BUD-FORCING AND IN VITRO CULTURE
FOR CONSERVATION OF OAK (QUERCUS L.)

by

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# TABLE OF CONTENTS

LIST OF TABLES ........................................................................................................ vii
LIST OF FIGURES .................................................................................................... viii
ABSTRACT ................................................................................................................ ix

Chapter

1 INTRODUCTION TO OAK CONSERVATION .................................................. 1

1.1 Oaks: Importance and Status ...................................................................... 1
1.2 *In Situ* and *Ex Situ* Plant Conservation .............................................. 2

1.2.1 Seed Banks ............................................................................................. 4
1.2.2 Living Collections .................................................................................. 4
1.2.3 Cryopreservation ................................................................................... 6
1.2.4 *In Vitro* Culture ................................................................................... 8

REFERENCES ........................................................................................................ 11

2 EFFECT OF BAP, A CYTOKININ, ON BUD-FORCING OF TWELVE OAK (*QUERCUS* L.) SPECIES FOR PRODUCTION OF MICROPROPAGATION EXPLANTS ........................................... 14

2.1 Abstract ......................................................................................................... 14
2.2 Introduction .................................................................................................... 15

2.2.1 Forcing Methods ..................................................................................... 15
2.2.2 Dormancy, Environment, and Hormones ............................................. 17
2.2.3 Objectives and Hypotheses ................................................................... 19

2.3 Materials and Methods ................................................................................ 20
2.4 Results and Discussion ................................................................................ 23
2.5 Conclusion .................................................................................................... 28

REFERENCES ........................................................................................................ 34
LIST OF TABLES

Table 2.1. Average length, width, and bud count of *Quercus* cuttings used in the bud-forcing experiment ................................................................. 21

Table 2.2. Least Squares Regression of treatment, week, and interaction between treatment and week of the nine *Quercus* species not exhibiting early, severe dieback in the bud-forcing experiment. A Dunnett’s Test also compared the control to the treatments for species showing no significant interaction between treatment and week (Dunnett, 1955). ... 24

Table 2.3. Section, distribution, native habitat, and bud morphology of *Quercus* species used in the bud-forcing experiment................................. 30

Table 3.1. Percentages of explants exhibiting pre-existing leaf expansion, bud enlargement, shoot production, and callus in micropropagation experiment by *Quercus* species, section, and medium. ................. 56

Table 3.2. Connecting letters report of the Tukey-Kramer HSD (Honestly Significant Difference) Test comparing mean survival time (days) and standard error by *Quercus* species for micropropagation experiment. Species not connected by the same letter are significantly different...... 59

Table 3.3. Section, IUCN (International Union for Conservation of Nature) Red List Status, NatureServe Global Status, distribution, and native habitat of *Quercus* species used in micropropagation experiment ...................... 65
LIST OF FIGURES

Figure 2.1. *Quercus* bud activity evaluation scale from 0-4 with 0 = no development and 4 = target stage for shoot tip micropropagation ........... 22

Figure 2.2. *Quercus imbricaria* buds, day 35, across all treatment levels, as an example of a species responding significantly to BAP application....... 26

Figure 2.3. *Quercus rubra* buds, day 35, across all treatment levels, as an example of a species not responding significantly to BAP application.. 26

Figure 2.4. Mean bud activity over time of the twelve *Quercus* species used in the bud-forcing experiment ........................................................................ 33

Figure 3.1. Nutrient millimolarity comparison of Woody Plant Medium (Lloyd and McCown, 1981) and Gressoff and Doy Medium (Gresshoff and Doy, 1972)........................................................................................................ 45

Figure 3.2. Example of a young shoot tip (*Quercus canbyi*) ready to be harvested as an explant for the *in vitro* culture experiment ................. 52

Figure 3.3. Images of *Quercus* explant growth responses in the *in vitro* culture experiment: 1) expansion of pre-existing leaves in *Q. boyntonii*, 2) bud enlargement in *Q. engelmannii*, 3) shoot production in *Q. gambelii*, and 4) callus production in *Q. dumosa* ........................................ 55

Figure 3.4. Percent of *Quercus* explants surviving over time (days) in micropropagation experiment by (a) *Quercus* section, (b) medium, and (c) origin type......................................................................................... 58

Figure 3.5. Percent of *Quercus* species affected by contamination in micropropagation experiment. Column width varies according to the sample size (n) .................................................................................. 60
ABSTRACT

Oaks (*Quercus* L.) are globally iconic trees, prized for their contributions as a keystone species, strong, rot-resistant wood, and landscape value. Despite their importance, many species of *Quercus* are under threat from a range of issues, such as habitat loss and attacks from pests and diseases. Conservation efforts for this genus can be complex and challenging, especially since *Quercus* species are considered “recalcitrant” due to the inability of their seeds, acorns, to survive seed banking, an important conservation method. One method to support conservation efforts is *in vitro* culture (tissue culture) using newly-flushed shoot tips. Two experiments were conducted to determine 1) the viability of bud-forcing *Quercus* and effectiveness of 6-benzylaminopurine (BAP), a cytokinin, on stimulating bud break and shoot elongation of *Quercus* and 2) the responses of species from the three North American *Quercus* sections (*Lobatae*, *Quercus*, and *Protobalanus*) on two different media in *in vitro* culture.

Natural shoot emergence in the spring is a narrow and somewhat unpredictable time window, but forcing bud break of cuttings can lengthen this window in a controlled environment. Experiment 1 involved dormant cuttings collected from 12 *Quercus* species placed into flasks of distilled water. Flasks were placed in a greenhouse with weekly BAP treatments applied by paint brush at either 0, 100, or 500 ppm. Results varied by species. The BAP treatment at 100 or 500 ppm significantly increased the rate of bud break and shoot elongation for four of the *Quercus* species (*imbricaria*, *macrocarpa*, *pagoda*, and *variabilis*) and significantly
decreased the rate of bud break and shoot elongation in *Q. falcata*. There was no significant effect from BAP application on the remaining seven species. A majority of the *Quercus* species reached the target stage for micropropagation with all treatments, indicating that forcing bud break is a viable option for *Quercus*, but the rate of bud development in some species may be enhanced by BAP application.

In Experiment 2, newly-flushed shoots were collected in the spring from 12 species of *Quercus* representing the three North American *Quercus* sections (*Lobatae*, *Quercus*, and *Protobalanus*) (different species from Experiment 1). The shoot explants were grown *in vitro* on one of two media formulations: Gresshoff and Doy (GD) basal salts and vitamins or Lloyd and McCown Woody Plant (WP) basal salts with Murashige and Skoog (MS) vitamins. Responses of growth, survival, and contamination varied by species, but these differences were concealed when grouped by section. Growth responses of leaf expansion, bud expansion, shoot production, and callus production were observed in nine of the species. Explants grown on WP/MS media had significantly more growth responses and longer survival times than those on GD media.
Chapter 1

INTRODUCTION TO OAK CONSERVATION

1.1 Oaks: Importance and Status

Oaks, *Quercus* L., are a large, diverse genus of approximately 500 species of deciduous and evergreen woody trees and shrubs (Oldfield and Eastwood, 2007). *Quercus* species naturally occur in a wide variety of habitats in the Northern Hemisphere, primarily across North America, Europe, and Asia, but still touching every continent except Australia and Antarctica (Manos et al., 1999; Nixon, 2006). Oaks are members of the Fagaceae, and most taxonomists divide *Quercus* into two subgenera, *Quercus* and *Cyclobalanopsis*, with the former broken down into four sections: *Quercus* (white oaks), *Lobatae* (red or black oaks), *Protobalanus* (golden cup or intermediate oaks), and *Cerris* (Turkey oaks) (Manos et al., 1999). Sections *Lobatae* and *Protobalanus* are both found in the Americas, with *Protobalanus* only present in the southwest United States and Northern Mexico (Manos et al., 1999; Nixon, 2006). Section *Quercus* is widespread throughout the Americas, Europe, Asia, and into North Africa, and section *Cerris* is also found in Europe, Asia, and North Africa, but not the Americas (Manos et al., 1999; Nixon, 2006). Subgenus *Cyclobalanopsis* is exclusively found in southeastern Asia (Manos et al., 1999).

Oaks are iconic around the world, prized economically for their strong, hard, rot-resistant wood, and aesthetically for their ornamental value in the landscape. Perhaps most important are the numerous ecological contributions, such as wildlife food and habitat, that this keystone genus provides as a part of many ecosystems. For
example, it has been determined that 534 species of Lepidoptera caterpillar species are supported by oaks in the United States mid-Atlantic region alone (Tallamy and Shropshire, 2009). Despite their importance in both wild and cultivated landscapes, many species of *Quercus* are under threat from a range of biotic and abiotic issues (Kramer and Pence, 2012; Oldfield and Eastwood, 2007). These include habitat loss, over-harvesting, competition with invasive species, climate change, and attacks from pests such as *Phytophthora ramorum*, the fungus responsible for Sudden Oak Death (Oldfield and Eastwood, 2007).

The Red List of Oaks, published in 2007, was the first attempt at a genus-wide evaluation of the threat level of the roughly 500 currently identified species of *Quercus* and used the internationally-accepted criteria of the International Union for Conservation of Nature (IUCN), version 3.1 (Oldfield and Eastwood, 2007). Only about one-third of these species had enough data to be fully evaluated, nearly 7% of the species were too low on data to be fully evaluated, and over half of the species lacked data to such an extent that they were unable to be evaluated at all. Of the 175 *Quercus* species able to be fully evaluated, roughly 45% were categorized as critically endangered, endangered, vulnerable, or near threatened.

1.2 *In Situ* and *Ex Situ* Plant Conservation

Research and conservation efforts pertaining to *Quercus* species are key to preserving biodiversity, saving threatened species, and preventing additional species from reaching a threatened status. The Global Strategy for Plant Conservation (GSPC) was adopted by the Convention on Biological Diversity (CBD) and sets forth 16 targets “with the ultimate goal of halting the current and continuing loss of plant diversity” (CBD, 2010). Target 3 of the GSPC specifically calls attention to the need
for additional information and research into methods for the conservation of plant diversity, while additional targets set goals of having at least 75% of threatened species conserved both in situ (Target 7) and ex situ (Target 8) (CBD, 2010).

In situ and ex situ are the two main approaches to plant conservation (Razdan, 2003; Tuxhill and Nabhan, 2001). In situ conservation involves the protection and management of plant species within their natural habitat, while ex situ conservation involves protecting plants species in settings outside of their natural habitat (Kramer and Pence, 2012; Razdan, 2003; Tuxhill and Nabhan, 2001). In situ conservation can include areas such as national parks, wildlife refuges, nature reserves, and private lands, and allows for the continued adaptation and evolution of species and populations in the wild, as well as the promotion and preservation of ecological processes and interactions (Tuxhill and Nabhan, 2001).

While in situ conservation is largely agreed by conservationists to be the ultimate priority, this method alone is not sufficient to preserve and protect plant species, given the rapid pace of habitat loss and degradation worldwide (CBD, 2010; Guerrant et al., 2004). Ex situ conservation is meant to be complementary to in situ efforts to support and restore wild populations, and involves the protection of plants and germplasm outside of their natural habitat (Guerrant et al., 2004; Pence, 2011; Tuxhill and Nabhan, 2001). This approach is only a safety net, and should be considered a temporary measure, not the ultimate solution, to saving threatened plant species (Guerrant et al., 2004; Kramer and Pence, 2012). Ex situ methods support in situ conservation through the preservation of, and convenient access to, rich concentrations of plant germplasm for research, breeding, and education (Guerrant et al., 2004; Tuxhill and Nabhan, 2001). There are many forms and approaches to ex situ
conservation, including seed banks, living collections, cryopreservation, and \textit{in vitro} culture (Guerrant et al., 2004; Kramer and Pence, 2012; Razdan, 2003).

1.2.1 Seed Banks

Seed banking is a method of drying and then storing seeds long-term at low temperatures for the purpose of securing plant species genetic diversity (Kramer and Pence, 2012; Razdan, 2003). While the most efficient and effective conservation strategy for many plant species, the process cannot be applied to \textit{Quercus} species (Bonner, 2003; Pence, 2011; Razdan, 2003). \textit{Quercus} species are labeled “exceptional”, due to their “recalcitrant” seeds (acorns), which do not tolerate desiccation below what is a relatively high critical moisture content of 20-35% (Bonner, 2003; Pence, 2011). This sensitivity to moisture loss presents a challenge for conserving threatened \textit{Quercus} species by eliminating the possibility of long-term germplasm storage in seed banks (Kramer and Pence, 2012; Pence, 2011). Without the option to seed bank acorns, increased importance is placed on different approaches to \textit{Quercus} conservation, such as living collections, cryopreservation, and \textit{in vitro} culture (Kramer and Pence, 2012; Pence, 2011).

1.2.2 Living Collections

Living collections are composed of plants cultivated in field or controlled environments outside of their natural habitat, frequently at botanic gardens and arboreta (Guerrant et al., 2004; Hawkes et al., 2012). One of the biggest advantages of this \textit{ex situ} conservation method is that it allows for easily available material and permits active evaluation and research while the plants are being conserved (Hawkes et al., 2012). Additionally, living collections can be used for public display and
education on plant conservation, especially in the context of botanic gardens and arboreta. Despite the advantages, living collections come with many challenges that can limit their use. These include a high cost of maintenance, labor, materials, facilities and land, as well as risk of damage from pests, disease, and natural hazards (Guerrant et al., 2004; Hawkes et al., 2012). Another potential disadvantage to living collections involves the method of building the collections through seed or vegetative propagation. For various threatened species, including many *Quercus*, material for these types of propagation is not readily available and the collection of that material could be a threat to the species itself (Kramer and Pence, 2012; Pence, 2010).

