in the coldest area of its native habitat in Hokkaido, Japan (Japan Meteorological Agency 2012, GBIF Secretariat 2019), while *J. cinerea* can survive temperatures as low as -40 °C (Dirr 2009). The relative cold and heat tolerances of *J. cinerea, J. ailantifolia*, and their F1 (*J.* × *bixbyi*) and backcross hybrids were evaluated in chapter 3. Hybrids were found to be intermediate to their progenitors' cold and heat tolerance levels, but closer to *J. cinerea* in cold tolerance. However, the electrolyte leakage methods with excised tissue used to evaluate cold tolerance in this study only allowed for a relative, rather than absolute, comparison of the species (Haase 2011). In addition to assessing damage, a further study using whole plants would also allow for evaluation of survival and phenology. The objective of this study was to understand the cold tolerance, budbreak, and thus potential suitability of *J.* × *bixbyi* as an alternative to planting *J. cinerea,* as well as any cold tolerance variation within *J. cinerea*. It was hypothesized that *J. cinerea* would have greater survival and less damage than *J.* × *bixbyi* and that *J. cinerea* would break bud later than *J.* × *bixbyi*. Within *J. cinerea*, it was hypothesized that individuals from lower (colder) USDA hardiness zones (USDA 2012), would have greater cold tolerance than those from higher (warmer) zones. To address these hypotheses, a whole-plant freeze test was conducted using one-year-old seedlings of the two species.

4.3 Materials and methods

4.3.1 Plant material

Seeds of *J. cinerea* and *J.* × *bixbyi* (F1 hybrids) were obtained from orchards managed by the Hardwood Tree Improvement and Regeneration Center (HTIRC) in West Lafayette, IN and USDA-ARS National Clonal Germplasm Repository (NCGR) in Corvallis, OR in fall 2017. Seeds and seedlings were cared for as described in Brennan and Jacobs (2020; Appendix C). Seeds were stratified in a cooler at approximately 3.8 °C for 120 days, beginning November 2017. In mid-March 2018, prior to planting, seeds were pre-germinated in growth chambers (TC2, Environmental Growth Chambers, Chagrin Falls, OH) to rogue out inviable seeds. Germination began two to three weeks later for a majority of the seeds. Upon germination, seedlings were planted in Metro-Mix 560 (Sun Gro Horticulture Distribution, Inc., Agawam, MA) in TP414 "Tall One" pots (Stuewe & Sons, Inc., Corvallis, OR) and placed in a greenhouse at 24.5/23.3 °C average day/night temperatures. Seedlings were each watered 2-3 times a week and fertilized once a week beginning in May with 365 mL of 16.5N-2.2P-13.5K fertigated water through August. In late November 2018, the seedlings were transferred back to the growth chambers for cold acclimation at 7 °C until the beginning of the experiment (approximately 50 days of acclimation).

Ultimately, 124 *J. cinerea* and 115 *J.* × *bixbyi* one-year-old seedlings were selected for the experiment beginning January 2019. There were eight families within *J. cinerea*, each represented by 15 or 16 seedlings (based on availability). *Juglans cinerea* parent material was originally sourced from provenances in USDA Plant Hardiness Zones 4 (average annual extreme minimum temperature of -34.4 °C) to 6 (average annual extreme minimum temperature of -23.3 °C) (USDA 2012), with at least two families per hardiness zone (Table D.1). Although *J. cinerea* occurs in zones 3-7, only zones 4, 5, and 6 were represented in this study, which represents a majority of the species' population. There were six families of *J.* × *bixbyi*, each with 15-35 seedlings, also sourced from provenances in hardiness zones 4, 5, and 6 (but were not analyzed by zone; see Statistical Analysis) (Table D.1). All seedlings were genetically analyzed to confirm species identity using nuclear satellite marker methods (Hoban et al. 2008).

4.3.2 Whole-plant freeze test

Freeze treatments were applied to the whole seedling from late January to early February 2019 following the methodology of Haase (2011). Each seedling received a single frost cycle. We carried out frost cycles for different target temperatures: -10, -20, -30, and -38 °C. The frost cycles were programmed in freezing chamber with a programmable temperature controller (40-12, ScienTemp, Adrian, MI). Each cycle lasted ~15.5 h: for the first 9 h, starting at 7 ºC, the temperature was dropped until reaching the target temperature, which was held for 2 h, lastly, the temperature was raised again to 7 °C during the last 4.5 h. The cooling rate was different among frost cycles according to the target temperature, but in all cases, it was less than 5 °C h^{-1}, while the warming rates were less than 10 °C h^{-1}. Those rates were selected to avoid bias in frost damage by either rapid freezing or thawing (Lassoie and Hinckley 1991). Frost cycles were applied in groups of 11-12 randomly divided seedlings, except for the -38 °C target temperature where only 5-6 seedlings were used, each as a function of freezer capacity, for a total of 18 treatment batches. In each cold treatment there were 6-13 seedlings per hardiness zone and per cold treatment of *J. cinerea*, and 22-26 seedlings per cold treatment of *J.* × *bixbyi*.

Immediately prior to freeze treatments, height of the stem was measured from cotyledon scar to tip. Then, each seedling's pot was wrapped in recycled denim home insulation (R19 cut to half thickness, UltraTouch, Chandler, AZ) to prevent the soil temperature from dropping below 0 °C. In each frost cycle one in-soil temperature probe (HOBO Data Logger U12-013 with Sensor TMC1-HD, Onset, Bourne, MA) was inserted in a pot of the lower position of the freezer. Soil temperature was -0.08±0.01 ºC in the -38 ºC treatment. A small fan was located inside the freezer to prevent temperature stratification. In each frost cycle three probes were located at lower, middle, and upper levels of the freezer to control air temperatures. In all the cases, minimum temperatures were target temperature ±0.7ºC. Following treatments, seedlings were placed back into the growth chambers at 7 °C. An additional set of 6-12 seedlings per species/hardiness zone of *J. cinerea* and 23 seedlings of *J.* × *bixbyi* were kept in the growth chamber conditions at 7 °C for the entire treatment period as a control group. In late February, all seedlings were subjected to a two-step acclimation process prior to moving them to the greenhouse, consisting of four days at 11 °C with 7 h photoperiod, followed by four days at 16 °C with 8 h photoperiod. Then seedlings were moved back to the greenhouse in March 2019 at 24.5 /23.3°C average day/night temperatures.

4.3.3 Measurements

Seedlings were monitored for 82 days following the initiation of warming conditions, until there was no new budbreak activity on any individual for at least 10 days. Observation of budbreak phenology was evaluated every two days. Stage and date of budbreak was recorded for the most developed bud on each seedling based on the following scale: 0 = no activity, 1 = bud swelling and development of greenish color, 2 = splitting apart of external bud leaves, 3 = initiation of leaf unfurling and expansion with leaves in vertical orientation, and 4 = leaves fully or mostly unfurled and angled out from the main stem (Figure 4.1). At the end of the monitoring period, seedlings were marked as "dead" if there was no visible growth (no bud development or leaves). To further confirm that these individuals were not alive, stems were scraped to ensure that the cambium was black in color. For living individuals, damage was calculated as proportion of dieback of the total stem height with the following equation:

$$Dieback\ Damage = \frac{(Stem\ height\ of\ highest\ bud\ burst) - (Total\ stem\ height)}{(Total\ stem\ hieght)} \times 100 \qquad (\%) \qquad (1)$$

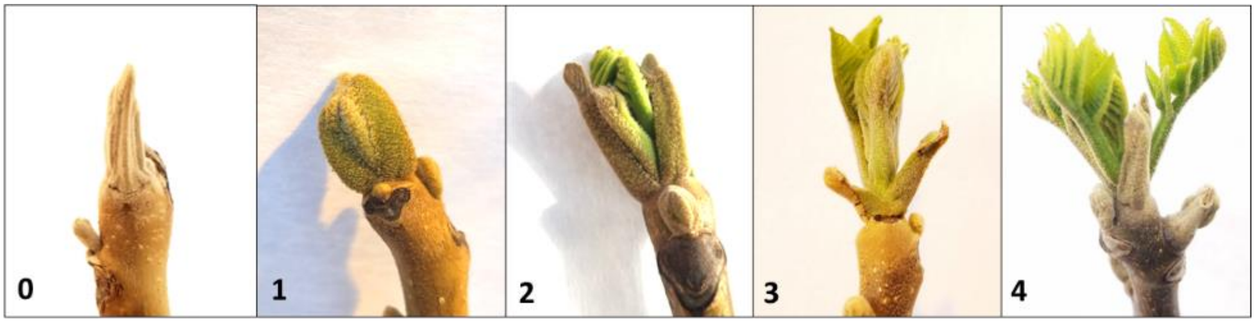

Figure 4.1. Phenological scale used to track budbreak of *Juglans cinerea* and *Juglans* × *bixbyi* seedlings.

4.3.4 Statistical analysis

All data analyses were conducted in R v. 3.4.3 (R Core Team 2019). A logistic regression model was used to analyze survival data using base R. Temperature and species (*J. cinerea* by hardiness zone and *J.* × *bixbyi* as one group) were included as fixed effects of interest. A large enough sample size could not be obtained for each zone to permit analysis of *J.* × *bixbyi* by zone, so they were analyzed together. Seedling height was included as a fixed effect to improve the model and account for its variation. Dieback damage was analyzed with a linear model using generalized least squares with R package 'nlme' (Pinheiro et al. 2019). The dieback damage variable was natural-log-transformed to meet the assumption of normally distributed errors. All predictor variables used for the damage model were the same as for survival analysis. Budbreak was analyzed using a Cox proportional hazards regression mixed effects model using R package 'coxme' (Therneau 2020). Temperature and species were included as fixed effects of interest in the budbreak model, as in the survival and damage models, however, height did not improve the model and was not included. Growth chamber (3-level variable) was also included as a fixed effect for budbreak model improvement but was not of interest. When main effects were significant, Tukey's HSD with R package 'emmeans' (Lenth et al. 2019) was used for post-hoc pairwise contrasts within significant variables ($\alpha = 0.05$).

4.4 Results

4.4.1 Survival

Both temperature (X^2_4 = 20.90, *P* = 0.0003) and species group (X^2_3 = 18.14, *P* = 0.0004) affected survival. No temperature × species group interaction was evident for survival (X^2_{12} = 7.90, *P* = 0.793). At and above -30 °C, seedling survival was above 60% in all species and hardiness zones (Figure 4.2a). Below -30 °C, survival dropped to 25-50% in all *J. cinerea* hardiness zone groups and 88% in *J.* × *bixbyi*. There was no significant difference in survival between *J. cinerea* and *J.* × *bixbyi* at treatment temperatures of -30, -20, -10 or 7 °C. At the -38 °C treatment level, however, *J.* × *bixbyi* exhibited greater survival than *J. cinerea* from hardiness zones 5 (*P* = 0.008) or 6 (*P* = 0.027) but was not statistically different *J. cinerea* from hardiness zone 4. None of the *J. cinerea* groups differed from each other for survival at -38 °C.

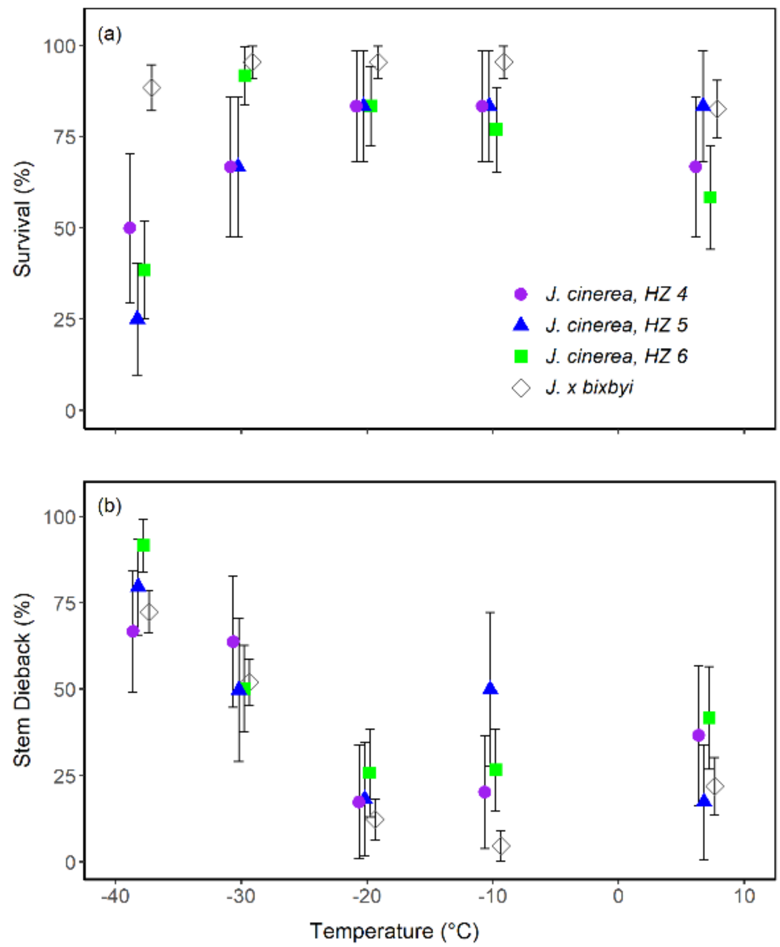

Figure 4.2. (a) Survival and (b) stem dieback damage of seedlings of *Juglans cinerea* from different USDA hardiness zones (HZ) and its hybrid, *Juglans* × *bixbyi*, after exposure to five different cold treatments. Data are means and SE.

4.4.2 Stem dieback damage

Temperature affected stem dieback damage (X^2_4 = 147.791, P < 0.0001), but species group did not (X^2_3 = 0.355, P = 0.950). No temperature × species group interaction was evident for stem dieback (X^2_{12} = 11.809, P = 0.461). Dieback damage induced by the -30 °C treatment was 50% or greater for all species and groups, increasing to over 70% at -38 °C (Figure 4.2).

4.4.3 Budbreak phenology

Temperature, species group, and their interaction affected the number of days for seedlings to reach each budbreak stage (Table 4.1, Figure 4.3, Figure D.1). Budbreak was most rapid and uniform among seedlings treated at -20 °C. Above and below that temperature, fewer seedlings were able to break bud and fewer seedlings were able to finish the cycle and reach stage 4. Seedlings of *J.* × *bixbyi* had more rapid budbreak than did seedlings of *J. cinerea* of all hardiness zones; this result was observed across all phenological stages and temperatures. For example, *J.* × *bixbyi* initiated bud break (stage 1) of up to an average of 20 days earlier than *J. cinerea*, depending on the hardiness zones and treatment groups being compared. *Juglans cinerea* from coldest areas (hardiness zone 4) showed a higher percentage of seedlings able to break bud to completion (reach stage 4) at the coldest temperature treatment (-38 °C) than did those at higher temperature hardiness zones. Conversely, the rate of budbreak of *J. cinerea* from zone 4 was most delayed at the control treatment (7 °C).

Table 4.1. Effect of species group, temperature, and their interaction on the number of days to reach four consecutive stages of budbreak on seedlings of *Juglans cinerea* and its hybrid, *Juglans* × *bixbyi*, after exposure to five different cold treatments.

	Species			Temperature			Species × Temperature		
Stage	X^2	DF	*P*-value	X^2	DF	*P*-value	X^2	DF	*P*-value
1	44.19	3	< 0.0001	7.89	1	0.005	9.82	3	0.021
2	45.65	3	< 0.0001	7.87	1	0.005	10.46	3	0.015
3	50.09	3	< 0.0001	4.54	1	0.033	10.96	3	0.012
4	46.29	3	< 0.0001	5.16	1	0.023	11.46	3	0.009

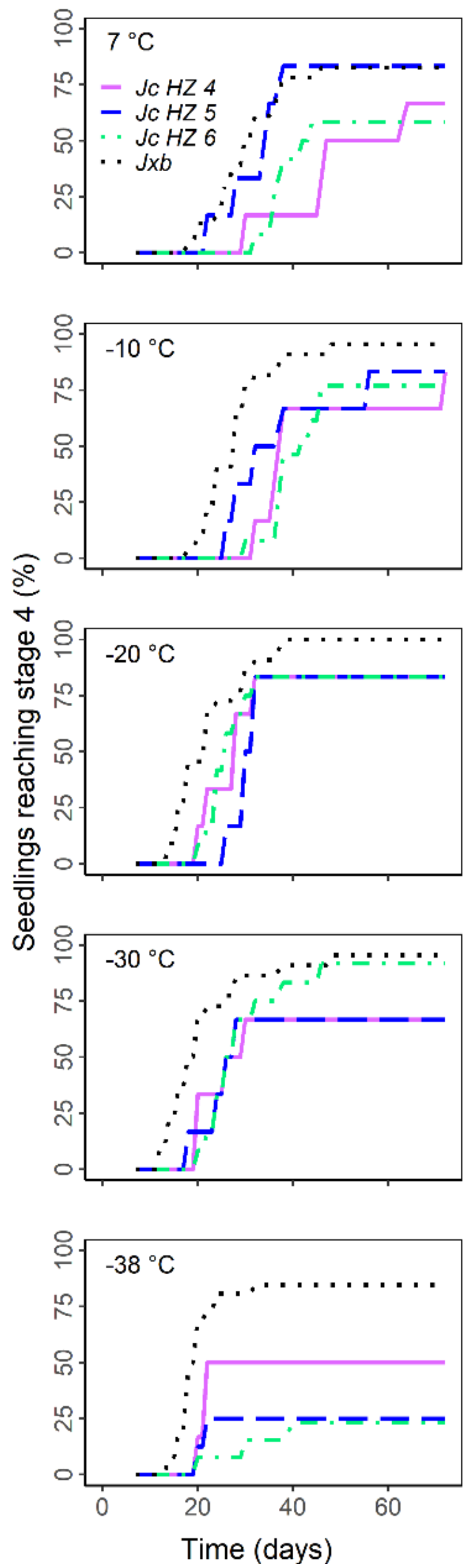

Figure 4.3. Stage 4 bud break of seedlings of *Juglans cinerea* (*Jc*) from different USDA hardiness zones (HZ) and its hybrid, *Juglans* × *bixbyi* (*Jxb*), after exposure to five cold treatments.

4.5 Discussion

4.5.1 Effect of temperature

Survival and damage did not exceed the LT_{50} for any species or group, except at the coldest treatment of -38 °C, and even then, none of the species group averages dropped below 25% survival (Figure 4.2), demonstrating the extreme cold tolerances of both *J. cinerea* and *J.* × *bixbyi*. This is consistent with red pine (*Pinus resinosa* Ait.), another exceptionally cold tolerant species, which did not even surpass 35% EL at -40 °C (Islam et al. 2009). Budbreak was most uniform and rapid when seedlings were exposed to the -20 °C treatment, and was most delayed at the control temperature (7 °C) (Figure 4.3). This outcome is consistent with observations in other species, where warmer winter temperatures led to delayed budbreak in red alder (*Alnus rubra* Bong.; Prevéy and Harrington 2018) and almond (*Prunus dulcis* (Mill.) D.A. Webb; Benmoussa et al. 2017). Studies evaluating the effects of increased warming in autumn and winter due to climate change on budbreak reported similar results in a wide variety of other forest tree species (Heide 2003, Lange et al. 2016). These studies have concluded that delayed budbreak due to warmer temperatures in autumn and winter can counteract potential early budbreak from early warm spring temperatures also associated with climate change. Thus, *J. cinerea* and *J.* × *bixbyi* may not ultimately experience drastically altered phenologies due to climate change, because of the counterbalancing effects of warmer autumns and winters *versus* early springs.

4.5.2 Provenance differences

There was no difference in survival or damage within *J. cinerea* from any hardiness zones (Figure 4.2). This is in contrast to the results of our previous study involving the species (Chapter 3), which found that stem segments from hardiness zones 4 and 5 experienced lower electrolyte leakage than those from zones 6 and 7 following freeze treatments. However, the provenance differences in that study, while significant, were small, differing by up to only 4% for the maximum damage at the lowest temperature tested. The contrasting results from these two studies could also be due to the greater and wider variety of genotypes included in Chapter 3 compared to the current study. Differences in the type of material being tested (whole plant versus excised tissue), age of material (seedlings versus mature trees), and type of cold acclimation (growth chamber versus field) could further explain differences (Neuner et al. 1997, Gusta et al. 2009).

While there were no differences in survival or damage among provenances of *J. cinerea*, differences were detected when budbreak was used as an indicator of cold hardiness (Table 4.1). Delayed budbreak is a strategy for avoidance of damage from late spring frosts, especially in colder climates (Vitasse et al. 2014, Muffler et al. 2016). Seedlings from the coldest area, hardiness zone 4, were most able to reach budbreak stage 4 at -38 °C compared to those from the two warmer zones (5 and 6). Conversely, at the two warmest temperatures (-10 and 7 °C), seedlings from hardiness zone 4 experienced the most delayed budbreak Figure 4.3). Spring phenology has also been shown to vary by provenance in numerous species, including Persian walnut (*Juglans regia* L.; Hemery et al. 2005), pecan (*Carya illinoinensis* (Wangenh.) K.Koch; Wood et al. 1998), and European silver fir (*Abies alba* Mill.; Mihai et al. 2018). This underscores the importance of choosing locally adapted planting material, particularly for restoration sites (Bischoff et al. 2008).

Although we were unable to analyze the timing of budbreak within hardiness zones of naturally occurring *J.* × *bixbyi* within *J. cinerea*'s range in North America, it is important to note that there was great variability in performance between different families (half-siblings) of this hybrid species. The genetic background of *J. ailantifolia* in North America is largely unknown, and this presents difficulty in accounting for its naturalized hybrids. Nevertheless, it is likely that such hybrids have contributed to the observed large variability among families. This phenomenon has also been reported in ecophysiological performance comparisons in other tree species and their hybrids, such as oak (*Quercus* L.; Himrane et al. 2004) and chestnut (*Castanea* Mill.; Pinchot et al. 2017). Given this variability, pre-screening of the cold tolerance of different hybrid families is encouraged to help gain a better understanding of their performance.

4.5.3 Species differences

Survival of *J.* × *bixbyi* was significantly higher than *J. cinerea* from hardiness zones 5 and 6, but not different from hardiness zone 4, the coldest zone. However, there was no difference in dieback damage among any of the species or zones (Figure 4.2). These results contrast with those of chapter 3, which found that the hybrids experienced greater damage than *J. cinerea*. However, it should be noted that in the aforementioned study, both hybrid types exhibited cold hardiness much closer to their cold hardiest progenitor, *J. cinerea,* than to *J. ailantifolia*. This suggests the possible dominance of cold hardiness genes, which would be consistent with the findings of the current study. In other ecophysiological traits, *J.* × *bixbyi* seedlings have been reported to be intermediate to both parent species in terms of both flood and drought tolerance (Crystal and Jacobs 2014). Hybrids of spruce (*Picea* Mill.; De La Torre et al. 2014) and willow (*Salix* L.; Fritz et al. 2006) were also reported as being intermediate to their progenitors. Given the dearth of research evaluating hybrids of tree species, only one study could be found with results consistent with the current study, where the hybrid pine species *Pinus densata* Mast. exhibited heterosis (hybrid vigor; Allendorf et al. 2013) and surpassed the physiological performance of both progenitors, *Pinus tabuliformis* Carr. and *Pinus yunnanensis* Franch. (Gao et al. 2009). To best fill the native ecological niche of *J. cinerea*, *J.* × *bixbyi* would ideally match closely the characteristics of its native progenitor. A strong increase in cold tolerance could impart a survival advantage for *J.* × *bixbyi*, thereby potentially disrupting the natural balance of the ecosystem (Burgess and Husband 2006). However, *J.* × *bixbyi* had greater survival than *J. cinerea* only from warmer provenances (5 and 6) and only at the lowest temperature treatment of -38 °C. Given that the average annual extreme minimum temperatures of zone 5 only reach as low as -29 °C (USDA 2012), it is extremely unlikely that temperatures as low as -38 °C would occur in those provenances. Further, there was no difference in survival at the warmer treatment temperatures more indicative of the naturally occurring temperatures in that region. Thus, in terms of survival, it is not anticipated that hybrids would have a cold tolerance advantage if planted in the warmer parts of *J. cinerea*'s distribution.

While there was no differences in dieback damage and small differences in survival between *J.* × *bixbyi* and *J. cinerea*, large phenological differences were observed, with the hybrid species consistently breaking bud earlier than its native progenitor (Table 4.1, Figure 4.3). Early budbreak is a trait associated with invasive plants, and thus, *J.* × *bixbyi* could have a distinct advantage over its native progenitor species - and perhaps in the ecosystem as a whole - through an extended growing season, particularly with the advent of climate change (Wolkovich et al. 2013, Wolkovich and Cleland 2014). Conversely, earlier budbreak could negatively affect the success of *J.* × *bixbyi* in that delayed budbreak is a strategy to prevent cold damage through avoidance of injury from late spring frosts (Vitasse et al. 2014). Because the flowers of *Juglans* species emerge with the leaves at bud break in the spring (Rink 1990), the flowers of *J.* × *bixbyi* could be especially at risk from late spring frosts. The leaves can regrow in the same year at minimal cost to the plant, but loss of the flowers in *Juglans* equates to loss of reproductive ability, and thus seed crop, for the entire year. Further, the more rapid budbreak of *J.* × *bixbyi* could become an increasingly important consideration over time, as increasing occurrences of late spring frosts have been reported to be associated with climate change (Augspurger 2013, Muffler et al. 2016). Earlier budbreak in *J.* × *bixbyi* is also a concern in terms of its ability to fill the ecological niche that *J. cinerea* occupies, as the life cycles of native fauna are often synchronized to the phenology of the native flora in their habitat for feeding, pollination, and seed dispersal - processes which could be disrupted if phenological asynchrony occurs (Rathcke and Lacey 1985, Freitas and Bolmgren 2008). Going forward, future research should evaluate whether a disadvantage though greater vulnerability to late spring frosts, or an advantage through an extended growing season will be the more likely scenario - or whether these two points will ultimately counterbalance each over an extended period of time. Further, a full determination of the suitability of *J.* × *bixbyi* as a replacement for its *J. cinerea* progenitor must also include evaluation and comparison of other traits, both ecological (morphology, reproductive ability, etc.) and economic (growth form, wood quality, etc.).

4.6 Conclusion

While differences within and among species were observed, no species or hardiness zone group exceeded the LT_{50} until the coldest treatment of -38 °C, demonstrating the extreme cold tolerance of both *J. cinerea* and its hybrid, *J.* × *bixbyi*. Budbreak phenology was most uniform at the -20 °C treatment, rather than at the two warmest treatments (-10 and 7 °C), which may indicate the importance of low, non-lethal temperatures for these species. No survival or stem dieback differences were detected between provenances of *J. cinerea*. However, *J. cinerea* seedlings from the coldest provenances (hardiness zone 4) experienced more delayed budbreak at the two warmest treatments (-10 and 7 °C) than those from warmer provenances (hardiness zones 5 and 6), further supporting the importance of cumulative freezing days to reach optimum performance. Seedlings of *J.* × *bixbyi* and *J. cinerea* had similar dieback and survival, although in some cases *J.* × *bixbyi* surpassed the survival of *J. cinerea*. At the lowest treatment temperature (-38 °C) only, *J.* × *bixbyi* had greater survival than *J. cinerea* from warmer provenances (hardiness zones 5 and 6). Given that temperatures that low are extremely unlikely to occur in those areas, though, it is not anticipated that hybrids would have a survival advantage if planted there. Conversely, the consistently earlier budbreak of *J.* × *bixbyi* could cause the species to be more vulnerable to spring frosts and out-of-sync with the surrounding ecosystem. Consequently, this could limit restoration success using these hybrids and calls into question their ability to serve as an adequate substitute for *J. cinerea*.