A further drawback to living collections, especially in reference to *Quercus*, is the accurate representation of species. This is problematic, both in terms of accurate identification of species, especially given the vast number of *Quercus* species and the readiness of many to hybridize, and sufficient representation of threatened species (Kramer and Pence, 2012). The Global Survey of Ex Situ Oak Collections, performed by Botanic Gardens Conservation International (BGCI), states that of the 3,796 records of *Quercus* species held in *ex situ* living collections in botanic gardens across the globe, only 91 records of just 13 of the 29 critically endangered and endangered *Quercus* species were reported (BGCI, 2009). For this survey, a record was defined as “the presence of a single *Quercus* (Oak) taxon within a garden’s collection and may include multiple accessions and/or individuals” (BGCI, 2009). Looking specifically at North American *Quercus* species, Toppila (2012) surveyed living collections of North American *Quercus* species held within 344 botanic gardens worldwide and found that just 67 of 3192 records (2%) were of critically imperiled and imperiled taxa.
Given the results of these two surveys, it is clear that *ex situ* *Quercus* collections tend to be composed predominantly of secure species, while species in the greatest need of conservation are underrepresented. This brings up an important point when discussing living collections in that not all of them hold conservation value, more likely being created for ornamental display or commercial value (Guerrant et al., 2004). Even those that contain threatened species may not be actively managing for intra-species diversity and other conservation purposes, only holding a small number of genetically related individual specimens with little to no accession, source, or provenance information (Guerrant et al., 2004; Hawkes et al., 2012). In that regard, for living collections to be of high value for *ex situ* conservation, they must hold a large number of genetically diverse specimens of known wild origin and represent populations from across a species’ natural range (Guerrant et al., 2004; Hawkes et al., 2012). Additionally, these collections should be well maintained, with special attention to avoid crossing between collections (Hawkes et al., 2012).

1.2.3 **Cryopreservation**

Cryopreservation of seeds, pollen, embryonic axes, somatic and zygotic embryos, and vegetative tissue provides an additional approach to long-term storage of germplasm (Engelmann, 2004; Guerrant et al., 2004; Pence, 1995; Razdan, 2003). Specifically, the cryopreservation of embryos and embryonic axes allows for the preservation of genetic diversity in a manner similar to seed banking, while cryopreservation of vegetative tissues, such as shoot tips, allows for the creation of a “tissue bank” for propagation and restoration efforts (Pence, 2011). Cryopreservation involves the removal of water from tissue, either by drying or removal or replacement of water by chemical means (depending on tissue type) followed by exposure to liquid
nitrogen at -196°C (Engelmann, 2004; Guerrant et al., 2004; Razdan, 2003). Cellular
divisions and metabolic processes cease at this temperature, making long-term storage
possible (Engelmann, 2004; Razdan, 2003). The desiccation required for
cryopreservation is not always as great as for seed banking, depending on the system,
and the rapid cooling involved in the process allows for vitrification (Engelmann,
2004; Razdan, 2003). Vitrification is the direct transition of liquid water to an
amorphous phase (glass), which prevents the formation of damaging ice crystals
(Engelmann, 2004; Razdan, 2003). When certain tissue types, such as somatic
embryos and zygotic embryonic axes, are removed and thawed from cryopreservation,
the process is dependent on the subsequent in vitro culture of the tissue for its ultimate
success (González-Benito et al., 2002; Pence, 2010; Pence, 2011; Razdan, 2003).

Recent research on Quercus cryopreservation has looked at several species,
including Q. ilex (Gonzalez-Benito et al., 2002), Q. franchetii (Xia et al., 2014), Q.
gambelii (Xia et al., 2014), Q. macrocarpa (Pence, 1992), Q. robur (Jörgensen, 1990),
Q. rubra (Pence, 1992; Xia et al., 2014), and Q. suber (Fernandes et al., 2008;
Gonzalez-Benito et al., 2002; Valladares et al., 2004). Quercus tissue types
successfully cryopreserved thus far include somatic embryos (Fernandes et al., 2008),
pollen (Jörgensen, 1990), and embryonic axes (Gonzalez-Benito et al., 2002; Pence,
1992; Valladares et al., 2004; Xia et al., 2014). In studying four Quercus species
native to either the United States or China, Xia et al. (2014) found greater desiccation
tolerance and survival following cryo-exposure in excised embryonic axis samples
from areas with colder winters. This indicates that in Quercus embryonic axes,
survival against freezing temperatures may contribute to desiccation tolerance and
survival to cryo-exposure, rather than the ability to adapt to drought (Xia et al., 2014).
Cryopreservation allows for the long-term storage of germplasm that is protected from contamination, and with minimal space and maintenance requirements (Engelmann, 2004; Razdan, 2003). However, cryopreservation requires a significant initial capital investment, specially trained technicians, a regular supply of liquid nitrogen, and in the case of certain tissues, such as embryonic axes, *in vitro* supplies and maintenance for recovery (Guerrant et al., 2004; Pence, 2011; Razdan, 2003). While cryopreservation of embryo axes has shown potential for conservation of some *Quercus* species, further protocols must be developed to make it a viable option for genetic diversity preservation or tissue banking, especially with a genus as large and diverse as *Quercus*.

1.2.4 *In Vitro Culture*

*In vitro* culture, or tissue culture, involves growing different types of plant tissues, such as shoot tips or embryos, on nutrient media in a sterile, enclosed, and light-controlled system (Hartmann et al., 2002; Pence, 2010; Razdan, 2003). By using conditions to promote slow growth, such as low temperature and light levels, and by occasional sub-culturing, *in vitro* cultures can be maintained as *ex situ* conservation germplasm collections (tissue banks) for intermediate-term storage over several years (Engelmann and Engels, 2002; Guerrant et al., 2004; Pence, 2011; Razdan, 2003). Unlike living collections, *in vitro* collections are protected from pests, disease, and environmental hazards (Hartmann et al., Razdan, 2003). *In vitro* propagation can be employed to increase the number of individuals for both *ex situ* and *in situ* conservation, especially if a species is difficult to propagate through traditional methods or there are very few individuals available (Engelmann and Engels, 2002; Guerrant et al., 2004; Hartmann et al., 2002; Pence, 2011; Razdan, 2003).
Furthermore, *in vitro* culture can also be used for supplying material for cryopreservation, culturing cryopreserved material after thawing, and recovering and growing embryos after embryonic axis freezing (Hartmann et al., 2002; Pence, 2010; Pence, 2011; Razdan, 2003). However, mutations and somaclonal variation (genetic instability) can be a concern with tissues kept in *in vitro* culture (Guerrant et al., 2004; Hartmann et al., 2002; Razdan, 2003). Additionally, like cryopreservation, *in vitro* culture requires a significant initial capital investment, specially trained technicians, and specialized laboratory supplies (Guerrant et al., 2004; Hartmann et al., 2002; Pence, 2011). *In vitro* culture also requires continued maintenance through regular sub-culturing, thus making it a more costly storage method over time than cryopreservation (Guerrant et al., 2004; Hartmann et al., 2002; Pence, 2011).

*Quercus* species tend to be difficult to grow *in vitro*, especially due to their high tannin (phenolic) content (Kramer and Pence, 2012). Further, the range of *Quercus* species studied for *in vitro* culture is limited, especially with regards to threatened species, despite the strong need for research on these species (Kramer and Pence, 2012). Research has primarily focused on economically important species, such as *Quercus rubra* (Vieitez et al., 1993; Vieitez et al., 2009), *Quercus robur* (Chalupa, 1988; Favre and Juncker, 1987; Vieitez et al., 1994), and *Quercus suber* (Manzanera and Pardos, 1990; Romano et al., 1992). Only three studies were found investigating *in vitro* propagation of threatened *Quercus* species, conducted by Kramer and Pence (2012) studying *Quercus acerifolia*, *Quercus arkansana*, *Quercus boyntonii*, and *Quercus georgiana*; Kartsonas and Papafotiou (2007) studying *Quercus euboica*; and Tamta et al. (2008) studying *Quercus semecarpifolia*. Specific tissue types used in the *in vitro* culture of *Quercus* species include seedling material...
(Kartsonas and Papafotiou, 2007; Tamta et al., 2008; Vengadesan and Pijut, 2009; Vieitez et al., 1985), stump sprouts (Favre and Juncker, 1987; Manzanera and Pardos, 1990; Vieitez et al., 1993), and shoot tips from mature growth (Chalupa, 1988; Kramer and Pence, 2012; Romano, 1992; Vieitez, 1994; Vieitez et al., 2009).

Given the relevance of \textit{in vitro} culture in both \textit{ex situ} and \textit{in situ} conservation, but lack of specific protocols for different \textit{Quercus} species, especially those that are threatened, additional research on \textit{in vitro} methods is necessary to further aid conservation efforts (Kramer and Pence, 2012). To this end, two experiments were conducted to determine: 1) the effectiveness of 6-benzylaminopurine (BAP), a cytokinin, on bud-forcing and shoot elongation of \textit{Quercus} as a means of supplying material for \textit{in vitro} culture and 2) the responses of species from three North American \textit{Quercus} sections (\textit{Lobatae, Quercus}, and \textit{Protobalanus}) across two different \textit{in vitro} culture media.
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Chapter 2

EFFECT OF BAP, A CYTOKININ, ON BUD-FORCING OF TWELVE OAK (QUERCUS L.) SPECIES FOR PRODUCTION OF MICROPROPAGATION EXPLANTS

2.1 Abstract

Oak (Quercus L.) shoot tip micropropagation involves newly flushed shoots collected in the spring. This is a narrow and somewhat unpredictable time window, but forcing bud break of cuttings in a controlled environment can lengthen this window. The objectives of this experiment were to determine the feasibility of bud-forcing Quercus and the effectiveness of BAP (6-benzylaminopurine), a cytokinin, on Quercus bud break and shoot elongation. Dormant cuttings were collected from 12 species of Quercus: alba, bicolor, cerris, falcata, imbricaria, macrocarpa, macrocarpa var. macrocarpa, pagoda, palustris, rubra, texana, and variabilis. The cuttings were placed into flasks of distilled water in a greenhouse, with weekly treatment applications at 0, 100, or 500 ppm BAP, a cytokinin and plant hormone that promotes cell division. Cuttings were evaluated weekly and rated on a scale of 0 (no development) to 4 (target stage for shoot tip micropropagation). Results indicate that BAP treatment at 100 or 500 ppm significantly increased the rate of bud break and shoot elongation for four of the Quercus species: imbricaria, macrocarpa, pagoda, and variabilis, while significantly decreasing the rate in Q. falcata. There was no significant effect from BAP application on the remaining seven species. A majority of the species reached stage 4 with all treatments, indicating that forcing bud break is a
viable option for *Quercus*, but the rate in some species may be enhanced by BAP application.

2.2 Introduction

One tool to support the conservation of threatened *Quercus* species is *in vitro* culture using young, newly flushed shoots collected immediately after emergence in the spring (Kramer and Pence, 2012). This is a narrow and somewhat unpredictable time window for obtaining explants. However, forcing bud break of dormant buds on cuttings can increase the time range to collect young shoot explants and allow for shoot development in a controlled, clean environment, thus reducing the risk of contamination (Preece and Read, 2003). Further, many researchers, including plant conservationists, receive cuttings as sources for *in vitro* culture explants by mail (see Chapter 3). To prevent damage to young shoots, this material may be sent in a state of dormancy, before the buds have broken. The ability to increase the rate of bud break and elongation in these cuttings could prove useful improve the feasibility of *in vitro* research in these cases.

2.2.1 Forcing Methods

There are two primary methods to force dormant buds: stem-tip forcing in water or solution and shoot-forcing using large branch segments (Holsinger, 2006; Preece and Read, 2003). The first method involves the use of approximately 15-30 cm long by less than one cm wide mature stems from the ends of dormant branches (Barker et al., 1997; Preece and Read, 2003; Romano et al., 1992; Sánchez et al., 1997a; Sánchez et al., 1997b; Yang and Read, 1992). The stems are placed upright in a warm greenhouse or growth chamber (typically averaging around 23-27°C) with
their bases set in pure water or a water-based solution that may contain a combination of hormones, sugars, anti-microbial compounds, or other substances that force the dormant buds to break. Hormones may also be applied to the surface of the cuttings by spray or brush, instead of through the solution the cuttings bases are submerged in (Preece and Read, 2003; Romano et al., 1992; Sánchez et al., 1997a; Sánchez et al., 1997b).

The second method, shoot-forcing using large branch segments, requires large, thick older branches cut from the more juvenile phase portion of the tree and measuring approximately 1-8 cm wide (Henry and Preece, 1997; Holsinger, 2006; Preece and Read, 2003; Toribio et al., 2004; Vieitez et al., 1994). The branches are cut into segments that are usually around 30-50 cm long and placed horizontally onto a bed of perlite, vermiculite, or a mixture of the two, in a warm greenhouse or growth chamber (typically averaging around 23-25°C) with mist or drip irrigation and forced to flush shoots. Shoot-flushing using large branch sections takes advantage of the ‘cone of juvenility’ of a plant to produce juvenile material through dormant epicormic, latent, or axillary buds (Holsinger, 2006; Preece and Read, 2003). Although not as juvenile as that of the growth in seedlings, the cone of juvenility is the portion of an adult plant that has a younger ontogenetic (physiological) age and is usually located at the base and central portion of the plant (Hartmann et al., 2002; Wendling et al, 2014). Growth in the outer branch portions and upper parts of a plant is in a more mature state that is capable of flowering (Hartmann et al., 2002; Wendling et al., 2014).

Studies have found in vitro propagation using explants originating from material in the mature state to be more difficult to establish and grow in culture than using juvenile material (Hanus and Rohr, 1985; Kartsonas and Papatfutiou, 2007; Preece and Read,
2003; Sánchez and Vieitez, 1991; Vieitez et al., 2007). However, the large branch sections required for the shoot-flushing method may not be a realistic option for researchers with limited plant material availability. This includes plant conservationists who may be studying species of small size, or with an exceedingly limited number of specimens in existence. Harvesting large branches from these specimens for forcing could be quite damaging (Kramer and Pence, 2012). Thus, depending on the situation, forcing bud break and shoot flush on smaller, dormant stem tips may be a better option to avoid excessive removal of material from plant specimens.

### 2.2.2 Dormancy, Environment, and Hormones

Dormancy is described as the suspension of visible growth and development, where metabolism is greatly reduced and cell division is suppressed (Samish, 1954; Basler and Kormer, 2014). It is governed by a large, complex set of internal and external factors that are not all completely understood. Externally, in terms of environment, there are three important considerations when releasing dormancy and forcing bud break in cuttings: chilling, photoperiod, and temperature. The significance and role of each of these factors varies by species and likely provenance. *Quercus, Fagus, Acer,* and *Picea*, along with others growing in temperate climates, require certain levels of exposure to chilling temperatures before bud growth is able to resume, but *Picea* has been found to have a much smaller chilling requirement than other genera (Basler and Kormer, 2014). *Fagus* species are known to respond especially strongly to long photoperiods with increased bud growth rates, but are less sensitive to warm temperatures in the final stages of bud development. This is in contrast to *Quercus, Acer,* and *Picea,* which respond strongly to warm stages during
the final stages of bud development (Basler and Kormer, 2014). Given this, elevated temperatures, higher than that of the environment in which the material is collected, play a key role in much of the bud-forcing research involving *Quercus* and other temperate woody species (Holsinger, 2006; Romano et al., 1992; Vieitez et al., 2007; Yang and Read, 1993; Yang and Read, 1997). Dormant cuttings are harvested in the cooler winter months, especially in temperate climates, and then placed into greenhouses or growth chambers, averaging around 22-26°C (Holsinger, 2006; Romano et al., 1992; Vieitez et al., 2007; Yang and Read, 1993; Yang and Read, 1997).

Internally, hormones are an important set of factors in dormancy, bud break, and shoot elongation (Davies, 2010; Hartmann et al., 2002; Kramer and Kozlowski, 2012; Pallardy, 2010). Bud dormancy ends when the primary causes of this state, such as abscisic acid (ABA) and various other growth inhibitors active at chilling temperatures, decrease in the bud with the arrival of warmer spring temperatures (Davies, 2010; Kramer and Kozlowski, 2012; Pallardy, 2010). This point marks an increase in hormones, such as gibberellins and cytokinins, which promote cell elongation and cell division, respectively. Later in the spring, there is also an increase in auxin, another hormone involved with cell elongation (Davies, 2010; Kramer and Kozlowski, 2012; Pallardy, 2010).

Several studies have used applications of gibberellins, cytokinins, and/or auxins to promote bud break and shoot elongation in cuttings from various different woody species (Manzanera and Pardos, 1990; Read and Qiguang, 1987; Romano et al. 1992; Sánchez et al., 1997; Yang and Read, 1997). In *Castanea dentata* and *Acanthopanax sieboldianus* (now *Eleutherococcus sieboldianus*), it was found that the
application of GA₃, a gibberellin, and 6-benzylaminopurine (BAP), a cytokinin, in the forcing solution increased percent bud break and shoot elongation, and while GA₃ hastened bud break, BAP delayed bud break (Yang and Read, 1997). In the same study, IBA, an auxin, decreased percent and time to bud break. In *Castanea sativa* and *C. sativa X C. crenata* (Siebeld and Zucc hybrids), both cuttings sprayed and not sprayed with BAP produced shoots to the ideal stage of micropropagation within two weeks (Sánchez et al., 1997). However, the cuttings sprayed with BAP showed improved growth and reactivity *in vitro* compared to those not sprayed with BAP. An experiment by Read and Qiguang (1987) had similar *in vitro* results, including increased shoot proliferation, when BAP was introduced into the forcing solution with *Philadelphus coronarius* and *Dirca palustris*.

Forcing studies involving *Quercus* have used growth regulators, primarily the cytokinin BAP, for the promotion of bud break and shoot elongation in cuttings (Manzanera and Pardos, 1990; Romano et al., 1992; Yang and Read, 1997). However, this was generally conducted as a preliminary step to obtain young shoot explants for *in vitro* culture, which was the main focus of these studies, and the bud-forcing step of the research was generally not part of a formalized, controlled experiment. A study that specifically experimented with bud-forcing and shoot elongation of *Quercus alba*, found that GA₃ and BAP in combination increased percent bud break and promoted shoot elongation, but GA₃ and BAP alone hastened bud break and delayed bud break, respectively (Yang and Read, 1997).

### 2.2.3 Objectives and Hypotheses

There is a limited amount of formalized research involving the forcing of dormant buds of different *Quercus* species and the effectiveness of hormone
application in this forcing method. Given this, the first objective of this experiment was to determine the feasibility of forcing dormant buds on mature phase stem tips in order to obtain shoots as an explant source for in vitro culture on twelve different Quercus species. The second objective was to determine the effect of BAP on bud break and shoot elongation. The following hypotheses were developed: 1) Buds from a majority of the twelve Quercus species used in the experiment will reach the target stage for use as an explant in the in vitro culture of shoot tips and 2) Quercus cuttings treated with BAP at the 100 ppm and 500 ppm levels will have an accelerated rate of bud break and shoot elongation compared to Quercus cuttings not treated with BAP.

2.3 Materials and Methods

Dormant, mature phase cuttings of one- and two-year old growth were collected from the stem tips of 12 different species of Quercus: alba, bicolor, cerris, falcata, imbricaria, macrocarpa, macrocarpa var. macrocarpa, pagoda, palustris, rubra, texana, and variabilis (Table 2.3). All species used are considered of “least concern” by the IUCN Red List of Oaks (Oldfield and Eastwood, 2007) and “secure” by NatureServe (2015). Collection of material from cultivated trees took place at Longwood Gardens in Kennett Square, Pennsylvania, USA on February 16, 2015. High temperatures had been hovering around -10°C on and immediately before collection day and there was approximately 5 cm of snowfall accumulation. Nine to fifteen cuttings were taken from each tree with alcohol-sterilized pruners, and were then measured, sorted, and selected for consistency. Depending on species, the terminal cuttings averaged 14-27 cm in length and 2-6 mm in width with 5-22 buds each (Table 2.1). The cuttings were temporarily stored for three days in plastic bags in a dark cooler at 2.7°C until the start of the experiment on February 19, 2015.
Table 2.1. Average length, width, and bud count of *Quercus* cuttings used in the bud-forcing experiment.

<table>
<thead>
<tr>
<th>Species Name</th>
<th>Avg. Length (cm)</th>
<th>Avg. Width (mm)</th>
<th>Avg. # of Buds</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Quercus alba</em></td>
<td>15</td>
<td>4</td>
<td>18</td>
</tr>
<tr>
<td><em>Quercus bicolor</em></td>
<td>20</td>
<td>4</td>
<td>22</td>
</tr>
<tr>
<td><em>Quercus cerris</em></td>
<td>20</td>
<td>3</td>
<td>14</td>
</tr>
<tr>
<td><em>Quercus falcata</em></td>
<td>15</td>
<td>3</td>
<td>7</td>
</tr>
<tr>
<td><em>Quercus imbricaria</em></td>
<td>20</td>
<td>3</td>
<td>15</td>
</tr>
<tr>
<td><em>Quercus macrocarpa</em></td>
<td>27</td>
<td>6</td>
<td>14</td>
</tr>
<tr>
<td><em>Quercus macrocarpa var. macrocarpa</em></td>
<td>18</td>
<td>5</td>
<td>10</td>
</tr>
<tr>
<td><em>Quercus pagoda</em></td>
<td>15</td>
<td>3</td>
<td>15</td>
</tr>
<tr>
<td><em>Quercus palustris</em></td>
<td>14</td>
<td>4</td>
<td>14</td>
</tr>
<tr>
<td><em>Quercus rubra</em></td>
<td>15</td>
<td>4</td>
<td>10</td>
</tr>
<tr>
<td><em>Quercus texana</em></td>
<td>18</td>
<td>4</td>
<td>15</td>
</tr>
<tr>
<td><em>Quercus variabilis</em></td>
<td>16</td>
<td>3</td>
<td>5</td>
</tr>
</tbody>
</table>

The experiment was a randomized complete block design with factorial treatments 12 species, 3 BAP treatments (0, 100, and 500 ppm), and 3 replications, giving a total of 108 cuttings. The cuttings were rinsed thoroughly with distilled water and then the basal ends were recut at an angle under water to prevent air from entering the xylem. The cuttings were placed cut stem down into 500 mL Erlenmeyer flasks containing approximately 150 mL of distilled water. Flasks were equally spaced on 0.5 m² of a bench in a glass greenhouse with a heat set point of 20°C and a cooling set point of 26.5°C. Each week, immediately prior to treatment application, the stem bases were gently scrubbed by hand (wearing nitrile gloves) and rinsed under distilled water. The water in the flasks was then replaced with fresh distilled water.
Treatments were prepared and applied similar to a method by Romano et al. (1992), by dissolving 0 g/L, 0.1 g/L, or 0.5 g/L of BAP (PhytoTech Laboratories) into distilled water. Treatment solutions were applied weekly by paintbrush to the entire cutting until runoff. Once the cuttings were no longer dripping, they were placed back into the flasks of water. To account for environmental differences that may have existed between different parts of the experimental area, flasks were randomly rotated within each treatment block each week. Following this, each block was shifted to a different place around the greenhouse bench. The experiment lasted 42 days and each week, cuttings were evaluated and rated on a bud development scale of 0-4 with 0 = no development, 1 = slight bud swelling and elongation, 2 = moderate bud swelling and elongation with visible green coloration, 3 = bud break with partially visible leaf and/or inflorescence tips, 4 = at least one newly emerged leaf blade fully visible (target stage for shoot tip micropropagation) (Fig. 2.1). The Least Squares Regression method and Dunnett’s Test were used to analyze the *Quercus* bud break and shoot elongation responses (Dunnett, 1955).

![Figure 2.1](image)

*Figure 2.1. Quercus* bud activity evaluation scale from 0-4 with 0 = no development and 4 = target stage for shoot tip micropropagation
2.4 Results and Discussion

The first buds started visibly expanding on day 14 in *Q. bicolor* across all treatment levels and in *Q. macrocarpa* at the 500 ppm BAP treatment level. By day 28, all species, except the 0 ppm BAP-treated (control) cuttings of *Q. pagoda*, were responding across all levels. (None of the 0 ppm BAP-treated *Q. pagoda* cuttings responded by the conclusion of the experiment). On day 28, some of the buds of *Q. macrocarpa*, *Q. macrocarpa* var. *macrocarpa*, *Q. palustris*, *Q. rubra*, and *Q. texana* began reaching the target stage for micropropagation across all treatment levels, with *Q. imbricaria* and *Q. variabilis* beginning to attain the target stage only at the 100 and 500 ppm BAP treatment levels. The last species to reach stage 4 was *Q. falcata* on day 42, across all treatment levels. All *Quercus* species except *alba*, *bicolor*, *cerris*, and *pagoda* reached stage 4 with all treatments. *Q. bicolor* cuttings began showing signs of dieback on day 28 in the 0 and 100 ppm BAP treatment levels. All cuttings of *Q. bicolor* and *Q. alba*, and several of *Q. cerris* died in all treatment levels by day 35. Most cuttings of these species reached stage 2 or 3 before beginning to decline. The experiment was concluded on day 42 as cuttings from all species and treatments had either fully reached the target stage for shoot tip micropropagation, or exhibited severe signs of dieback.

The Least Squares Regression method was used to analyze the *Quercus* bud-forcing data. If the BAP treatment effect was shown to be significant and there was no significant interaction between treatment and week, then a Dunnett’s Test was performed to compare the two different treatments (100 and 500 ppm BAP) against the control (0 ppm BAP) (Dunnett, 1955). The responses from the twelve species varied to such an extent that the species could not be analyzed together, necessitating that each species be evaluated individually (Table 2.2). Many cuttings of *Q. alba*, *Q.
*bicolor*, and *Q. cerris* died midway through the experiment and these species were not included in the statistical analysis, although graphs of their responses are included with those of the rest of the species (Fig. 2.4).

**Table 2.2.** Least Squares Regression of treatment, week, and interaction between treatment and week of the nine *Quercus* species not exhibiting early, severe dieback in the bud-forcing experiment. A Dunnett’s Test also compared the control to the treatments for species showing no significant interaction between treatment and week (Dunnett, 1955).

<table>
<thead>
<tr>
<th>Species Name</th>
<th>Treatment</th>
<th>Week</th>
<th>Treatment*Week</th>
<th>R²</th>
<th>Dunnett’s Test¹</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Quercus falcata</em></td>
<td>.0406</td>
<td>&lt;.0001</td>
<td>.6771</td>
<td>.948</td>
<td>0 ppm BAP 1.600</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>100 ppm BAP 1.600</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>500 ppm BAP 1.267*</td>
</tr>
<tr>
<td><em>Quercus imbricaria</em></td>
<td>&lt;.0001</td>
<td>&lt;.0001</td>
<td>.0751</td>
<td>.961</td>
<td>0 ppm BAP 2.200</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>100 ppm BAP 2.867***</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>500 ppm BAP 2.933***</td>
</tr>
<tr>
<td><em>Quercus macrocarpa</em></td>
<td>.0147</td>
<td>&lt;.0001</td>
<td>.4784</td>
<td>.949</td>
<td>0 ppm BAP 2.600</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>100 ppm BAP 2.733</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>500 ppm BAP 3.067**</td>
</tr>
<tr>
<td><em>Quercus macrocarpa</em></td>
<td>.1530</td>
<td>&lt;.0001</td>
<td>.6479</td>
<td>.989</td>
<td>0 ppm BAP 2.800</td>
</tr>
<tr>
<td>var. macrocarpa</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>100 ppm BAP 2.667</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>500 ppm BAP 2.800</td>
</tr>
<tr>
<td><em>Quercus pagoda</em></td>
<td>&lt;.0001</td>
<td>&lt;.0001</td>
<td>N/A²</td>
<td>.761</td>
<td>0 ppm BAP .0000</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>100 ppm BAP .6000***</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>500 ppm BAP .5500***</td>
</tr>
<tr>
<td><em>Quercus palustris</em></td>
<td>.0045</td>
<td>&lt;.0001</td>
<td>.007</td>
<td>.988</td>
<td>0 ppm BAP --</td>
</tr>
<tr>
<td></td>
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<td></td>
<td></td>
<td></td>
<td>100 ppm BAP --</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>500 ppm BAP --</td>
</tr>
<tr>
<td><em>Quercus rubra</em></td>
<td>.0649</td>
<td>&lt;.0001</td>
<td>.014</td>
<td>.984</td>
<td>0 ppm BAP --</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>100 ppm BAP --</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>500 ppm BAP --</td>
</tr>
<tr>
<td><em>Quercus texana</em></td>
<td>&lt;.0001</td>
<td>&lt;.0001</td>
<td>.0001</td>
<td>1.00</td>
<td>0 ppm BAP --</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>100 ppm BAP --</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>500 ppm BAP --</td>
</tr>
<tr>
<td><em>Quercus variabilis</em></td>
<td>.0134</td>
<td>&lt;.0001</td>
<td>.1989</td>
<td>.969</td>
<td>0 ppm BAP 2.400</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>100 ppm BAP 2.733*</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>500 ppm BAP 2.733*</td>
</tr>
</tbody>
</table>

*p < 0.05
**p < 0.01
***p < 0.001
¹ Control was 0 ppm BAP
² All cuttings of *Q. pagoda* at the 500 ppm BAP treatment level died during the last week of the experiment, so an interaction value could not be calculated
The BAP treatment at 100 or 500 ppm compared to the control (0 ppm BAP) significantly increased the rate of bud break and shoot elongation in four of the *Quercus* species: *imbricaria* (Fig. 2.2), *macrocarpa, pagoda*, and *variabilis*. None of the cuttings of *Q. pagoda* reached the target bud development stage. However, the *Q. pagoda* cuttings receiving BAP treatments did show some bud development, while the control group did not respond at all. Interestingly, the BAP treatment at 500 ppm significantly decreased the rate of bud break and shoot elongation in *Q. falcata* compared to the control. The remaining seven species, *alba, bicolor, cerris, macrocarpa* var. *macrocarpa, palustris, rubra* (Fig. 2.3), and *texana*, either showed no significant effect from the BAP treatment, and/or showed a significant interaction between treatment and week. It is possible that endogenous cytokinin concentrations vary by *Quercus* species. Therefore, the optimal, and even inhibitory, levels of exogenous cytokinin application for increased rate of bud break and shoot elongation could vary depending on the different pre-existing cytokinin concentrations of the different species (Mok and Mok; 1994).
Figure 2.2. *Quercus imbricaria* buds, day 35, across all treatment levels, as an example of a species responding significantly to BAP application.