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CHAPTER 5. PERCEPTIONS OF LAND MANAGERS TOWARDS USING HYBRID AND GENETICALLY MODIFIED TREES

5.1 Abstract

Increased stress from global changes are impacting tree growth, productivity, and survival. As a result, many native tree species are at-risk of extinction. Hybridization and genetic modification are two possible methods for incorporating stress tolerance and are currently being explored for some at-risk tree species. However, many concerns, both ecological and economic, have been identified regarding the use of these biotechnologies. There is limited information on perceptions of hybrid and genetically modified (GM) trees, and even less information about what those responsible for widescale tree management think about using these biotechnologies. An online survey was administered to land managers in 46 organizations in Indiana, USA to gauge perceptions of hybrid and GM trees, as well as current use of hybrid trees. Land managers had stronger concern for ecological, rather than economic, issues, with "potential for invasiveness" being strongest. Agreement was highest for using hybrid and GM trees for "conservation and restoration of at-risk species", "timber production", and "non-timber products (fruit, syrup, etc.)" and lowest for "promoting biodiversity" and "reforestation and restoration of forests". However, land managers are not a uniform group, and perceptions varied by several characteristics, such as concern type, age, and the type of land they managed. Ecological concern and the type of land being managed most strongly predicted current hybrid use. Overall, our results indicate that if/when using hybrid or GM trees is deemed the appropriate choice for at-risk species restoration, a majority of land managers in Indiana would likely be agreeable to recommendations about using them. However, it was also made clear throughout the study that despite indicating agreement towards using hybrid and GM trees, most respondents still had strong ecological concerns about their suitability as a native species replacement. In order to alleviate these concerns, it will be essential that hybrids and GM trees be thoroughly vetted, with the results clearly communicated. Additionally, active engagement with land managers will be critical, as this is a population with a clear stake in the issue, and one that would ultimately be responsible for any widescale implementation of hybrid and GM trees.

5.2 Introduction

Global changes, such as climate change, habitat loss, and invasive organisms, are impacting tree growth, productivity, and survival (Parker and Gilbert 2004, Niu et al. 2014). Many native tree species are having trouble coping with these changes and are at-risk of extinction, a loss both ecologically and economically (Thomas et al. 2004, Bellard et al. 2016). Some species hold increased tolerance to certain stressors resulting from global change, and incorporation of the genes responsible for this tolerance could be an option in supporting at-risk species. One method of incorporation is through hybridization, the crossing of two different species (Allendorf et al. 2013). Breeding through hybridization with non-native species is currently being evaluated to aid in restoration of several tree species, such as the American chestnut (*Castanea dentata* (Marsh.) Borkh.; Clark et al., 2019), butternut (*Juglans cinerea* L.; Brennan et al. 2020 – Chapter 2), and American elm (*Ulmus americana* L.; Martín et al., 2019). A second method is genetic modification, or genetic engineering, defined here as adding, removing, or changing specific genes through biotechnology (FAO 2010). This approach has been explored much less often than hybridization for at-risk tree species restoration, but a well-known example is American chestnut (Jacobs et al. 2013). In addition to incorporation of stress-tolerance genes to support at-risk species, potential advantages of hybridization and genetic modification include accelerating the tree improvement process, improving tree growth and production, and helping solve problems with the food supply (Fernandez-Cornejo et al. 1999, Harfouche et al. 2012). However, many concerns, both ecological and economic, have also been associated with the use of both biotechnologies. These include the potential for invasiveness, genetic swamping (when genetic material transfers from one species to another through hybrids and overtime results in one species dominating over the other), high cost, and lack of performance knowledge (Vila et al. 2000, Allendorf et al. 2013, Tsatsakis et al. 2017, NASEM 2019).

As efforts continue to research, evaluate, and possibly implement widescale use of hybridized and genetically modified (GM) trees, it is crucial to concurrently understand societal perceptions to their use. Whether or not these types of trees are supported by scientific consensus as both safe and effective, the success of restoration efforts using hybrid and GM trees will be strongly dependent on land managers' and public perceptions and acceptance to their use (Hall 2007, Jacobs et al. 2013, Martín et al. 2019). Studies of perceptions towards genetic modification have been primarily focused on agronomic crops and food and found to be complex, divided, and often negative (Hallman et al. 2003, Costa-Font et al. 2008, Frewer et al. 2013). However, there is limited information on perceptions of GM trees, or of hybridization in general, which may differ due to the nature of the plant types and intended use. While some trees are used for food production like agronomic crops, trees are more widely used for various non-food consumption purposes. These include timber production, green infrastructure, wildlife habitat, restoration, and recreation, which could be considered more acceptable uses of hybridization and genetic modification than food production (Merkle et al. 2007, Gamborg and Sandøe 2010). Additionally, with regards to species restoration and conservation, the spread and proliferation of pest and disease resistance genes in trees to wild populations is seen by some stakeholders as not only positive, but a potential goal (Merkle et al. 2007, Strauss et al. 2009). Conversely, given that trees are generally able to spread their pollen and seed further than other types of plants, cultivated hybrid and GM trees in plantation settings may mate and spread into nearby native populations more effectively than agronomic crops, raising environmental concerns towards the safety of nearby ecosystems (Williams 2005, Hall 2007). Further, trees are much longer-lived than most agronomic food crop plants, which are typically annuals, and the long-term ecological implications of hybrid and GM trees could mean greater hesitancy and decreased acceptance to their use (Williams 2005, Hall 2007, Merkle et al. 2007, Gamborg and Sandøe 2010).

Jepson and Arakelyan (2017a) compared perceptions of GM native trees to those of agricultural food crops among informed (knowledgeable) members of the public in the UK. They found that while there was a strong correlation between acceptability of both types, there was stronger approval for GM trees planted in both natural woodland and forestry plantation settings than for GM food crops. However, in a second study by Jepson and Arakelyan (2017b) of the general public, the majority of study participants considered GM trees and food crops equally acceptable or unacceptable. Taken together, these two studies indicate that perceptions to the use of GM trees versus agronomic crops may vary by population.

More specifically for tree-related perceptions, Hajjar et al. (2014) evaluated acceptance of six different forest management strategies in response to climate change by the general public and community leaders in western Canada. In general, the researchers found decreasing acceptance by both the public and community leaders of strategies that involved more manipulation. However, while the strategy of using GM trees had the second lowest level of acceptance, it was still higher than the strategy of no intervention at all. This aligns with the findings of Jepson and Arakelyan

(2017a, 2017b), which presented a range of possible solutions to ash dieback in the UK, including the use of GM native ash (*Fraxinus* L. species) trees with increased disease tolerance. While the use of GM native trees was generally ranked low compared to more conventional planting and breeding strategies, respondents still preferred the use of GM trees over a strategy of no action. Acceptance towards the use of hybrids of native and non-native ash trees was also evaluated in these studies. The hybrid strategy was viewed more favorably than the use of GM trees, but not as highly as conventional breeding and re-planting methods. These studies have begun the process of unravelling the complex perceptions towards hybrid and GM tree use, however, more remains to be uncovered about the specific concerns or perceived benefits driving these perceptions.

It is also essential to investigate perceptions towards biotechnologies among specific populations, beyond the 'general public', as this approach can be too simplistic, limiting the ability to understand the complex motivations of different segments of the population (Fischhoff and Fischhoff 2001). As illustrated previously, there is currently a small body of literature available on the perceptions of the public and community leaders towards the use of biotechnology (Hajjar et al. 2014, Hajjar and Kozak 2015, Jepson and Arakelyan 2017a, 2017b, St-Laurent et al. 2018), however, information about perceptions of land managers, the people responsible for tree management, is very limited. Land managers are typically considered in regards to forestry and management of woodlands or tree plantations (Jepson and Arakelyan 2017a). Here, however, we consider land managers more broadly to also include those working with trees in urban areas, such as horticulturists, landscape architects, and urban foresters. Land managers not only work on public lands, but are often consulted for management of trees on private lands, further extending their impact. As an example, family forest owners collectively control 36% of U.S. forestlands (Butler et al. 2016). Generally, these landowners are not very active in engaging in traditional forestry activities (Butler et al. 2016), but when they do take such management actions, a significant number engage with professionals to help plan and execute their tree or land management decisions (Kilgore et al. 2015). Thus, land managers have a strong influence on what trees get planted and where, resulting in a significant impact on both our urban and natural landscapes. This makes it essential to understand not only their perceptions to hybrid and GM trees, but also how those perceptions translate to actual use (here, in the case of hybrid trees only, given current legal restrictions to GM tree use).

The overall goal of this exploratory research was to gauge land manager perceptions to the use of hybrid and GM trees, as well as current use of hybrid trees. Thus, our research questions were as follows:

1. What are the perceptions of land managers to using hybrid and GM trees?
2. What factors and characteristics of land managers are associated with their perceptions of hybrid and GM trees?
3. What factors and characteristics determine current use of hybrid and at-risk tree species among land managers?

The information generated from this study is meant to aid scientists in designing research to address these issues and in promoting and disseminating research results in a way that land managers find relevant in making their tree selection decisions.

5.3 Methods

5.3.1 Study population and recruitment

For the purposes of this study, land managers were defined as professionals and/or volunteers involved with tree selection, sale/distribution, management, and/or planting of trees in Indiana, USA. If an individual did not mark themselves as being involved with one or more of these tree work activities, or did not perform the activities in Indiana, then that individual was excluded from the study. As discussed previously, land managers are a wide and diverse group involved with multiple land and organization types, and are not accessible by any single, or even a few, venues. This makes developing a comprehensive sampling frame challenging. In order to reach as many land managers operating in Indiana as possible, participants were recruited through different professional and governmental organizations and groups. Participants were recruited directly by the researchers by assembling a list of publicly available email addresses for Indiana governmental organizations, district foresters, consulting foresters, land trusts, and other non-profit land management groups. The membership of many of the large organizations and professional societies were not publicly available online, but still constitute an important portion of land managers in Indiana. In order to reach these individuals, the leadership of these organizations distributed our survey to their membership on our behalf or gave us permission and access to contact them directly. For a complete list of the 46 organizations and professional groups reached by the survey invitation see Appendix E.

5.3.2 Data sources

Data for this study was collected using a mixed methods approach in two phases, interviews and survey. Prior to their implementation, the interview and survey recruiting emails, interview protocol, and survey questionnaire were approved by Purdue University's Institutional Review Board (protocol no. 1805020620). The first phase was qualitative and involved nine semi-structured in-person and phone interviews with Indiana land managers. These interviews were conducted in order to gain a preliminary understanding of the population and inform development of the subsequent survey. Initial interview participants were selected by identifying key and influential land managers during the process of assembling the sampling frame. Later interviewees were identified through recommendations made during the earliest of the interviews. All interviewees were recruited through an email invitation. Interviews lasted 45-120 minutes, depending on the interviewee, and involved questions about their work and experience with hybrid, GM, and at-risk plants.

The second phase of the study was quantitative and involved a survey as the primary method of capturing data. An online survey was created using Qualtrics and was distributed following the Tailored Design Method (Dillman et al. 2014). All participants able to be recruited directly received an initial email invitation and three waves of email reminders. Survey invitations distributed through organizational leadership took the form of an email invitation, an article in the organization's e-newsletter, and/or a post on the organization's Facebook page. Individuals contacted this way received either one or no reminders, depending on the discretion of the organization's leadership.

The survey was composed of 26 multiple choice and Likert scale questions divided into five sections: 1) work/volunteer background, 2) hybrid plants, 3) GM plants, 4) at-risk plants, and 5) demographic characteristics of the respondents. Section 1 included questions about the land manager's organization type, time working in the organization, land type managed, client type, and primary work purpose. Questions in sections 2 (hybrids trees) and 3 (GM trees) explored perceived ecological concerns, economic concerns, and production benefits. Section 2 also asked about current use of hybrid trees (GM tree use was not surveyed given legal restrictions to their use), as well as the participant's preferred hybrid type for restoration. Section 3 also included a question comparing perceptions on whether a GM version of a native species is still native, and a question comparing level of concern towards using hybrid versus GM trees. The fourth section asked about current use of at-risk tree species, and asked participants to express their agreement for using hybrid and GM trees for restoration and conservation, as compared to several other possible purposes. The fifth and final section asked basic demographic questions about age, gender, education, and county of residence. Since some of the issues covered in the survey may be less familiar to certain types of land managers, definitions were provided at the beginning of the survey and again as needed throughout the survey as in Table 5.1. The complete survey protocol is available in Appendix F.

Table 5.1. Definitions of concepts used in the survey

Concept	Definition
Hybrid	Offspring resulting from the cross of two *different species* (Allendorf et al. 2013)
Genetic modification	Adding, removing, or changing *specific* genes through biotechnology (Walter and Menzies 2010)
At-risk species	A species at risk of being lost from the landscape, due to threats such as exotic pests or pathogens, climate change, and habitat loss (USGS 2020)
Genetic swamping	Excessive introgression; when genetic material transfers from one species to another over time through hybrids and results in one species dominating over the other (ex: Chinese bittersweet over American bittersweet growing in the US) (Todesco et al. 2016)
Non-native species	Not native to the region prior to European settlement (NRCS 2020)

The survey was live and available February through April 2019. After removing responses from individuals who did not mark themselves as Indiana land managers through the initial screening question and those that did not complete any of the perceptions or use questions, we ultimately received 273 survey responses from Indiana land managers. We attempted to reach every land manager in the state of Indiana and in doing so, we often relied on organizational leadership to disseminate the survey invitation to their membership. In these cases, it was not possible to control the precise timing of when certain groups received the invitation, and if or when they received follow-up reminders. Thus, some participants received their second or third reminders, while at the same time, others were receiving the survey invitation for the first time. As such, a typical non-response bias check comparing early responses to later responses was not appropriate. Instead, we evaluated the responses for each land manager subgroup (characteristic) to ensure that each category of the different types of land managers was well represented. There was no comprehensive sampling frame available to work with, so experts in the field were also consulted to assure that the demographics seen in the survey matched with their experience with the population.

5.3.3 Data analysis

All statistical analysis was conducted using R version 3.6.2 (R Core Team 2019). Very little is known about the land manager population in Indiana, so this study was primarily exploratory in nature. As such, univariate descriptive statistics are used exclusively for quantifying perceptions (i.e., first research question). To assist with interpretation of data for the second and third research questions, dimensionality of variables related to ecological and economic concerns, tree improvement advantages, and purpose of use were reduced using a summated scale (Spector 1992), creating new composite variables (Table 5.2). Tree improvement advantage, ecological concern, and economic concern sub-variables were summated according to how they were grouped and presented in the survey protocol. The new composite variables created for purposes of use were grouped by creating a new restoration purpose variable, in addition to four others (provisioning, supporting, regulating, and cultural purposes) using the Ecosystems Services Framework as a guide (Millennium Ecosystem Assessment 2005). Correlations and Cronbach's Alpha (Cronbach 1951) were used to verify the internal consistency of each grouping. Due to the high number of individuals responding "Don't know" for the concern variables, the responses were converted to binary (1 = presence of concern, 0 = all else) before creating the summated scale for use in the cumulative link models. The new composite economic concern variable for both hybrid and GM trees was close but did not meet the suggested minimum of 0.7 for Cronbach's Alpha (Nunnally 1978), so caution is needed in interpretation involving these variables.

Table 5.2. Description of new, composite variables of level of concern or dis-/agreement regarding perceptions associated with hybrid and GM trees created to reduce dimensionality using a summated scale. The original variables averaged to create the composite variables are indented below each new variable.

Variable	Cronbach's Alpha Hybrid trees	Cronbach's Alpha GM trees
Strongly disagree to strongly agree		
Tree improvement advantages	0.86	0.92
-Accelerating the tree improvement process		
-Improved tree growth and production		
-Better resource-use efficiency		
-Greater site suitability and functionality		
-Greater stress and pest resistance		
-Decreased need for pesticide applications		
-Helping solve problems with the food supply		
Restoration purposes	0.84	0.86
-Reforestation and restoration of forests		
-Conservation and restoration of at-risk species (plant or animal)		
Supporting purposes	0.84	0.88
-Promoting wildlife/habitat creation		
-Promoting biodiversity		
Provisioning purposes	0.83	0.81
-Timber production		
-Non-timber production (fruit, nuts, syrup, etc.)		
Regulating purposes	0.85	0.87
-Regulating ecosystem services (storm water management, erosion control, carbon storage, clean air, etc.)		
-Green infrastructure and managed landscapes		
Cultural purposes	0.89	0.93
-Aesthetic value		
-Recreation		
Not concerned to very concerned		
Ecological concerns	0.72	0.83
-Changes induced in local ecosystem		
-Genetic swamping of native population		
-Potential for invasiveness		
-Negative effects on wildlife		
Economic concerns	0.62	0.65
-Low availability		
-Expensive to buy and produce		
-Lack of performance knowledge		

Responses regarding concern levels, hybrid type preference, whether a GM tree is native, and the concern comparison for hybrid versus GM trees were analyzed using Pearson's Chi-Square Test in base R to address the second research question about characteristics of land managers associated with perceptions. When any assumptions of Pearson's Chi-Square Test were violated, Fisher's Exact Test was conducted instead, using base R. To further address the second research question, perceptions of tree improvement advantages and purposes of use were analyzed using cumulative link models (also called ordinal regression or proportional odds models) with R package 'ordinal' (Christensen 2019), in conjunction with 'car' (Fox and Weisberg 2019), and 'RVAideMemoire' (Hervé 2020) to determine the likelihood ratio chi-square and *p*-value of variable effects. The analyses of land manager characteristics associated with current use of hybrid and at-risk tree species was conducted by calculating average marginal effects in logistic regression using R package 'mfx', which allows for the use of ordinal explanatory variables (Fernihough and Henningsen 2019).

5.4 Results

5.4.1 Profile of respondents

Land manager respondents ranged from 23-78 years in age, with a mean of 47 years old Table 5.3). Seventy-nine percent of respondents were male, with 86% having a bachelor's or graduate degree. Respondents' county of residency was nearly evenly split between northern (49%) and southern counties (51%) of Indiana. Respondents had spent an average of 14 years in their current organization, with a range of 1-47 years. Sixty-one percent of respondents managed natural lands for their tree management work. The predominant client types identified by survey respondents were general public (65%) and private landowners (60%). The most common primary work purposes (ranked as number one) were timber production (18%), promoting wildlife habitat/creation (17%), and restoration and reforestation (15%). Extended information about the work purposes of respondents is included in Table G.1.

Table 5.3. Demographic and land manager characteristics of survey respondents.

Characteristics (unit if applicable)	Type of variable (categorical or continuous)	% or mean (SD)	n
Age (years)	23-40	36.8%	250
	41-59	37.6%	
	60-78	25.6%	
Gender	Male	79.1%	249
Education	High school degree or equivalent	2.0%	253
	Some college	5.5%	
	Associate's degree	6.3%	
	Bachelor's degrees	57.7%	
	Graduate degree	28.5%	
Residency (region of Indiana)	Northern half	49.0%	251
Organization type	Federal government	2.2%	270
	State government	30.0%	
	Local government	11.9%	
	Non-profit	20.7%	
	For-profit	30.0%	
	University/educational	5.2%	
Time in organization (years)	Continuous (range: 1-47)	14.36 (11.98)	271
Land manager type	Natural lands	60.7%	272
	Urban lands	23.2%	
	Both natural and urban lands about equally	16.2%	
Primary purpose of work (ranked as #1)	Timber production	18.3%	273
	Non-timber production	0.7%	
	Aesthetic	9.9%	
	Recreation	5.1%	
	Promoting wildlife/habitat creation	16.8%	
	Restoration and reforestation	14.7%	
	Conservation of at-risk species and communities	3.3%	
	Regulating ecosystem services	5.1%	
	Promoting biodiversity	5.5%	
	Green infrastructure and managed landscapes	8.1%	
	Communicating and providing advice on tree/forest management policies and programs	10.3%	
	Nursery production	2.2%	
Client types	General public	64.1%	270
	Homeowners	31.9%	
	Private landowners	60.0%	
	Green industry	14.1%	
	Other land managers	31.5%	
	Government	43.0%	
	Institutions and businesses	3.7%	

5.4.2 *Overall concerns about hybrid vs. GM trees*

We asked land managers how they think about using hybrid compared to GM trees in a hypothetical sense. The majority of survey respondents reported having similar levels of concerns with the use of both hybrid and GM trees (43%). Thirty-five percent reported having more concerns about GM than hybrid trees, while 11% reported having more concerns about hybrid than GM trees. Ten percent respondents reported "don't know". Of all the land manager characteristics, age and education were the only two variables statistically significantly associated with respondents' relative concern of hybrid versus GM trees. Specifically, younger respondents (23-40 years old) reported having more concerns about hybrid trees, while those in the older age groups (41-59 and 60-78 years old) reported having more concerns about GM trees ($\chi^2 = 13.92$; $p = 0.030$). Those with more education (bachelor's or graduate degree) were more likely to report having similar levels of concerns with the use of both hybrid and GM trees, while those with less education (high school degree, some college, or an associate's degree) were more likely to report having more concerns about one than the other ($\chi^2 = 14.025$; $p = 0.029$). Respondents' relative concern of hybrid versus GM trees was also associated with their level of ecological concern. Specifically, those having less ecological concern about GM trees were also less concerned about GM trees in general when compared to hybrid trees (Fisher's exact $p = 0.0004$). All other variable relationships were non-significant. For the full statistics of association of land manager characteristics with this concern comparison see Table G.2.

5.4.3 *Hybrid tree perceptions and current use*

Perceived hybrid tree improvement advantages

Generally, there was more agreement than disagreement regarding the tree improvement advantages of hybrid trees (Figure 5.1a). Respondents had the greatest agreement (agree or strongly agree) with using hybrid trees for "greater stress and pest resistance" (70%) and "improved tree growth and production" (62%). In contrast, respondents had the least agreement (strongly disagree or disagree) with using hybrid trees for "greater site suitability and functionality" (19%) and "better resource-use efficiency" (19%). It should be noted, however, that disagreement was similarly low across all advantage variables. Those working in for-profit organizations were more likely to agree with tree improvement advantages of hybrid trees than those in state government (χ^2 = 14.706; p = 0.012), but age, gender, education, region of residency in Indiana, and land type managed were not associated with agreement regarding tree improvement advantages of hybrid trees (see Table 5.4 for full statistical values).

Concerns about hybrid trees

Ecological concerns about hybrid trees (potential for invasiveness, negative effects on wildlife, genetic swamping, and ecosystem changes) were consistently stronger than economic concerns (performance, expense, and availability) (Figure 5.1b). The greatest ecological concern about hybrid trees was the potential for invasiveness (68% strongly concerned), while negative effects of wildlife was the least important ecological concern (i.e., 15% of respondents chose "not concerned"). In contrast, the greatest economic concern was the lack of performance knowledge (24% strongly concerned), while low availability was the least important economic concern (i.e., 61% of respondents chose "not concerned").

Of various land manager characteristics and perceptions (see Table 5.2 for the list of variables), their level of agreement with tree improvement advantages was the only variable significantly associated both with ecological and economic concerns, but the direction of the relationship depended on the type of concern (Table 5.4). A higher level of ecological concerns was associated with less agreement with hybrid trees having tree improvement advantages (χ^2 = 8.693; p = 0.003). Conversely, a higher level of economic concerns was associated with greater agreement with hybrid trees having tree improvement advantages (χ^2 = 27.640; p < 0.0001). There was also a significant negative association between ecological concern about hybrid trees and age, with decreasing concern with increasing age (χ^2 = 12.142; p = 0.016). The level of ecological concerns about hybrid trees was also associated with organization type, with those working in university and educational organizations having less concern than those in other organization types (Fisher's exact p = 0.011). The level of ecological concerns was not associated with the level of economic concerns about hybrid trees among our respondents. Further, gender, education, region of residency in Indiana, and land type managed were not associated with either ecological or economic concerns about hybrid trees (see Table 5.4 for all statistical values for non-significant relationships).

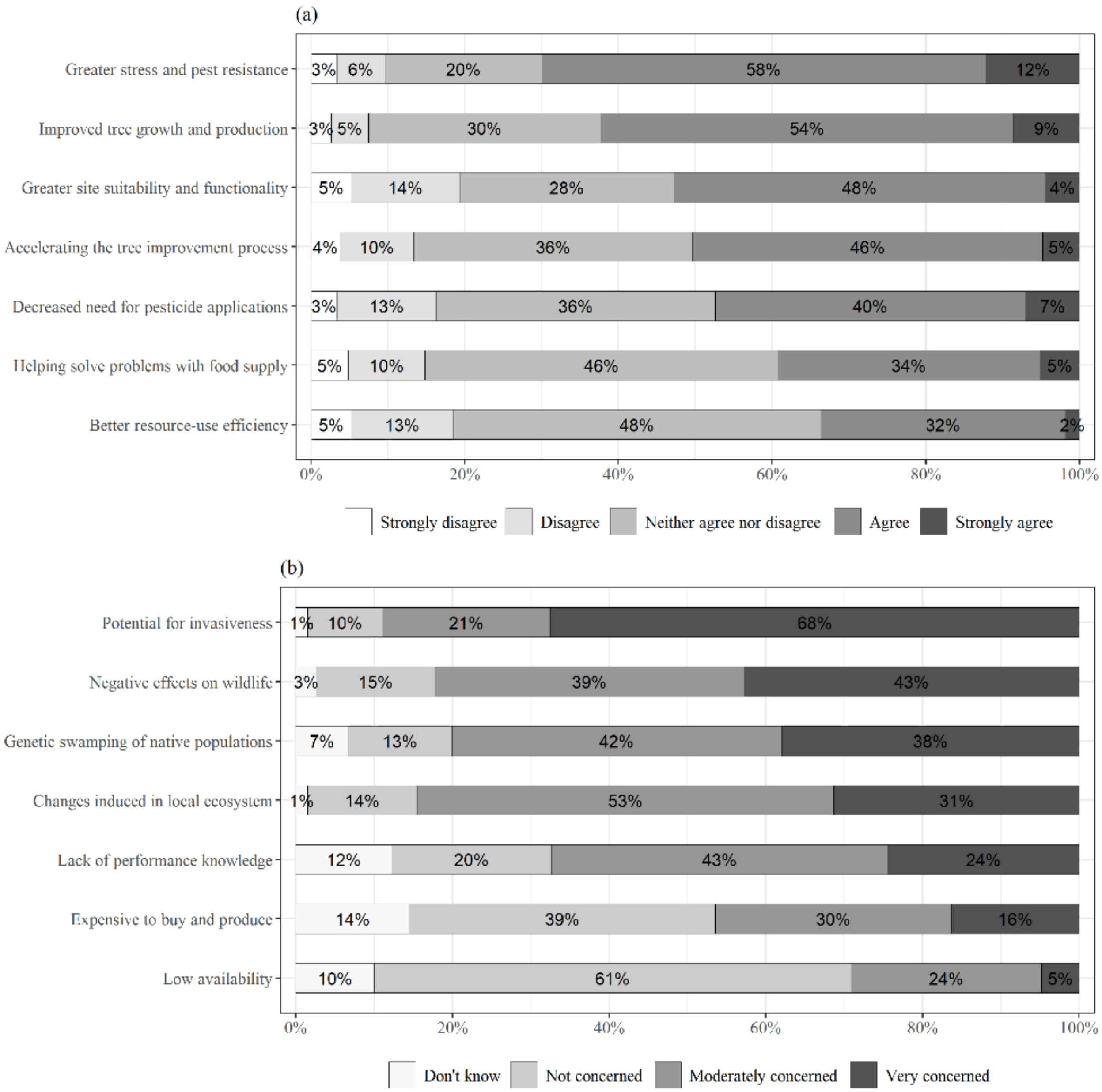

Figure 5.1. Survey respondents' perceived (a) tree improvement advantages of hybrid trees, in order of decreasing agreement, and (b) concerns, in order of decreasing concern (n=271).

Table 5.4. Summary of statistical relationships between land manager characteristics and perceptions of hybrid trees.

	Agreement (greater)	Concern (greater)		Agreement towards using for different purposes (greater)				
	Tree improvement advantages	Ecological	Economic	Restoration purposes	Provisioning purposes	Supporting purposes	Regulating purposes	Cultural purposes
Age (older)	NS $\chi^2 = 2.838$, $p = 0.092$	**–** **$\chi^2 = 12.142$,** **$p = 0.016$**	NS $\chi^2 = 4.227$, $p = 0.376$	NS $\chi^2 = 0.014$, $p = 0.906$	NS $\chi^2 = 0.021$, $p = 0.885$	NS $\chi^2 = 0.154$, $p = 0.694$	NS $\chi^2 = 0.659$, $p = 0.417$	NS $\chi^2 = 0.680$, $p = 0.410$
Gender (male)	NS $\chi^2 = 2.264$, $p = 0.133$	NS $\chi^2 = 11.466$, $p = 0.144$	NS $\chi^2 = 0.599$, $p = 0.741$	+ **$\chi^2 = 5.745$,** **$p = 0.017$**	NS $\chi^2 = 0.700$, $p = 0.403$	+ **$\chi^2 = 5.083$,** **$p = 0.024$**	NS $\chi^2 = 0.144$, $p = 0.704$	NS $\chi^2 = 0.011$, $p = 0.197$
Education (higher)	NS $\chi^2 = 0.699$, $p = 0.705$	NS Fisher's exact $p = 0.183$	NS $\chi^2 = 0.135$, $p = 0.998$	NS $\chi^2 = 1.075$, $p = 0.584$	NS $\chi^2 = 0.085$, $p = 0.958$	NS $\chi^2 = 4.354$, $p = 0.113$	NS $\chi^2 = 2.735$, $p = 0.255$	NS $\chi^2 = 0.172$, $p = 0.918$
Region of residency in Indiana (north)	NS $\chi^2 = 1.745$, $p = 0.186$	NS $\chi^2 = 4.776$, $p = 0.092$	NS $\chi^2 = 4.108$, $p = 0.134$	NS $\chi^2 = 0.009$, $p = 0.926$	NS $\chi^2 = 1.057$, $p = 0.304$	NS $\chi^2 = 0.013$, $p = 0.860$	NS $\chi^2 = 1.739$, $p = 0.187$	NS $\chi^2 = 0.732$, $p = 0.392$
Organization type	**for-profit: +** **state gov.: –** **$\chi^2 = 14.706$,** **p = 0.012**	**educational: –** **than other types** **Fisher's exact** **$p = 0.183$**	NS Fisher's exact $p = 0.600$	NS $\chi^2 = 4.212$, $p = 0.519$	NS $\chi^2 = 1.858$, $p = 0.868$	NS $\chi^2 = 6.932$, $p = 0.226$	NS $\chi^2 = 6.492$, $p = 0.261$	NS $\chi^2 = 2.721$, $p = 0.743$
Land type managed (more urban)	NS $\chi^2 = 0.252$, $p = 0.882$	NS $\chi^2 = 2.843$, $p = 0.241$	NS Fisher's exact $p = 0.613$	NS $\chi^2 = 1.991$, $p = 0.369$	NS $\chi^2 = 2.872$, $p = 0.238$	NS $\chi^2 = 0.913$, $p = 0.633$	+ **$\chi^2 = 4.755$,** **$p = 0.032$**	+ **$\chi^2 = 5.066$,** **$p = 0.026$**
Tree improvement (greater agreement)	N/A	– **$\chi^2 = 8.693$,** **$p = 0.003$**	+ **$\chi^2 = 27.640$,** **$p < 0.0001$**	+ **$\chi^2 = 50.695$,** **$p < 0.0001$**	+ **$\chi^2 = 53.129$,** **$p < 0.0001$**	+ **$\chi^2 = 68.492$,** **$p < 0.0001$**	+ **$\chi^2 = 78.790$,** **$p < 0.0001$**	+ **$\chi^2 = 57.826$,** **$p < 0.0001$**
Ecological concern (greater)	– **$\chi^2 = 8.693$,** **$p = 0.003$**	N/A	NS $\chi^2 = 7.510$, $p = 0.111$	– **$\chi^2 = 7.295$,** **$p = 0.007$**	– **$\chi^2 = 20.735$,** **$p < 0.0001$**	– **$\chi^2 = 10.566$,** **$p = 0.001$**	NS $\chi^2 = 1.769$, $p = 0.184$	– **$\chi^2 = 9.751$,** **$p = 0.002$**
Economic concern (greater)	+ **$\chi^2 = 27.640$,** **$p < 0.0001$**	NS $\chi^2 = 7.510$, $p = 0.111$	N/A	+ **$\chi^2 = 8.657$,** **$p = 0.003$**	NS $\chi^2 = 0.752$, $p = 0.386$	+ **$\chi^2 = 7.987$,** **$p = 0.005$**	NS $\chi^2 = 2.737$, $p = 0.098$	NS $\chi^2 = 1.906$, $p = 0.167$

Attitudes towards using hybrid trees for different purposes

Overall, there was more agreement than disagreement regarding the use of hybrid trees for various purposes (Figure 5.2). The top three purposes for using hybrid trees with the greatest agreement (agree or strongly agree) among land managers were "non-timber production" (63%), "timber production" (59%), and "conservation and restoration of at-risk species" (57%). The purposes for using hybrid trees with the greatest disagreement (strongly disagree or disagree) were "promoting biodiversity" (32%), "reforestation and restoration of forests" (30%), and "promoting wildlife habitat" (27%).

Two land manager characteristics were associated with their attitudes towards using hybrid trees for different purposes. Specifically, if a land manager managed urban land, they were more likely to agree with using hybrid trees for the purpose of generating regulating ($\chi^2 = 4.755$; $p = 0.032$) and cultural ecosystem services (ES; $\chi^2 = 5.066$; $p = 0.026$). Self-identifying as a male was also associated with greater agreement with using hybrid trees for restoration ($\chi^2 = 5.745$; $p = 0.017$) and generating supporting ES ($\chi^2 = 5.083$; $p = 0.024$). Age, education, region of residency in Indiana, and organization type were not associated with agreement regarding the use of hybrid trees for various purposes (see Table 5.4 for statistical values). In addition to these bivariate relationships, there was a strong positive relationship between land managers perceiving tree improvement advantages of hybrid trees and their agreement with using hybrid trees for all five purposes: restoration ($\chi^2 = 50.695$; $p < 0.0001$), provisioning ES ($\chi^2 = 53.129$; $p < 0.0001$), supporting ES ($\chi^2 = 68.492$; $p < 0.0001$), regulating ES ($\chi^2 = 78.790$; $p < 0.0001$), and cultural ES ($\chi^2 = 57.826$; $p < 0.0001$) (Table 5.4). Land managers' level of economic concerns was also positively associated with agreement with using hybrid trees for restoration ($\chi^2 = 8.657$; $p = 0.003$) and supporting ES purposes ($\chi^2 = 7.987$; $p < 0.0001$). However, the level of ecological concerns was negatively associated with agreement with using hybrid trees for restoration ($\chi^2 = 7.295$; $p = 0.007$), provisioning ES ($\chi^2 = 20.735$; $p < 0.0001$), supporting ES ($\chi^2 = 10.566$; $p = 0.001$), and cultural ES ($\chi^2 = 9.751$; $p = 0.002$), but not for regulating ES ($\chi^2 = 2.737$; $p = 0.098$).

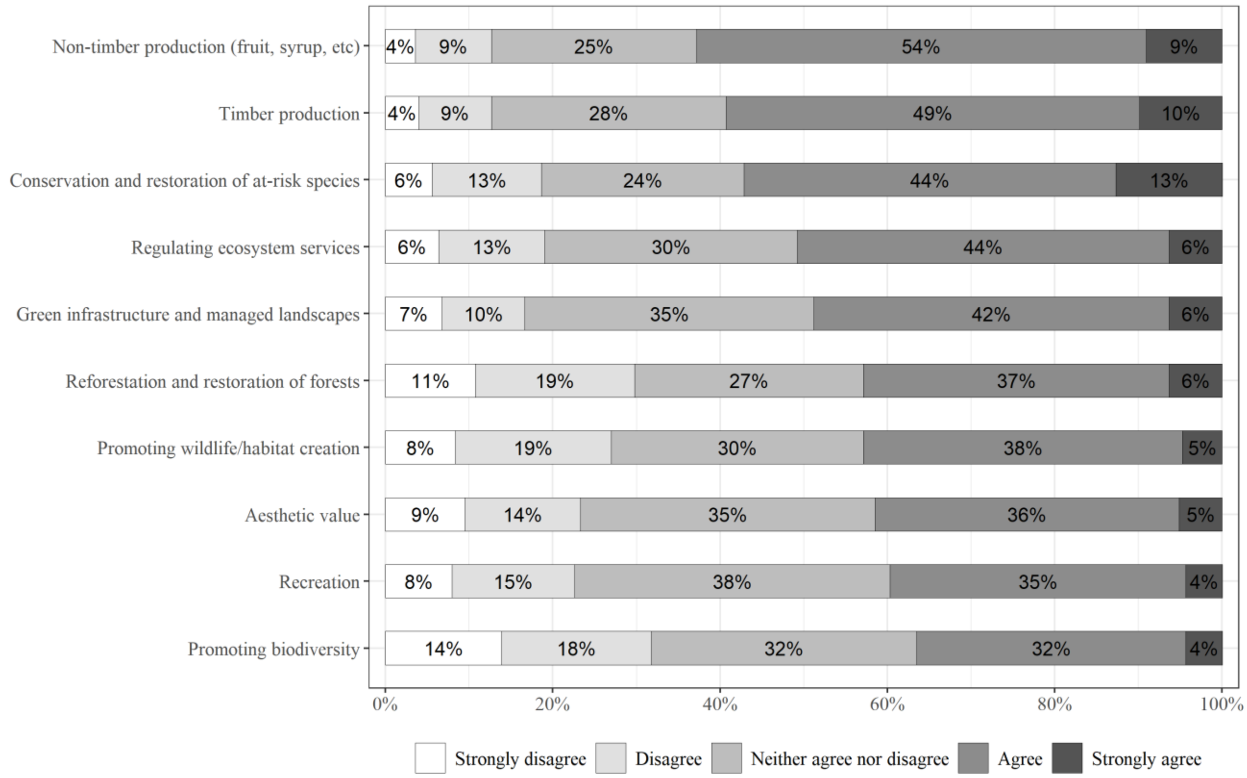

Figure 5.2. Survey respondents' attitudes towards using hybrid trees for various purposes, in order of decreasing agreement (n=253).

Hybrid type preference for restoration

We provided respondents with two hybrid types for use in restoration. Seventy-four percent of respondents preferred the native species × native species hybrid type for use in restoration, while none of the respondents preferred the native species × non-native species hybrid type. Nine percent of respondents preferred either type, while 13% preferred neither type. Four percent of respondents selected "don't know". There was a significant association between hybrid type preference and age, with respondents in the older age bracket (60-78 years old) indicating stronger preferences for both hybrid types than those in the younger age brackets (23-40 and 41-59 years old; Fisher's exact $p = 0.026$). Gender was also associated with preference, with more female respondents than male respondents preferring neither hybrid types (Fisher's exact $p = 0.006$). No other land manager characteristics were associated with hybrid type preference; for the full statistics see Table G.3. Additionally, the more ecologically concerned respondents were about hybrid trees, the more likely they preferred neither hybrid types; relatedly, the less ecologically concerned respondents were about hybrid trees, the more likely they preferred either hybrid type (Fisher's exact $p = 0.002$). Those who were less agreeable with the tree improvement advantages of hybrid trees were also less likely to prefer either hybrid type (Fisher's exact $p < 0.0001$).

Current use of hybrid trees

Twenty-nine percent of land managers reported using hybrid trees in their work, while 62% reported not using them, and 9% did not know whether they were using hybrid trees or not. A list of the specific hybrid species respondents reported using in their work is included in Table G.4. Land managers in local government or non-profit organizations were more likely to use hybrid trees than those in state government ($p = 0.040$ and 0.035, respectively; Table 5.5). The type of land managed was also significantly associated with whether hybrid trees were used; those managing urban lands were more likely to use hybrid trees ($p = 0.001$). Those with higher levels of ecological concerns were less likely to use hybrid trees ($p = 0.001$), while the level of economic concern was not significantly associated with hybrid tree use. There was also no relationship between hybrid tree use and age, gender, education, region of Indiana, or agreement with tree improvement advantages of hybrid trees.

Table 5.5. Logistic estimates of the empirical models for estimating use of hybrid trees by land managers in Indiana. Average marginal effects (AME) were used with standard error (SE). The reference level for organization type was state government (n=230).

Explanatory variables	AME	SE	*p*-value
Age (greater)	0.001	0.002	0.780
Gender: female	-0.039	0.059	0.511
Education (greater)	0.115	0.064	0.070
Region of Indiana: south	-0.002	0.051	0.970
Organization: local government	**0.218**	**0.106**	**0.040**
Organization: for-profit	0.101	0.074	0.175
Organization: non-profit	**0.162**	**0.077**	**0.035**
Organization: university/educational	0.113	0.154	0.463
Land type managed (more urban)	**0.183**	**0.056**	**0.001**
Agreement of tree improvement advantages of hybrid trees (greater)	0.204	0.106	0.055
Ecological concern of hybrid trees (greater)	**-0.484**	**0.133**	**0.0003**
Economic concern with hybrid trees (greater)	0.140	0.075	0.064

Note: Data reported for all ordinal variables represents the linear relationship between levels. None of the higher order relationships (ex: quadratic) tested significant and were excluded to conserve space.

5.4.4 GM tree perceptions

Perceived GM tree improvement advantages

Overall, there was more agreement than disagreement regarding the tree improvement advantages of GM trees (Figure 5.3a). Respondents had the greatest agreement (agree or strongly agree) with using GM trees for the purposes of "greater stress and pest resistance" (68%) and "improved tree growth and production" (61%). In contrast, respondents had the least agreement (strongly disagree or disagree) with using GM trees for "greater site suitability and functionality" (19%) and "herbicide tolerance" (19%). It should be noted that disagreement was fairly consistent across the tree improvement advantages. While gender, education, region of residency in Indiana, and land type managed were not associated with agreement regarding tree improvement advantages of GM trees (see Table 5.6 for full statistical values), age was negatively associated with agreement with GM trees having tree improvement advantages (χ^2 = 4.602; p = 0.032).

Concerns about GM trees

Trends in level and type of concerns about GM trees closely followed those about hybrid trees. Ecological concerns about GM trees were consistently stronger than economic concerns (Figure 5.3b). The greatest concern about GM trees overall was the potential for invasiveness with 69% of respondents indicating strong concern. The greatest economic concern about GM trees was the lack of performance knowledge (36% strongly concerned). Low availability was the least important economic concern (i.e., 54% of respondents chose "not concerned").

As with hybrid trees, the only land manager characteristic and perception variable significantly associated with both ecological and economic concerns about GM trees was the level of agreement with tree improvement advantages, with the direction of the relationship depending on the type of concern (Table 5.6). A higher level of ecological concerns about GM trees was associated with less agreement with GM trees having tree improvement advantages (χ^2 = 5.454; p = 0.020). Conversely, a higher level of economic concerns was associated with greater agreement with GM trees having tree improvement advantages (χ^2 = 20.415; p < 0.0001). The level of economic concerns about GM trees was positively associated with age, with concern increasing with age (χ^2 = 9.958; p = 0.041). The level of ecological concerns was not associated with the level of economic concerns about GM trees (see Table 5.6), nor any land manager characteristics (i.e., gender, education, region, organization type, land type managed) (see Table 5.6 for all statistical values of non-significant relationships).

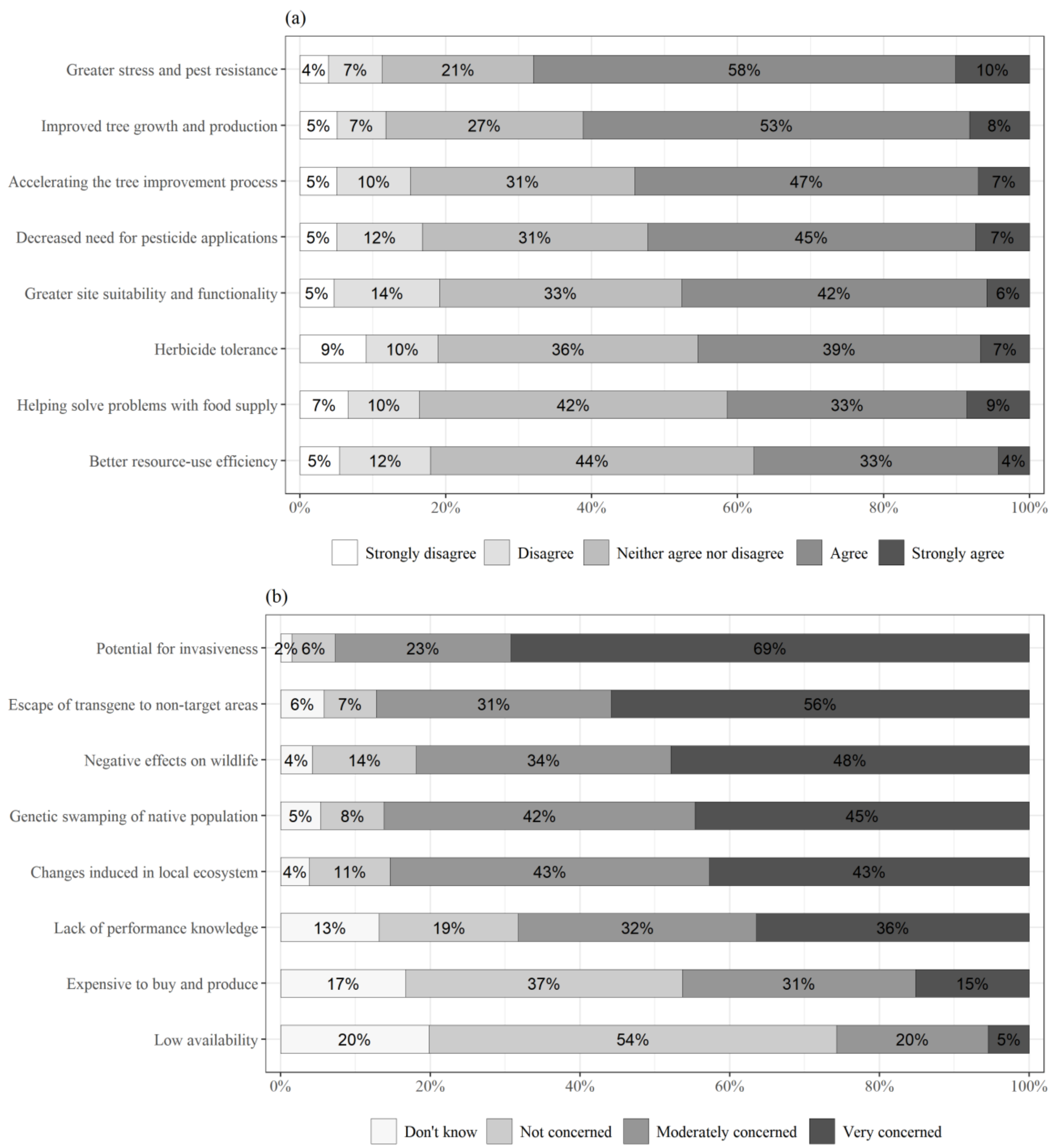

Figure 5.3. Survey respondents perceived (a) tree improvement advantages of GM trees, in order of decreasing agreement, and (b) concerns, in order of decreasing concern (n=260).

Table 5.6. Summary of statistical relationships between land manager characteristics and perceptions of GM trees.

	Agreement (greater)	Concern (greater)		Agreement towards using for different purposes (greater)				
	Tree improvement advantages	Ecological	Economic	Restoration purposes	Provisioning purposes	Supporting purposes	Regulating purposes	Cultural purposes
Age (older)	**–** **χ^2 = 4.602,** **p = 0.032**	NS χ^2 = 0.749, p = 0.945	**+** **χ^2 = 9.958,** **p = 0.041**	NS χ^2 = 0.396, p = 0.529	NS χ^2 = 0.059, p = 0.809	NS χ^2 = 0.488, p = 0.485	NS χ^2 = 0.413, p = 0.520	**+** **χ^2 = 4.792,** **p = 0.029**
Gender (male)	NS χ^2 = 3.004, p = 0.083	NS χ^2 = 0.320, p = 0.989	NS χ^2 = 1.418, p = 0.492	NS χ^2 = 0.899, p = 0.343	NS χ^2 = 0.039, p = 0.844	NS χ^2 = 0.472, p = 0.492	NS χ^2 = 1.890, p = 0.169	NS χ^2 = 0.067, p = 0.795
Education (higher)	NS χ^2 = 1.273, p = 0.529	NS Fisher's exact p = 0.227	NS Fisher's exact p = 0.660	NS χ^2 = 1.068, p = 0.586	NS χ^2 = 1.232, p = 0.540	NS χ^2 = 0.733, p = 0.679	NS χ^2 = 0.850, p = 0.654	NS χ^2 = 0.254, p = 0.881
Region of residency in Indiana (north)	NS χ^2 = 1.737, p = 0.187	NS χ^2 = 0.460, p = 0.795	NS χ^2 = 2.123, p = 0.346	NS χ^2 = 0.204, p = 0.651	NS χ^2 = 0.025, p = 0.874	NS χ^2 = 0.209, p = 0.650	NS χ^2 = 0.018, p = 0.894	NS χ^2 = 2.572, p = 0.109
Organization type	NS χ^2 = 7.083, p = 0.215	NS Fisher's exact p = 0.235	NS Fisher's exact p = 0.457	NS χ^2 = 1.964, p = 0.854	NS χ^2 = 3.044, p = 0.693	NS χ^2 = 1.631, p = 0.898	NS χ^2 = 5.304, p = 0.380	NS χ^2 = 3.900, p = 0.564
Land type managed (more urban)	NS χ^2 = 0.837, p = 0.360	NS χ^2 = 4.183, p = 0.124	NS Fisher's exact p = 0.099	**–** **χ^2 = 4.646,** **p = 0.035**	NS χ^2 = 1.880, p = 0.391	NS χ^2 = 2.705, p = 0.259	NS χ^2 = 0.036, p = 0.982	NS χ^2 = 0.568, p = 0.753
Tree improvement advantages (greater agreement)	N/A	**–** **χ^2 = 5.454,** **p = 0.020**	**+** **χ^2 = 20.415,** **p < 0.0001**	**+** **χ^2 = 85.111,** **p < 0.0001**	**+** **χ^2 = 70.992,** **p < 0.0001**	**+** **χ^2 = 73.369,** **p < 0.0001**	**+** **χ^2 = 61.989,** **p < 0.0001**	**+** **χ^2 = 47.996,** **p < 0.0001**
Ecological concern (greater)	**–** **χ^2 = 5.454,** **p = 0.020**	N/A	NS Fisher's exact p = 0.383	**–** **χ^2 = 9.099,** **p = 0.003**	**–** **χ^2 = 10.760,** **p = 0.001**	**–** **χ^2 = 12.328,** **p < 0.0001**	NS χ^2 = 1.989, p = 0.158	NS χ^2 = 0.304, p = 0.581
Economic concern (greater)	**+** **χ^2 = 20.415,** **p < 0.0001**	NS Fisher's exact p = 0.383	N/A	**+** **χ^2 = 5.936,** **p = 0.015**	**+** **χ^2 = 5.596,** **p = 0.018**	**+** **χ^2 = 5.138,** **p = 0.023**	**+** **χ^2 = 8.002,** **p = 0.005**	NS χ^2 = 3.418, p = 0.065

Attitudes towards using GM trees for different purposes

Overall, there was more agreement than disagreement for using GM trees for various purposes (Figure 5.4). There was greatest agreement (agree or strongly agree) among land managers with using GM trees for "conservation and restoration of at-risk species" (56%), "timber production" (54%), and "non-timber production" (47%). The purposes for using GM trees with the greatest disagreement (strongly disagree or disagree) were "promoting biodiversity" (39%), "reforestation and restoration of forests" (34%), and "promoting wildlife habitat" (33%).

As with hybrid trees, two land manager characteristics were associated with their attitudes towards using GM trees for different purposes. Respondents who managed urban land were less likely to agree with using GM trees for restoration purposes (χ^2 = 4.646; p = 0.035), but the type of land managed was not associated with agreement for the other purposes (see Table 5.6). Age was only associated with agreement with using GM trees for cultural ES, with agreement increasing with age (χ^2 = 4.792; p = 0.029). Neither gender, education, region of residency in Indiana, or organization type was associated with agreement for using GM trees for any purposes (see Table 5.6 for statistics). In addition, agreement with the tree improvement advantages of GM trees was strongly and positively associated with agreement with using GM trees for all five purposes: restoration (χ^2 = 85.111; p < 0.0001), provisioning ES (χ^2 = 70.992; p < 0.0001), supporting ES (χ^2 = 73.369; p < 0.0001), regulating ES (χ^2 = 61.989; p < 0.0001), and cultural ES (χ^2 = 47.996; p < 0.0001) (Table 5.6). There was also a positive relationship between land managers' level of economic concerns and their agreement with using GM trees for restoration (χ^2 = 5.936; p = 0.015), provisioning ES (χ^2 = 5.596; p = 0.018), supporting ES (χ^2 = 5.138; p = 0.023), and regulating ES (χ^2 = 8.002; p = 0.005), but not for cultural ES (χ^2 = 3.418; p = 0.065). Conversely, the level of ecological concerns was negatively associated with agreement for using GM trees for restoration (χ^2 = 9.099; p = 0.003), provisioning ES (χ^2 = 10.760; p = 0.001), and supporting ES (χ^2 = 12.328; p < 0.0001), but not for regulating ES (χ^2 = 1.989; p = 0.158) and cultural ES (χ^2 = 0.304; p = 0.581).

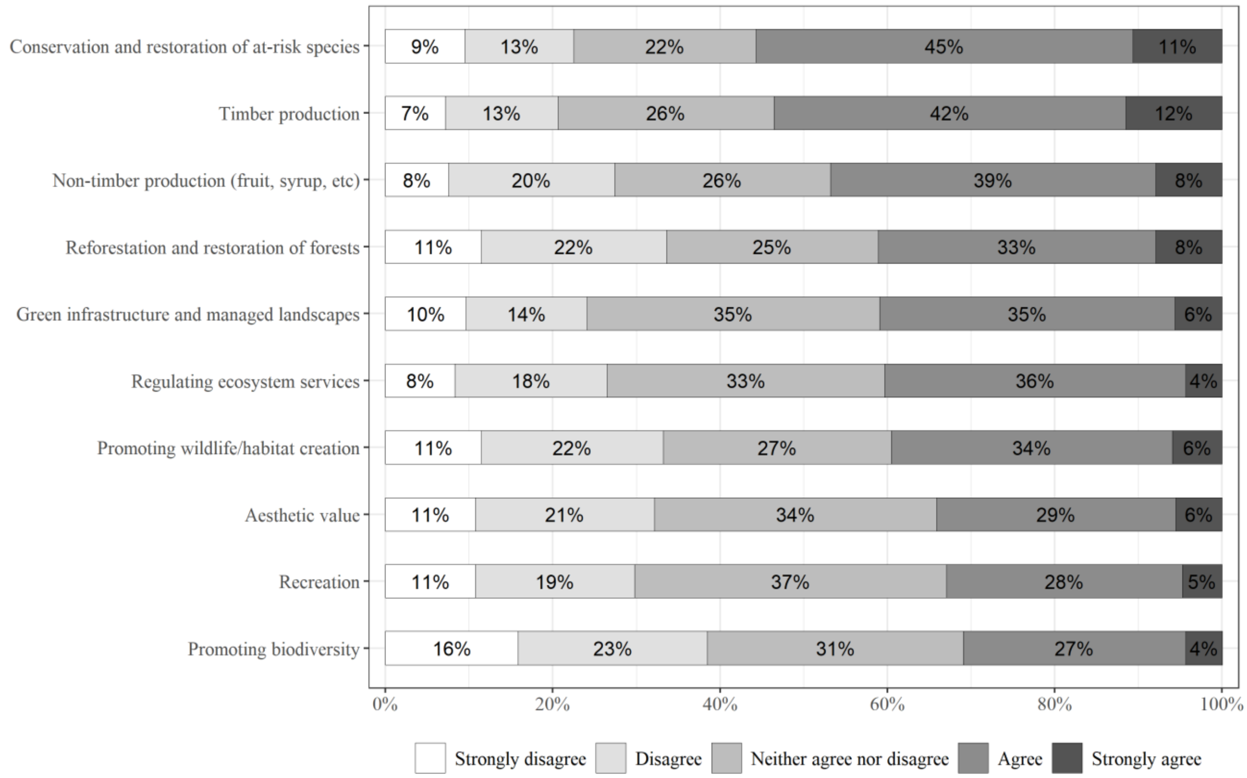

Figure 5.4. Survey respondents' attitudes towards using GM trees for various purposes, in order of decreasing agreement (n=253).

Perceived nativity of GM trees

Forty-seven percent of respondents perceived a GM version of a native species as not being native, while 26% said that it would still be native and 27% reported "don't know". Respondents with higher levels of ecological concerns about GM trees were more likely to perceive a GM version of a native species as not native, while those with lower levels of ecological concerns were more likely to perceive it as native (Fisher's exact $p < 0.0001$). Further, respondents with stronger agreement with the tree improvement advantages of GM trees were more likely to perceive a GM version of a native species as still being native ($\chi^2 = 38.488$; $p < 0.0001$). No other land manager characteristics or perceptions were associated with perceived nativity of GM trees (for the full statistical values see Table G.5).

5.4.5 *Current use of at-risk trees*

Twenty-three percent of respondents used at-risk tree species (see Table 5.1 for definition) in their work, while 60% did not use them and 18% reported not knowing whether they used at-risk tree species or not. A list of the specific at-risk species used by respondents is included in the Table G6. It is interesting to note that several species, like oaks and hickories, that were frequently reported are, in fact, not ranked as at-risk either locally or throughout their range. Official designations of whether a species is at-risk range-wide wide or locally were determined through the International Union for Conservation of Nature Red List of Threatened Species (IUCN 2019), NatureServe Explorer (NatureServe 2017), USDA PLANTS Database (USDA 2019), and Indiana Department of Natural Resources (IDNR 2019). None of the land manager characteristic or perception variables in the logistic model for predicting the use of at-risk tree species tested significant (see Table G.7 for full statistics).