Figure 2.3. *Quercus rubra* buds, day 35, across all treatment levels, as an example of a species not responding significantly to BAP application.

The *Quercus* sections, distributions, native habitats, bud morphologies, and average cutting length, width, and bud counts (Tables 2.1 and 2.3) were evaluated for
each of the species used in the experiment to determine if there was a response pattern among the species. No pattern could be found in these characteristics among the species that responded to the BAP treatment with a significantly faster rate of bud break and elongation, compared to those that did not. The four species that responded significantly were not concentrated in one or two of the *Quercus* sections, but instead represented all three sections used in the experiment (*Lobatae, Quercus*, and *Cerris*); this was the same situation for those that did not respond. Distributions were also investigated and found to be wide-ranging across North America, Europe, and Asia for both those that responded significantly to the BAP treatment, and those that did not. Native habitats of species in both response groups were also quite diverse, from lowlands to highlands, with sand to clay and well- to poorly drained soils. The bud morphologies varied for both the species that responded significantly to the BAP treatment and those that did not, with a mixture of sizes, pointed and blunt tips, and bud scales with and without hairs. The average cutting length, width, and bud counts varied for both those that did not respond to the treatment and those that did.

However, it was noted that *Q. bicolor* and *Q. macrocarpa* were the only species to exhibit bud development at day 14, a week earlier than the other species used in the experiment. Both species are in the *Quercus* section, have overlapping distributions in north- and central-eastern USA and south-central and eastern Canada, similar bud morphologies (imbricate, broadly ovate, pubescent), and similar bud sizes (3-6 mm long). *Q. macrocarpa* was also one of the first species to reach level 4, but as *Q. bicolor* cuttings died early, perhaps due to bacteria growth in the stem. Nevertheless, it is unclear whether the similarities noted between these species are notable observations or not.
2.5 Conclusion

The effect of BAP on *Quercus* bud-forcing and elongation varied by species. No BAP-response patterns were found through a comparison of species distributions, habitat, bud morphologies, cutting measurements, and cutting bud counts, although it was noted that two species in the *Quercus* section first exhibited bud development a week earlier than the other ten species. However, a majority of the species reached stage 4 with all treatments. This indicates that forcing bud break in *Quercus* without BAP application is a viable option, but the rate may be enhanced with some species, such as *Q. imbricaria, Q. macrocarpa, Q. pagoda*, and *Q. variabilis*, by the application of BAP.

A potential limitation to this experiment is that the stem bases were not cut regularly, due to the wide range of cutting stem length between species. While some of the species had longer growth with enough length that would have allowed for regular basal cutting (ex. *Q. macrocarpa* and *Q. cerris*), regularly cutting the bases of the shorter stems could have resulted in a loss of key buds for tracking experimental responses (ex. *Q. rubra* and *Q. falcata*). Despite regular water changes and stem base cleanings, the lack of basal cutting could have allowed bacterial and fungal growth to disrupt the flow of water in the xylem and negatively affect development. If regular basal cutting is not an option, future work could incorporate a floral preservative to help prevent bacterial and fungal growth.

Future research in bud-forcing of *Quercus* could investigate the application of significantly greater BAP concentrations, increasing the application frequency, or using different application methods (adding BAP to the water the cutting bases were submersed in, rather than applying to the cutting surface). Further work could also compare rate of bud break and elongation on cuttings harvested at different times of
the year, perhaps earlier in the winter or even the fall, to establish the full time range feasible for bud-forcing. Alternative hormone types and combinations could also be applied, such as other cytokinin forms, including zeatin and thidiazuron, or gibberellins. Ultimately, it would be beneficial to determine the effects that hormones used in *Quercus* bud-forcing for explant production have on establishment, growth, and proliferation once *in vitro.*
Table 2.3. Section, distribution, native habitat, and bud morphology of *Quercus* species used in the bud-forcing experiment.

<table>
<thead>
<tr>
<th>Species Name</th>
<th>Common Name</th>
<th>Distribution (Oldfield and Eastwood, 2007)</th>
<th>Native Habitat</th>
<th>Bud Morphology</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Quercus alba</em>&lt;sup&gt;1&lt;/sup&gt; L.</td>
<td>White Oak</td>
<td>Central &amp; Eastern USA; Southeastern Canada</td>
<td>Occurs on a variety of sites, but prefers deep, moist, well-drained soils and slopes (IUCN, 2001)</td>
<td>Imbricate, broadly ovate, blunt, 3-6 mm long (Dirr, 2009)</td>
</tr>
<tr>
<td><em>Quercus bicolor</em>&lt;sup&gt;1&lt;/sup&gt; Willd.</td>
<td>Swamp White Oak</td>
<td>North- and Central-Eastern USA; Southeastern Canada</td>
<td>Found on a diversity of soil types in swamp forests, moist slopes, &amp; poorly drained uplands (IUCN, 2001)</td>
<td>Imbricate, broadly ovate, with pale pubescence on upper half, 3-6 mm long (Dirr, 2009)</td>
</tr>
<tr>
<td><em>Quercus cerris</em>&lt;sup&gt;3&lt;/sup&gt; Liebm.</td>
<td>Turkey Oak</td>
<td>Central &amp; Southern Europe; Asia Minor</td>
<td>Adaptable to a wide variety of sites and has naturalized in many different habitats throughout Europe (More and White, 2013)</td>
<td>Imbricate, ovoid, with distinct stipules that extend beyond the length of the bud, 5 mm long (McGinn, 2010)</td>
</tr>
<tr>
<td><em>Quercus falcata</em>&lt;sup&gt;2&lt;/sup&gt; Michx.</td>
<td>Southern Red Oak</td>
<td>Eastern &amp; Central USA</td>
<td>Poor, dry upland sites with sandy or clay loam soils (IUCN, 2001)</td>
<td>Imbricate, ovoid, acute, with pubescence concentrated on upper half, 6 mm long (Dirr, 2009)</td>
</tr>
<tr>
<td><em>Quercus imbricaria</em>&lt;sup&gt;2&lt;/sup&gt; Michx.</td>
<td>Shingle Oak</td>
<td>Eastern &amp; Central USA</td>
<td>A diversity of well-drained sites, including uplands, mesic bottomlands, &amp; stream beds (IUCN, 2001)</td>
<td>Imbricate, conical to broadly ovate, sharp-pointed or blunt*, pubescent, often with stipules, 3-6 mm long (Dirr, 2009)</td>
</tr>
<tr>
<td><em>Quercus macrocarpa</em>&lt;sup&gt;1&lt;/sup&gt; Michx.</td>
<td>Bur Oak</td>
<td>Northeastern &amp; Central USA; South-Central &amp; Eastern Canada</td>
<td>Very widely distributed and adapted, growing in a variety of habitats, preferably in limestone or calcareous clay (Gucker, 2011)</td>
<td>Imbricate, conical to broadly ovate, sharp-pointed or blunt*, pubescent, often with stipules, 3-6 mm long (Dirr, 2009)</td>
</tr>
<tr>
<td><em>Quercus macrocarpa</em>&lt;sup&gt;1&lt;/sup&gt; Michx. var. <em>macrocarpa</em></td>
<td>Mossycup Oak</td>
<td>North- and Central-Eastern USA; South-Central &amp; Eastern Canada</td>
<td>Very widely distributed and adapted, growing in a variety of habitats, preferably in limestone or calcareous clay (Gucker, 2011)</td>
<td>Imbricate, conical to broadly ovate, sharp-pointed or blunt*, pubescent, often with stipules, up to 6 mm long (Kalma and Wu, 2007)</td>
</tr>
<tr>
<td>Species</td>
<td>Common Name</td>
<td>Distribution</td>
<td>Habitat</td>
<td>Leaf Characteristics</td>
</tr>
<tr>
<td>-------------------------</td>
<td>-------------------</td>
<td>---------------------------------------------------</td>
<td>---------------------------------------------------------------------------------------------------</td>
<td>----------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td><em>Quercus pagoda</em>² Raf.</td>
<td>Cherrybark Oak</td>
<td>East-Central &amp; South Eastern USA</td>
<td>Moist lowlands, mesic slopes, and bottomland forests (IUCN, 2001)</td>
<td>Imbricate, ovoid, sharp-pointed, pubescent on upper half, 3-6 mm long (Seiler et al., 2015)</td>
</tr>
<tr>
<td><em>Quercus palustris</em>² Münnch.</td>
<td>Pin Oak</td>
<td>East-Central &amp; South Eastern USA; South-Central Canada</td>
<td>Bottomland forests and along rivers with wet, acidic soils (IUCN, 2001)</td>
<td>Imbricate, conical to ovate, sharp-pointed, 3-6 mm long (Dirr, 2009)</td>
</tr>
<tr>
<td><em>Quercus rubra</em>² L.</td>
<td>Northern Red Oak</td>
<td>Eastern &amp; Central USA; Southeastern Canada</td>
<td>Most common on mesic slopes and well-drained uplands (IUCN, 2001)</td>
<td>Imbricate, oval to ovate, sharp-pointed, sometimes with hairs concentrated at apex and around scale margins, 6-9 mm long (Dirr, 2009)</td>
</tr>
<tr>
<td><em>Quercus texana</em>² Buckl.</td>
<td>Nuttall's Oak</td>
<td>South-Central USA</td>
<td>Prefers flood plains, streams, &amp; bottomlands with wet clay soils, but tolerates drier sites as well (IUCN, 2001)</td>
<td>Imbricate, pubescent, with ciliate scale margins, 3-6 mm long (Dirr, 2009)</td>
</tr>
<tr>
<td><em>Quercus variabilis</em>³ Blume</td>
<td>Chinese Cork Oak</td>
<td>China, Japan, &amp; Korea</td>
<td>Warm temperate deciduous forests and lowlands (Box and Fujiwara, 2015)</td>
<td>Imbricate, narrow conical, sharp-pointed, with hairs on scale margins and occasional stipules, 5 mm long (Héardot, 2015)</td>
</tr>
</tbody>
</table>

Sections: ¹*Quercus* (White Oaks), ²*Lobatae* (Black or Red Oaks), and ³*Cerris* (Turkey Oaks)

*The material for this species used in the experiment exhibited mostly blunt buds.*
Figure 2.4. Mean bud activity over time of the twelve *Quercus* species used in the bud-forcing experiment.
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Chapter 3

EVALUATION OF DIFFERENT MEDIA FOR THE IN VITRO CULTURE OF TWELVE NORTH AMERICAN SPECIES OF OAK (QUERCUS L.)

3.1 Abstract

Oaks (Quercus L.) are valued for their ecological, economical, and aesthetic contributions, however many oak species are threatened by issues including habitat loss and attacks from pests and diseases. Oak conservation can be complex since acorns do not preserve well under traditional seed banking techniques. In vitro culture can be a useful tool in the conservation of Quercus, however limited protocols exist for this genus of plants. Young shoot tips from mature phase cuttings were cultured in vitro to determine whether there is a taxonomic response pattern in explants from twelve Quercus species representing the three North American Quercus sections (Lobatae, Quercus, and Protobalanus) across two different media. In spring of 2015, cuttings from twelve Quercus species were obtained: arkansana, boyntonii, canbyi, chrysolepis, dumosa, engelmannii, gambelii, graciliformis, palmeri, texana, tomentella, and vacciniifolia. Shoot-tip explants collected from the cuttings were placed into one of two different media formulations: Lloyd and McCown Woody Plant (WP) basal salts with Murashige and Skoog (MS) vitamins, or Gresshoff and Doy (GD) basal salts and vitamins. Responses of growth, survival, and contamination varied significantly by species, but these differences were concealed when grouped by section. Growth responses of leaf expansion, bud expansion, shoot production, and callus production were observed in nine of the Quercus species: arkansana,
graciliformis, texana, boyntonii, dumosa, engelmannii, gambelii, tomentella, and vacciniifolia. Explants grown on the WP medium had significantly more growth responses and longer survival times than those on the GD medium. The study provides a deeper understanding into how responses can vary by Quercus species and cannot necessarily be generalized by taxonomic section. The research has also shown the effects on survival time and growth of explants on different medium types and supports a recommendation of using WP media instead of GD media for the in vitro propagation of Quercus.

3.2 Introduction

Oaks (Quercus L.) are globally iconic trees, prized for their contributions as a keystone species, strong, rot-resistant wood, and landscape value. Despite their importance, many species of Quercus are under threat from a range of global issues, such as habitat loss, competition from invasive species, and attacks from pests and disease (Kramer and Pence, 2012; Oldfield and Eastwood, 2007). Conservation of this genus can be quite complex since Quercus acorns do not preserve well under traditional techniques, such as seed banking (Bonner, 2003). One tool to support conservation efforts is in vitro culture (Hartmann et al., 2002; Pence, 2010; Razdan, 2003). Also known as tissue culture, this method involves growing different types of plant tissues, such as shoot tips or embryos, on nutrient media in a sterile, enclosed, and temperature- and light-controlled system (Hartmann et al., 2002; Pence, 2010; Razdan, 2003).

By using conditions to promote slow growth, including low temperature and light levels, and by occasional sub-culturing, in vitro cultures can be maintained as ex situ conservation germplasm collections for intermediate-term storage over several
years (Engelmann and Engels, 2002; Guerrant et al., 2004; Pence, 2011; Razdan, 2003). *In vitro* propagation can be employed to increase the number of individuals for both *ex situ* and *in situ* conservation, especially if a species is difficult to propagate through traditional methods or there are very few individuals available (Engelmann and Engels, 2002; Guerrant et al., 2004; Hartmann et al., 2002; Pence, 2011; Razdan, 2003). Furthermore, *in vitro* culture can also be used for supplying material for cryopreservation, culturing cryopreserved material after thawing, and recovering and growing embryos after embryonic axis freezing (Hartmann et al., 2002; Pence, 2010; Pence, 2011; Razdan, 2003).

### 3.2.1 *In Vitro* Culture Methods

*In vitro* culture is most commonly used to generate shoots, callus, or somatic embryos (embryos formed from vegetative cells) (Hartmann et al., 2002; Razdan, 2003). Shoot formation is used for virus elimination, rejuvenation, micrografting, regeneration of plants with different ploidy levels, and perhaps most commonly for micropropagation (Hartmann et al., 2002; Razdan, 2003). These methods can involve the use of many types of source tissues, including meristems, shoot tips, buds, nodal segments, leaf pieces, and root segments. Seedling formation can be accomplished using seeds, which may be difficult to germinate using traditional methods, such as those of orchid species; or by isolating embryos, ovaries, or ovules for research, propagation, micrografting, or controlled breeding (Hartmann et al., 2002; Razdan, 2003). Callus formation can take place using any type of vegetative tissue, and can be used to generate suspension cultures or protoplasts (isolated single cells without a cell wall) for research, breeding, and genetic transformation (Hartmann et al., 2002; Razdan, 2003). Somatic embryo formation (somatic embryogenesis) can be direct or
indirect (arising from embryogenic callus) and is frequently used for clonal regeneration or recovery of plants that have been genetically transformed (Hartmann et al., 2002; Razdan, 2003). *In vitro* plantlet formation through micropropagation, or the outgrowth of lateral shoots *in vitro*, can be a useful tool for many of the methods of germplasm preservation mentioned previously and is the focus of this study (Hartmann et al., 2002; Pence 2010; Razdan, 2003).

Micropropagation research has been conducted on several genera and species within the Fagaceae, the family that includes *Quercus* (Pijut et al., 2011). In *Fagus grandifolia* (Barker et al., 1997) and *F. sylvatica* (Meier and Reuther, 1994), micropropagation has been successful using buds from mature trees and shoot tips from seedlings and root sprouts. Seedling shoot tips have also been used successfully in the micropropagation of *Nothofagus leoni* (Pastur and Arena, 1999) and *N. obliqua* (Pastur & Arena 1995). *Castanea sativa* and *C. sativa x C. crenata*, and have been successfully micropropagated using nodal segments and shoot tips from stump sprouts, epicormics shoots, and crown branches of mature trees, in addition to seedling shoot tips and plantlets formed from cultured embryonic axes (Sanchez et al., 1991; Sanchez et al., 1997a; Sanchez et al., 1997b; Vieitez et al., 2007). Shoot tips from stump sprouts and seedlings have been successfully used in the micropropagation of *C. dentata* (Xing et al., 1997).

*Quercus* micropropagation research has predominantly focused on economically important species, such as *Q. robur* (Chalupa, 1988; Favre and Juncker, 1987; Juncker and Favre, 1994; Vieitez et al., 1994), *Q. rubra* (Schwarz and Schlarbaum, 1993; Vengadesan and Pijut, 2009; Vieitez et al., 1993), and *Q. suber* (Manzanera and Pardos, 1990; Romano et al., 1992). Only three studies were found
investigating *in vitro* propagation of threatened *Quercus* species, conducted by Kramer and Pence (2012) studying *Q. acerifolia*, *Q. arkansana*, *Q. boyntonii*, and *Q. georgiana*; Kartsonas and Papafotiou (2007) studying *Q. euboica*; and Tamta et al. (2008) studying *Q. semecarpifolia*. *Quercus* micropropagation using shoot tips and nodal segments from seedlings has been studied extensively (Chalupa, 1988; Favre and Juncker, 1987; Kartsonas and Papafotiou, 2007; Manzanera and Pardos, 1990; Schwarz and Schlarbaum, 1993; Vengadesan and Pijut, 2009; Tamta et al., 2008). There are many additional studies that involve the use of other types of juvenile explant sources as well, including stump sprouts (Chalupa, 1988; Favre and Juncker, 1987; Manzanera and Pardos, 1990), epicormic shoots (Chalupa, 1988; Vieitez et al., 1993; Vieitez, 1994), and basal branches (Chalupa, 1988). Studies involving the use of mature phase material are less common (Kartsonas and Papafotiou, 2007; Kramer and Pence, 2012; Romano et al., 1992; Vieitez et al., 1993).

### 3.2.2 Ontogenetic Age of Explant Source Material in *In Vitro* Culture

Ontogenetic age in plants refers to the physiological, or developmental, phase of a plant or sections of a plant, from juvenile to mature (adult capable of flowering), rather than the actual, chronological age of a plant (Hartmann et al., 2002; Wendling et al., 2014). Juvenile phase plant material can be found in seedlings and adventitious shoots induced to grow from roots, as well as the ‘core of juvenility’ in an adult plant, which includes basal branches, stump sprouts, and the central portion of the plant (Hartmann et al., 2002; Razdan, 2003; Wendling et al., 2014). Material from the adult phase is most commonly found in the upper and outside branches of plants (Hartmann et al., 2002; Razdan, 2003; Wendling et al., 2014). Explants originating from material in the mature state tend to be more difficult to establish and grow in culture than explants.
from juvenile phase material (Hartmann et al., 2002; Preece and Read, 2003; Razdan, 2003).

For species in the Fagaceae, micropropagation using explants retaining a high degree of juvenility has been compared with explants originating from the new growth of mature material in adult trees. Barker et al. (1997) used shoot tips from seedlings and stump sprouts to compare with young shoot tips from mature branches in *Fagus grandifolia*. In *Castanea sativa* and *C. sativa x C. crenata*, Sánchez and Vieitez (1991), Sánchez et al. (1997b), and Vieitez et al. (2007) compared the response of shoot tip explants taken from basal stems, stump sprouts, epicormic shoots, and seedlings of trees to those from the upper, mature portion. Kartsonas and Papafotiou (2007) used shoot tips from *Quercus euboica* seedlings and compared their response in micropropagation to those taken from mature phase plants. For *Quercus rubra*, Vieitez et al. (1993) compared responses of explants from seedling and mature phase shoot tips. Each of these studies indicated a greater response (increased bud break, higher multiplication rates, and better establishment in culture) with juvenile material compared to the mature material.

However, juvenile material may not always be feasible to obtain, especially for threatened *Quercus* species. Acorn production for seedlings can be low and verification of parentage may be hard to determine, since *Quercus* hybridize easily (Kramer and Pence, 2012; Oldfield and Eastwood, 2007). Depending on the circumstances, the basal branches may or may not be present on individual plants. Acquiring stump sprouts is not practical when there are only a limited number of individuals available, since the primary method of production of these sprouts, coppicing, is to cut a tree down to a stump (Rong et al., 2013; Wendling et al., 2014).
Wounding or removal of root material for induction of adventitious shoots could injure and harm the plant by creating portals for pests and pathogens and depleting the tree’s carbohydrate storage (Jim, 2003; Kramer and Kozlowski, 2012). Therefore, determining, a reliable method of in vitro culture involving mature phase material would be useful in overcoming the challenges of obtaining juvenile phase plant material from threatened species.

3.2.3 In Vitro Culture Media

In vitro media are generally composed of gelling agents, basal salts, and organic compounds (Hartmann et al., 2002; Razdan, 2003). There are two main options for gelling agents: agar, formed from certain species of red algae and gellan gum (gel), a polysaccharide obtained from Pseudomonas bacteria (Hartmann et al., 2002; Razdan, 2003). Most research on Quercus micropropagation has used agar-based media (Juncker and Favre, 1994; Kartsonas and Papafotiou, 2007; Kramer and Pence, 2012; Romano et al., 1992; Vieitez et al., 1994; Vieitez et al., 2009), and occasionally gel-based media (Kramer and Pence, 2012; Tamta et al., 2008; Vengadesan and Pijut, 2009). The basal salts (inorganic salts) used in in vitro culture media provide the macronutrients (nitrogen, phosphorus, potassium, sulfur, calcium, magnesium) and micronutrients (chlorine, boron, cobalt, copper, manganese, iron, zinc, molybdenum, and nickel), with many different formulations available (Hartmann et al., 2002; Razdan, 2003). The two predominant basal salt media formulations used in micropropagation of Castanea species (Vieitez et al., 2007; Xing et al., 1997) and Quercus species (Kramer and Pence, 2012; Vieitez et al., 2012) are Lloyd and McCown Woody Plant Medium (WP) (Lloyd & McCown, 1981) and Gresshoff and Doy Medium (GD) (Gresshoff and Doy, 1972). A millimolar comparison of select
nutrients in WP and GD is shown in Figure 3.1., with one of the most significant differences between the two formulations being the greater nitrogen and lower sulfur levels in the GD formulation. A variety of basal salt media formulations, including both WP and GD, have been used in the micropropagation of other genera in the Fagaceae, such as *Fagus* (Barker et al., 1997; Meier and Reuther, 1994) and *Nothofagus* (Pastur and Arena 1995; Pastur and Arena 1999).

**Figure 3.1.** Nutrient millimolarity comparison of Woody Plant Medium (Lloyd and McCown, 1981) and Gressoff and Doy Medium (Gresshoff and Doy, 1972)

There are also many types of organic compounds used in *in vitro* culture media, including vitamins, carbohydrates, hormones, and other substances, such as those to prevent browning (Hartmann et al., 2002; Razdan, 2003). Vitamins are often included with the basal salt media formulations, and generally include thiamin, nicotinic acid, glycine, pyridoxine, and inositol (Hartmann et al., 2002; Razdan, 2003).
The most commonly used carbohydrate source used in *in vitro* media is sucrose (Harmann et al., 2002), but the effectiveness of glucose and fructose has also been evaluated for members of the Fagaceae. Explants of *Fagus sylvatica* (Cuenca and Vieitez, 2000) and *Castanea sativa* (Vieitez et al. 2007) grown on media containing glucose or fructose initiated greater numbers of shoots than explants grown on media containing sucrose. However, explants on the glucose or fructose containing media also showed poorer shoot length and increased risk of hyperhydricity compared to explants grown using sucrose (Cuenca and Vieitez, 2000; Vieitez et al. 2007).

Romano et al. (1995) studying *Quercus suber* found similar results in comparing the three carbohydrate sources, but the study also found that glucose was the most effective carbon source to promote optimum rooting.

Hormones, or plant growth regulators, are used to control organ and tissue development, with cytokinins and auxins being the two most important and most commonly used classes of hormones for *in vitro* culture (Hartmann et al., 2002; Razdan, 2003). Cytokinins are responsible for cell division and are most commonly used to promote shoot initiation and proliferation, while auxins are responsible for cell elongation and are most often used to promote rooting (Hartmann et al., 2002; Razdan, 2003). Common forms of cytokinins include 6-benzylaminopurine (BAP; 6-benzyladenine), zeatin, and kinetin, while indole-3-acetic acid (IAA), indole-3-butyric acid (IBA), and α-naphthaleneacetic acid (NAA) are the most common forms of auxins. In general, a higher ratio of cytokinin to auxin induces more shoot growth, while a lower cytokinin to auxin ratio promotes greater root growth. Balanced levels of cytokinins and auxins often result in growth of callus, or a mixture of shoots and roots (Hartmann et al., 2002; Razdan, 2003).
Several researchers working with species in the Fagaceae have recommended the addition of lower concentrations of cytokinins, generally in the form of BAP at or near 0.89µM, to the micropropagation media for maximum shoot multiplication for *Fagus* (Barker et al., 1997), *Nothofagus* (Pastur and Arena, 1995; Pastur and Arena, 1999), *Castanea* (Tetsumara and Yamashita, 2004; Vieitez et al., 2007), and *Quercus* (Chalupa, 1988; Favre and Juncker, 1987; Juncker and Favre, 1994; Manzanera and Pardos, 1990; Ostrolucká et al., 2007; Puddephat et al., 1997; Romano et al., 1992; Vieitez et al., 1993). With increasing concentrations of cytokinin, not only was shoot proliferation decreased, but the prevalence of hyperhydricity was also increased in several studies (Juncker and Favre, 1994; Pastur and Arena, 1995; Pastur and Arena, 1999; Puddephat et al., 1997; Romano et al., 1992; Tetsumara and Yamashita, 2004; Vieitez et al., 2007). Hyperhydricity is a condition where explants take on a translucent, water-soaked appearance and grow abnormally (Debergh et al., 1992; Hartmann et al., 2002). [It should be noted that the term ‘vitrification’ is sometimes used to label this condition as well, but the use of this particular term is discouraged to avoid confusion with its use in cryopreservation (Debergh et al., 1992)]. This condition involves the excess uptake of water that inhibits lignin and cellulose synthesis (Hartmann et al., 2002; Purohit et al., 2011).

Research has also determined that the addition of auxin to the medium, especially in the form of IBA, for at least a seven day period, promoted optimum rooting with the greatest number and length of roots in members of the Fagaceae, but the specific concentrations recommended vary by species. Rooting in *Nothofagus obliqua* (Pastur and Arena, 1995) was optimal at 0.66 µM IBA, while *N. leoni* (Pastur and Arena, 1999) responded best to 1.23 µM IBA. For *Castanea*, a genus that is
notoriously difficult to root, much higher concentrations of auxin at 14.76 µM IBA were recommended (Sánchez et al., 1997b; Vieitez et al., 2007). For *Quercus cerris* (Ostrolucká et al., 2007), *Q. robur* (Chalupa, 1988; Ostrolucká et al., 2007), *Q. rubra* (Ostrolucká et al., 2007), and *Q. virgiliana* (Ostrolucká et al., 2007), lower auxin levels through 1.0-2.5 µM IBA were recommended. However, Manzanera and Pardos (1990) found that higher levels at 4.92 µM IBA for juvenile phase material and as much as 24.6 µM IBA for adult phase material produced optimum rooting in *Q. suber*. Clearly, *Quercus* species rooting *in vitro* can vary greatly by species and ontogenetic age.