5.5 Discussion

5.5.1 *Perceived advantages and concerns of hybrid and GM trees*

Land managers in our study reported having similar overall levels of concern, as well as specific ecological and economic concerns about both hybrid and GM trees. Importantly, our results also suggest that land mangers tend to be more concerned about the ecological impacts of hybrid and GM trees than economic impacts. This is somewhat counterintuitive because hybrid trees have been actively used and planted for centuries, in addition to naturally forming from introduced non-native species crossing with native species. As such, their ecological impacts have been documented across a wide range of landscapes (Burgess and Husband 2006, Zalapa et al. 2009, Gaskin 2016, Cronk and Suarez-Gonzalez 2018). In contrast, potential ecological implications of using GM trees have yet to be extensively evaluated, given the limited situations in which they are legally allowed to be grown (Sedjo 2010). Yet, our results suggest similar levels and types of concerns about hybrid and GM trees.

To understand these results, we look at the case of American chestnut hybrids. Backcross breeding species conservation efforts have been made to preserve as many of the traits from the native progenitor as possible to enable them to serve as an adequate substitute for the native progenitor species, and with some success (Diskin et al. 2006, Knapp et al. 2014). However, American chestnut hybrid breeding programs are still in-progress, so long-term effects are yet unknown. Ecological issues associated with other hybrid trees with a non-native progenitor, including invasiveness and genetic swamping, have also been documented, such as with the white mulberry (*Morus alba* L.; Burgess and Husband 2006), Callery pear (*Pyrus calleryana* Decne.; Culley and Hardiman 2007), and Siberian elm (*Ulmus pumila* L.; Zalapa et al. 2009). Together, these studies have painted an ecologically checkered history of hybrids, which can shape land managers' perceptions of hybrid trees. Social science research has shown that humans have stronger experiential, rather than analytical, processing systems and that personal experiences can be a strong factor motivating behaviors such as mitigating climate change (Weber 2006, Marx et al. 2007) and accepting gene technology (Connor and Siegrist 2010). Given the magnitude of invasive species in Indiana (IN Invasive Species Task Force 2008), it is likely that many land managers have experienced the ramifications of invasive exotic species first-hand in their own work. As such, studies documenting ecological impacts of hybrid trees combined with land managers' own experiences with invasive exotic species may be important in shaping land managers' perceptions of hybrid trees, particularly their potential ecological impacts.

In this context, however, our results also suggest that land manager acceptance of hybrid and GM trees will likely involve careful consideration of their potential utility in light of possible risk, as new information becomes available. Specifically, our results show that land managers appear to have concluded that some risks, such as species extinction or not meeting the production demands of a growing population, may be larger than those associated with the use of hybrid and GM trees, as illustrated in the following quotes left by two survey respondents in an open-comment box:

> (1) *"The risk of using hybrid or genetically modified trees for restoring at-risk species entails a risk of its own. Therefore the risk of using and not using these trees must be carefully weighed against one another, including the risk of unintended consequences."*

> (2) *"The risk and rewards of both hybrid and GM trees is still very infantile. Our current knowledge is equivalent to corn genetics in the 40's or soybean genetics in the 60's! The big differences in in the decades needed for evaluation and decision and the many scores of species/sub-species considered."*

Our study also identifies a high percentage of land managers expressing neutrality or "don't know", illustrating the magnitude of "the unknown" associated with hybrid and GM trees. It was repeatedly conveyed through the preliminary interviews and many comments at the end of the survey, that one way to address land manager concerns and mitigate ecological risk would be for extensive, long-term research and testing to measure what is currently unknown. This is consistent with the results of St-Laurent et al. (2018) regarding the use of assisted migration, where respondents indicated a high degree of uncertainty and need for additional research. These calls for extensive research and evaluation likely indicate the high degree of trust placed in scientists and researchers by land managers, and moving forward, the importance of ecological studies of hybrid and GM trees.

Our study also shows a high level of agreement among land managers that both hybrid and GM trees have tree improvement advantages, such as "greater stress and pest resistance" and "improved tree growth and production". Simultaneously, many land managers also felt ambiguous about the various tree improvement advantages of hybrid and GM trees. As mentioned previously, this could possibly be due to the still infant nature of the biotechnologies, and that land managers may simply not have enough experience or information to form an opinion. This is partially consistent with results from a study involving another informed population, tree scientists in Europe, who ranked tree conservation through increased disease resistance as the most important benefit of GM trees (out of eight), but increased production-related benefits were rated more intermediately (Kazana et al. 2016). The reverse situation was true, in relation to GM agronomic crops, for another informed population, farmers in Sweden, who ranked higher yields as the top benefit and greater pest resistance intermediately (Lehrman and Johnson 2008). It is possible that tree scientists may be more ecologically minded (biocentric), farmers more economically minded (anthropocentric), and land managers may be more intermediate, particularly given the diversity of fields within the population (McFarlane and Boxall 2003, St-Laurent et al. 2018).

5.5.2 Attitudes towards using hybrid and GM trees for various purposes

Our study shows a high level of agreement with using both hybrid and GM trees for "non-timber production (fruit, syrup, etc.)", "timber production", and "conservation and restoration of at-risk species" purposes, and a low-level agreement with using them for "promoting biodiversity" and "reforestation and restoration of forests". Our results are consistent with what have been reported by Jepson and Arakelyan (2017a, 2017b), who found that informed and general publics tended to rank the use of hybrid and GM trees for ash restoration very closely. Interestingly, the latter purpose ranked number one in agreement for GM tree use but was third for hybrid trees.

Genetic modification of endangered trees, such as the American chestnut, thus far has only involved changing one or a few genes (Jacobs et al. 2013), however, thousands of genes will be different from the conservation target species in a hybrid plant. For example, even though we do not yet know the total number of genes in American chestnut, we can estimate that it has roughly 37,000 genes, based on the number of genes in Chinese chestnut, *Castanea mollissima* Blume (Xing et al. 2019). Consequently, even the current 15/16 American chestnut backcross hybrid with Chinese chestnut would still have roughly 2,300 genes from the non-native progenitor and exactly what functions and traits each of those genes controls is largely unknown, as discussed in the following survey comment:

> *"One of the biggest concerns and hold-ups, at least for me, is the philosophical question of whether or not a hybrid (American x Chinese chestnut backcross, for example) is truly an equivalent replacement for the at risk native. Even a 15/16 native hybrid has considerable genetic "wiggle room." There are concerns over creating new invasive species, diluting what little remaining pure genetic stock there may be, and overlooking more difficult to determine things such as microbiological and insect interactions with plants."*

Further, with at-risk species conservation, the function of that species in its native ecosystem is being conserved as well, as demonstrated in this comment:

> *"Hybrid trees should be avoided, including their use in proximity to related native trees (to avoid genetic swamping). If in genetic engineering a particular gene can be precisely modified to prevent sensitivity of the tree species to exotic pathogens, it might be acceptable if it can be shown that the modified tree species bears no changes in morphology and "behavior" in the natural landscape. Micro-surgical genetic changes to a species appears to be much better than hybridization, especially with exotic species, e.g., Chinese/American chestnut crosses."*

While species conservation was rated in the top three uses for both tree types, the larger magnitude of non-native genes in a hybrid could be perceived by land managers as a slightly greater risk to an ecosystem than using GM trees, resulting in the slightly different rankings.

Our study also shows a high level of disagreement among land managers with using hybrid and GM trees for "promoting biodiversity" and for "reforestation and restoration of forests". Land managers might perceive that these two particular purposes could be accomplished more readily with existing conventional tree improvement and planting techniques. Traditional tree management methods have widely been perceived as more acceptable and less risky than use of newer biotechnologies by both informed (Jepson and Arakelyan 2017a) and general publics (Hajjar et al. 2014, Hajjar and Kozak 2015, Jepson and Arakelyan 2017a, 2017b, St-Laurent et al.

2018). Further, traditional breeding, silvicultural, and replanting methods have already shown success in restoring forests and promoting biodiversity within them (ex: Sweeney et al. 2002, Stanturf et al. 2014, da Cruz et al. 2020). However, when it comes to saving at-risk tree species from new and swift-acting threats such as non-native pests and diseases, traditional breeding that does not use interspecific hybrids or GM trees, has not shown sufficient success to save species, such as American chestnut (Jacobs et al. 2013) and butternut (Michler et al. 2006). As such, land managers have to navigate between using conventional methods to foster a healthy, diverse forest, such as replanting in more suitable sites and using new biotechnologies to save species from swift, new threats in their work.

Further, biotechnologies, such as hybridization and genetic modification, have already shown effectiveness in growing agronomic crops and operate at a much quicker timeline than traditional breeding (FAO 2000). The speed of the method was shown to be an important factor in a study that measured acceptance of GM technology compared to more traditional methods for saving ash trees in the UK (Jepson and Arakelyan 2017a). Acceptance for using GM technology rose by nearly 20%, while it decreased by 13-20% for some of the conventional breeding methods, after the expected timeline of each method was indicated. GM and hybrid technologies may be perceived as being more critical now for both saving threatened trees species from newly introduced, fast-spreading pests and diseases, as well as for meeting the production demands of a rapidly growing population.

5.5.3 At-risk tree species

While very accurate in their reporting of hybrid species, some land managers reported some common species, like oaks and hickories, as being at-risk, even though these species are widely classified as being secure. It is possible that they were perceived that way because the oak-hickory forest type in general is transitioning to maple-beech in parts of Indiana due to factors such as fire suppression (Nowacki and Abrams 2008), ungulate browse (McWilliams et al. 2018), and invasive plants (Schulte et al. 2011). Less than a quarter of respondents reported using at-risk tree species in their work and no significant characteristics were associated with at-risk tree use. It is possible that the biggest factor determining actual use of at-risk tree species is the specific work purpose of land managers, rather than just the organization type. This is something we attempted to measure in this survey, but unfortunately, there are many possible tree work purposes (at least 12 levels) and ultimately not enough cases in many of the levels for an accurate statistical analysis by this variable. Future work involving a larger sample size might be more successful in measuring and analyzing this variable.

5.5.4 The effect of land manager characteristics in shaping their perceptions

Overall, our results suggest a trend of younger land managers being more willing to accept newer technology than older land managers (here, genetic modification being newer than hybridization), which aligns with what has been found in other research. In relation to ash tree conservation in the UK, Jepson and Arakalyan (2017a, 2017b) found that younger generations not only viewed GM technology more positively than older generations, but also had greater support for methods that involved higher degrees of scientific intervention in general. While St-Laurent et al. (2018) also found a similar age tendency in terms of the use of GM technology as a climate change forest adaptation strategy, Hajjar et al. (Hajjar et al. 2014) did not. However, decreasing acceptance with increasing age has been reported in several studies related to GM food (Hallman et al. 2003, Onyango and Nayga 2004, Schläpfer 2008). Further, not only is this age trend observed with biotechnologies related to plants, but it is also mirrored more broadly in perceptions to newer technologies (Chau and Hui 1998, Morris 2000, Kwateng et al. 2019). This can be explained in part by the tendency of younger individuals to be more aware of the benefits of new technology (Venkatesh et al. 2003, Yousafzai and Yani-de-Soriano 2012), which was supported in the current study by the negative association of age and agreement towards the tree improvement advantages of GM trees. However, it should be noted that age was not a significant predictor of current use of hybrid trees, so perception differences by age may not translate to actual use of GM trees either (if/when they are legally allowed to be grown widescale).

Gender was only significant when examining land managers' perceptions towards hybrid trees. Specifically, self-identifying as female was only associated with greater disagreement with using hybrid trees for restoration and supporting ES purposes, and female land managers preferred neither type of hybrid trees be used. Interestingly, none of the variables for GM tree perceptions differed significantly by gender. In the only two studies that could be referenced evaluating perceptions to both hybrid and GM trees at the same time, Jepson and Arakelyan found either no difference by gender (2017a) or that males had more acceptance than females for more interventionist approaches, including hybridization and genetic modification (2017b), using similar survey instruments. The population assessed in the former study (no gender perception differences) was an informed public that included landowners, land managers, naturalists, and gardeners, but the target population of the second study was the general public. Scientific, particularly biological, knowledge (not simply education level) has been documented to increase acceptance of GM food and gene technology (Moerbeek and Casimir 2005, Connor and Siegrist 2010, Mielby et al. 2012), and it has also been reported that males tend to have both more knowledge and acceptance of GM foods and gene technology than females (Moerbeek and Casimir 2005, Connor and Siegrist 2010). While our survey did not measure scientific knowledge, we assumed that a moderate to high level is required to specialize in the land management profession, thus it is possible that differences by gender in our study were few to non-existent because the scientific knowledge levels of both females and males were similar.

In terms of type of land managed, land managers responsible for urban lands agreed more with using hybrid trees for regulating and cultural ES purposes than those managing natural lands. This is consistent with urban land managers' greater actual usage of hybrids, as both of these types of purposes more strongly involve human-impacted landscapes (i.e., urban areas). Urban land managers are under intense pressure to plant durable and aesthetic trees, while also maintaining biodiversity (Ordóñez and Duinker 2013, Conway and Vecht 2015). These modern urban forest management pressures are strongly rooted in past and current experiences with massive pest outbreaks decimating populations of common street tree species, such as American elm with Dutch elm disease in the early to mid-1900s (Hubbes 1999) and quite recently various ash species (*Fraxinus* L.) with the emerald ash borer (Poland and McCullough 2006). These massive tree mortality events have quite dramatically illustrated the importance of having a diverse urban tree population with increased resiliency and decreased vulnerability to any single threat. This has led to widespread adoption by urban land managers of the 10-20-30 Formula proposed by Santamour (1990), which states that urban forests should contain "no more than 10% of any single tree species, no more than 20% of species in any tree genus, and no more than 30% of species in any tree family." While this rule is broadly supported in concept, it is difficult to execute in practice (Kendal et al. 2014). Clearly, the need to meet the 10-20-30 Formula requirements, pressure to have tough species that will tolerate difficult urban conditions, ability to fulfill societal needs and functions, and ultimately, and species availability from suppliers, all interact to limit the tree species options of urban land managers. Many hybrid tree species have been (and are still being) developed specifically to expand the urban tree species selections with increased tolerance to urban conditions and stressors, while also meeting the other requirements of suitable urban trees (Bassuk et al. 2009). Thus, it follows that in the current study, urban land managers would both perceive the use of hybrid trees more positively (particularly for urban purposes), as well as use them more often than those who manage natural lands.

While urban land managers tended to perceive hybrids more positively, those managing more natural lands had greater agreement for the use of GM trees for restoration purposes. This could relate to the relative percentages of genes from the target native species and non-target species contained within a GM species (as discussed at length previously section 5.5.2 "Attitudes towards using hybrid and GM trees for various purposes"). In restoration, not only are species being restored, but also the function of the ecosystem. Natural land managers might be considering that, given the higher percentage of genes from the target species contained within a GM version of that species (compared to a hybrid), that tree type might have a greater amount of native adaptations that would allow for more successful restoration.

While age, gender, and type of land managed played a significant role in our study, no other land manager characteristic variables did. Previous studies have shown mixed results regarding education, which was not significant in shaping hybrid and GM tree perceptions in our study. This is consistent with the results of Hajjar et al. (2014) and St-Laurent et al. (2018) who also found no difference in acceptance of GM trees, or any other forest management strategy, by education. However, Jepson and Arakelyan (2017a, 2017b) found a positive relationship between acceptance to the use of GM trees and education. Land managers are part of an informed population that requires a certain level of education and experience in order to successfully perform their tree management duties, so differences by level of education may not actually exist given the common knowledge baseline. Additionally, northern parts of Indiana are dominated by agricultural landscapes where the use of hybrid annual crops is common (IN Geological and Water Survey 2011), while southern parts of the state are more dominated by hardwood forested landscapes of primarily the oak/hickory forest type (Gormanson and Kurtz 2017). Yet, there was also no difference in perceptions or current use by land managers from northern versus southern counties in Indiana. This could be because this survey was administered to people who primarily manage tree lands, rather than crop lands, so differences by region might only be seen by people who manage both tree and crop lands, which was not assessed in this survey. Finally, organization type was only associated with two variables regarding perceptions of hybrid trees and none regarding perceptions of GM trees. Organization type was a significant variable in predicting current use of hybrid trees, with land managers working in state government organizations using less than those in local government and non-profit organizations. State government land managers disagreed significantly more on the tree improvement advantages of hybrids and so may see less benefit to their use. Further, according to our survey, Indiana state government land managers predominantly manage natural lands (84%), compared to those in local government (13%) and non-profit (55%) organizations. As discussed previously, respondents managing natural lands had more negative perceptions with the use of hybrid trees, which may be why state government land managers were then less likely to use hybrid trees.

5.5.5 Perceptions of 'nativeness'

'Nativeness', or the level of native genes contained within a species, was clearly a concept of importance to most Indiana land managers. Nearly three-quarters of respondents preferred the native species × native species hybrid type, while none of the respondents indicated preference for the native species × non-native species hybrid type. For GM trees, the majority (47%) of land managers felt that a GM version of a native species was no longer native, while 27% did not know, indicating that nativeness is an issue with which many land managers are, understandably, still struggling. In both the hybrid and GM tree nativeness questions, the levels of ecological concerns and agreements with tree improvement advantages played important, but inverse, roles, with those having greater ecological concern and less agreement on advantages preferring neither hybrid type and perceiving GM trees as not native. Perceptions of nativeness and naturalness have contributed to forming perceptions of not just GM trees (Hajjar and Kozak 2015, Jepson and Arakelyan 2017b), but also GM food crops (Mielby et al. 2013, Siegrist et al. 2016) and even GM wildlife (Kohl et al. 2019) as well. However, that begs the question: what is "nativeness" and how can it be conceptualized here?

The concept of nativeness, and how to define it, is intriguing in that it is both ecologically and philosophically rooted. For example, what delineates whether a species arrived in a particular geographic location through human intervention, or whether it arrived there on its own? Do species transported by both indigenous peoples and European settlers (for instance), count as native? Where are humans even native to? Because of this complexity, there is considerable discussion and debate on the topic (e.g., Woods and Moriarty 2001, Warren 2007, Knights 2008). Yet, there is no single, widely agreed upon definition. In light of the current discussion of hybrid and GM trees, perhaps the most compelling case is that of Woods and Moriarity (2001), who describe five possible criteria for distinguishing native from non-native species, summarized here:

1. <u>The Human Introduction Criterion</u> – whether human activity indirectly or directly introduced a species to the area
2. <u>The Evolutionary Criterion</u> – whether a species originally evolved in the area
3. <u>The Historical Range Criterion</u> – whether a species has historically grown in the area
4. <u>The Degradation Criterion</u> – whether a species degrades and harms the environment of the area
5. <u>The Community Membership Criterion</u> – whether a species is an integrated component of the community or ecosystem of the area

Woods and Moriarity (2001) are clear to state that meeting or not meeting any single criterion does not automatically classify a species as being native or non-native, but rather how a species meets the most applicable cluster of these criteria given its specific circumstance.

To explore this situation for hybrid and GM trees, it might be helpful to consider the perception of nativeness of these tree types on a spectrum. On the more native end, humans are simply modifying a pre-existing species so that the majority of it will still be the same as the unhybridized version, just slightly improved so that it can survive in its changed environment. If viewed from this perspective, hybrid and GM trees would probably meet criteria 1, 2, 3, and 5. Conversely, on the other extreme, the non-native end, simply by the fact that humans intervened to combine part of one species with another to form hybrid and GM trees, we are essentially creating entirely new species - that have no 'track record' in the target restoration area. If viewed from this second perspective, these tree types would certainly *not* meet criteria 1, 2, 3, and 5 for nativeness. From either the more native or non-native perspective, it is still unclear whether hybrid and GM trees of at-risk native species fall in or outside of the fourth criterion pertaining to degradation. As discussed previously, unintentional hybridization of native with non-native tree species has been documented in many instances as being ecologically detrimental (e.g., Burgess and Husband 2006, Culley and Hardiman 2007, Zalapa et al. 2009). However, whether that will be the case for the hybrid and GM trees currently in development *specifically* for species restoration, rather than as an unintended, uncontrolled consequence (i.e., escape from cultivation), is still largely unknown. Further, the goal of using these tree types for restoration is actually to integrate into and support the ecosystem through reintroduction of a declining species, thus meeting the fourth criterion – but again, whether that goal will be met or not is still to be determined. In our study, land managers expressed strong ecological concerns, which in turn, affected perceptions of nativeness. Thus, learning more about the ecological consequences to using hybrid and GM trees developed specifically for restoration would help determine whether they degrade or support their ecosystem, while also aiding in determinations of nativeness.

5.6 Conclusion

The goal of this research was not to make a judgement as to whether hybrid or GM trees should be used. What is does do, though, is provide a clue as to the perceptions of land managers to these tree types and how willing land managers would respond to recommendations on their use. Respondents' strongest concern was overwhelmingly about the "potential for invasiveness" with these tree types, and all ecological type concerns were stronger than economic concerns. The most agreed upon tree improvement advantages of hybrid and GM trees were for "greater stress and pest resistance" and "improved tree growth and production", while disagreement was similarly low across the different advantages. Respondents agreed most with using hybrid and GM trees for conservation and restoration of at-risk species, timber production, and non-timber products (fruit, syrup, etc.) and disagreed most with using them for promoting biodiversity and reforestation and restoration of forests. However, we found that land managers are not a uniform group, and perceptions varied by several characteristics, such as concerns, age, and the type of land they managed. Ecological and economic concerns were shown to have an inverse effect on other perceptions; those having greater ecological concern perceived hybrid and GM trees more negatively, while those with greater economic concern perceived them more positively. While greater ecological concern was negatively associated with current hybrid use, economic concerns did not significantly predict use. Younger land managers tended to view GM trees more positively than hybrid trees, while the reverse relationship was true for older land managers. Respondents managing more urban lands were found to not only perceive hybrids more positively, but were also more likely to use them in their work than those managing natural lands. Overall, the majority of land managers in our study agreed with a variety of advantages and purposes of use for hybrid and GM trees. Thus, if or when using these tree types is deemed the right choice for restoring at-risk species (and approved legally), most land managers in Indiana would likely be agreeable to recommendations about using these tree types. However, it was also made very clear throughout the study that despite indicating a high degree of agreement towards using hybrid and GM trees, respondents still had very strong ecological concerns about their use and suitability as a native replacement. In order to alleviate these concerns, it will be essential that the traits of hybrids and GM trees be thoroughly vetted, particularly ecologically, with the results of these evaluations clearly communicated. Additionally, active engagement with land managers, such as consultation on the specific types of research needed, will be critical; this is a population that has a clear stake in the issue, and would ultimately be responsible for any widescale implementation of hybrid and GM trees.

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CHAPTER 6. SYNTHESIS AND FUTURE PATHWAYS

6.1 Overview

Within the last 200 years, global change has contributed to over 500 plant species extinctions and over 17,500 plant species reaching threatened status (IUCN 2020), illustrating the monumental need for broad plant conservation and restoration efforts. The overarching goal of this research was to holistically consider restoration of a threatened tree species and link together research from different fields to aid in solving the challenge. In order to accomplish this, butternut (*Juglans cinerea* L.), threatened by butternut canker disease (BCD), was used as a case study for evaluating the use of its hybrids as a potential restoration tool. Studies in biotechnical (chapter 2), ecophysiological (chapters 3 and 4), and societal (chapter 5) disciplines were conducted to understand the suitability of using hybrids to restore butternut.

6.2 Chapter 2: Hybrid breeding for restoration of threatened forest trees: Incorporating disease tolerance in *Juglans cinerea*

Preliminary evidence from wild populations has indicated that butternut hybrids resulting from crosses with Japanese walnut (*Juglans ailantifolia* Carr.) could be more tolerant to BCD than unadmixed butternut (Boraks and Broders 2014), however, putative tolerance in the hybrids had not been formally tested in a controlled study. Thus, the goal of this first study was to gain a better understanding of BCD in butternut and its hybrids in a controlled setting. Specifically, the objectives were to evaluate potential BCD tolerance within and between the two butternut types, as well as any differences in canker growth between different *Ocj* isolates. Differences in canker presence/absence and size were observed by fungal isolate, which could help explain some of the differences in BCD severity seen between butternut populations. Smaller and fewer cankers and greater genetic gains were seen in hybrid families, demonstrating that hybrids warrant further evaluation as a possible breeding tool for developing BCD-resistant butternut trees.

6.3 Chapter 3: Can cold and heat tolerances suggest adaptive limitations for *Juglans cinerea* restoration using hybrids?

Even with increased disease tolerance, the hybrids must possess closely similar ecophysiological tolerances to butternut to be an effective replacement. Growing in areas that can reach as low as -40 °C (Dirr 2009), one of the most notable characteristics of butternut is its extreme cold hardiness. Japanese walnuts, however, are native to in an overall warmer ecosystem, which rarely drops below -20 °C in even the coldest areas (Japan Meteorological Agency 2012). This indicates a potential disparity in extreme temperature tolerances between butternut, Japanese walnut, and their hybrids. Additionally, it is critical to understand any variation of extreme temperature tolerance within a species, which would allow for potential range-wide variation in adaptations to be uncovered. This, in turn, would aid restoration efforts through the selection and use of properly adapted trees (Bischoff et al. 2008). Thus, the objective of chapter 3 was to compare relative cold and heat tolerances within butternut from different USDA plant hardiness zone provenances and between butternut, Japanese walnut, and their hybrids. Cold tolerance differed more than heat tolerance among butternut provenances and across species. Within butternut, trees from colder areas exhibited less cold damage than those from warmer areas and while differences in heat damage between hardiness zones occurred, they did not follow a clear trend. Consequently, at the intraspecific level, cold and heat tolerance were not correlated. However, at the interspecific level, cold and heat tolerance were negatively correlated. Butternut exhibited greatest cold tolerance, butternut exhibited greatest heat tolerance, and hybrids were intermediate. Thus, the utility of hybrids for restoration could be limited at the ecophysiological extremes of species' distributions.

6.4 Chapter 4: Cold hardiness traits indicate a lack of ecological similarity between a progenitor forest tree species and its hybrids

Since cold hardiness is such a quintessential trait of butternut, a further cold test was conducted using whole plants for chapter 4, with the objective of understanding differences in survival, damage, and budbreak within butternut and between butternut and its hybrids. No species or hardiness zone group exceeded the LT_{50} until the coldest treatment of -38 °C, demonstrating the extreme cold tolerance of both butternut and its hybrids. Budbreak phenology was most uniform at the -20 °C treatment, possibly indicating the importance of low, non-lethal temperatures for these species. No survival or damage differences were detected in provenances of butternut, although seedlings from the coldest provenances (zone 4) experienced more delayed budbreak at the two warmest treatments than those from warmer provenances (zones 5 and 6). Interspecific differences were not observed in dieback, but were in survival and budbreak. The hybrids had greater survival than butternut from warmer provenances at the -38 °C treatment, but given that temperatures that low are extremely unlikely to occur in those provenances, it is not anticipated to give the hybrids an advantage if planted there. However, the earlier budbreak of the hybrids could cause it to be asynchronous to butternut's ecosystem and more vulnerable to spring frosts, calling into question the ability of hybrids to serve as an adequate restoration substitute for butternut.

6.5 Chapter 5: Perceptions of land managers towards using hybrid and genetically modified trees

If hybrids, as well as genetically modified (GM) trees, are shown to hold effective disease tolerance and are supported by science as an ecologically suitable replacement for using the native progenitor species, the success of restoration efforts using these hybrids will ultimately depend on land managers, the people responsible for replanting efforts. Gaining an understanding of their perceptions to using these biotechnologies in trees, prior to attempting to recommend and implement their use, would aid in proactively addressing potential concerns so that the restoration process is more effective. The objective of chapter 5 was to gauge land manager perceptions to the use of hybrid and GM trees, as well as current use of hybrid trees. Land managers had stronger concern for ecological, rather than economic, issues, with "potential for invasiveness" being the strongest concern. Agreement was highest for using hybrid and GM trees for "conservation and restoration of at-risk species", "timber production", and "non-timber products (fruit, syrup, etc.)" and lowest for "promoting biodiversity" and "reforestation and restoration of forests". However, land managers are not a uniform group, and perceptions varied by several characteristics, such as concern type, age, and the type of land they managed. Ecological concern and the type of land being managed most strongly predicted current hybrid use. Overall, the majority of land managers agreed with a variety of advantages and purposes of use for hybrid and GM trees. Thus, if or when using these tree types is deemed the right choice for restoring at-risk species (and approved legally), most land managers in Indiana would likely be agreeable to recommendations about using these tree types.

6.6 Synthesis

The BCD tolerance screening of chapter 2 indicate that butternut hybridization with the BCD-tolerant Japanese walnut may have transferred disease tolerance genes into the hybrids. This aligns with work in other species, such as American chestnut (Steiner et al. 2017, Clark et al. 2019) and American elm (Griffin et al. 2017, Pinchot et al. 2017) where hybridization with a non-native species was used to incorporate disease tolerance. However, while the hybrids appear to hold greater BCD tolerance, the results of chapters 3 and 4 indicate that their potential as an ecological substitute for butternut is mixed. The results of the comparative study on excised tissues in chapter 3 indicate that hybrids were no different than butternut for heat tolerance and while different, still fairly close, in cold tolerance (especially when compared to their Japanese walnut progenitor). Yet, the results of chapter 4 tell a slightly different story about cold tolerance, with hybrids out-performing butternut in some metrices, and under-performing in others. This illustrates that while relative, comparative studies are valuable for building an initial understanding of ecophysiological tolerances within and between different species, experiments using the whole plant will provide the most realistic information. More central to the goals of the present studies, though, is that the results also suggest that when progenitors come from contrasting ecosystems, their hybrids might be ecologically different compared to their progenitors. This is consistent with results seen for the drought and flood tolerance of butternut hybrids (Crystal and Jacobs 2014), as well as for hybrids of chestnut (Pinchot et al. 2017) and oak (Himrane et al. 2004) species. Indeed, the results of chapter 5 suggest that while Indiana land managers were overall in agreement to using both hybrid and GM trees to restore at-risk species, they still had strong ecological concerns about the suitability of their use – concerns which were partially validated by chapter 3 and 4 results. It is important to note, though, that consistent with the results of Crystal and Jacobs (2014) and Crystal et al. (2016), great variation was seen within the ecological traits of butternut hybrids, with some families performing very similarly to their butternut progenitor. Consequently, it may be possible to develop disease tolerant, ecologically similar hybrids with careful selection and backcross breeding. Diskin et al. (2006) and Knapp et al. (2014) have shown that, at least in terms of phenotypic and photosynthetic traits, this is possible in American chestnut hybrids. Thus, in order to alleviate land manager concerns, it will be essential that hybrid and GM trees be thoroughly vetted, with the results clearly communicated. Additionally, active engagement with land managers will be critical, as this is a population with a clear stake in the issue, and which would ultimately be responsible for any widescale implementation of hybrid and GM trees.

6.7 Future pathways

The results of these studies collectively indicate that while there is potential for using hybrids for restoration of butternut, a great deal of work remains to fully address and understand the issue. In terms of potential BCD resistance, even longer-term field studies of at least 5-10 years will be necessary to be able to see potential survival and growth differences between butternut and its hybrids infected with *Ophiognomonia clavigignenti-juglandacearum*, the causal fungus of BCD. As with work currently being conducted on hybrids of American chestnut (Steiner et al. 2017, Clark et al. 2019) and American elm (Griffin et al. 2017), the durability of BCD tolerance in promising hybrids will need to be tested in multiple types of environments and field sites. Also similar to work with American chestnut (Diskin et al. 2006, Knapp et al. 2014), this would need to coincide with careful breeding and selection for hybrids that not only have the greatest BCD tolerance, but are otherwise most similar to butternut. Traits such as reproductive potential, invasiveness, growth rate, form, and wood quality compared to the unadmixed species will be critical to evaluate in order to find hybrids with the greatest ecological, economic, and cultural similarity (Allendorf et al. 2013, Woodcock et al. 2017). During this entire process, inclusion of land managers and other stakeholders will be essential. Researchers evaluating hybrid and GM trees should not only clearly and regularly communicate the results of their work, but also seek to engage and include land managers and other stakeholders in guiding future research. Not only will this provide useful direction for restoration projects, but will also build greater ownership and involvement, and thus, greater success for the projects.

6.8 Conclusion

Although valuable for its large, energy-rich seed masts, veneer-quality wood, and cultural and medicinal properties, butternut is seriously threatened by BCD. More broadly, plant species around the globe are threatened or going extinct at an alarming rate (IUCN 2020). Meeting the challenge of restoring these species will require concerted, collaborative efforts across multiple disciplines. I hope this dissertation illustrates that building research specifically to link with work in other fields allows for a fuller, more comprehensive evaluation of the broader issue, while also providing greater illumination of future steps required for restoring a threatened species.

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APPENDIX A. SUPPLEMENTARY DATA FOR CHAPTER 2

Table A.1. Plant material used in the butternut canker disease tolerance screening by species (*Juglans cinerea*) or hybrid, family (accession number assigned to each family by the Hardwood Tree Improvement and Regeneration Center), origin (location where the original mother tree scion or seed material was collected from), and number of trees.

Species/Hybrid	Family	Origin	Trees (no.)
J. cinerea	709	Caledonia, MN	10
J. cinerea	712	Arlington, WI	8
J. cinerea	713	Rochester, MN	8
J. cinerea	714	Rochester, MN	9
J. cinerea	715	Rochester, MN	6
J. cinerea	716	Rochester, MN	9
J. cinerea	717	Whitewater, WI	8
J. cinerea	718	Whitewater, WI	10
J. cinerea	722	Nicolet NF, WI	10
J. cinerea	723	Whitewater, WI	8
J. cinerea	726	Mazaska Lake, MN	8
J. cinerea	727	Rochester, MN	9
J. cinerea	728	M. Twain NF,MO	7
J. cinerea	730	M. Twain NF,MO	10
J. cinerea	733	Perch River, NY	10
J. cinerea	736	Berlin, VT	9
J. cinerea	738	Trade Lake, WI	8
J. cinerea	741	Whitewater, WI	10
J. cinerea	742	Stratford, NH	8
J. cinerea	743	'Creighton', PA	9
J. cinerea	744	'Painter', IA	9
J. cinerea	746	Whitewater, WI	10
J. cinerea	747	Bark River, MI	10
Hybrid	702	New Paris, IN	6
Hybrid	704	Plymouth, IN	9
Hybrid	706	New Paris, IN	3
Hybrid	707	Brimfield, IN	7
Hybrid	708	Steuben Co., IN	7
Hybrid	710	Madison, WI	10
Hybrid	711	Madison, WI	9
Hybrid	731	Clover Lick, WV	11
Hybrid	732	Loudon, NH	9
Hybrid	734	Sanford, ME	9
Hybrid	735	Sanford, ME	10
Hybrid	748	Chequam NF, WI	6
Hybrid	750	Ankeny, IA	10

APPENDIX B. SUPPLEMENTARY DATA FOR CHAPTER 3

Table B.1. *Juglans* trees at the Hardwood Tree Improvement and Regeneration Center (West Lafayette, IN, USA) selected for the study. F1 hybrids are *J. cinerea* × *J. ailantifolia* and backcross hybrids are F1 × *J. cinerea* (Backcross-*Jc*). “State” refers to US state were propagation material originated. “Zone” refers to USDA Plant Hardiness Zone of the material collection site.

Accession #	Family	Species	State	Zone
51-2010-08-21	OS-71 R	*J. cinerea*	WI	4
51-2010-12-25	OS-140 R	*J. cinerea*	MN	4
51-2010-13-24	OS-54 R	*J. cinerea*	WI	4
51-2010-14-66	OS-97 R	*J. cinerea*	NY	4
51-2010-19-44	OS-52 SP	*J. cinerea*	MN	4
51-2010-20-46	OS-141 R	*J. cinerea*	WI	4
51-2010-21-75	1458	*J. cinerea*	VT	4
51-2010-22-69	1457	*J. cinerea*	VT	4
51-2010-08-65	1469	*J. cinerea*	WI	5
51-2010-11-68	1469	*J. cinerea*	WI	5
51-2010-15-72	826 M	*J. cinerea*	WI	5
51-2010-17-65	1390	*J. cinerea*	MI	5
51-2010-21-25	1434	*J. cinerea*	PA	5
51-2010-08-45	1481	*J. cinerea*	WV	6
51-2010-09-77	1379	*J. cinerea*	MI	6
51-2010-11-30	1348	*J. cinerea*	KY	6
51-2010-12-73	1361	*J. cinerea*	KY	6
51-2010-16-23	1407	*J. cinerea*	MO	6
51-2010-16-77	1351	*J. cinerea*	KY	6
51-2010-17-27	1407	*J. cinerea*	MO	6
51-2010-19-50	1407	*J. cinerea*	MO	6
51-2010-20-37	1354	*J. cinerea*	KY	6
51-2010-22-23	1430	*J. cinerea*	PA	6
51-2010-22-57	OS-86 R	*J. cinerea*	MO	6
51-2010-23-29	1417	*J. cinerea*	NJ	6
51-2010-06-56	1454	*J. cinerea*	VA	7
51-2010-09-26	1447	*J. cinerea*	TN	7
51-2010-10-25	1439	*J. cinerea*	TN	7
51-2010-10-42	1451	*J. cinerea*	VA	7
51-2010-13-72	1441	*J. cinerea*	TN	7
51-2010-14-70	1445	*J. cinerea*	TN	7
51-2010-15-73	1450	*J. cinerea*	VA	7
51-2010-15-77	1262	*J. cinerea*	AL	7
51-2010-18-44	1448	*J. cinerea*	VA	7
51-2010-20-52	1265	*J. cinerea*	AL	7

Accession #	Family	Species	State	Zone
51-2010-23-32	OS-97 R	Backcross-*Jc*	NY	4
51-2010-07-56	OS-8 R	Backcross-*Jc*	WI	5
51-2010-16-61	1466	Backcross-*Jc*	WI	5
51-2010-17-31	731	Backcross-*Jc*	WV	5
51-2010-18-36	735 M	Backcross-*Jc*	ME	5
51-2010-20-42	1466	Backcross-*Jc*	WI	5
51-2010-07-73	OS-101B SP	Backcross-*Jc*	CT	6
51-2010-08-22	1394	Backcross-*Jc*	MI	6
51-2010-15-28	1268	Backcross-*Jc*	CT	6
51-2010-18-75	1381	Backcross-*Jc*	MI	6
51-2010-22-32	OS-101B SP	Backcross-*Jc*	CT	6
51-2010-20-71	1267	Backcross-*Jc*	AL	7
51-2010-10-26	1364	F1	ME	4
51-2010-15-61	1365	F1	ME	4
51-2010-06-54	735 M	F1	ME	5
51-2010-11-16	OS-7 R	F1	WI	5
51-2010-11-77	731	F1	WV	5
51-2010-14-46	1369	F1	MI	5
51-2010-19-19	OS-8R	F1	WI	5
51-2010-20-63	OS-7 R	F1	WI	5
51-2010-21-69	707	F1	IN	5
51-2010-09-62	1268	F1	CT	6
51-2010-16-27	1407	F1	MO	6
51-2010-19-16	1551	F1	WV	6
51-2010-09-54	1551	*J. ailantifolia*		
51-2010-10-48†	1551	*J. ailantifolia*		
51-2010-12-61†	1551	*J. ailantifolia*		
51-2010-16-37	707	*J. ailantifolia*		
51-2010-23-27	707	*J. ailantifolia*		
53-2011-04-15	1728	*J. ailantifolia*		
53-2011-06-17	1732	*J. ailantifolia*		
53-2011-07-26	1732	*J. ailantifolia*		
53-2011-13-25	1727	*J. ailantifolia*		
53-2011-13-32	1733	*J. ailantifolia*		
53-2011-14-35	1734	*J. ailantifolia*		
53-2011-16-27	1733	*J. ailantifolia*		
53-2011-17-46	1733	*J. ailantifolia*		

†Tree used in heat test but died before cold test was conducted.

Table B.2. Maximum photochemical efficiency of PSII (F_V/F_M) of *Juglans* leaflets in the field and immediately prior to the heat test. Data are averages and SEs (n = 24).

Species/Hybrid	$(F_V/F_M)_{field}$	$(F_V/F_M)_{pre}$
J. cinerea	0.784±0.005	0.766±0.008
Backcross-*Jc*	0.794±0.005	0.791±0.009
F1	0.791±0.003	0.781±0.018
J. ailantifolia	0.783±0.003	0.763±0.011

APPENDIX C. SEED PROPAGATION PROTOCOL FOR PURE AND HYBRID BUTTERNUT (*JUGLANS CINEREA*)

A version of this work was previously published as follows:
Brennan AN, Jacobs DF (2020) Seed propagation protocol for pure and hybrid butternut (*Juglans cinerea* L.). Tree Planters' Notes 63:39–50. https://www.rngr.net/publications/tpn/63-1/seed-propagation-protocol-for-pure-and-hybrid-butternut-juglans-cinerea-l.

Abstract

Butternut (*Juglans cinerea* L.) is a native, eastern North American hardwood tree with economic and ecological value. It is severely threatened by butternut canker disease, which is rapidly killing the species range-wide. Hybrids of butternut and butternut canker-resistant Japanese walnut (*Juglans ailantifolia* Carr.) have been proposed as an alternative to planting pure butternut. Information on pure and hybrid butternut seed harvest, preparation, stratification, germination, planting, and initial seedling care is lacking. Methods and results are described from a project growing these species at Purdue University, forming a seed propagation protocol for the species. Germination was first observed 14 days after stratification. After 17 days, 64 percent of seeds germinated using the current method. Alternate methods to those used in this project are provided when possible, so growers can tailor protocols at different scales.

Introduction

Butternut (*Juglans cinerea* L.) is a medium-sized, exceptionally cold-hardy (USDA zone 3) hardwood tree native to Eastern North America (Dirr 2009, Rink 1990). The economically valuable wood of this species is easily worked and rot-resistant, making it ideal for furniture, paneling, veneer, and carving (Goodell 1984, Michler et al. 2005, Ostry et al. 1994). Butternut also holds ecological value as a mast species, providing energy-rich food for wildlife (and humans) with its large, oily kernels (Ostry et al. 1994). However, butternut canker disease, caused by the fungus *Ophiognomonia clavigignenti-juglandacearum* ([Nair, Kostichka, & Kuntz] Broders & Boland), has caused rapid declines in butternut populations since its discovery in 1967 (Broders and Boland 2011). The species is now classified as "endangered" by the International Union for Conservation of Nature (Stritch and Barstow 2019) and is listed under Canada's Species At Risk

Act (SARA) (Environment Canada 2010). In the United States, butternut has a conservation status of either “critically imperiled,” “imperiled,” or “vulnerable” in 21 States (NatureServe 2019). While butternut was never as widely produced as the closely related black walnut (*Juglans nigra* L.), the severity and prevalence of butternut canker disease has recently made butternut less viable for nurseries to produce and sell.

Butternut is readily able to hybridize with Japanese walnut (*Juglans ailantifolia* Carr.) and the resulting hybrids have naturalized in some parts of butternut’s range (Hoban et al. 2009). Researchers have only recently begun comparing the biology and performance of pure and hybrid butternuts. Crystal and Jacobs (2014) found that the hybrids were intermediate to butternut and Japanese walnut in terms of drought and flood stress tolerance. Morphologically, the hybrids have shown great variability and can hold the phenotypical features of either of the progenitor species (Crystal et al. 2014). The hybrids have also shown initial tolerance to butternut canker disease (Boraks and Broders 2014, Orchard et al. 1982), and are now being proposed by some as a possible alternative for butternut restoration (Boraks and Broders 2014, Michler et al. 2005).

Detailed and illustrated guidelines on the care of pure and hybrid butternut seeds and seedlings would aid in both restoration and research efforts, while also making it easier for growers to propagate and increase butternut in the landscape. This article contains seed-propagation protocols for pure and hybrid butternut, including information on seed harvest, preparation, stratification, germination, planting, and initial seedling care. Pure and hybrid butternut seedlings were recently grown at Purdue University (West Lafayette, IN) for a project comparing their cold tolerances and phenology. Specific details from the seed propagation portion of the project are recorded here, but alternative methods are also included for use by growers at different scales with varying resources.

Step 1: Seed harvest, preparation, and stratification

Harvesting

Harvest butternut and hybrid butternut fruits after ripening in autumn, preferably before they fall to the ground (Bonner 2008, Woeste et al. 2009, Young and Young 1992). For our project, fruits were harvested from September to October 2017 from the U.S. Department of Agriculture National Germplasm Repository (NCGR) and from six orchards of the Hardwood Tree Improvement and Regeneration Center (HTIRC) at Purdue University (Table C.1). Fruits were stored in plastic ventilated bags to allow airflow (Figure C.1).

Figure C.1. Freshly harvested butternut and hybrid butternut seeds placed in ventilated plastic bags prior to preparation for stratification. (Photo by A.N. Brennan 2017)

Table C.1. Butternut and hybrid butternut seed and germination information for Purdue University project. Seeds were harvested in fall 2017 and germinated in spring 2018.

Accession	Name	Orchard	Species	Origin	Quantity	Avg. Wt/nut (g)	No. Germ.	% Germ.
PI 666982	CJUG 1. 002 PL: Ayres	NCGR (Corvallis, OR)	butternut	MI	42	12.5	40	95.2
PI 666983	CJUG 4. 002 Chamberlin	NCGR (Corvallis, OR)	butternut	NY	33	13.0	33	100.0
PI 666987	CJUG 9. 001 PL: Herrick	NCGR (Corvallis, OR)	butternut	IA	29	22.7	24	82.8
PI 666992	CJUG 14. 001 PL: Booth	NCGR (Corvallis, OR)	butternut	NY	53	9.1	44	83.0
# 719	Part: 9906 OS-23 Slocums Woods	HTIRC (Walla Walla, WA)	butternut	WI	39	13.9	37	94.9
# 856	Hadley #1' Dave Hadley	HTIRC (West Lafayette, IN)	butternut	MI	40	12.8	35	87.5
03-713	Prog. OS-14 - #2097	HTIRC (West Lafayette, IN)	butternut	WI	37	11.4	31	83.8
PI 666997	CJUG 42. 001 Collier #2	NCGR (Corvallis, OR)	butternut	WV	34	16.8	32	94.1
# 968	Haberle # 1	HTIRC (West Lafayette, IN)	butternut	KY	40	15.5	36	90.0
# 979	Rickey #2 - Chilicothe	HTIRC (West Lafayette, IN)	butternut	OH	40	14.3	30	75.0
# 1073	Maxwell #5	HTIRC (West Lafayette, IN)	butternut	OH	40	12.3	37	92.5
# 1090	Hoosier #2	HTIRC (Huntingburg, IN)	butternut	IN	40	18.3	39	97.5
# 1083	Part: 9903 Indiana -Hoosier # 3/HNF	HTIRC (Walla Walla, WA)	butternut	IN	40	20.0	40	100.0
# 701	11th Road Hyb. Marshall Co	HTIRC (Plymouth, IN)	hybrid	IN	86	11.4	71	82.6
# 1093	Kellogg Comp. Hyb	HTIRC (West Lafayette, IN)	hybrid	MI	43	13.7	29	67.4
OS-222	‘LaCrosse’ Hybrid	HTIRC (West Lafayette, IN)	hybrid	WI	40	14.9	29	72.5
HYB 212	‘Vrana’ Fulton Co.	HTIRC (Plymouth, IN)	hybrid	IN	84	15.1	78	92.9
# 2033	Prog. No. 1-OS-191 / HTI #750	HTIRC (Wanatah, IN)	hybrid	IA	20	16.9	7	35.0
# 1000	Norristown # 2	HTIRC (West Lafayette, IN)	hybrid	IN	42	12.1	31	73.8
# 696	'Bountiful' grafts	HTIRC (Vera, MO)	hybrid	MO	79	12.3	77	97.5
TOTAL					**901**		**780**	**86.6**

Fruits can be planted directly into the ground (direct seeding) immediately after harvest or after removal of the green husks. As it requires fewer steps, direct seeding can be more efficient, allowing you to skip stratification and pre-germination, and may be most useful for large-scale plantings. Stratification and pre-germination, however, allow for more control over the entire process, protection from predation, and the ability to screen out nonviable seeds and unhealthy seedlings prior to planting. If direct planting the seeds, make sure the fruits are covered with a 1- to 2-in (2.5- to 5-cm) layer of soil and consider using screens to protect the planted seeds from rodent predation (Bonner 2008). See step 3 in this article for information on site selection and seedling care if direct planting.

Husk removal

While not necessary, removing the husks before stratifying and storing the seeds is helpful for preventing mold growth (Bonner 2008, Woeste et al. 2009). Remove the husks when they are firm, yet slightly soft; after this point, they can become too soft and quite difficult to remove (Bonner 2008, Young and Young 1992). We removed husks in our project within approximately 1 month of harvest.

Remove the major portion of the husk using any form of abrasion that can safely remove the husks without cracking the shell (Hartmann et al. 2002, Woeste et al. 2009). Possible methods conducted on a hard surface (driveway, garage floor, etc.) include: pounding with a metal rake (Figure C.2A), running over with a light- to mid-weight vehicle (Figure C.2B), and stomping and twisting while wearing hard-soled shoes (Figure C.2C). The husk can also be manually peeled off. Another method is to remove by repeated abrasion over a raised, metal grill-like structure that allows the husks to fall through, but the seeds to remain above (Figure C.2D). Throughout the husking process, a garden hose or similar object can be used to set a perimeter and provide a barrier to prevent seeds from rolling away (Figure C.2). Be advised that skin and clothes that come in contact with the husk and seed during this process are likely to become stained. Once the majority of the husk is removed, a power-washer or garden hose can be used to remove remaining bits of husk, but is not necessary (Woeste et al. 2009) (Figure C.3).

Figure C.2. Husks of butternut and hybrid butternut seeds can be removed by (A) pounding with a metal rake, (B) running over with a light- to mid-weight vehicle, (C) stomping and twisting while wearing hard- soled shoes, and (D) repeated abrasion over a raised metal grill-like structure that also allowed the husks to fall through to the floor. A garden hose was used in A, B, and C to contain the seeds and prevent them from rolling away. (Photos by A.N. Brennan 2017)

Figure C.3. Power-washing can be used to remove the final bits of husk from butternut and hybrid butternut seeds. (Photo by A.N. Brennan 2017)

Rogueing and sanitization

Within a few weeks of husk removal, prepare the seeds for stratification. To rogue out nonviable seeds, submerse the seeds in water and discard those that float (Woeste et al. 2009). For our project, we sanitized seeds in November 2017 with a 1:10 bleach:water solution to help prevent fungal and bacterial growth (Fraedrich and Cram 2012, Reil et al. 1998). Dip and swoosh batches of seeds using a large colander in a bucket of the bleach solution for approximately 15 seconds (Figure C.4A) followed by a 15-second rinse under plain water (Figure C.4B).

Figure C.4. Sanitizing butternut seeds prior to stratification can be accomplished by (A) placing them in a colander and immersing for 15 seconds in a 1:10 bleach:water solution followed by (B) rinsing under plain water. (Photos by A.N. Brennan 2017)

Stratification preparation and storage

For our project, we placed cleaned seeds in moist, but not wet, sand (just enough so no water could be squeezed out by hand from a fistful of sand) (Figure C.5A and B). Other stratification media, such as peat, sphagnum moss, or vermiculite can also be used (Reil et al. 1998, Woeste et al. 2009). Ensure that each seed is completely surrounded by the medium (Figure C.5C) and that a small amount of airflow can pass through the container—enough so that the seeds can respire, but not enough to dry out the medium (Woeste et al. 2009). We accomplished this by drilling small holes (3/32-in [2.4-mm] drill bit) into inverted cake-storage containers with loose-fitting lids (Figure C.5D). If preparing multiple seed batches, make sure to appropriately label containers.

Figure C.5. To prepare for stratification, sanitized butternut seeds can be (A) placed in a single layer on a shallow layer of moist sand using inverted cake containers. (B) Seeds should be covered with another shallow layer of sand, ensuring that each seed is surrounded by the moist sand. (C) This process is repeated for three layers of seeds. (D) The finished container should be covered with a loose-fitting lid to allow for a small amount of air circulation. Small holes can also be drilled near the top to further aid in circulation. (Photos by A.N. Brennan 2017)

Store the seed containers in a cool area, such as a cooler or well-insulated garage or shed, just above freezing (34 to 41 °F [1 to 5 °C]) for stratification (Bonner 2008, Woeste et al. 2009). Juglans seeds are very attractive to wildlife, so ensure they are stored such that wildlife cannot access them (Bonner 2008, Woeste et al. 2009). For our project, seeds were stored in a walk-in cooler at 37 to 41 °F (2.8 to 5.0 °C) (Figure C.6).

Figure C.6. Butternut and hybrid butternut seeds packed in moist sand in inverted and non-airtight containers and stored in a walk-in cooler for stratification. (Photo by A.N. Brennan 2017)

Stratification duration and monitoring

Stratify the seeds for 90 to 120 days (Bonner 2008, Young and Young 1992). We stratified the seeds for our project for 120 days and removed them from cool conditions in mid-March 2018. Check seeds weekly throughout the stratification period for mold growth and to ensure the sand is not drying out. If mold growth does occur, discard the moldy sand, re-sanitize the affected seeds as described previously, and replace them in a new batch of moist sand. Other techniques, such as the application of fungicides or hydrogen peroxide can also be used, although fungicides may negatively affect germination and should be used with caution (Cram and Fraedrich 2012). If the sand is too dry, add just enough water to keep the sand moist, but not wet. In our project, we noticed dark-brown staining in the sand surrounding some of the seeds (Figure C.7). We took a small sample of seeds from different batches, including from those where the surrounding sand was stained, and cracked them open with a hammer to check the endosperm health. All endosperms from the samples looked healthy: bright cream to nearly white and a bit "gummy" (Figure C.8). Given this, we suspected the brown staining to be leached tannins from the seed itself, particularly from any bits of remaining husk.

Figure C.7. Brown staining (circled) in the moist sand surrounding butternut seeds after 45 days in stratification is suspected to be leached tannins from the seed and leftover husk pieces. (Photo by A.N. Brennan 2017)

Figure C.8. To ensure seed health in the middle of stratification, a small sample of butternut seeds were cracked open to reveal healthy, cream- to nearly white-colored endosperm. (Photo by A.N. Brennan 2017)

Step 2: Seed germination

Upon completion of the stratification period in early spring, seeds can be planted directly into the ground or moved to warmer conditions for pre-germination before planting. Germinating the seeds in ideal conditions before planting into pots or in the field will encourage more expedient and uniform germination and allow for selection of the most viable and healthy seedlings.

Germination container and medium selection

Use moderately shallow, broad containers or trays, at least 7-in (17.8-cm) deep to ensure adequate depth for fast-growing roots. We used plastic storage containers (16.75-in length by 11.88-in width by 7.00-in height [42.5-cm by 30.2-cm by 17.8-cm]) and drilled nine small holes in the bottom of each container to allow for drainage of excess water (Figure C.9). Fill the trays a little more than halfway with moist, but not wet, sand, peat, perlite, vermiculite, or soil, exclusively or in a combination (Bonner 2008). We used a 50:50 sand:perlite mixture (Figure C.10A).

Figure C.9. Plastic storage containers are useful for germinating butternut and hybrid butternut seeds prior to planting. In this example, small holes were drilled in the bottom to allow for drainage. (Photo by A.N. Brennan 2018)

Figure C.10. (A) A germination tray prepared for butternut seeds with a moist germination medium of 50:50 sand:perlite. (B) The seeds are placed on top of the medium lengthwise, on their sides. (C and D) Seeds are then covered with a shallow layer of medium. (Photos by A.N. Brennan 2018)

Preparing seeds for germination

Place the seeds in the substrate-filled trays. Lay each seed on its side, lengthwise (Figure C.10B). Butternuts have hypogeal (underground) germination, so it is important to then cover the seeds with a shallow layer (approximately 1 in [2.5 cm]) of substrate (Figures C.10C and D) (Rink 1990). Make sure there is enough room for the radicle (first seedling root) to emerge and grow downwards until transplanting or outplanting (otherwise, when the radicle reaches the bottom of the container, it will grow horizontally and “tangle” with other roots, making it difficult to extract for planting). Label the container to identify the seed batch and cover it to help retain moisture but still allow a small amount of airflow. We used the loosely fitting lids that came with the storage containers (Figure C.11), though other covers, such as loosely applied plastic wrap or tightly fitting lids with small holes drilled into them, could also be used.

Figure C.11. Covered germination trays of butternut seeds in a growth chamber. (Photo by A.N. Brennan 2018)

Germination conditions

Place the seed trays into warm conditions (68 °F [20 °C] up to 86 °F [30 °C]) (Bonner 2008, Young and Young 1992). Light is optional for germination of *Juglans* species (Bonner 2008, Young and Young 1992). A greenhouse or growth chamber is ideal for providing warm, consistent temperatures, but if neither of these is available, germination heating mats can be used. These mats take up a small amount of space and are relatively inexpensive and easy to obtain from online vendors. *Juglans* seeds can also be germinated at room temperature, although it will take longer and may not be as uniform. In our project, we placed the seed trays into growth chambers (Figure C.11) with 8 hours of 86 °F (30 °C) day temperature alternated with 16 hours of 68 °F (20 °C) nighttime temperature (Bonner 2008). No light was used.

Check the germination containers every 4 days to ensure a consistently moist, but not wet, medium; add water as needed. At the same time, monitor for germination and fungal growth (the bleach sanitation method described previously will help prevent this). If serious fungal growth occurs, consider discarding the affected seeds or try treating them with a hydrogen peroxide solution (Fraedrich and Cram 2012). A general fungicide is also an option but could negatively impact germination (Fraedrich and Cram 2012).

Germination

Seeds begin to germinate by cracking open at the main seam along the length of the shell. Soon afterwards, the radicle emerges from the crack (Figures C.12A-C) followed by the hypocotyl hook (curved stem that breaks through the surface of the growing medium) (Figure C.12D). The hook will straighten so that the epicotyl (terminal shoot) is on top (Figure C.12E). Seeds from the same family tend to germinate at a similar time, though there can be some variation in developmental speed (Figure C.12F).

In our project, germination was first observed after 14 days (late March). Generally, 50 to 80 days are required for the majority of seeds to germinate and a germination rate of about 65 percent is expected (Bonner 2008, Young and Young 1992). Our method, however, resulted in a majority of seeds (64 percent) germinating by 17 days and 86.6 percent germinated within 45 days (Table C.1).

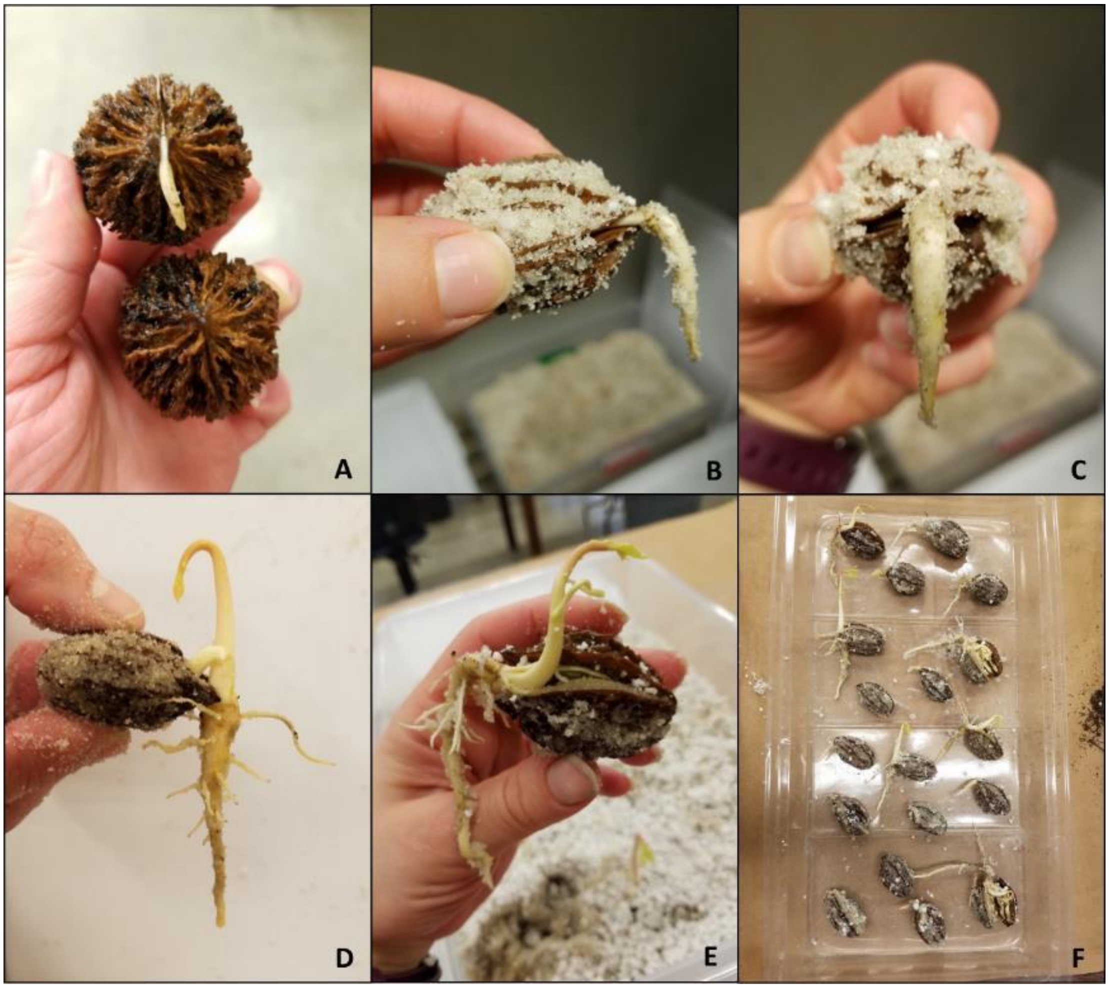

Figure C.12. Germination of pure and hybrid butternut seeds begins with (A) a crack along the seam of the shell (B and C) from which the radicle will emerge. After the radicle emerges, (D) the hypocotyl hook will push out of the seed and the growing medium. (E) Eventually, the hypocotyl will straighten so that the epicotyl is pointing upwards. While related seeds will tend to germinate at a similar time, there is still some variation, (F) which can be seen by the different developmental stages of seeds of the same family. (Photos by A.N. Brennan 2018)

Step 3: Planting the seedlings

Planting in the field

Once the radicle is visible, germinated seeds can be carefully removed and planted directly in the ground or into pots. If planting directly in the ground, well-drained, rich loamy soils are ideal for butternut, but the species may also tolerate rocky, dry soils (Cogliastro et al. 1997, Rink 1990). Butternuts are shade-intolerant and must be planted in full sun (Rink 1990). Care must also be taken to protect the young seedlings from herbivore damage (particularly deer) by using fencing or tree shelters (Woeste et al. 2009). Once butternut seedlings are planted in the field, they generally require very little maintenance as long as the previously listed conditions are met. If the seedlings are planted on a particularly dry site or during a dry year, it is advisable to check if additional watering is required every few weeks during the first year of establishment.

Planting in pots

If planting butternut germinants into pots, start with 1-gal (3.8 L) or larger tree pots. We use TP414 "Tall One" pots (Stuewe & Sons, Inc., Corvallis, OR). Depending on individual growth rates, the seedlings may need to be transplanted into larger pots later in the growing season. Fill the pots with a coarse, well-draining medium that is predominantly bark and/or coir mixed with peat, perlite, and/or vermiculite, and a wetting agent. We used Metro-Mix 560 (Sun Gro Horticulture Distribution, Inc., Agawam, MA) for our project.

Plant the pre-germinated seedlings, radicle pointed down, about 1- to 2-in (2.5- to 5-cm) deep (Bonner 2008), so that the medium just lightly covers the seed shell (Figure C.13). A layer of vermiculite or perlite can also be added to the top of the pots to help retain moisture and prevent weed growth. Immediately after planting the germinated seeds, water well with unfertilized water (until water drains out the bottom).

Figure C.13. Germinated butternut seeds, not yet covered, placed in pots (bottom right corner) and seeds that have already been covered with a shallow layer of potting mix (top left corner with red markers). (Photo by A.N. Brennan, 2018)

Place the pots into a rack or other support structure (such as milk crates or inverted cow panels on supports) that will keep the long, narrow tree pots in an upright position. For our project, pots were placed in a greenhouse on a metal grid supported by a wooden frame and legs (Figure C.14). Butternut and butternut hybrid seedlings grow very quickly (Figure C.15), so will need to be spaced apart as they grow to accommodate the vigorous growth.

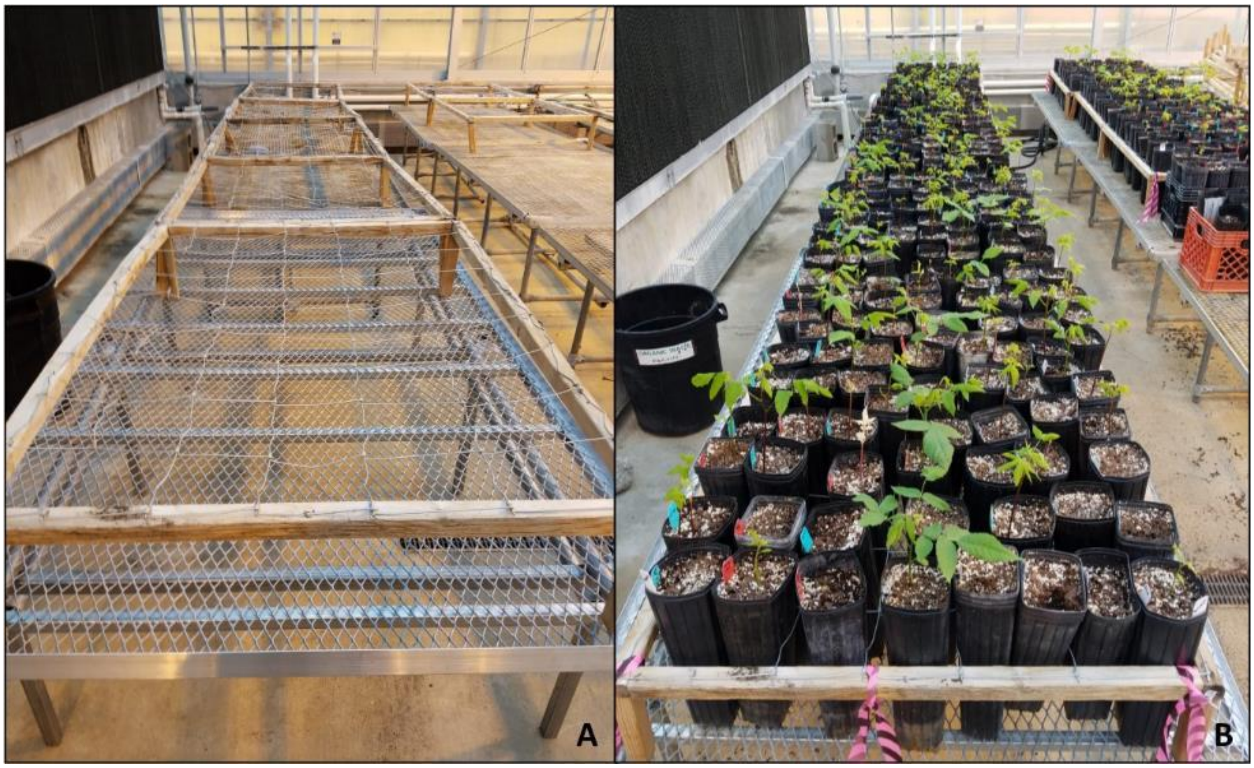

Figure C.14. (A) Metal grids supported by a wooden frame and legs were used to support (B) containers of butternut and hybrid butternut seedlings. The seedlings were spaced more widely as they grew. (Photos by A. N. Brennan, 2018)

Figure C.15. Butternut seedlings, (A) 1-week post-germination and (B) 6-weeks post-germination. (Photos by A. N. Brennan, 2018)

Step 4: Culturing seedlings in pots during the first growing season

Irrigation

Allow the medium to dry out somewhat, but not completely, between watering sessions. For our project, plants were watered when the medium turned from dark brown/nearly black (freshly watered) to light brown and felt dry below the top 1 to 2 in (2.5 to 5 cm). Regularly monitor the top few inches of the medium and check the moisture level from the bottom of the pots. Monitoring moisture levels is especially important until a deeper root system develops beyond the first few inches of growing medium. It is also important not to overwater, which can encourage damping-off. This fungal disease, especially prevalent in seedlings, causes the base of the stem to rot and the seedling to collapse (James 2012).

Fertilization

Once seedlings have grown their first two or three true leaves, begin fertilizing them once a week. For field-grown hardwood seedlings, fertilization can be beneficial, but is not required for survival (Jacobs et al. 2005). If growing the seedlings in pots, however, fertilization is important due to the closed nature of the growth system. Pay special attention to the amount of nitrogen added. At least once monthly, irrigate beyond field capacity with clear water to rinse the substrate, thereby preventing salinization buildup.

Since there is currently no literature describing fertilizer regimes for butternut or hybrid butternut, we used the recommended nitrogen rate (luxury consumption point) for the closely related black walnut (Nicodemus et al. 2008) which is 1,200 mg N/seedling by the end of the growing season. The fertilizer concentration in our greenhouse fertigation water was 150 mg N/L. Thus, to apply 1,200 mg N/seedling by the end of the growing season, we needed to apply a total of 8 L (or 8,000 ml) fertigation per plant. By dividing the total fertigation needed by the 22 weeks in the growing period (May to September), we determined that the application rate should be 365 mL of fertigation water per seedling each week.

Conclusion

Using our seed propagation methods, we found that overall, pure and hybrid seeds were both able to germinate quickly and uniformly. Eighty-six percent of the seeds germinated in 45 days; however, 64 percent had germinated by day 17, illustrating that this method can be used to germinate a majority of the seeds in just over 2 weeks. Our methods were also successful in producing strong, healthy seedlings, with all surviving through their first growing season (the duration of the project). The methods implemented in our project were designed specifically for our own research efforts, but additional methods were also provided to allow the protocol to be versatile for a variety of purposes and scales. This protocol is a valuable tool for butternut land managers and researchers wishing to use genetically diverse, seed-propagated material, while also supporting efforts to evaluate the suitability of hybrid butternuts as an alternative to the pure species.

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APPENDIX D. SUPPLEMENTARY DATA FOR CHAPTER 4

Table D.1. *Juglans* plant material used in the whole-plant freeze test by species, family (accession number), orchard where seeds were obtained from, provenance (state where the original mother tree scion or seed material was collected from), USDA Plant Hardiness Zone of the provenance, and number of seedlings.

Species	Orchard[1]	Family	Provenance	Zone	Seedlings (no.)
J. cinerea	HTRIC (Walla Walla, WA)	719	WI	4	15
J. cinerea	HTRIC (West Lafayette, IN)	856	MI	5	16
J. cinerea	HTRIC (West Lafayette, IN)	968	KY	6	16
J. cinerea	HTRIC (Walla Walla, WA)	1083	IN	6	15
J. cinerea	HTRIC (Huntingburg, IN)	1090	IN	6	16
J. cinerea	NGCR (Corvallis, OR)	PI 666982	MI	5	16
J. cinerea	NGCR (Corvallis, OR)	PI 666983	NY	4	15
J. cinerea	NGCR (Corvallis, OR)	PI 666997	WV	6	15
J. × bixbyi	HTRIC (Plymouth, IN)	212	IN	5	16
J. × bixbyi	HTRIC (West Lafayette, IN)	222	WI	4	17
J. × bixbyi	HTRIC (Vera, MO)	696	MO	6	35
J. × bixbyi	HTRIC (Plymouth, IN)	701	IN	5	15
J. × bixbyi	HTRIC (West Lafayette, IN)	1000	IN	6	16
J. × bixbyi	HTRIC (West Lafayette, IN)	1093	MI	6	16

[1]*HTIRC = Hardwood Tree Improvement and Regeneration Center; NCGR = National Clonal Germplasm Repository*

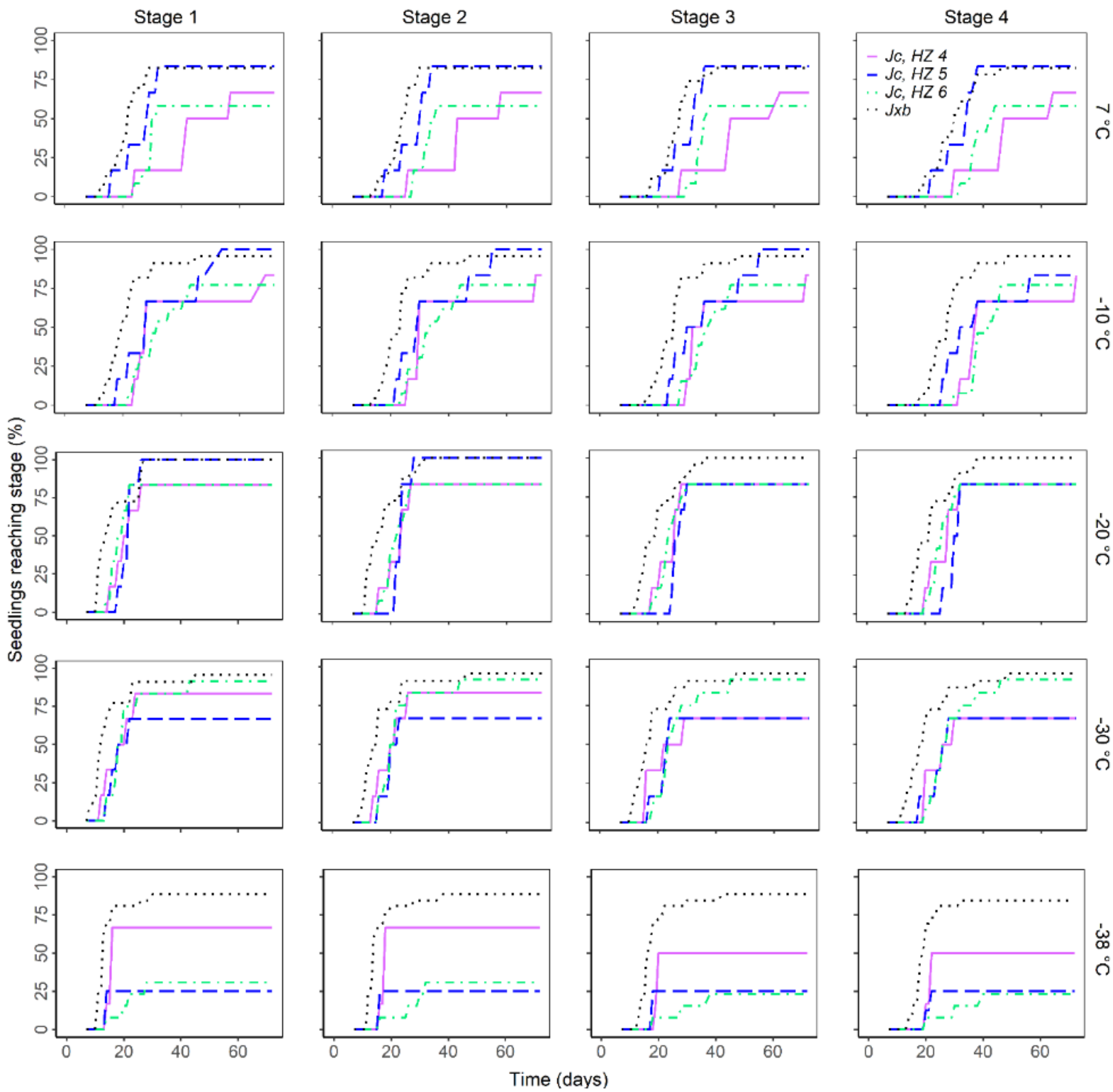

Figure D.1. Four bud break stages of seedlings of *Juglans cinerea* (*Jc*) from different USDA hardiness zones (HZ) and its hybrid, *Juglans* × *bixbyi* (*Jxb*), after exposure to five cold treatments.

APPENDIX E. INDIANA LAND MANAGEMENT ORGANIZATIONS AND PROFESSIONAL GROUPS REACHED BY SURVEY INVITATION FOR CHAPTER 5

- Contacted directly through publicly available email addresses:
  - District foresters (listed through Indiana Department of Natural Resources)
  - Consulting foresters (listed through Indiana Forestry and Woodland Owners Association)
  - Certified foresters (listed through Society of American Foresters)
  - United States Forest Service
  - Natural Resources Conservation Service
  - Indiana Department of Transportation
  - Land trusts (identified through the Land Trust Alliance)
    - ACRES Land Trust
    - Central Indiana Land Trust
    - Clear Lake Township Land Conservancy
    - Friends of the Panhandle Pathway, Inc.
    - George Rogers Clark Land Trust
    - La Porte County Conservation Trust
    - Little River Wetlands Project
    - Mud Creek Conservancy, Inc.
    - Oak Heritage Conservancy
    - Openlands
    - Ouabache Land Conservancy
    - Oxbow, Inc.
    - Red-tail Land Conservancy
    - Shirley Heinze Land Trust, Inc.
    - Sycamore Land Trust
    - The Hillside Trust
    - Three Valley Conservation Trust
    - Wawasee Area Conservancy Foundation
    - Wood-Land-Lakes Resource Conservation and Development
    - Woodland Savanna Land Conservancy
    - Ducks Unlimited, Inc.
    - The Conservation Fund
    - The Nature Conservancy
  - Indiana Forest Alliance
  - Indiana Hardwood Lumbermen's Association
  - Indiana Woodland Steward
  - National Association of Conservation Districts - Forest Resource Policy Group Board
  - Pheasants Forever – Indiana chapters
  - Ducks Unlimited – Indiana contacts
  - The Walnut Council – Indiana contacts

    - Indiana Tree Farm
    - The American Chestnut Foundation - Indiana Chapter
    - Keep Indianapolis Beautiful

- Contacted indirectly through leadership or directly with leadership permission:
    - Indiana Department of Natural Resources (IDNR) staff
    - IDNR Community and Urban Forestry Program participants
    - Society of American Foresters – Indiana chapter
    - Indiana Arborist Association
    - Indiana Parks and Recreation Association
    - Association of Landscape Architects – Indiana chapter
    - Indiana Nursery and Landscape Association

APPENDIX F. SURVEY PROTOCOL FOR CHAPTER 5

Perceptions of land managers to the use of hybrid and genetically modified trees

Introduction

Thank you for taking the time to help us with this survey research study. This study examines how land managers (professionals involved with tree selection, distribution, and planting) perceive hybrid, genetically modified, and at-risk tree species. We are seeking your feedback about these issues through a research survey. Your feedback will contribute to understanding the perceptions and concerns of land managers to help target restoration efforts.

The survey is divided into five sections about 1) your work, 2) hybrid plants, 3) genetically modified plants, 4) at-risk plants, and 5) general questions about yourself. When you answer the survey questions, please use the definitions below for the following terms:

- **Hybrid:** Offspring resulting from the cross of two *different species*
- **Genetic swamping (excessive introgression)**: When genetic material transfers from one species to another over time through hybrids and results in one species dominating over the other (ex: Chinese bittersweet over American bittersweet growing in the US)
- **Genetic modification:** Adding, removing, or changing specific genes through biotechnology
- **At-risk species**: A species at risk of being lost from the landscape, due to threats such as exotic pests or pathogens, climate change, and habitat loss
- **Non-native:** Not native to the region prior to European settlement

Section 1 of 5: General Information about Your Work

To begin, this is a general section that will allow us to understand how trees are involved with your work.

1. ***Screening Question/Skip Logic:*** This survey is targeted towards Indiana land managers, defined here as professionals and/or volunteers involved with tree selection, sale/distribution, management, and/or planting of trees in Indiana. Using this definition, do you consider yourself a land manager? (Select one.)
    a. Yes -> *continues to rest of survey*
    b. No -> End of survey, thank you for your time.

Section 1 of 5: General Information about Your Work (second page)

2. What type of organization do you *primarily* work or volunteer for in your tree work capacity? (Select one.)
    a. Federal government
    b. State government
    c. Local government
    d. Non-profit

   e. For-profit
   f. University/educational
   g. Other (please specify):______________

3. How long have you been with your current, primary organization? (Enter number of years; round to the nearest year.)
   a. _____________ years

4. Who are your clients in your current, primary organization? (Select all that apply.)
   a. General public
   b. Homeowners
   c. Private landowners
   d. Green industry (nursery growers, landscapers, landscape architects/designers, etc.)
   e. Other land managers
   f. Government
   g. Other (please specify):______________

5. Do you consider yourself an urban or a natural land manager? (Select one.)
   a. Mostly a natural land manager
   b. Mostly an urban land manager
   c. About equally a natural and urban land manager

6. Please rank the top 1-3 primary purposes of your tree-related work. Type "1" next to your first choice, "2" next to your second choice (if applicable), and "3" next to your third choice (if applicable).
   a. Timber production
   b. Non-timber production (fruit, nuts, syrup, etc.)
   c. Nursery production
   d. Aesthetic
   e. Recreation
   f. Promoting wildlife/habitat creation
   g. Restoration and reforestation
   h. Conservation of at-risk species (plant or animal)
   i. Regulating ecosystem services (storm water management, erosion control, carbon storage, clean air, etc.)
   j. Promoting biodiversity
   k. Green infrastructure and managed landscapes
   l. Communicating and providing advice on tree and forest management policies and programs
   m. Other (please specify):______________

<u>Section 2 of 5: Your Thoughts on Hybrid Plants</u>
Now we would like to ask you a few questions to know your thoughts on ***hybrid plants*** (offspring resulting from the cross of two different species).

7. Do you use **hybrid trees** in your plantings or planting recommendations? (Select one.)
    a. Yes (display logic)
        i. 7b. What are the **<u>top 1-3</u> hybrid tree species** you have used?
            1. 1st highest used hybrid tree species:______________
            2. 2nd highest used hybrid tree species (if applicable):______________
            3. 3rd highest used hybrid tree species (if applicable):______________
    b. No
    c. I don't know

8. The following is a list of items that have been discussed in the literature as potential impacts of hybrid plants. Please indicate your level of concern for each of the following items in regards to **hybrid plants** (Likert scale: Not concerned, Moderately concerned, Very concerned, or Don't know):
    a. Changes induced in local ecosystems
    b. Genetic swamping of native population (excessive introgression)
    c. Potential for invasiveness (having too much advantage over native species and outcompeting them)
    d. Negative effects on wildlife (ex: decreasing food or habitat availability)

9. The following is a list of potential issues with obtaining hybrid plants for your plantings. Please indicate your level of concern for each of the following items in regards to **hybrid plants** (Likert scale: Not concerned, Moderately concerned, Very concerned, Don't know):
    a. Low availability
    b. Lack of performance knowledge (insufficient information available on how the hybrid will perform in different conditions and sites)
    c. Expensive to produce and buy

10. Please indicate your level of agreement or disagreement that each of the following items is a potential benefit of **hybrid plants** (Likert scale: Strongly disagree, Disagree, Neither agree nor disagree, Agree, Strongly agree):
    a. Accelerating the tree improvement process
    b. Better resource-use efficiency
    c. Greater site suitability and functionality
    d. Decreased need for pesticide applications
    e. Greater aesthetic value
    f. Greater stress and pest resistance
    g. Improved tree growth and production
    h. Saving at-risk species (plant or animal)
    i. Supporting wildlife
    j. Helping solve problems with food supply

11. Imagine two different **hybrid plants**: (1) the first hybrid plant resulted from a cross of a native species with another native species, and (2) the second hybrid plant resulted from a cross of a native species with a non-native species. If given the choice, which of the two types of hybrid plants would you use in restoration? (Select one.)
    a. Prefer to use first hybrid plant (native/native hybrid)
    b. Prefer to use second hybrid plant (native/non-native hybrid)
    c. Would use either
    d. Would not use either
    e. Don't know

<u>Section 3 of 5: Your Thoughts on Genetically Modified Plants</u>

This next set of questions will focus on ***genetically modified plants*** (plants with specific genes added, removed, or changed through biotechnology). Note that with the exception of three fruit tree varieties, current US federal regulations do not allow genetically modified *trees* to be planted outside of APHIS-approved field trials where genetically modified tree species and varieties are currently being evaluated.

12. The following is a list of items that have been discussed in the literature as potential impacts of **genetically modified plants**. Please indicate your level of concern for each of the following items in regards to genetically modified plants (Likert scale: Not concerned, Moderately concerned, Very concerned, or Don't know):
    a. Changes induced in local ecosystems
    b. Genetic swamping of native population (excessive introgression)
    c. Potential for invasiveness (having too much advantage over native species and outcompeting them)
    d. Negative effects on wildlife (ex: decreasing food or habitat availability)
    e. Escape of transgene(s) to non-target areas

13. The following is a list of potential issues with obtaining genetically modified plants for your plantings (if they were legally available to purchase). Please indicate your level of concern for each of the following items in regards to **genetically modified plants** (Likert scale: Not concerned, Moderately concerned, Very concerned, or Don't know):
    a. Lack of availability
    b. Lack of performance knowledge (insufficient information available on how the hybrid will perform in different conditions and sites)
    c. Expensive to produce and buy

14. Please indicate your level of agreement or disagreement that each of the following items is a potential benefit of **genetically modified plants** (Likert scale: Strongly disagree, Disagree, Neither agree nor disagree, Agree, Strongly agree):
    a. Accelerating the tree improvement process
    b. Better resource-use efficiency
    c. Greater site suitability and functionality
    d. Decreased need for pesticide applications
    e. Greater aesthetic value

f. Greater stress and pest resistance
g. Improved tree growth and production
h. Saving at-risk species (plant or animal)
i. Supporting wildlife
j. Helping solve problems with food supply
k. Herbicide tolerance

15. If a native species was genetically modified, would you still consider it a native species? (Select one.)
    a. Yes
    b. No
    c. I don’t know

16. Hypothetically, how do you think about using **hybrid versus genetically modified trees**? (Select one.)
    a. I have more concerns with the use of hybrid trees than the use of genetically modified trees.
    b. I have more concerns with the use of genetically modified trees than the use of hybrid trees.
    c. I have similar levels of concerns with the use of both hybrid and genetically modified trees.
    d. I don’t know.

Section 4 of 5: Your Thoughts on At-Risk Trees

Here we would like to ask you what you think about ***at-risk tree species*** (those at risk of being lost from the landscape) and how you might use them in your work.

17. Do you use **at-risk tree species** in your plantings or planting recommendations? (Select one.)
    a. Yes (display logic)
        i. 17b. What are the **top 1-3** **at-risk tree species** you have used?
            1. 1st highest used at-risk tree species:______________
            2. 2nd highest used at-risk tree species (if applicable):______________
            3. 3rd highest used at-risk tree species (if applicable):______________
    b. No
    c. I don’t know

18. What do you think about using **hybrid trees** for restoring **at-risk tree species**? (Select one.)
    a. I think it is always appropriate to do this
    b. I can imagine some cases when this is appropriate
    c. I think it is never appropriate to do this
    d. I don’t know

19. Please indicate your level of agreement or disagreement with using ***hybrid trees*** for each of the following purposes (Likert scale table: Strongly disagree, Disagree, Neither agree nor disagree, Agree, Strongly agree):
    a. Timber production
    b. Non-timber production (fruit, nuts, syrup, etc.)
    c. Aesthetic value
    d. Recreation
    e. Promoting wildlife/habitat creation
    f. Reforestation and restoration of forests
    g. Conservation and restoration of at-risk species (plant or animal)
    h. Regulating ecosystem services (storm water management, erosion control, carbon storage, clean air, etc.)
    i. Promoting biodiversity
    j. Green infrastructure and managed landscapes

20. What do you think about using **genetically modified trees** for restoring **at-risk tree species**? (Select one; display logic for the "maybe" response.)
    a. I think it is always appropriate to do this
    b. I can imagine some cases when this is appropriate
    c. I think it is never appropriate to do this
    d. I don't know

21. Please indicate your level of agreement or disagreement with using ***genetically modified trees*** for each of the following purposes (Likert scale table: Strongly disagree, Disagree, Neither agree nor disagree, Agree, Strongly agree):
    a. Timber production
    b. Non-timber production (fruit, nuts, syrup, etc.)
    c. Aesthetic value
    d. Recreation
    e. Promoting wildlife/habitat creation
    f. Reforestation and restoration of forests
    g. Conservation and restoration of at-risk species (plant or animal)
    h. Regulating ecosystem services (storm water management, erosion control, carbon storage, clean air, etc.)
    i. Promoting biodiversity
    j. Green infrastructure and managed landscapes

Section 5 of 5: General Questions about You

In this last question, we would like to ask some general questions about you.

22. What county do you live in? (Dropdown menu; select one.)
    a. [Dropdown list of 92 Indiana counties, plus "Don't live in Indiana" and "Prefer not to answer"]

23. What is your age? (Enter number of years.)
    a. ____________

24. What is your gender? (Select one.)
    a. Male
    b. Female
    c. Other
    d. Prefer not to answer

25. What is the highest level of education you have completed? (Select one.)
    a. Did not graduate high school
    b. High school graduate or equivalent
    c. Some college, no degree
    d. Associate's degree
    e. Bachelor's degree
    f. Graduate degree

Conclusion

26. (Optional.) Before we end, is there anything else that you would like to share regarding the use of hybrid or genetically modified trees for restoring at-risk species? (Free response).

____

Your responses have been recorded. We thank you for your time spent taking the survey.

If you would like to receive a copy of the final report for this study, please follow this link to be taken to a separate page to sign up.

---SEPARATE SURVEY SO MAIN SURVEY RESPONSES ARE NOT TIED TO EMAIL ADDRESS---

If you would like to receive a copy of the **final report** for this study, please enter your preferred **email address** here. (Your email address will *only* be used for sending you the report and will *not* be connected to your survey responses.)

____

APPENDIX G: SUPPLEMENTARY DATA FOR CHAPTER 5

Table G.1. Ranking of work purposes of Indiana land manager survey respondents (n=273).

Work purpose	No. times ranked #1	% Ranked No. 1	No. times in top 3
Timber	50	18.3	88
Non-timber production	2	0.7	9
Aesthetic	27	9.9	80
Recreation	14	5.1	59
Promoting wildlife/habitat creation	46	16.9	149
Restoration and reforestation	40	14.7	111
Conservation of at-risk species and communities	9	3.3	46
Regulating ecosystem services	14	5.1	48
Promoting biodiversity	15	5.5	70
Green infrastructure and managed landscapes	22	8.1	64
Communicating and providing advice on tree/ forest management policies and programs	28	10.3	50
Nursery production	6	2.2	12

Table G.2. Land manager characteristics associated with a direct concern comparison of hybrid versus GM trees (n=259). (Possible responses: "similar levels of concern for both hybrid and GM trees", "more concern with hybrid trees, more concern with GM trees", and "don't know".)

Land manager characteristic	Statistical values	Relationship
Age	$\chi^2 = 13.92$; $p = 0.030$	Younger respondents (23-40 years old) held more concern about GM trees, while those in the older age groups (41-59 and 60-78 years old) held more concern for GM trees.
Gender	$\chi^2 = 1.017$; $p = 0.797$	NS
Education	$\chi^2 = 14.025$; $p = 0.029$	Those with more education (bachelor's or graduate degree) more often held similar levels of concern for both tree types, while those with less education (high school degree, some college, or an associate's degree) were more often concerned about either hybrid or GM trees specifically.
Region of residency in Indiana	$\chi^2 = 2.371$; $p = 0.499$	NS
Organization type	Fisher's exact $p = 0.480$	NS
Land type managed	Fisher's exact $p = 0.566$	NS
Agreement with tree improvement advantages-hybrid trees	Fisher's exact $p = 0.666$	NS
Agreement with tree improvement advantages-GM trees	Fisher's exact $p = 0.079$	NS
Level of ecological concern-hybrid trees	Fisher's exact $p = 0.913$	NS
Level of ecological concern-GM trees	Fisher's exact $p = 0.004$	Those having less ecological concern about GM trees were also less concerned about GM trees compared to hybrid trees.
Level of economic concern-hybrid trees	Fisher's exact $p = 0.710$	NS
Level of economic concern-GM trees	Fisher's exact $p = 0.389$	NS

"NS" indicates a statistically nonsignificant relationship.

Table G.3. Land manager characteristics associated with their hybrid type preference for restoration (n=271). (Possible responses: "native species × native species", "native × non-native species", "either type", "neither type", and "don't know".)

Land manager characteristic	Statistical values	Relationship
Age	Fisher's exact $p = 0.026$	Land managers in the oldest age bracket (60-78 years old) preferred either type of hybrid more than those in the younger age brackets (23-40 and 41-59 years old).
Gender	Fisher's exact $p = 0.006$	More females preferred neither type of hybrid than males.
Education	Fisher's exact $p = 0.053$	NS
Region of residency in Indiana	$\chi^2 = 1.071$; $p = 0.784$	NS
Organization type	Fisher's exact $p = 0.277$	NS
Land type managed	Fisher's exact $p = 0.705$	NS
Agreement with tree improvement advantages-hybrid trees	Fisher's exact $p < 0.0001$	Those who strongly disagreed or disagreed with the tree improvement advantages of hybrid trees preferred neither type of hybrid more than those who agreed or strongly agreed.
Ecological concern-hybrid trees	Fisher's exact $p = 0.002$	The greater the ecological concern held, the more neither type of hybrid was preferred; the less ecological concern held, the more either type of hybrid was preferred.
Economic concern-hybrid trees	Fisher's exact $p = 0.060$	NS

"NS" indicates a statistically nonsignificant relationship.

Table G.4. Hybrid tree species that survey respondents reported using on the lands they manage in Indiana. The % of respondents using a specific hybrid species was calculated based on the total number of respondents who indicated that they use hybrids in their plantings (n=79).

Scientific name	Common name	% of respondents using species
Acer × spp	Maple hybrids	33
A. × *freemanii*	Freeman maple	11
A. × *freemanii* 'Jeffersred'	Autumn Blaze maple	3
A. truncatum × *A. platanoides*	Shangtung maple × Norway maple	1
Aesculus × *carnea* 'Briotii'	Briotii red horse chestnut	1
Amelanchier × spp	Serviceberry hybrids	4
A. × *grandiflora* 'Autumn Brilliance'	Autumn Brilliance serviceberry	3
Betula × spp	Birch hybrids	1
Castanea × spp	Chestnut hybrids	20
C. dentata × spp	American chestnut hybrids	3
Corylus × spp	Hazelnut hybrids	1
Diospyros × spp	Persimmon hybrids	1
Fagus × spp	Beech hybrids	1
Fraxinus × spp	Ash hybrids	3
Gleditsia × spp	Honeylocust hybrids	4
Juglans × spp	Walnut hybrids	25
J. × *bixbyi*	Butternut x Japanese walnut	16
J. nigra × spp	Black walnut hybrids	3
Magnolia × spp	Magnolia hybrids	4
M. × *soulangeana*	Saucer magnolia	3
Malus × spp	Apple hybrids	4
Pinus rigida × *taeda*	Pitch pine x loblolly pine	4
Platanus × *acerifolia*	London planetree	16
P. × *acerifolia* 'Morton Circle'	Exclamation London planetree	4
Populus × spp	Poplar hybrids	5
Prunus × spp	Cherry hybrids	3
Pyrus × spp	Pear hybrids	1
Quercus × spp	Oak hybrids	16
Section *Quercus* spp	White oak group hybrids	5
Q. bicolor × *robur*	Swamp white oak × English oak	1
Q. robur × *Q. alba* 'Crimschmidt'	Crimson Spire oak	1
Section *Lobatae* spp	Red oak group hybrids	3
Salix × spp	Willow hybrids	1
Thuja × 'Green Giant'	Green Giant Arborvitae	3
Ulmus × spp	Elm hybrids	32
U. americana × spp	American elm hybrids	3

Scientific name	Common name	% of respondents using species
U. japonica × *U. wilsoniana* 'Morton'	Accolade elm	4
U. parvifolia × spp	Chinese elm hybrids	5
U. parvifolia × *U. minor* 'Frontier'	Frontier elm	5
U. × 'Homestead'	Homestead elm	1
Zelkova × spp	Zelkova hybrids	1

Table G.5. Land manager characteristics associated with perceptions of whether a GM-version of a native species is still native (n=259). (Possible responses: "yes", "no", "I don't know".)

Land manager characteristic	Statistical values	Relationship
Age	$\chi^2 = 3.404$; $p = 0.493$	NS
Gender	$\chi^2 = 1.557$; $p = 0.459$	NS
Education	$\chi^2 = 5.073$; $p = 0.280$	NS
Region of residency in Indiana	$\chi^2 = 3.679$; $p = 0.159$	NS
Organization type	Fisher's exact $p = 0.429$	NS
Land type managed	$\chi^2 = 8.658$; $p = 0.070$	NS
Agreement with tree improvement advantages- GM trees	$\chi^2 = 38.488$; $p < 0.0001$	Those who agreed or strongly agreed with the tree improvement advantages of GM trees were more likely to perceive a GM-version of a native species as native; those who strongly disagreed or disagreed with the advantages were more likely to perceive it as not being native.
Ecological concern- GM trees	Fisher's exact $p < 0.0001$	Those with greater ecological concern about GM trees were more likely to say that a GM-version of a native species is not native; those with less ecological concern were more likely to select that it is not native.
Economic concern- GM trees	$\chi^2 = 3.982$; $p = 0.137$	NS

"NS" indicates a statistically nonsignificant relationship.

Table G.6. At-risk tree species that survey respondents reported using on the lands they manage in Indiana. Official designations of whether a species is at-risk range-wide wide or locally were determined through the IUCN Red List of Threatened Species (IUCN 2019), NatureServe Explorer (NatureServe 2019), USDA Plants (USDA 2019), and Indiana Department of Natural Resources (IDNR 2019). The % of respondents using a specific at-risk species was calculated based on the total number of respondents who indicated that they use at-risk tree species in their plantings (n=58).

Scientific name	Common name	% of respondents using species	Native range	At-risk range-wide?	At-risk locally only?
Acer griseum	Paperbark maple	2	central China	×	
Aesculus hippocastanum	Horse chestnut	2	eastern Europe	×	
Alnus incana subsp. rugosa	Speckled alder	2	Canada, northeastern US		IL, IN
Betula spp.	Birch spp.	3	North America, Europe, Asia		
B. papyrifera	Paper birch	2	North America		IL, IN
Carya spp.	Hickory & pecan spp.	10	North America, Asia		
C. illinoensis	Pecan	5	eastern US		
C. ovata	Shagbark hickory	3	eastern North America		
Castanea dentata	American chestnut	43	eastern US	×	
Castanea ozarkensis	Ozark chinquapin	2	southeastern US	×	
Chionanthus virginicus	White fringetree	2	eastern US		OH
Cladrastis kentukea	Yellowwood	7	North America		IL, IN
Diospyros virginiana	Common persimmon	2	eastern & midwestern US		CT, NY
Fraxinus spp.	Ash species	9	North America, Europe, Asia		
F. americana	White ash	2	eastern & central North America	×	
F. pennsylvanica	Green ash	2	eastern & central North America	×	
Ginkgo biloba	Ginkgo	2	China	×	
Gymnocladus dioicus	Kentucky coffeetree	2	eastern US		NY
Juglans cinerea	Butternut	40	eastern North America	×	
Magnolia acuminata	Cucumber magnolia	2	eastern US		FL, IN
Oxydendrum arboreum	Sourwood	2	eastern US		IN, MD
Pinus banskiana	Jack pine	2	northeastern US, Canada		IL, NH, VT

Scientific name	Common name	% of respondents using species	Native range	At-risk range-wide?	At-risk locally only?
Prunus americana	American plum	2	central & eastern US, southern Canada	×	NH, VT
Quercus spp.	Oak spp.	21	Americas, Asia, Europe, & northern Africa		
Q. alba	White oak	3	eastern US, southeastern Canada		
Q. bicolor	Swamp white oak	3	northeastern US		
Q. palustris	Pin oak	2	eastern US		
Q. imbricaria	Shingle oak	2	eastern US		
Q. montana	Chestnut oak	2	eastern US		
Q. rubra	Northern red oak	2	eastern US, southeastern Canada		
Sassafras albidum	Sassafras	2	eastern US		ME
Taxodium distichum	Bald cypress	2	eastern US		IN
Ulmus americana	American elm	10	eastern US, southeastern Canada	×	

Table G.7. Logistic estimates of the empirical models for estimating use of at-risk trees species by land managers in Indiana. Average marginal effects (AME) and standard error (SE) were used. The reference level for organization type was state government (n=234).

Explanatory variables	AME	SE	*p*-value
Age (greater)	0.001	0.002	0.714
Gender: female	-0.058	0.062	0.345
Education (greater)	0.076	0.064	0.230
Region of Indiana: south	0.019	0.055	0.726
Organization: local government	0.145	0.118	0.220
Organization: for-profit	0.100	0.079	0.210
Organization: non-profit	0.012	0.084	0.884
Organization: university/educational	0.306	0.168	0.068
Land type managed (more urban)	-0.067	0.055	0.228

APPENDIX H: PHOTOS OF RESEARCH

All photos are taken by Andrea Brennan, unless otherwise noted.

Figure H.1. A mature butternut tree, *Juglans cinerea*, in the wild with its characteristic flat-topped silver-ridged bark.

Figure H.2. Butternut seeds (a) still in the husk and (b) with husk removed to expose shell.

Chapter 2: Hybrid breeding for restoration of threatened forest trees: Incorporating disease tolerance in *Juglans cinerea*

Figure H.3. Inoculation of butternut and hybrid butternut trees began with (1) drilling a 6 mm hold through the bark and slightly into the cambium, (2) filling the hole with an agar plug of butternut canker disease fungal inoculum, and (3) taping over the hole. Photo credits: James McKenna.

Figure H.4. Butternut tree attempting to callus over canker wounds (pencil for scale).

Chapter 3: Cold, but not heat, tolerance suggests adaptive limitations for *Juglans cinerea* restoration using hybrids

Figure H.5. Plot of butternut, Japanese walnut (*Juglans ailantifolia*), and their hybrids in Martell Forest, West Lafayette, IN, USA where samples were collected for both relative cold and heat tests.

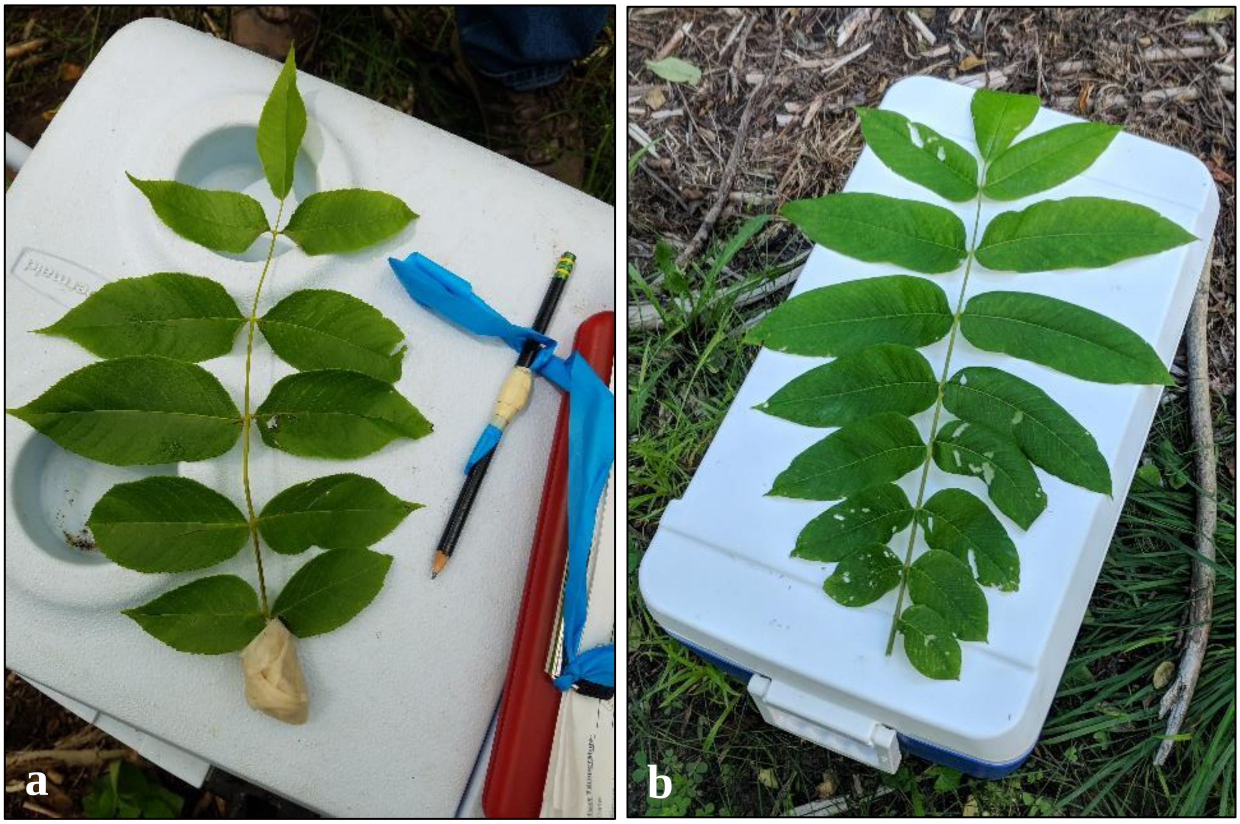

Figure H.6. (a) Butternut and (b) Japanese walnut leaf samples for heat test.

Figure H.7. Leaf sample collection and preparation involved wrapping the petiole base in a wet paper towel and securing the paper towel with a rubber band. The petiole base was then inserted into a small amount of water in the corner of a labeled 2-gal plastic bag. A small amount of warm air was breathed into the bag prior to sealing to provide light air cushioning and support for temporary storage in the cooler. (b) A small binder clip was used to fasten the side corners and provide additional support so the sample would stay upright in the cooler.

Figure H.8. Laboratory bench set-up for heat test from left to right: sample coolers, fluorimeter, leaflet prep areas, sample pole mounts, and post-batch sample processing.

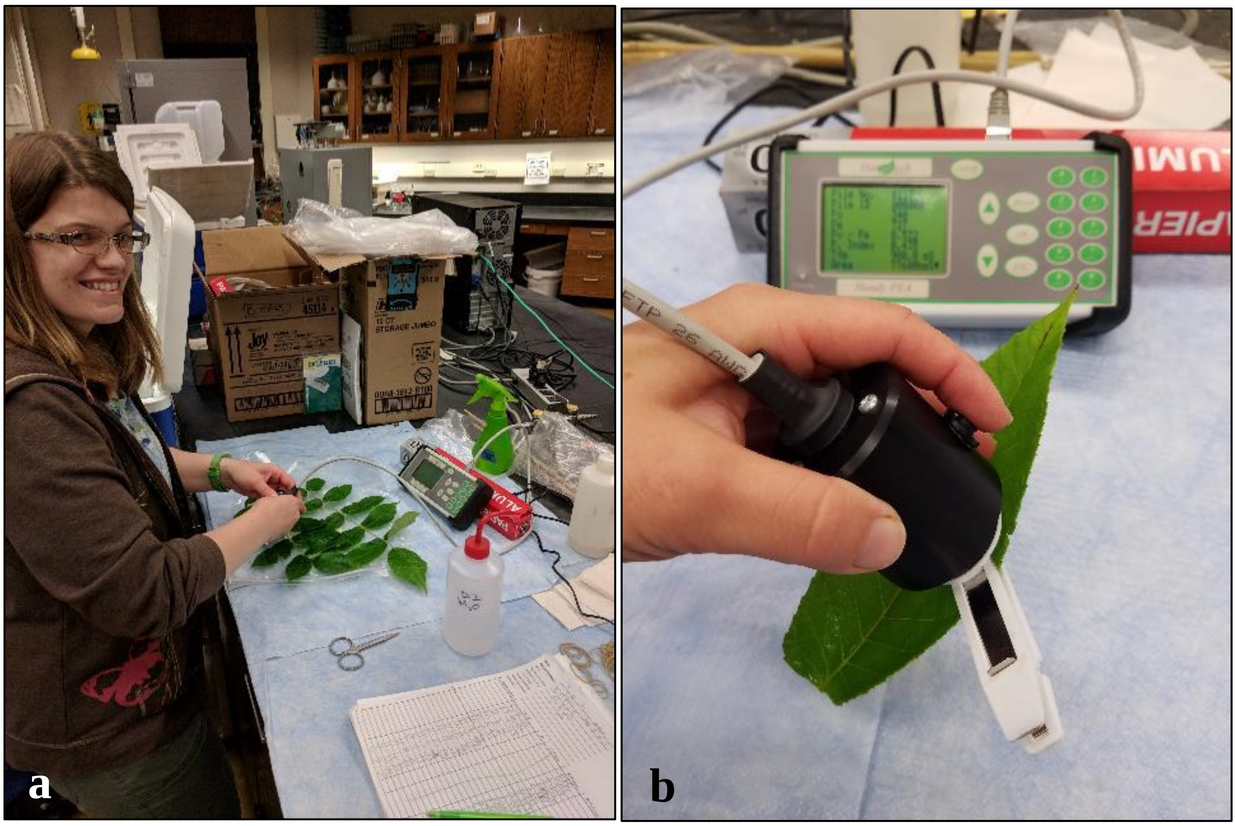

Figure H.9. Taking pre-test chlorophyll fluorescence measurements using a fluorimeter. Photo credit (a): James Warren.

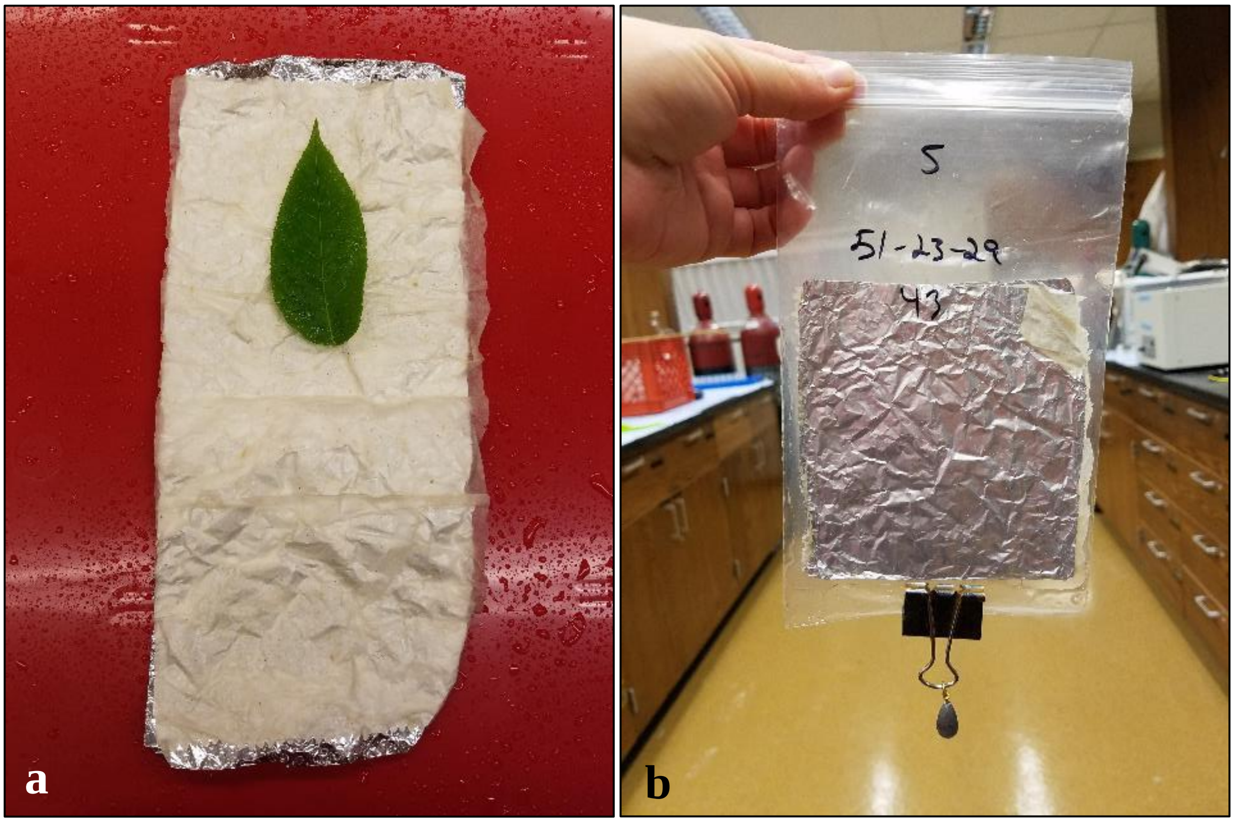

Figure H.10. Sample preparation immediately prior to the heat test involved (a) placing a leaflet on the top half of a moist paper towel and aluminum foil and (b) then folding the bottom half of the paper towel/roil over the top and placing into a labeled plastic bag with the air gently squeezed out and weighted at the bottom with a binder clip and fishing weight.

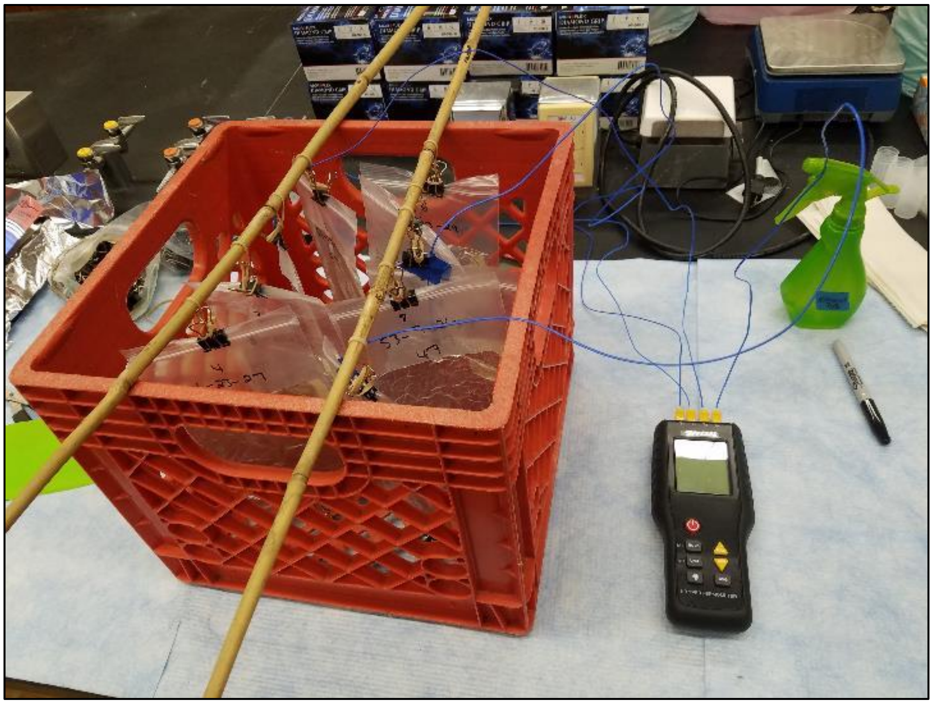

Figure H.11. Prepared sample bags mounted onto bamboo rods with rubber bands. Four samples had thermocouples placed directly with the leaflet prior to sealing into the bag.

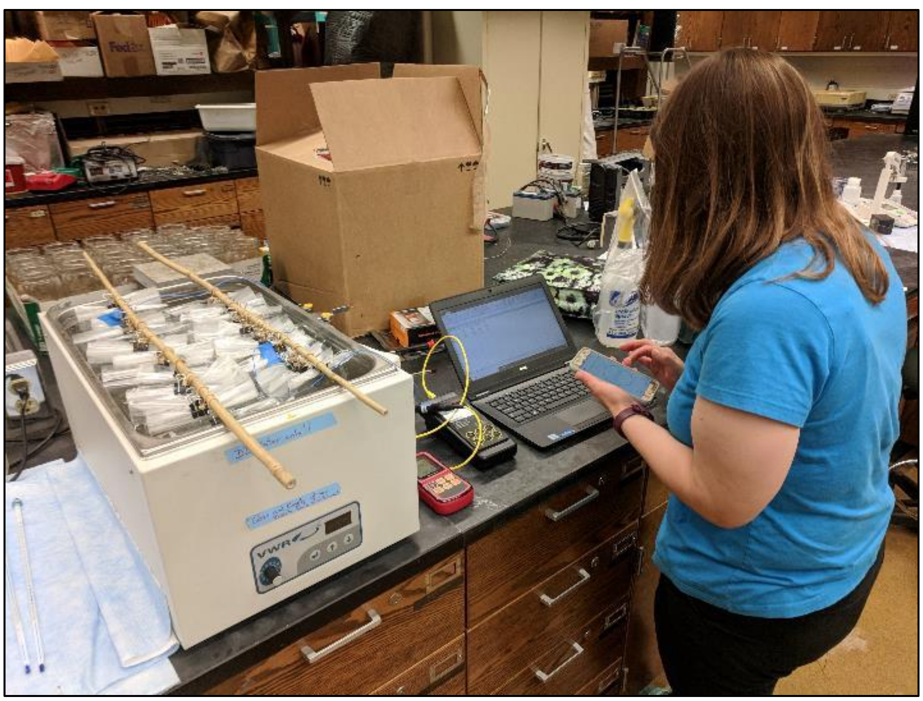

Figure H.12. When the hot water bath reached the target temperature, two sample poles were suspended over the bath. The temperature of the sample and water thermocouples was recorded every 5 minutes.

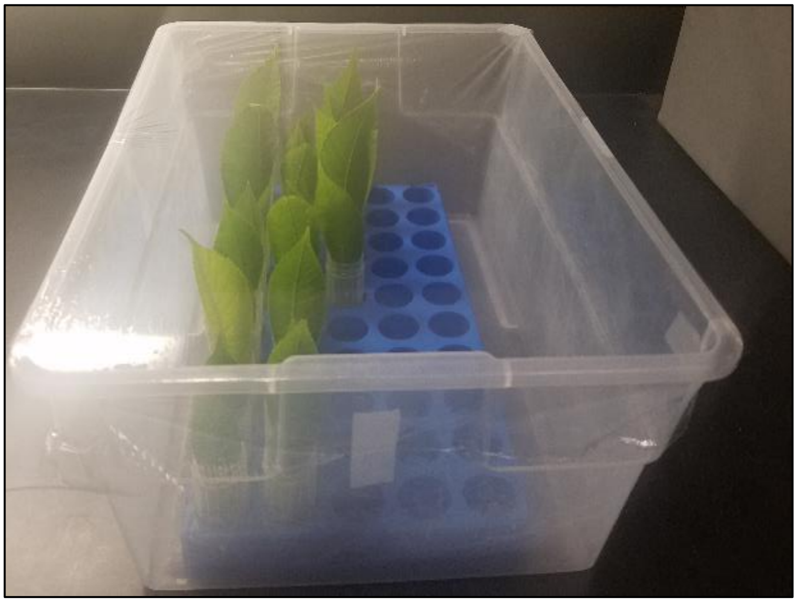

Figure H.13. Following heat treatment, leaflets were removed from the bag and packaging and placed into plastic vials with water at the bottom. The vial racks were then placed into a plastic container sealed with plastic wrap to prevent moisture stress on the samples. Samples were allowed to recover overnight in the dark followed by post-treatment fluorescence measurements.

Figure H.14. Browned leaflets following treatment at the highest temperature (54 °C).

Figure H.15. The bases of dormant twig samples were wrapped in a wet paper towel, which was secured with a rubber band. (a) Twig bases were then placed into the corner of a labeled plastic bag with a small amount of water in the corner. The air was not pressed out of the bag prior to sealing. (b) Sample bags were then placed into a rack to keep them upright inside the cooler.

Figure H.16. An especially impressive example of a single year’s growth from a butternut tree (the horizontal blue bars denote the top and bottom of the twig sample).

Figure H.17. Twig samples were cut into five 1-in. segments, one for each cold treatment.

Figure H.18. Stem segments were then placed into vials of water, sealed with caps. Photo credit (b): James Warren.

Figure H.19. Prepared samples were then placed into a pre-chilled programable freezer with thermocouples and data loggers to receive cold treatments.

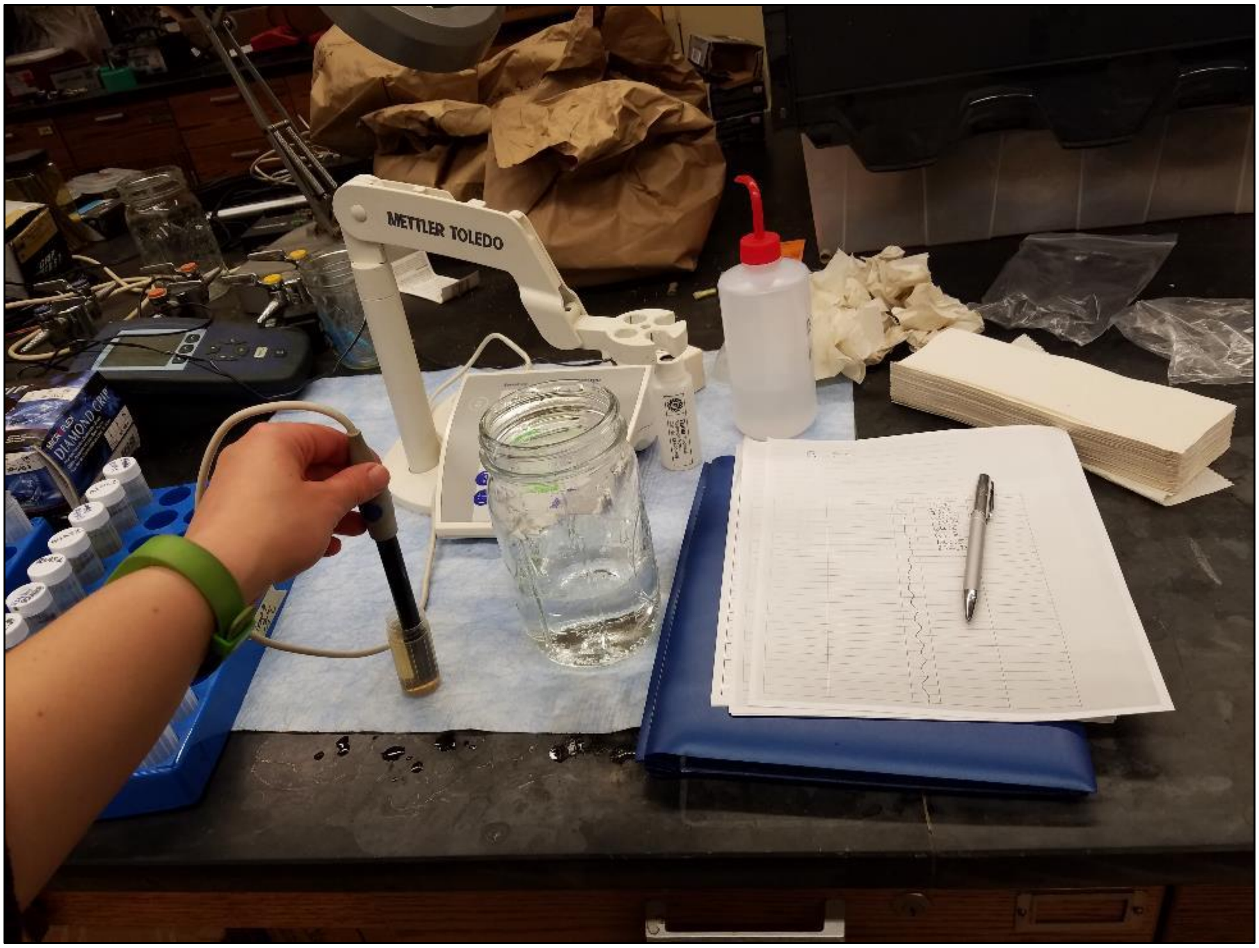

Figure H.20. Electrolyte leakage (EL) into the water was assessed using a conductivity meter after complete thaw and again following autoclaving to induce 100% EL.

Chapter 4: Cold hardiness traits suggest a lack of ecological similarity between a progenitor forest tree species and its hybrids

For additional photos of seed and seedling culture, see Appendix C.

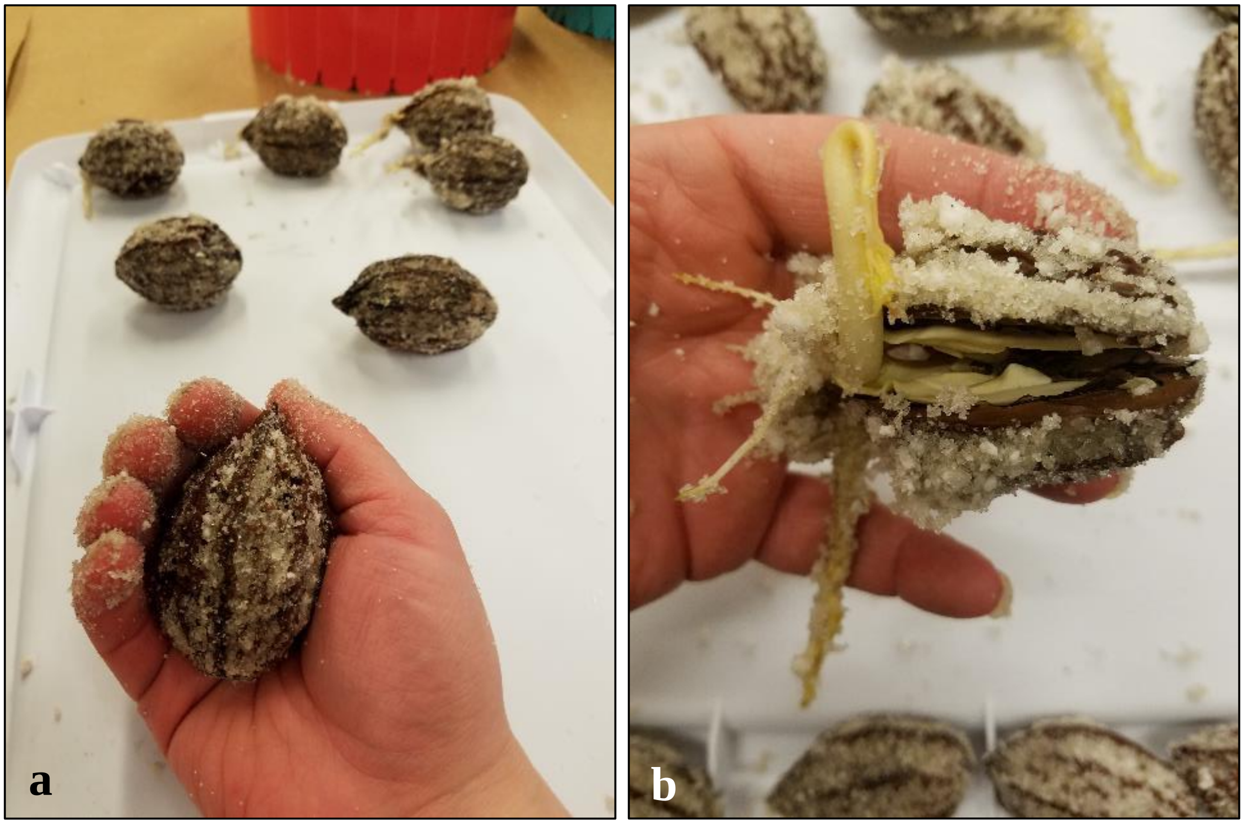

Figure H.21. (a) An exceptionally massive family of butternut seeds germinating for the whole-plant freeze test and (b) an interesting germinant that pushed its shell apart to expose the inside of the seed.

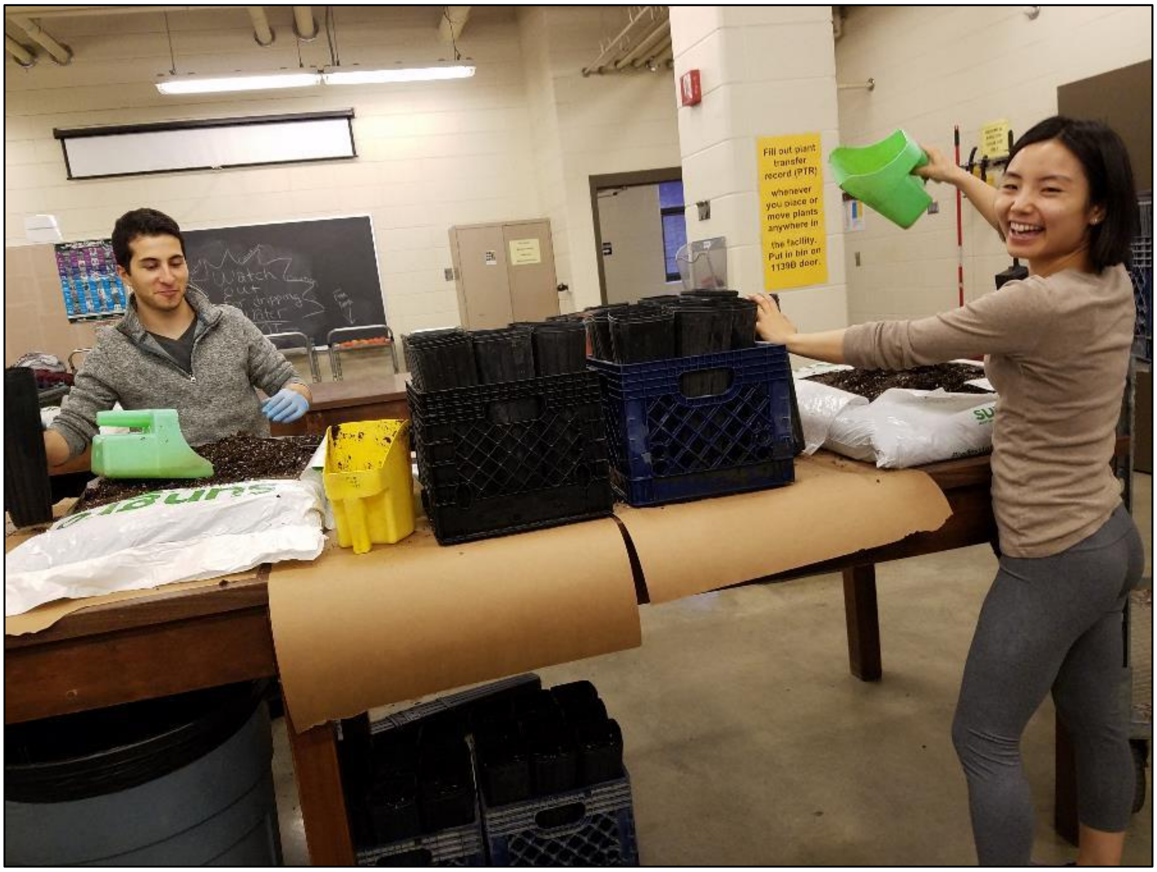

Figure H.22. It was “all hands on deck” to get the rapidly germinating butternut and hybrid butternut seeds planted quickly enough. Pictured are fellow FNR graduate student Akane Abbasi (right) and her husband Sahand Abbasi (left). Not pictured, but also absolutely crucial to the planting process: Paul Brennan (my dad), James Warren (HTRIC), Aziz Ebrahimi (FNR graduate student) and Emily Thyroff (FNR graduate student).

Figure H.23. Seedlings growing in the greenhouse (a) on 04/13/2018 (2-3 weeks post-germination) and (b) just 12 days later on 04/25/2018 (seedlings were ultimately spaced out more as they grew).

Figure H.24. Some hybrid butternut seedlings exhibited (a) variegated and (b) completely white foliage. Some variegated seedlings eventually grew out of their initial variegation, but the entirely white ones started declining after a few weeks when their endosperm reserves began to be exhausted. (Neither the variegated nor white seedlings were used in the actual experiment.)

Figure H.25. Butternut seedling at just 1.5 months old. Photo credit: James Warren.

Figure H.26. Fellow FNR graduate student and butternut enthusiast, Megan Zagorski, generously helping move seedlings from the greenhouse to growth chambers in the fall.

Figure H.27. Seedlings were placed into growth chambers in late fall and winter for cold hardening.

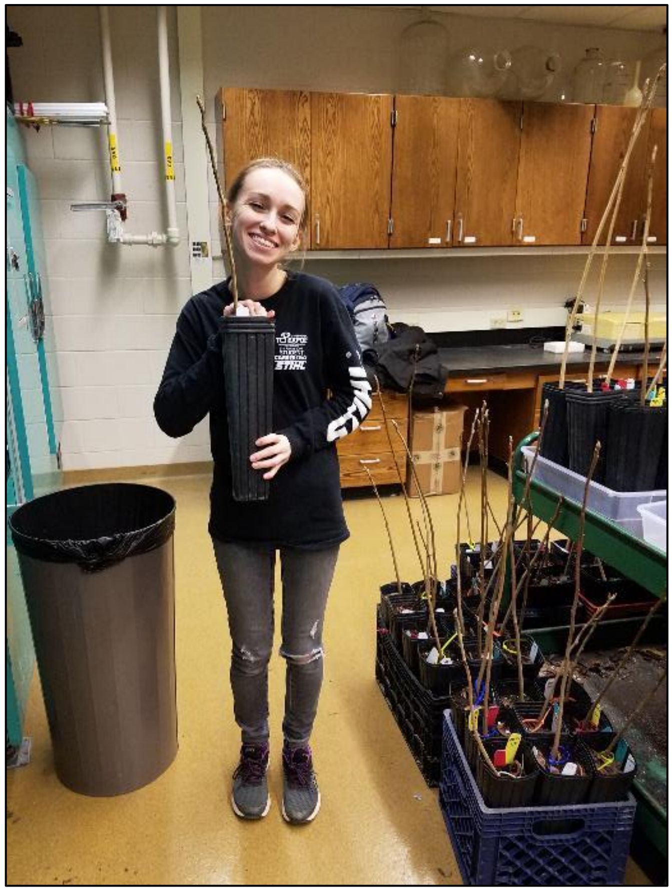

Figure H.28. Another fellow butternut enthusiast and FNR undergraduate student, Elizabeth Brewer, was invaluable for assisting with seedling care and preparation and data collection. She also led the effort to “rehome” surviving seedlings upon completion of the experiment and was able to plant some at her own home.

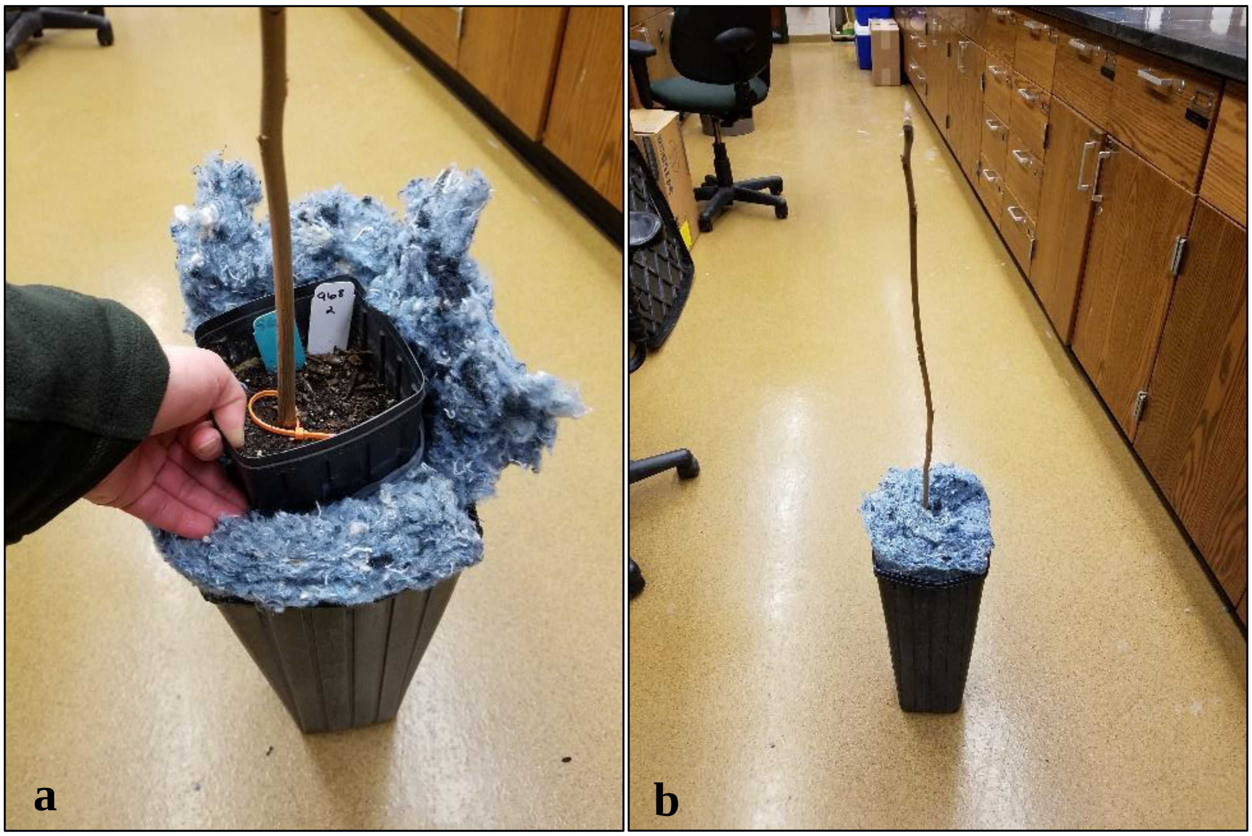

Figure H.29. To mimic the natural insulation received by plants from the ground, larger pots were lined with recycled denim home insulation and the smaller seedling pots were placed inside.

Figure H.30. For the freeze treatments, seedlings were placed on a custom-made PVC pipe rack (constructed with help from James Warren) inside a programable freezer with a small fan to evenly distribute air temperatures, along with numerous soil and air thermocouples and data loggers to monitor temperatures.

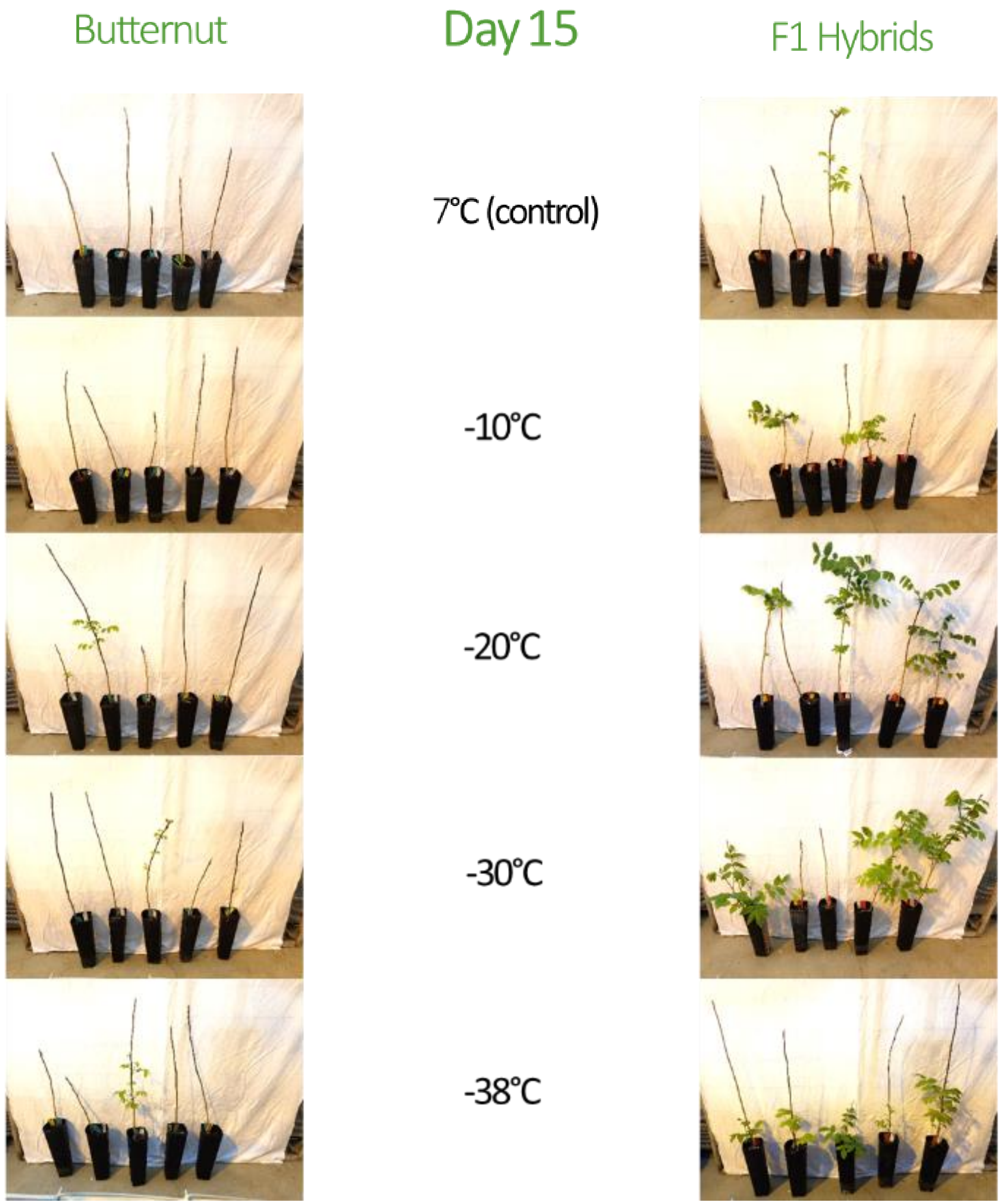

Figure H.31. Budbreak of five randomly chosen seedlings of butternut and F1 hybrid butternut treated at each temperature at 15 days following removal of dormancy conditions.

Figure H.32. Budbreak phenology was tracked for 82 days before the experiment was ended and final measurements were taken.

Figure H.33. (a) James Warren (US Forest Service) and (b) Aziz Ebrahimi (fellow FNR graduate student) frequently helped me with all aspects of my research, including here, with final survival and damage measurements and clean-up. I cannot properly express the magnitude of appreciation I have for that.