Activated charcoal is another organic compound sometimes added to *in vitro* media (Hartmann et al., 2002; Pan and van Staden, 1998; Thomas, 2008). Activated charcoal has several uses for *in vitro* culture, one of which is the adsorption of phenolic compounds exuded by explants (Hartmann et al., 2002; Pan and van Staden, 1998; Thomas, 2008). Phenolic exudation is harmful to the explants and poses a significant challenge to the *in vitro* culture of *Quercus* species (Kramer and Pence, 2012). Activated charcoal was used to adsorb phenolic compounds in *Quercus* micropropagation research conducted by Ostrolucká et al. (2007) and Vieitez et al. (2009). Studies have also found the use of activated charcoal increases rooting rates in micropropagation of *Fagus* (Barker et al., 1997), *Castanea* (Vieitez et al., 2007), and *Quercus* (Ostrolucká et al., 2007; Vieitez et al., 2009). This is likely due in large part to the darkness the charcoal adds at the base of the shoot, which allows for the accumulation of photosensitive auxins (Pan and van Staden; 1998). However, at the same time, activated charcoal, with its very fine network of pores, also adsorbs growth regulators in large quantities (Hartmann et al., 2002; Nissen and Sutter, 1990; Pan and
van Staden, 1998; Thomas, 2008). The specific concentrations of additional growth regulators needed to compensate for this adsorption are still undetermined, making frequent transfers to fresh media the most common approach currently used to mitigate the harmful effects of phenolic compounds to explants (Pan and van Staden, 1998; Thomas, 2008).

3.2.4 Objectives and Hypotheses

With approximately 500 *Quercus* species in the world (Oldfield and Eastwood, 2007), it would be useful to be able to predict *Quercus* responses by group and have micropropagation protocols that could be generalized across groups of related species. The first objective of this study was to determine whether there is a taxonomic response pattern (growth responses, survival time, and contamination) in micropropagation among the three North American *Quercus* sections: *Lobatae* (black or red oaks), *Quercus* (white oaks), and *Protobalanus* (golden cup or intermediate oaks) (Nixon, 2002). The second goal of this study was to provide a protocol for the micropropagation of mature phase *Quercus* material, when no juvenile phase material is available, as is often the case with threatened species (Kramer and Pence, 2012; Pence, 2010). The third objective of this research was to determine whether WP (Lloyd and McCown, 1981) or GD (Gresshoff and Doy, 1972), the two most commonly used media in the micropropagation of *Quercus* species, is more effective in the establishment, growth, and multiplication of *Quercus* explants.

3.3 Materials and Methods

This micropropagation experiment focused predominantly on establishment and stabilization, stage I of the micropropagation process (Hartmann et al., 2002).
Twelve *Quercus* species were used in the experiment, including both threatened and non-threatened species representing the three different *Quercus* sections of North America: *Lobatae* (black or red oaks), *Quercus* (white oaks), and *Protobalanus* (golden cup or intermediate oaks) (Manos et al., 1999; Nixon, 2006). Emphasis was placed on species native to the southern United States and adjacent Mexico. Research by Xia et al. (2014) has shown that *Quercus* species from areas with warmer winters may not be as successful in cryopreservation, having less desiccation tolerance and survival following cryo-exposure of excised embryonic axis samples. This increases the importance of developing additional tools to support germplasm preservation for species growing in the warmer southern regions of North America. The *Quercus* species chosen for the experiment were: *arkansana, boyntonii, canbyi, chrysolepis, dumosa, engelmannii, gambelii, graciliformis, palmeri, texana, tomentella,* and *vacciniifolia* (Table 3.3).

Living collections in nine U.S. public gardens and arboreta containing these twelve species were identified by working with the Program Director of Botanic Gardens Conservation International (BGCI) U.S. and through the PlantSearch Database (BGCI, 2014). Depending on availability, it was possible to obtain material from 1-3 different institutions and from 2-5 different individual specimens for each species. (Specific information regarding institutions donating *Quercus* material and the associated plant accessions is listed in Appendix A.) For the public gardens and arboreta willing to donate material, it was requested that cuttings be collected in early spring, just as the *Quercus* buds were breaking, and then express-mailed to Longwood Gardens in Kennett Square, Pennsylvania, USA. (The specific protocol for collecting
and sending cuttings, modified from methods used in Kramer and Pence (2012), is included in Appendix B.)

Upon arrival, the bases of all cuttings were recut and immediately placed into either 250 mL Erlenmeyer flasks or 400 mL beakers of distilled water. Twigs that arrived with emerged leaves were allowed to sit in distilled water for at least 15 minutes to regain turgidity. Young shoots with at least one fully open leaf blade were then collected from the cuttings to be used as explants (Fig. 3.2). The excised shoots were surface-sterilized with a 1:10 dilution of commercial bleach (Clorox) with 0.05% Tween 20 (a surfactant; PhytoTech Laboratories) for 10 minutes with stirring. Explants were then rinsed by placement into sterilized, distilled water in standard 25 x 150 mm glass culture tubes. The tubes were then temporarily closed and inverted 3-5 times and allowed to sit for at least one minute.

Twigs without emerged leaves were placed in greenhouse conditions, with water changed twice weekly, until leaf emergence. Shoots with at least one young leaf blade fully open were removed and surface-sterilized as described previously. Immediately prior to culturing, explant bases were recut to remove the exposed tissue adversely affected by the bleach during sterilization. If the leaf blades of the young shoots were greater than 1 cm in length, they were cut down to 1 cm to prevent excess transpiration and water loss from the explant.
Figure 3.2. Example of a young shoot tip (*Quercus canbyi*) ready to be harvested as an explant for the *in vitro* culture experiment

*Quercus* explants were grown in culture using two different media formulations: 1) Lloyd and McCown Woody Plant (WP) Medium basal salts (1981) (PhytoTech Laboratories) with Murashige and Skoog (MS) vitamins (1962) (PhytoTech Laboratories), or 2) Gresshoff and Doy (GD) basal salts and vitamins (1972) (PhytoTech Laboratories). The WP basal salt/MS vitamin media will hereafter be referred to as “WP” media. Both media formulations were amended with 3% sucrose (PhytoTech Laboratories), 0.6% agar (PhytoTech Laboratories), 0.89 µM BAP (PhytoTech Laboratories), and 100mg/L benlate ([benomyl; methyl 1-(butylcarbomoyl)-2benzimidazolecarbamate; a fungicide; Sigma-Aldrich]. Benlate was included in the media because past research has shown that the chemical is able to prevent fungal growth, which can be a significant challenge when growing *Quercus* species *in vitro* (Favre and Juncker, 1987; Kartsonas and Papafotiou, 2007; Kramer and Pence, 2012; Romano et al., 1992; Schwarz and Schlarbaum, 1993). The explants were transferred on to fresh media two to three times a week for the first three weeks
due to visible exudation of harmful phenolic compounds, and then once every one to two weeks thereafter.

Depending on material availability, there were 12-54 explants of each of the twelve *Quercus* species, split between the two media types (GD and WP), for a total of 419 explants (Table 3.1). Due to low material availability, a sample number as high as originally anticipated was not obtained for some of the species, such as *Q. dumosa* and *Q. palmeri*. Additionally, despite receiving many cuttings of *Q. chrysolepis* and *Q. vacciniifolia* from several public gardens and arboreta, it was noted by each of the donor organizations that their specimens of these species were exhibiting poor growth that season; this was reflected in the low number of explants for *Q. chrysolepis* and *Q. vacciniifolia*. Each explant was grown separately in approximately 15 mL of medium in a standard 25 x 150 mm culture tube with a translucent polypropylene closure sealed with Parafilm. Cultures were grown in 22.5-25.5°C ambient temperature. Photosynthetically active radiation (PAR) was provided by cool white fluorescent bulbs at approximately 20-25 μmol/m²/sec under constant light, 24 hours a day. Data on growth responses, contamination, and survival times was collected each time the explants were transferred onto fresh media. The term “growth responses” is used collectively to refer to expansion of pre-existing leaves, bud enlargement, shoot production, and callus production. Growth responses and contamination were evaluated using contingency analysis and survival times were evaluated using one-way Analysis of Variance (ANOVA). An alpha level of 0.05 was considered significant for all statistical analyses.
3.4 Results

Growth responses, survival time, and contamination were tracked for each explant. The primary variables investigated were species, section, and medium type. For six of the twelve species (Q. arkansana, Q. boyntonii, Q. canbyi, Q. gambelii, Q. tomentella, and Q. vacciniifolia) material origin was also a variable considered. These six species were the only ones in which material originating both from parent plants of wild provenance and from parent plants of commercial origin was available.

3.4.1 Growth Responses

The growth responses monitored were pre-existing leaf expansion, bud enlargement, shoot production, and callus production (Fig. 3.3). Growth responses varied by species and were low among all species, with 41 out of 419 explants exhibiting growth responses, in nine of the species: Q. arkansana, Q. graciliformis, Q. texana, Q. boyntonii, Q. dumosa, Q. engelmannii, Q. gambelii, Q. tomentella, and Q. vacciniifolia (Table 3.1). The most commonly observed growth response was expansion of pre-existing leaves, which was seen in 20 explants and across five species. Bud enlargement was the second most common growth response and was observed in 12 explants from five species. While some enlarged buds eventually broke and developed into new shoots, most explants exhibiting this response turned brown and died before the buds could further develop. Callus was seen in 10 explants from five species. When callus growth occurred, it grew from a number of different areas (leaves, stems, petioles, meristems, etc.). The exception to this was Q. texana, where callus growth was only noted on the bases of petioles. The primary target growth response, production of new shoots, was the least common with only five observations of this growth response in two species: Q. arkansana and Q. gambelii.
Growth of new shoots was noted on average around the 30\textsuperscript{th} day for the *Q. arkansana* explants and on average around the 60\textsuperscript{th} day for *Q. gambelii* explants. Only one explant (*Q. gambelii*) produced multiple shoots.

**Figure 3.3.** Images of *Quercus* explant growth responses in the *in vitro* culture experiment: 1) expansion of pre-existing leaves in *Q. boyntonii*, 2) bud enlargement in *Q. engelmannii*, 3) shoot production in *Q. gambelii*, and 4) callus production in *Q. dumosa*
Table 3.1. Percentages of explants exhibiting pre-existing leaf expansion, bud enlargement, shoot production, and callus in micropropagation experiment by *Quercus* species, section, and medium.

<table>
<thead>
<tr>
<th>Quercus Section/Species Name</th>
<th>No. of Explants (down)</th>
<th>% Exhibiting:</th>
<th>GD</th>
<th>WP</th>
<th>GD</th>
<th>WP</th>
<th>GD</th>
<th>WP</th>
<th>GD</th>
<th>WP</th>
<th>GD</th>
<th>WP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Medium (across)</td>
<td>Pre-Existing Leaf Expansion</td>
<td>Bud Enlargement</td>
<td>Shoot Production</td>
<td>Callus</td>
<td>All Combined</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lobatae</td>
<td>192</td>
<td>3.1</td>
<td>3.1</td>
<td>-</td>
<td>2.1</td>
<td>1.0</td>
<td>2.1</td>
<td>1.0</td>
<td>6.3</td>
<td>7.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Q. arkansana</td>
<td>54</td>
<td>11.1</td>
<td>11.1</td>
<td>-</td>
<td>3.7</td>
<td>3.7</td>
<td>7.4</td>
<td>-</td>
<td>-</td>
<td>14.8</td>
<td>22.2</td>
<td></td>
</tr>
<tr>
<td>Q. canbyi</td>
<td>50</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Q. graciliformis</td>
<td>52</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>3.8</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>3.8</td>
<td>-</td>
<td>3.8</td>
<td></td>
</tr>
<tr>
<td>Q. texana</td>
<td>36</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>11.1</td>
<td>-</td>
<td>11.1</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Quercus</td>
<td>140</td>
<td>1.4</td>
<td>8.6</td>
<td>-</td>
<td>10.0</td>
<td>2.9</td>
<td>4.3</td>
<td>5.7</td>
<td>5.7</td>
<td>20.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Q. boyntonii</td>
<td>30</td>
<td>6.7</td>
<td>13.3</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>6.7</td>
<td>6.7</td>
<td>13.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Q. dumosa</td>
<td>18</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>33.3</td>
<td>22.2</td>
<td>33.3</td>
<td>22.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Q. engelmannii</td>
<td>44</td>
<td>-</td>
<td>4.5</td>
<td>-</td>
<td>27.3</td>
<td>-</td>
<td>-</td>
<td>4.5</td>
<td>-</td>
<td>31.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Q. gambelii</td>
<td>48</td>
<td>-</td>
<td>12.5</td>
<td>-</td>
<td>4.2</td>
<td>-</td>
<td>8.3</td>
<td>-</td>
<td>-</td>
<td>12.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Protobalanus</td>
<td>87</td>
<td>7.0</td>
<td>9.1</td>
<td>4.7</td>
<td>2.3</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>11.6</td>
<td>11.4</td>
<td></td>
</tr>
<tr>
<td>Q. chrysolepis</td>
<td>12</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Q. palmeri</td>
<td>20</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Q. tomentella</td>
<td>30</td>
<td>20.0</td>
<td>26.7</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>20.0</td>
<td>26.7</td>
<td></td>
</tr>
<tr>
<td>Q. vaccinifolia</td>
<td>25</td>
<td>-</td>
<td>-</td>
<td>16.7</td>
<td>7.7</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>16.7</td>
<td>7.7</td>
<td></td>
</tr>
<tr>
<td><strong>All Combined</strong></td>
<td><strong>419</strong></td>
<td><strong>3.3</strong></td>
<td><strong>6.2</strong></td>
<td><strong>1.0</strong></td>
<td><strong>4.8</strong></td>
<td><strong>0.5</strong></td>
<td><strong>1.9</strong></td>
<td><strong>2.4</strong></td>
<td><strong>2.4</strong></td>
<td><strong>7.2</strong></td>
<td><strong>12.4</strong></td>
<td></td>
</tr>
</tbody>
</table>

Dash (-) indicates a value of zero

Growth responses were evaluated statistically through contingency analysis. Explants affected by contamination were excluded from the analyses. Unfortunately, there were not enough growth responses to conduct a statistical analysis of the growth response data by species. When grouped by section (*Lobatae, Quercus*, and *Protobalanus*), growth response data was analyzed with a Pearson’s Chi-Square Test for Independence. Growth response was found to be independent of section (p = 0.35). Using the same test, a relationship was found between growth response and medium, with there being significantly more explants having growth responses on the WP medium than those on the GD medium (p = 0.03). For the six species where material origin could be compared, origin was not significant (p = 0.15).
3.4.2 Survival Time

Survival time was calculated excluding explants that were affected by contamination. The survival time distribution was skewed right and not normally distributed, which affects the reliability of ANOVA. To normalize the distribution, the natural log of each of the survival times was used for this analysis. Overall, mortality was high across all species, sections, media, and origin types (Fig. 3.4), with an overall mean survival time of just over 30 days. Survival time varied significantly by Quercus species when analyzed through a one-way ANOVA (p < 0.0001). Explants of Q. dumosa and Q. vaccinifolia had the longest mean survival times, while those of Q. palmeri and Q. chrysolepis had the shortest. A comparison of the explant survival time means by species using a Tukey-Kramer HSD (Honestly Significant Difference) Test can be found in the connecting letters report in Table 3.2.
Figure 3.4. Percent of *Quercus* explants surviving over time (days) in micropropagation experiment by (a) *Quercus* section, (b) medium, and (c) origin type
Table 3.2. Connecting letters report of the Tukey-Kramer HSD ( Honestly Significant Difference) Test comparing mean survival time (days) and standard error by *Quercus* species for micropropagation experiment. Species not connected by the same letter are significantly different.

<table>
<thead>
<tr>
<th>Quercus Name</th>
<th>Mean Survival Time (Days)</th>
<th>Standard Error</th>
</tr>
</thead>
<tbody>
<tr>
<td>Q. dumosa</td>
<td>A</td>
<td>84.3</td>
</tr>
<tr>
<td>Q. vacciniifolia</td>
<td>A B</td>
<td>55.3</td>
</tr>
<tr>
<td>Q. canbyi</td>
<td>A B C</td>
<td>48.7</td>
</tr>
<tr>
<td>Q. arkansana</td>
<td>A B</td>
<td>41.6</td>
</tr>
<tr>
<td>Q. graciliformis</td>
<td>A B</td>
<td>40.8</td>
</tr>
<tr>
<td>Q. tomentella</td>
<td>A B</td>
<td>36.4</td>
</tr>
<tr>
<td>Q. boyntonii</td>
<td>A B C</td>
<td>27.7</td>
</tr>
<tr>
<td>Q. engelmannii</td>
<td>B C</td>
<td>25.6</td>
</tr>
<tr>
<td>Q. gambelii</td>
<td>A B C</td>
<td>23.9</td>
</tr>
<tr>
<td>Q. texana</td>
<td>B C</td>
<td>20.7</td>
</tr>
<tr>
<td>Q. chrysolepis</td>
<td>B C</td>
<td>14.0</td>
</tr>
<tr>
<td>Q. palmeri</td>
<td>C</td>
<td>12.7</td>
</tr>
</tbody>
</table>

The differences in survival times among the species were masked when grouped by section, and there was no significant difference found in mean survival time between the three sections (*Lobatae, Quercus,* and *Protobalanus*) using a one-way ANOVA (p = 0.25). However, medium was shown to be a significant variable, using a one-way ANOVA, with the explants growing on the WP medium living over 13 days longer on average than those on the GD medium (p = 0.0013). Material origin also tested as a significant variable in survival time for the six species where material origin could be compared, using a one-way ANOVA (p = 0.004). Explants originating from material with wild provenance lived on average nearly 20 days longer compared to those of commercial origin.
3.4.3 Contamination

The overall contamination rate was 48%, but varied significantly by species using Pearson’s Chi-Squared Test (p < 0.0001). Of the twelve Quercus species, explants of Q. canbyi, Q.gambelii, Q. graciliformis, and Q. vacciniifolia showed the greatest amount of contamination, with rates of 92%, 85%, 64%, and 60%, respectively (Fig. 3.5). A subsequent test using logistic regression and a contrast of the log odds indicated that the contamination rates of these four species were significantly different than those of the other eight species in the experiment (p < 0.0001). Using Pearson’s Chi-Squared Test, no significant differences were found analyzing contamination by section (p = 0.21), medium (p = 0.16), or material origin (p = 0.12).

Figure 3.5. Percent of Quercus species affected by contamination in micropropagation experiment. Column width varies according to the sample size (n)
3.5 Discussion

Growth responses, survival times, and contamination levels varied by *Quercus* species, but no significant patterns emerged when grouped by section. This could indicate that the differences between *Quercus* species are great enough that their responses in tissue culture are unlikely to be predictable by section. To our knowledge, this is the first attempt to compare *in vitro* responses of *Quercus* species by section. The level of growth responses, especially new shoot production, was low overall, as also reported by Kartsonas and Papafotiou (2007), Kramer and Pence (2012), Romano et al. (1992), and Vieitez et al. (1993) when using mature phase *Quercus* shoot tips. The explants responded with the greatest number of growth responses on the WP media formulation, compared to the GD media formulation. This was also seen by Kramer and Pence (2012) in *Q. acerifolia*, *Q. arkansana*, *Q. boyntonii*, and *Q. georgiana*, and by Meier and Reuther (1994) in *Fagus sylvatica*, but contrary to finding of Romano et al. (1992), who found *Quercus suber* explants on GD media grew more than those on WP media.

The longest survival times were seen on the explants growing on the WP medium formulation over those on the GD medium formulation. This was also observed by Meier and Reuther (1994) in *Fagus sylvatica*, although the results were not considered significant. No other published research formally tracking and comparing *in vitro* survival times of species in the Fagaceae was found for comparison. The most significant distinction in nutrient content between the two formulations is the greater amount of nitrogen, including in the form of ammonium (NH$_4$), in the GD media (Figure 3.1). Although not formally tracked, many explants that died exhibited hyperhydricity several days before death occurred. In addition to gelling agent type and excess cytokinins (discussed in the chapter introduction),
another cause of hyperhydricity can be excessive ammonium (Hartmann et al., 2002; Razdan, 2003). This could have been an issue for the explants growing on the GD media, with the higher levels of ammonium.

There was a significant difference in the contamination rates by species. Contamination rates were highest with *Q. canbyi, Q. gambelii, Q. graciliformis*, and *Q. vacciniifolia* and these were significantly different from the other eight species in the experiment. Explant material for these four species were each represented by multiple tree specimens. Additionally, only cuttings from *Q. graciliformis* were sourced from one location, while the other three species were represented by material obtained from at least two different sources each. These two points make contamination linked to specific sources an unlikely cause of the greater amounts of contamination seen in these species. This variability in contamination levels between *Quercus* species and with explants from different trees and sources was also reported by Kramer and Pence (2012). The species with the highest levels of contamination would likely benefit from more rigorous sterilization protocols and perhaps increased levels of fungicide in the medium (Gamborg and Phillips, 2013).

### 3.6 Conclusion

While this study has provided useful information that could be applied to future research, it has several limitations. Due to low material availability for certain species, such as *Q. dumosa* and *Q. palmeri*, or poor growth of certain species at donor organizations, such as *Q. chrysolepis* and *Q. vacciniifolia*, a sample number as high as originally anticipated was not obtained for some of the species. While material origin was analyzed for the six species where cuttings originating both from parent plants with wild provenance and from parent plants of commercial origin were available, the
experiment was not originally designed to examine this variable. In the future, researchers that formally design an experiment examining material of known, wild provenance versus material originating from highly cultivated commercial stock could provide insight into the effects of cultivation on *Quercus* grown *in vitro*. Additionally, while hyperhydricity was noted, it was not formally tracked. Researchers of *Quercus* (Juncker and Favre, 1994; Puddephat et al., 1997; Romano et al., 1992), *Nothofagus* (Pastur and Arena, 1995; Pastur and Arena, 1999), and *Castanea* (Tetsumara and Yamashita, 2004; Vieitez et al., 2007) observed that excess cytokinins corresponded with increased hyperhydricity. However, future researchers should also consider investigating the role of other factors known to affect the prevalence of hyperhydricity in *Quercus* species grown *in vitro*, such as ammonium in the media (Hartmann et al., 2002; Razdan, 2003), which may have played a role in this experiment.

Through this experiment and research by Kramer and Pence (2012), *in vitro* evaluation of *Quercus* may find greater success (greater numbers of growth responses and longer survival times) with mature phase material using the WP medium formulation over GD, the other commonly used formulation in *Quercus in vitro* culture. Future research could explore specific nutrient concentrations in the media, especially to determine if low nitrogen and high sulfur content, both attributes of the WP formulation, are important factors in the *in vitro* culture of *Quercus*. Several weeks into culture, many of the explants would appear healthy and growing, but even just a few days later would begin to rapidly decline, exhibiting hyperhydricity and/or turning brown. These explants would ultimately die within a week of having been observed growing vigorously. It would be meaningful to determine if *Quercus* species require a different set of nutrients *in vitro* once they have established and begin visibly
growing. Additional studies could investigate other environmental factors, such as the effect of light, temperature, or the use of closures with gas-permeable membranes that would allow for the release of humidity, another factor in the occurrence of hyperhydricity (Hartmann et al., 2002; Razdan, 2003). While no patterns emerged by grouping *Quercus* species by section in this study, further studies could group species in other ways, such as by environmental adaptations, which might help predict how different *Quercus* species will respond to *in vitro* culture.

This *Quercus* study has provided a deeper understanding into how responses can vary by species and cannot necessarily be generalized by taxonomic section. The research has also shown the effects of different medium types on survival time and growth of explants. Each of these insights has strong potential to guide future research on the *in vitro* culture of *Quercus* species. Ultimately, though, mortality was high in the experiment, with the average explant surviving just over 30 days. Further research is necessary to define a protocol using mature phase material that is reliable and efficient enough to effectively support the conservation of threatened *Quercus* species.
Table 3.3. Section, IUCN (International Union for Conservation of Nature) Red List Status, NatureServe Global Status, distribution, and native habitat of *Quercus* species used in micropropagation experiment.

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Lobatae</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Quercus canbyi</em></td>
<td>Canby Oak</td>
<td>Not Yet Assessed</td>
<td>Not Yet Assessed</td>
<td>USA: TX; Mexico (Hélardot, 2015)</td>
<td>Dry, rocky soils (Hélardot, 2015)</td>
</tr>
<tr>
<td><em>Quercus texana</em></td>
<td>Nuttall's Oak</td>
<td>Least Concern</td>
<td>Secure</td>
<td>USA: AL, AR, IL, KS, KY, LA, MS, MO, OK, TN, TX (NatureServe, 2015)</td>
<td>Prefers flood plains, streams, &amp; bottomlands with wet clay soils, but tolerates drier sites as well (IUCN, 2001)</td>
</tr>
<tr>
<td><strong>Quercus</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Quercus dumosa</em></td>
<td>California Scrub Oak</td>
<td>Endangered</td>
<td>Imperiled</td>
<td>USA: CA; Mexico: Baja California (Oldfield and Eastwood, 2007)</td>
<td>Dry chaparral &amp; coastal scrub (NatureServe, 2015)</td>
</tr>
<tr>
<td><em>Quercus engelmannii</em></td>
<td>Engelmann Oak</td>
<td>Vulnerable</td>
<td>Vulnerable</td>
<td>USA: CA; Mexico: Baja California (Oldfield and Eastwood, 2007)</td>
<td>Sclerophyllous scrub (Oldfield and Eastwood, 2007)</td>
</tr>
<tr>
<td>Species</td>
<td>Distribution</td>
<td>Conservation Status</td>
<td>USA Distribution</td>
<td>Habitat</td>
<td></td>
</tr>
<tr>
<td>---------</td>
<td>--------------</td>
<td>---------------------</td>
<td>------------------</td>
<td>---------</td>
<td></td>
</tr>
<tr>
<td><strong>Quercus gambelii</strong> Nutt.</td>
<td>Gambel Oak</td>
<td>Least Concern</td>
<td>Secure</td>
<td>USA: AZ, CO, NM, NV, OK, SD, TX, UT, WY; northern Mexico (Oldfield and Eastwood, 2007; NatureServe, 2015)</td>
<td>Dry hills, slopes, &amp; canyons (NPIN, 2015)</td>
</tr>
<tr>
<td><strong>Protobalanus</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Quercus chrysolepis</strong> Liebm.</td>
<td>Canyon Live Oak</td>
<td>Least Concern</td>
<td>Secure</td>
<td>USA: AZ, CA, NM, NV, OR; Mexico (Oldfield and Eastwood, 2007)</td>
<td>Canyons, slopes, &amp; mountainous regions (USDA-NRCS, 2016)</td>
</tr>
<tr>
<td><strong>Quercus palmeri</strong> Engelm.</td>
<td>Palmer Oak</td>
<td>Not Yet Assessed</td>
<td>Apparently Secure</td>
<td>USA: AZ, CA, NM; Mexico: Baja California (NPIN, 2015)</td>
<td>Dry canyons &amp; slopes; chaparral; pinyon-juniper woodlands (NPIN, 2015)</td>
</tr>
<tr>
<td><strong>Quercus tomentella</strong> Engelm.</td>
<td>Island Oak</td>
<td>Vulnerable</td>
<td>Vulnerable</td>
<td>USA: CA; Mexico: Guadalupe Island, Baja California (Oldfield and Eastwood, 2007)</td>
<td>Dry forest areas (Oldfield and Eastwood, 2007)</td>
</tr>
<tr>
<td><strong>Quercus vacciniifolia</strong> Kell.</td>
<td>Huckleberry Oak</td>
<td>Not Yet Assessed</td>
<td>Apparently Secure</td>
<td>USA: CA; NV; OR (NatureServe, 2015)</td>
<td>Dry, windy, often rocky, montane chaparral fields (Howard, 1992)</td>
</tr>
</tbody>
</table>
REFERENCES


Bonner, F. 2003. Care and collection of acorns: A practical guide for seed collectors and nursery managers. USDA Forest Service Natl. Seed Lab., Dry Branch, GA.


Chapter 4

CONCLUSION

The large and diverse genus of *Quercus* is valued economically, aesthetically, and perhaps most importantly, for its innumerable ecological contributions (Manos et al., 1999; Nixon, 2006). Despite their importance, many species of *Quercus* are under threat from a range of issues, including habitat loss and attacks from pests and diseases (Kramer and Pence, 2012; Oldfield and Eastwood, 2007). Research and conservation efforts pertaining to *Quercus* species are key to preserving biodiversity, saving threatened species, and preventing additional species from reaching a threatened status. *In situ* (within natural habitat) and *ex situ* (outside of natural habitat) are the two main approaches to plant conservation (Kramer and Pence, 2012; Tuxhill and Nabhan, 2001). *Ex situ* methods support *in situ* conservation through the preservation of, and convenient access to, rich concentrations of plant germplasm (Guerrant et al., 2004; Pence, 2011; Tuxhill and Nabhan, 2001).

Seed banking is a method of drying and then storing seeds long-term at low temperatures for the purpose of securing plant species genetic diversity (Kramer and Pence, 2012; Razdan, 2003). While seed banking is the most efficient and effective conservation strategy for many plant species, the process cannot be applied to *Quercus* species (Bonner, 2003; Kramer and Pence, 2012; Pence, 2011; Razdan, 2003). *Quercus* species are labeled “exceptional”, due to their “recalcitrant” seeds (acorns), which do not tolerate the desiccation required for seed banking (Bonner, 2003; Pence, 2011). This sensitivity to moisture loss presents a challenge for conserving threatened
Quercus species by eliminating the possibility of long-term germplasm storage in seed banks (Kramer and Pence, 2012; Pence, 2011). Without the option to seed bank acorns, increased importance is placed on different approaches to Quercus conservation, such as living collections, cryopreservation (of both embryonic and vegetative tissues), and in vitro culture (Kramer and Pence, 2012; Pence, 2011).

Living collections, which are composed of plants cultivated outside of their natural habitat, allow material to be easily available for research, evaluation, and public display and education (Guerrant et al., 2004; Hawkes et al., 2012). Potential drawbacks to living collections include accurate genetic representation of species, and risks of damage from pests, diseases, and natural hazards (Guerrant et al., 2004; Hawkes et al., 2012; Kramer and Pence, 2012). Additionally, there is the high cost of space and maintenance, one of the most significant challenges with the use of living collections for long-term preservation (Guerrant et al., 2004; Hawkes et al., 2012; Kramer and Pence, 2012; Pence, 2011).

Cryopreservation involves the removal of water from tissue, either by drying or removal or replacement of water by chemical means, followed by exposure to liquid nitrogen (Engelmann, 2004; Guerrant et al., 2004; Razdan, 2003). The cryopreservation of embryos and embryonic axes allows for the preservation of genetic diversity in a manner similar to seed banking, while cryopreservation of vegetative tissues, such as shoot tips, allows for the creation of a “tissue bank” for propagation and restoration efforts (Pence, 2011). Cryopreservation is a long-term storage method that protects germplasm from contamination with minimal space and maintenance requirements (Engelmann, 2004; Razdan, 2003). However, this method requires a significant initial capital investment, specially trained technicians, and a
regular supply of liquid nitrogen (Guerrant et al., 2004; Pence, 2011; Razdan, 2003). While cryopreservation has shown potential for Quercus conservation, its success has varied by species (Xia et al., 2014), and shoot tip cryopreservation of Quercus species has yet to be reported. Further protocols must be developed to make it a viable option for genetic diversity preservation or tissue banking, especially with a genus as large and diverse as Quercus (Pence, 2010; Pence, 2011). Additionally, when certain tissue types, such as somatic embryos and zygotic embryonic axes, are removed and thawed from cryopreservation, the process is dependent on the subsequent in vitro culture of the tissue for recovery (González-Benito et al., 2002; Pence, 2010; Pence, 2011; Razdan, 2003).

In vitro culture (tissue culture) involves growing different types of plant tissues, such as shoot tips or embryos, on nutrient media in a sterile, enclosed, and light- and temperature-controlled system (Hartmann et al., 2002; Pence, 2010; Razdan, 2003). By using conditions to promote slow growth, such as low temperature and light levels, and by occasional sub-culturing, in vitro cultures can be maintained as ex situ conservation germplasm collections (tissue banks) for intermediate-term storage over several years (Engelmann and Engels, 2002; Guerrant et al., 2004; Pence, 2011; Razdan, 2003). In vitro collections are protected from pests, disease, and environmental hazards and can be employed to increase the number of individuals for both ex situ and in situ conservation (Engelmann and Engels, 2002; Guerrant et al., 2004; Hartmann et al., 2002; Pence, 2011; Razdan, 2003). This is especially important if a species is difficult to propagate through traditional methods or there are very few individuals available (Engelmann and Engels, 2002; Guerrant et al., 2004; Pence, 2011). Furthermore, in vitro culture can also be used for supplying material for
vegetative cryopreservation, culturing cryopreserved material after thawing, and recovering and growing embryos after embryonic axis freezing (Hartmann et al., 2002; Pence, 2010; Pence, 2011; Razdan, 2003). Like cryopreservation, though, \textit{in vitro} culture necessitates a significant initial capital investment, specially trained technicians, and specialized laboratory supplies (Guerrant et al., 2004; Hartmann et al., 2002; Pence, 2011). \textit{In vitro} culture also requires continued maintenance through regular sub-culturing, thus making it a more costly storage method over time than cryopreservation (Guerrant et al., 2004; Hartmann et al., 2002; Pence, 2011).

However, if \textit{Quercus} germplasm cannot be preserved through seed banking, and living collections and cryopreservation protocols are inadequate, then \textit{in vitro} culture becomes the only alternative available for the conservation of threatened \textit{Quercus} species.

The current range of \textit{Quercus} species studied for \textit{in vitro} culture is, unfortunately, limited and is primarily focused on economically important, rather than threatened, species (Kramer and Pence, 2012; Pence, 2011). This means that the current protocols have been developed for the wide-scale production of thousands of plants for agricultural or horticultural use and sale of a few important species, rather than preservation of threatened species (Pence, 2011). Additionally, these protocols primarily involve the use of juvenile phase material, generally obtained from seedlings and stump sprouts, which is not a realistic option for threatened \textit{Quercus} species (Kramer and Pence, 2012). Acorn production for seedlings can be low and verification of parentage may be hard to determine, since \textit{Quercus} hybridize easily (Kramer and Pence, 2012; Oldfield and Eastwood, 2007). Acquiring stump sprouts is not practical when there are only a limited number of individuals available, since the
primary method of production of these sprouts, coppicing, is to cut a tree down to a stump (Rong et al., 2013; Wendling et al., 2014). Therefore, current in vitro culture protocols for Quercus that focus on economically important species and use juvenile material are generally insufficient to support threatened species that need to be cultured in vitro on a smaller scale using mature material (Pence, 2011).

To help develop an in vitro culture protocol that is effective and efficient for the conservation of threatened Quercus species, the experiments of this work focused on mature phase material over a broad spectrum of species. Through the bud-forcing experiment, it was determined that forcing bud break in Quercus for the production of in vitro shoot tip explants without BAP application is a viable option, but the rate may be enhanced with some species by the application of BAP. Results of the in vitro culture experiment show that explants on the WP medium had a greater number of growth responses and longer survival times than those grown on the GD medium. However, in both experiments, significant variability was observed in the responses of the different species. Additionally, no response patterns were found in vitro through the grouping of species by taxonomic section.

With approximately 500 currently identified species of Quercus (Oldfield and Eastwood, 2007), the variability seen in these experiments underlines the need for increased attention to conservation of the many threatened Quercus species, rather than just the few economically important species. Broadly, future work should investigate further factors, beyond taxonomic section, such as environmental adaptations, that could help predict how different Quercus species will respond to different conservation methods. This will most effectively and efficiently contribute to the preservation of Quercus.
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Bonner, F. 2003. Care and collection of acorns: A practical guide for seed collectors and nursery managers. USDA Forest Service Natl. Seed Lab., Dry Branch, GA.

Box, E.O. and K. Fujiwara. 2015. Warm-temperate deciduous forests around the Northern Hemisphere. Springer.


Holsinger, Jr., A.C. 2006. Influence of benzyladenine on shoot forcing and tissue culture of *Juglans nigra* L. and *Quercus rubra* L. Southern Illi. Univ., Carbondale, IL, MS Thesis. UMI 1461934.


## Appendix A

PUBLIC GARDENS AND ARBORETA DONATING *QUERCUS* CUTTINGS FOR *IN VITRO* CULTURE RESEARCH

<table>
<thead>
<tr>
<th><em>Quercus</em> Species</th>
<th>Identifier</th>
</tr>
</thead>
</table>
|                  | Arnold Arboretum of Harvard University  
(Boston, Massachusetts, USA) |
| *Q. arkansana*    | 279-86*A   |
| *Q. arkansana*    | 477-86*B   |
| *Q. arkansana*    | 477-86*D   |
| *Q. texana*       | 293-86*B   |
|                  | Bartlett Tree Research Laboratories Arboretum  
(Charlotte, North Carolina, USA) |
<p>| <em>Q. arkansana</em>    | 2002-288<em>A |
| <em>Q. arkansana</em>    | 2002-289</em>A |
| <em>Q. boyntonii</em>    | 2002-036<em>A |
| <em>Q. boyntonii</em>    | 2002-037</em>A |
| <em>Q. canbyi</em>       | 2012-0266<em>A |
| <em>Q. canbyi</em>       | 2012-0266</em>B |
| <em>Q. chrysolepis</em>  | 2012-0131<em>A† |
| <em>Q. chrysolepis</em>  | 2012-0131</em>B† |
| <em>Q. chrysolepis</em>  | 2012-0351<em>C† |
| <em>Q. gambelii</em>     | 2009-0096</em>A |
| <em>Q. gambelii</em>     | 2014-0435<em>A |
| <em>Q. graciliformis</em>| 2012-0506</em>A |
| <em>Q. graciliformis</em>| 2012-0506<em>B |
| <em>Q. graciliformis</em>| 2012-0506</em>C |
| <em>Q. tomentella</em>   | 2012-0355<em>B |
| <em>Q. tomentella</em>   | 2012-0355</em>C† |
| <em>Q. vacciniifolia</em>| 2011-0133<em>A† |
| <em>Q. vacciniifolia</em>| 2012-0356</em>B |</p>
<table>
<thead>
<tr>
<th>The Huntington Library, Art Collections, and Botanical Gardens (San Marino, California, USA)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Q. engelmannii</em> Site Native (by Mausoleum)</td>
</tr>
<tr>
<td><em>Q. engelmannii</em> Site Native (by Tea Garden)</td>
</tr>
<tr>
<td>JC Raulston Arboretum at North Carolina State University (Raleigh, North Carolina, USA)</td>
</tr>
<tr>
<td><em>Q. canbyi</em> 070776</td>
</tr>
<tr>
<td><em>Q. emoryi</em> 120177</td>
</tr>
<tr>
<td>Morris Arboretum of the University of Pennsylvania (Philadelphia, Pennsylvania, USA)</td>
</tr>
<tr>
<td><em>Q. boyntonii</em> 2009-067*B</td>
</tr>
<tr>
<td><em>Q. texana</em> 1935-6725*A</td>
</tr>
<tr>
<td>The Morton Arboretum (Lisle, Illinois, USA)</td>
</tr>
<tr>
<td><em>Q. gambelii</em> 145-75*2</td>
</tr>
<tr>
<td><em>Q. gambelii</em> 145-75*8</td>
</tr>
<tr>
<td><em>Q. gambelii</em> 488-81*13†</td>
</tr>
<tr>
<td>Rancho Santa Ana Botanic Garden (Claremont, California, USA)</td>
</tr>
<tr>
<td><em>Q. chrysolepis</em> 10495*E1</td>
</tr>
<tr>
<td><em>Q. chrysolepis</em> 14489*B1†</td>
</tr>
<tr>
<td><em>Q. palmeri</em> 8326*B1</td>
</tr>
<tr>
<td><em>Q. palmeri</em> 8326*Z†</td>
</tr>
<tr>
<td><em>Q. vacciniiifolia</em> 8155*A3</td>
</tr>
<tr>
<td>The Santa Barbara Botanic Garden (Santa Barbara, California, USA)</td>
</tr>
<tr>
<td><em>Q. dumosa</em> Site Native (1)</td>
</tr>
<tr>
<td><em>Q. dumosa</em> Site Native (2)</td>
</tr>
<tr>
<td><em>Q. engelmannii</em> 74-239-4</td>
</tr>
<tr>
<td><em>Q. engelmannii</em> 74-239-5</td>
</tr>
<tr>
<td><em>Q. palmeri</em> 41-109-1</td>
</tr>
<tr>
<td><em>Q. tomentella</em> 44-153-1A</td>
</tr>
<tr>
<td><em>Q. tomentella</em> 44-153-1B</td>
</tr>
<tr>
<td><em>Q. vacciniiifolia</em> 85-058-1</td>
</tr>
<tr>
<td>Species</td>
</tr>
<tr>
<td>------------------</td>
</tr>
<tr>
<td><em>Q. canbyi</em></td>
</tr>
<tr>
<td><em>Q. chrysolepis</em></td>
</tr>
<tr>
<td><em>Q. palmeri</em></td>
</tr>
</tbody>
</table>

† Accession material was not able to be used in the experiment as it either did not survive mail transport in good condition or did not break bud
‡‡ Species removed from experiment due to low material availability
Appendix B

PROTOCOL FOR COLLECTING AND SENDING *QUERCUS* CUTTINGS FOR IN VITRO CULTURE RESEARCH

1. Take cuttings in early morning when the tree is well-watered to ensure buds and shoots contain as much moisture as possible when they are shipped.

2. Identify branches of new growth with swollen or newly broken leaf buds. (For each species, the goal is to have 10 buds/young shoots per tree from at least 2 different trees for the experiment.)

3. Use sterilized pruning shears to cut the stem of selected branches about 2 inches below the new growth at a 45 degree angle.

4. Place cuttings directly into plastic bags with moist paper towels. Cuttings from one tree can go together into one bag; place cuttings from different trees in separate bags.

5. Seal plastic bag and write identifying information, including species name, tree accession number (or other identifier), and institution name, using permanent marking pen.

6. Ship material overnight to Longwood Gardens.
Adapted from: