QUERIES IN *QUERCUS*: FROM THE DEVELOPMENT OF A TISSUE CULTURE CLONAL PROPAGATION SYSTEM TO THE EVALUATION OF STRESS TOLERANT HYBRID WHITE OAKS FOR THE URBAN ENVIRONMENT

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QUERIES IN *QUERCUS*: FROM THE DEVELOPMENT OF A TISSUE CULTURE CLONAL PROPAGATION SYSTEM TO THE EVALUATION OF STRESS TOLERANT HYBRID WHITE OAKS FOR THE URBAN ENVIRONMENT

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A research program was developed by Miles Schwartz Sax with a focus on researching two key priority areas for the Urban Horticulture Institute (UHI). The first priority area was the creation of a tissue culture system for the clonal propagation of hybrid white oaks. The second priority area was the development of evaluation methods to assess hybrid white oaks and other tree species for tolerance to stresses commonly found in the urban environment.

A tissue culture system was developed for the clonal propagation of hybrid white oaks between 2014 to 2018. There were 1,966 buds from 34 genotypes of UHI hybridized white oaks that were attempted in establishment and trialed in a tissue culture system. Individual genotype was the single largest factor affecting successful establishment, multiplication, stabilization and rooting of oaks in a tissue culture system. Fourteen lines of hybrid oaks were identified as having the capacity to stabilize and grow continuously in the tissue culture environment. Multiplication efficiency rates were dependent on individual genotype. This research showed the ability to re-establish genotypes of interest that had been previously established and stabilized in tissue culture. Re-establishment allowed for the possibility of the development of a direct establishment to rooting method that bypasses the time-consuming multiplication phase. A series of studies were undertaken with a focus on refining and simplifying the tissue culture process. These

included the use of phenolic binding agent PVP to reduce damage to explants by oxidization, the modification of the subculturing cycles, and the use of isolated terminal buds for explant multiplication.

In addition to trialing hybrids oaks, a series of oak species (*Q. bicolor, Q. gambelii, Q. macrocrpa, Q. garryana*) were trialed in a tissue culture system. This study examined how initial shoot and bud position on the stock plant affected establishment and multiplication rates of oaks in tissue culture. This study found that initial shoot position had no effect on tissue culture efficacy for trees up to four years old. As with hybrid oaks' successful establishment, multiplication and rooting rates were highly dependent on genotype. This study represents the first time that *Q. macrocarpa* and *Q. garryana* were successfully used in a tissue culture system.

Lastly, an experiment was undertaken that observed the gas exchange response and changes in turgor loss points of two tree species (*Quercus bicolor and Betula pendula*) grown under well-watered and drought conditions in limited soil volume. In response to soil water deficit conditions, *Q. bicolor* reduced its turgor loss point (osmotic adjustment) and maintained gas exchange rates. In contrast, *B. pendula* shed its leaves, did not reduce turgor loss point, and had varied gas exchange responses. Both species displayed contrasting physiological strategies to cope with drought by showing either an avoidance or tolerance mechanism.

BIOGRAPHICAL SKETCH

Miles Schwartz Sax holds a Bachelor of Science in Environmental Conservation Studies from the University of New Hampshire from 2008. He was awarded a Master of Professional Studies Degree in 2014 from Cornell University in Public Garden Leadership. Between finishing his master's degree and starting his Ph.D., he was the recipient of Cornell University's Frederick Dreer Award, which allowed him to gain international horticulture experience at Stellenbosch University Botanical Garden in Stellenbosch, South Africa.

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LIST OF ABBREVIATIONS

- UHI – Urban Horticulture Institute
- EtOH – Ethanol
- DI – Deionized
- RPM – Revolutions per minute
- BAP – 6-Benzylaminopurine
- PVP – polyvinylpyrrolidone
- MES – 2-(*N*-morpholino) ethanesulfonic acid
- IBA – Indole-3-butyric acid
- K-IBA - Indole-3-butyric acid, potassium salt
- PGR-free – Plant growth regulator free
- PPM – Plant Preservative Mixture
- $AgNO_3$ – Silver nitrate
- Zea – Zeatin
- AWHC – Available water holding capacity
- VPO – Vapor pressure osmometer
- PAR – Photosynthetic active radiation
- VPD – Vapor Pressure Deficit

LIST OF SYMBOLS

- Ψ_{π} – osmotic potential
- $\Psi_{\pi 100}$ – osmotic potential *at* full turgor.
- $\Delta\Psi_{\pi 100}$ – Osmotic adjustment

INTRODUCTION

The development of hybrid white oaks for the urban environment has been a long-term project for Cornell's Urban Horticulture Institute (UHI). The project has been composed of a series of breeding, propagation and plant evaluation trials, experiments and graduate research over the last two decades. The initial research that started this project was the successful clonal propagation of a series of oak species using a modified stool bed to develop rooted trees (Griffin and Bassuk 1996). These preliminary methods showed that asexual propagation of oaks by rooting was possible and promoted continued research to further develop the method and laid the foundation for a breeding program.

A three-year breeding program ran from 2004-2006 and 345 unique genotypes of hybrid white oaks were developed by crossing 40 parent species native to North America, Europe and Asia. The goal of this breeding project was to develop stress adapted (cold hardiness, pest and disease resistance, drought, salt and high pH tolerance) clonally propagated oaks for the urban environment. Paternal germplasm selection focused on species that grow under natural conditions that may be analogous to the urban environment such regions with high soil pH or frequent droughts. Maternal trees were selected as mature cold hardy trees that were growing at Cornell Botanic Garden in the F.R. Newman Arboretum. Acorns (seed) produced from controlled and open crosses generated a series of hybrid seedlings that have since been grown at Cornell's Blue Grass Lane field research site (USDA plant hardiness zone 5b [-15°F to -10°F]). These stock plants have been the primary germplasm used for evaluation, selection, propagation and tissue culture of hybrid white oaks for UHI.

Clonal propagation of oaks has long been recognized as a major challenge to selection, breeding and improvement of the genus *Quercus* for horticultural and forestry applications.
Growing *Quercus* from seed has been the primary means of propagation in North America with nurseries typically harvesting acorns from elite trees (Dirr and Heuser 2006). Due to both

hybridization among *Quercus* species and a high degree of intraspecific variation within species, oaks can express high variability in their phenotypes (Nixon, 1993). As a result, a high degree of variability in phenotypes and environmental adaptations are present in the oak nursery stock. Alternative propagation methods have been trialed in the nursery trade such as rooting by cutting and grafting propagation.

The grafting of oaks is reported as a viable method to produce asexual clones. The two primary successful methods are pot grafting onto seedling stock and field grafting in regions of the world with cool, moist spring weather (Obdržálek, 2006) (Dirr and Heuser, 2006). Successful grafting requires using the same (or closely rated) species for rootstock as scion. Grafting incompatibility has been commonly recognized as an issue with oaks (Santamour and Coggeshall 1996, Dirr and Heuser, 2006). Grafting incompatibility research in the 1980s attempted to use peroxidase isoenzymes as a means of characterizing different oak species' grafting compatibility (Santamour, 1988). Graft incompatibility has been delayed for as much as seven years in studies with *Q. rubra* (Coggeshall, 1996). Coggeshall et al. (2008) suggested that grafting incompatibility is variable by oak section with lower occurrences of failures within the white (*Quercus*) section. Likewise, the English Oak (*Quercus robur*) has over 262 named cultivars, many of which were propagated by grafting and do not display issues of incompatibility into their mature life phase (International Oak Society, 2018). Through meetings and tours of nurseries with the International Plant Propagators Society and the International Oak Society, the author has seen hot callus grafting used to increase grafting success in oaks. Hot callus grafting has been used to increase grafting success in species notably difficult to graft such as *Fagus grandifolia* (Carey et al. 2013) and *Juglans regia* (Gandey, 2009).

Propagation by cuttings has been achieved for Texas ecotypes of *Quercus virginiana* (Morgan, 1979). Tree Introduction Inc. has a series of rooted oaks including selections for the species *Quercus* virginiana, *Quercus* lyrata 'Highbeam®', *Quercus* nuttailii 'Highpoint'®, *Quercus*

phellos ‘Hightower’® and *Quercus* shumardii ‘Panache’® (Tree Introduction, 2011). The methods used to achieve propagation by rooting is not clear or commonly reported in the scientific literature.

In 1996, UHI’s first work in clonal propagation demonstrated an increased induction of rooting from a series of oak species (*Q. acutissima*, *Q. bicolor*, *Q. macrocarpa*, *Q. palustris*, *Q. robur*, *Q. rubra*) by adding an etiolation treatment and hormone application step into the stool bed protocol. Although the experiment showed varying rooting success based on species, the study also demonstrated the ability to generate rooted oaks with these methods (Griffin and Bassuk, 1996). From these initial findings, research continued to improve the method and achieve higher success rates for rooting oaks. Examples of this research include: the investigation of the effects of etiolation, root restriction, root pruning and light sources on the rooting success of a variety of *Quercus* species (*Q. macrocarpa*, *Q. bicolor*, *Q. palustris*, *Q. acutissima*, *Q. imbricaria*) (Hawver and Bassuk, 2000); the effects of severely cutting back stock plants and air layer on rooting of *Q. macrocarpa* and *Q. bicolor* (Amissah and Bassuk 2005); the study of the anatomical differences in stems of oaks that have high rooting ability (Amissah et al. 2008); the exploration of how cutting back stock plants to various heights, etiolation and exposure to different types of light effect rooting in oak (Amissah and Bassuk, 2009). From these previous studies a hybrid layering and stool bed system was developed and used to successfully propagation hybrid oaks from the UHI breeding program (Denig et al. 2013).

The development of the stool bed method for hybrid oak propagation cleared a horticultural barrier for selection and development. After generating enough clonally propagated oaks for replicable experiments, a series of selection and evaluation studies were undertaken. Select hybrid oaks were screened in a potted greenhouse experiment to determine their tolerance of high pH soils and to identify which parental lineages impart an alkaline tolerance characteristic (Denig, et al. 2014). This research identified twenty genotypes of hybrid oaks that show a degree

of high pH tolerance.

To assess the hybrid oaks under field conditions, clonally propagated trees were planted in the urban tree collection in the F.R. Newman Arboretum at Cornell Botanic Garden. The soils at this location have a pH of 8.0 and exhibit poor structure, both of which are common in urbanized landscapes. Alkaline tolerant clonal oaks were grown in this location to assess their performance under field conditions and were monitored on an annual basis to assess photosynthetic capacity and chlorosis using a SPAD meter between 2015 and 2017 (Appendix 5.12). Hybrid white oaks were additionally distributed to Schichtel's Nursery Inc. in Springville, New York for long term field evaluation.

In 2015 and 2016, stock plants grew over the growing season and were then evaluated for pest (scale, Japanese beetles, aphids and a Cynipid wasp galls) and disease (anthracnose and powdery mildew) prevalence (Appendix 5.7-5.9). Disease and pest pressure were quantitatively assessed and genotypes exhibiting high degrees of resistance were selected as candidates for tissue culture establishment.

While stool bed and rooting methods proved successful for the clonal propagation of hybrid white oaks, the number of individuals produced on an annual cycle was relatively low, reducing their commercial viability. To overcome this limitation, a series of experiments have been conducted by the author and members of the Bassuk lab in an attempt to develop tissue culture protocols for the UHI's hybrid white oaks. The following dissertation reports the efforts undertaken towards the goal of developing and evaluating clonally propagated hybrid white oaks for the urban environment. The dissertation is divided into section as follows: the development and trialing of a tissue culture system for UHI hybrid oak; the development of a direct to rooting method for UHI hybrid oaks; trialing of four oaks species in a tissue culture system; the osmotic adjustment and gas exchange response of two tree species under drought and well-watered conditions.

1. CHAPTER 1

DEVELOPMENT OF A TISSUE CULTURE PROPAGATION SYSTEM FOR HYBRID WHITE OAKS

1.1. Introduction

To develop clonally propagated hybrid white oaks, a tissue culture system was developed and trialed. First efforts were undertaken by Bryan Denig starting in 2014 with the successful establishment and maintenance of hybrid white oaks (Appendix 5.5) in a tissue culture system using methods from Vieitez et al. 2009. From this work a series of hybrid white oak lines were successfully established by B. Denig. These established lines included clones 06-1500-1, 06-1500-6 and 06-1819-1 and were used as a stock plants for experimentation in modification of the tissue culture system. A second round of initial establishment experiments was undertaken in 2015 by the author of this dissertation. These trials had low success establishment rates due to high contamination, low multiplication rates, and using relatively few starting nodes in establishment. As a result, 2015 functioned as a foundational year in the development of sterile techniques by the author and piloting tissue culture methods. In 2016, efforts were renewed and many of the challenges from 2015 were overcome. This progress resulted in the successful establishment, multiplication and rooting of a series of hybrid oaks in tissue culture. Additionally, experimentation and modification of methods improved efficiency and efficacy. Results reported in Chapter 1 are the combination of a series of studies conducted between 2016 and 2018.

1.2. Methods

1.2.1. Establishment

1.2.1.1. Hybrid Oak Selection

Hybrid white oaks were selected annually for tissue culture based on field observation and their performance in evaluation studies. In 2015, focus was placed on trialing genotypes that had shown high alkaline tolerance (Denig et al. 2014) and rooting capacity using the stool bed method (Denig et al. 2013). In 2016, a series of genotypes from the 2015 criteria were used along with genotypes that exhibited a high degree of pest and disease resistance in the 2015 growing season (Appendix 5.7-5.8). In 2017, some genotypes were selected that fulfilled the criteria from previous years. Additional genotypes were selected based on performance at Schichtel's Nursery, having showed pest and disease resistance in the 2016 growing season. In the 2018 growing season, hybrid oaks were selected that had successfully established in previous years. Studies in 2018 focused on developing a direct rooting method. Therefore, genotypes that had performed well in previous years and also had desirable horticultural qualities were used for these experiments. Table 1.2 reports hybrid genotype lines and years they were established in tissue culture.

1.2.1.2. Harvesting explant material from stock plants

The selected genotypes were harvested from stool bed grown stock plants for establishment in tissue culture. Stool bed stock plants were coppiced annually in April, removing the previous year's growth. The coppicing technique forces new juvenile shoots to emerge from a position close to the root shoot interface (Denig et al 2013). The one-year old stems were retained and placed in a 16-inch tall flower vase in two liters of water in a growth chamber. The growth chamber had a 12-hour photo period starting at 10:00 am, with an average temperature of 70°F,

ambient CO_2 and 200-500 µmol/m^2/s light from T5 cool white fluorescent bulbs. The stems were maintained in this condition until buds broke dormancy. The flush of new shoots from these buds were harvested after approximately four to six weeks upon reaching a length of 5 mm or greater. A second source of shoots used for establishment were collected between mid-May to early June when new shoots emerged from the coppiced field grown stock plants. Shoots were harvested directly from the field once they were 10 cm or greater in height. Field harvested shoots were stored in 50 ml falcon tubes in a cooler with crushed ice when being transferred to the laboratory for decontamination. Shoots that were taller than the falcon tube were cut into sections and placed in the same tube.

1.2.1.3. Disinfestation

Standard protocols were followed. Each shoot was placed in individually labeled 50 ml falcon tubes and stored on crushed ice while being transported to the tissue culture laboratory. In the laboratory, 50 ml of 70% ethanol (EtOH) was poured into each falcon tube. Up to eight individual tubes with EtOH were placed on orbital shaker set to 200 RMP for one minute. After rotational shaking, all tubes were moved into a laminar flower hood where EtOH was discarded. In the flow hood, 50 ml of a Clorox® bleach (7.4% sodium hypochlorite) and Tween 80 solution (20% bleach, 80% DI H_2O with 2-3 drops of Tween 80 per 100 ml) were added to each falcon tube and capped. Falcon tubes were placed on an orbital shaker set to 200 RPM for a period of 15 minutes before returning to the flow hood where the bleach/tween solution was discarded. The cultures and all tubes were rinsed three times with autoclaved sterile deionized water.

1.2.1.4. Establishment in tissue culture

Disinfected shoots were cut into one bud segments that were 50-100 mm long. Buds were harvested sequentially, starting at the proximal end of the shoot to the cut surface, proceeding

towards the terminal bud. Individual buds were placed upright into 25x150 mm culture tubes filled with 15ml establishment media. Oak establishment media consists of Woody Plant Media 2.41 g/L, BAP 0.5 mg/L, sucrose 30 g/ L, agar 5.5 g/L and pH adjusted to 5.6±0.1. After three days, individual buds were moved from one side of the culture tube to the opposite side when necessary to avoid oxidized phenolics that had exuded from cut surfaces. If phenolic secretion and oxidation continued after initial movement, buds were then moved to a new test tube with establishment media. Explants were transferred to fresh media every two weeks. After six weeks individual buds that elongated to a length equal to or greater than 5 mm were moved to the multiplication phase. Establishment was conducted using this method between the years 2014 to 2018.

1.2.2. Multiplication

1.2.3. Standard BAP Multiplication Media

When moved to the multiplication phase, the apical dome, including 2 mm of the terminal bud, and all leaves were removed from shoots that grew. Trimmed shoots were placed horizontally in a 111 ml baby food jar with 25 ml of multiplication media. Horizontal shoot placement allowed for lateral buds to develop and elongate into new shoots. Oak multiplication media was made of Woody Plant Media 2.41g/L, BAP 0.5 mg/L, sucrose 30 g/ L, agar 5.5 g/L and pH adjusted to 5.6 ± 0.1. Shoots were subcultured and transferred to fresh media at two-week intervals. After a period of six weeks, new shoots were harvested, and the multiplication cycle repeated. Over successive multiplication cycles, individual lines would either reduce the number of shoots produced and decline or increase the number of shoots and stabilize. Stabilized lines had the capacity to generate an equal or greater number of new shoots than the previous multiplication round and allowed for indefinite multiplication cycles. Stabilized genotype lines are reported in

Table 1.4. Stabilization lines were used to conduct multiplication, rooting and acclimatization experiments.

1.2.3.1. ZeatinPVP/50 ml Media

Modification of the multiplication media included experimentation with the cytokinin zeatin and the phenolic binding compound polyvinylpyrrolidone (PVP)(mol. wt 40,000). PVP was assessed in multiplication media to determine its effect on preventing oxidation of phenolic compounds released when shoots were mechanically damaged during the multiplication protocol. The combined zeatin PVP (ZeatinPVP) media was composed of Woody Plant Media 2.41 g/l, zeatin 200 ul/L, sucrose 30 g/L, MES 0.5 g/L, PVP40 0.5 g/L, Phytoblend 5.5 g/L, pH adjusted to 5.6 ± 0.1. For the first two weeks of the multiplication phase, shoots were placed in 25 ml of a ZeatinPVP media in 111 ml baby food jars. For the control treatment, 25 ml of the standard BAP media was used. After two weeks in culture, prior to the first subculturing transfer, the media was assessed to detect phenolic oxidation. Phenolic exudation was characterized by an absence or presence of oxidized phenolic compounds which appeared as a browning of the media. After the phenolic assessment, shoots in the ZeatinPVP treatment were transferred to a new baby food jar with 50 ml of standard BAP multiplication media and allowed to develop for four weeks with no further subculturing. When in control media, shoots were subculture and transferred to new media at the standard two-week interval. At the end of the six-week multiplication period both treatments were assessed for contamination, number of shoots produced and shoot length.

1.2.3.2. Transfer Time for 1-5 and 2-2-2 weeks

An experiment was designed to determine if the number of times a shoot was subcultured during the six-week multiplication phased affected the number of shoots produced and shoot length. Hybrid oak genotypes 05-830-50, 06-1500-1, 06-1819-1, 06-1821-2 were stabilized and grown

in the standard BAP multiplication media. Control treatment for the study included subculturing at two-week intervals for a total of six weeks, denoted as 2-2-2. The alternative treatment was subculturing shoots after one week and maintaining the shoots in this media for five additional weeks, denoted as 1-5. For each genotype, the experiment was replicated with forty individual shoots and data was collected at the end of the six-week multiplication cycle. Data collected included percent of cultures contaminated, number of shoots produced and shoot length.

1.2.3.3. Shoot and Tip

When beginning a new multiplication cycle, shoot tips were removed from shoots. Standard tissue culture methods used in this study and Vieitz et al. 2009 dictate the removal and disposal of the terminal bud. An experiment was conducted to determine if the removed terminal buds could be used in the multiplication phase. Five genotypes (05-830-50, 06-1500-1, 06-1500-6, 06-1819-1, 06-1821-3) were used for the experiment. Shoots tips were approximately 3 mm long, containing the terminal bud and a variable number of lateral buds. Two shoots and their accompanying two tips were placed in a single jar with 25 ml of standard multiplication media. Shoots and tips were subculture at two-week intervals and assessed after six weeks. The experiment was replicated over several multiplication cycles. At the end of each multiplication cycle number of shoots, and shoot length were assessed and averaged by genotype.

1.2.4. Rooting

1.2.4.1. Standard Rooting Method

Shoots greater than 5 mm in height with living terminal buds were selected at the end of the multiplication cycle and moved to rooting. Total shoot length and the total number of leaves were recorded prior to rooting. Idole-3-butyric acid (IBA) rooting media was used for rooting induction phase. IBA root induction media was comprised of Woody Plant Media 2.41 g/L, IBA

25 mg/L, sucrose 30 g/L, phytoblend 5.5 g/L and pH was adjusted to 5.6 ± 0.1. Explants were maintained for a period of seven days in IBA induction media before transfer to media without plant growth regulator (PGR) for two weeks. PGR-free media was comprised of Woody Plant Media 2. 41 g/L, active charcoal 4 g/L at pH 5.7, sucrose 30 g/L, phytoblend 5.5 g/L and pH was adjusted to 5.6 ± 0.1. For both induction and PGR-free phases, 15 ml of media was used per culture tubes (25 x 150 mm). Each shoot was placed in a single tube during the rooting phase. After the two weeks in the PGR-free media, root development was assessed. Measurements included occurrence of shoot tip necrosis, total number of roots produced and root length.

1.2.4.2. K-IBA vs IBA

For two genotypes (06-1500-6 & 06-1821-3) a trial experiment was conducted to determine the effectiveness of K-IBA compared to IBA for the root induction phase. K-IBA rooting media was the same as induction media except for the substitution of K-IBA for IBA at a rate of 25 mg/L. Explants were kept in IBA or K-IBA media for two days before transfer to PGR-free media for 30 days. Number of roots and root length were assessed at the end of the period.

1.2.5. Statistics

Statistical analysis was conducted using JMP Pro v14.0. Primary statistics used for data analysis included chi-square, Fisher's exact two tailed test, ANOVA, mixed effect models and Tukey HSD matching letter analysis. An alpha level of 0.05 was used for hypothesis testing to determine statistical significance. For Tukey HSD tests matching letters indicate equal mean values. Data transformations were used for data that did not have normal distributions as well as use of negative binomial distributions for specific tests. For mixed effect models with multiple comparisons, a Bonferroni correction was used to normalize p-values. Specific examples are as follows. The number of shoots in Table 1.2 was analyzed using a negative binomial model and

average shoot length was log transformed. Mean shoot length in Table 1.9 and root length Table 1.11 were normalized with a log transformation. A square root transformation was used for the number of shoots in Table 1.7 and Table 1.8. Residuals of all other data was normally distributed without transformations.

1.3. Results

1.3.1. Establishment

Stock plant source material for establishment of hybrid oak genotypes was collected either from force flushed stems in a growth chamber or newly emerged shoots from the field stock plants. Contamination rates were lower in shoots sourced from the growth chamber than from the field. Differences in contamination rates varied by year (Table 1.1).

Table 1.1: Contamination Rates During Establishment by Year and Stock Plant Source Location

Year	n	Field	n	Growth Chamber	Fisher's Exact Two Tailed
2016	545	19%	605	5%	<.0001
2017	248	54%	219	21%	<.0001
2018	162	49%	187	7%	<.0001
Total	955		1011		

From 2016 to 2018, a total of 1,966 buds were used for the establishment phase. Successful establishment rates were not dependent on source location of stock plants (field grown or growth chamber). The number of starting buds varied by genotype (Table 1.2). Of the starting 1,966 buds attempted in culture, 585 (30%) were responsive and grew in the establishment media (Table 1.2). Buds were counted as responsive if a new shoot emerged from the initial bud and elongated to a length of 5 mm or greater. Individual bud responsiveness was variable depending on genotype and ranged from 0-100%. The average number of shoots produced per individual starting bud and average shoot length were also highly dependent on genotype (Table 1.2)

Table 1.2: Hybrid White Oak Establishment by Genotype 2016-2018

Genotype	Year	Starting nodes	Contam.[z]	Num. buds responsive	% Buds responsive	Avg. num. shoots	95% conf. int	Avg. shoot length[y]	95% conf. int.
04-566-3	'17-'18	73	40%	35	48%	1.48	+/-(1.81, 1.19)	12.01	+/-(14.13, 10.22)
04-568-1	'16	74	0%	32	43%	2.78	+/-(3.25, 2.35)	7.82	+/-(9.26, 6.6)
04-572-1	'16	14	21%	1	7%	1	+/-(3.09, 0.06)	16.01	+/-(41.73, 6.14)
04-576-3	'16-'17	67	39%	13	19%	1.14	+/-(1.63, 0.73)	7.23	+/-(9.42, 5.54)
04-577-1	'16	37	0%	6	16%	1	+/-(1.72, 0.48)	9.13	+/-(13.5, 6.18)
05-806-1	'16	9	11%	1	11%	1	+/- (3.09, 0.06)	9	+/-(23.47, 3.46)
05-830-2	'16	30	67%	2	7%	1	+/-(2.36, 0.22)	6.01	+/-(11.82, 3.05)
05-830-50	'16,'18	79	27%	29	37%	2.26	+/-(2.7, 1.86)	13.14	+/-(15.7, 11)
05-853-1	'16	88	2%	18	20%	1.63	+/-(2.12, 1.21)	6.86	+/-(8.6, 5.48)
05-854-15	'17-'18	61	41%	11	18%	1.14	+/-(1.68, 0.71)	13.1	+/-(17.49, 9.81)
05-854-18	'17	42	33%	8	19%	1.64	+/-(2.4, 1.03)	8.34	+/-(11.7, 5.94)
05-854-22	'16	32	0%	6	19%	1.55	+/-(2.42, 0.88)	6.96	+/-(10.29, 4.71)
05-854-5	'16	34	18%	8	24%	2.15	+/-(3.01, 1.44)	13.53	+/-(18.99, 9.64)
05-874-3	'16	36	6%	2	6%	1	+/-(2.36, 0.22)	6.01	+/-(11.82, 3.05)
05-878-1	'18	37	30%	7	19%	1	+/-(1.66, 0.51)	15.18	+/-(21.8, 10.57)
05-899-2	'16	24	0%	2	8%	2	+/-(3.81, 0.78)	3	+/-(5.91, 1.53)
05-905-1	'16	60	12%	2	3%	2	+/-(3.81, 0.78)	6.01	+/-(11.82, 3.05)
05-905-3	'16	87	32%	4	5%	1.46	+/-(2.52, 0.69)	4.17	+/-(6.73, 2.58)
05-906-3	'16-'17	17	24%	5	29%	1	+/-(1.8, 0.44)	8.05	+/-(12.35, 5.24)
05-922-1	'17	87	13%	5	6%	1.56	+/-(2.52, 0.83)	6.65	+/-(10.21, 4.33)
06-1500-1	'18	69	41%	33	48%	1.95	+/-(2.33, 1.6)	9.83	+/-(11.61, 8.32)
06-1500-6	'18	59	29%	37	63%	1.5	+/-(1.82, 1.21)	13.18	+/-(15.43, 11.26)
06-1673-5	'16	36	0%	2	6%	3	+/-(5.15, 1.44)	6.01	+/-(11.82, 3.05)
06-1673-7	'16	39	0%	16	41%	2.57	+/-(3.21, 2)	6.11	+/-(7.77, 4.81)
06-1733-11	'16	36	11%	20	56%	1.42	+/-(1.85, 1.04)	9.98	+/-(12.37, 8.06)
06-1733-17	'17	37	46%	13	35%	1.53	+/-(2.1, 1.06)	8.06	+/-(10.52, 6.18)
06-1801-1	'17	18	44%	6	33%	1.96	+/-(2.92, 1.19)	8	+/-(11.84, 5.41)
06-1802-1	'16-'18	96	31%	33	34%	1.6	+/-(1.96, 1.29)	6.66	+/-(7.86, 5.63)
06-1804-1	'18	123	5%	80	65%	4.02	+/-(4.37, 3.69)	8.44	+/-(9.39, 7.58)
06-1805-1	'16	14	0%	0	0%	0	+/-(0, 0)	0	+/-(0, 0)
06-1810-2	'17-'18	57	21%	29	51%	2.11	+/-(2.54, 1.72)	11.97	+/-(14.3, 10.02)
06-1811-3	'16	14	0%	6	43%	3.62	+/-(4.89, 2.54)	9.44	+/-(13.96, 6.39)
06-1812-2	'16	116	18%	57	49%	1.42	+/-(1.67, 1.19)	8.37	+/-(9.51, 7.38)
06-1814-2	'17	35	29%	4	11%	1.22	+/-(2.2, 0.53)	7.62	+/-(12.3, 4.72)
06-1819-1	'17	52	35%	16	31%	2.18	+/-(2.78, 1.66)	11.56	+/-(14.69, 9.1)
06-1821-3	'16,'18	196	24%	36	18%	2.65	+/-(3.08, 2.26)	8.42	+/-(9.88, 7.18)

[z] Contam. stands for the percent of original starting buds that were contaminated, [y]shoot length is in mm.

Table 1.3: Hybrid Oak Genotype Identification Numbers and Maternal and Paternal Parentage

Genotype	Maternal	Paternal
04-566-3	*Q. bicolor*	*Q. muehlenbergii*
04-568-1	*Q. bicolor*	*Q. turbinella*
04-572-1	*Q. macrocarpa*	*Q. turbinella*
04-576-3	*Q. macrocarpa*	*Q. gambelii*
04-577-1	*Q. muehlenbergii*	*Q. prinoides*
05-806-1	*Q. montana*	*Q. lyrata*
05-830-2	*Q. bicolor*	*Q. muehlenbergii*
05-830-50	*Q. bicolor*	*Q. rugosa*
05-853-1	*Q. muehlenbergii*	*Q. aliena var. acutiserrata*
05-854-15	*Q. muehlenbergii*	*Q. fusiformis*
05-854-18	*Q. muehlenbergii*	*Q. fusiformis*
05-854-22	*Q. muehlenbergii*	*Q. fusiformis*
05-854-5	*Q. muehlenbergii*	*Q. fusiformis*
05-874-3	*Q. muehlenbergii*	*Q. lyrata*
05-878-1	*Q.* muehlenbergii	*Q.* virginiana
05-899-2	*Q. 'Ooti'*	*Q. fusiformis*
05-905-1	*Q. macrocarpa 'Ashworth Strain'*	*Q. michauxii*
05-905-3	*Q. macrocarpa 'Ashworth Strain'*	*Q. michauxii*
05-906-3	*Q. macrocarpa 'Ashworth Strain'*	*Q. michauxii*
05-922-1	*Q. 'Ooti'*	*Q. fusiformis*
06-1500-1	*Q. × warei 'Long' (Regal Prince®)*	Open
06-1500-6	*Q. × warei 'Long' (Regal Prince®)*	Open
06-1673-5	*Q. macrocarpa*	Open
06-1673-7	*Q. macrocarpa*	Open
06-1733-11	*Q. bicolor*	*Q. aliena var. acutiserrata*
06-1733-17	*Q. bicolor*	*Q. aliena var. acutiserrata*
06-1801-1	*Q. bicolor*	*Q. muehlenbergii*
06-1802-1	*Q. bicolor*	*Q. glauca*
06-1804-1	*Q. bicolor*	*Q. vaseyana*
06-1805-1	*Q. bicolor*	*Q. chapmanii*
06-1810-2	*Q. bicolor*	*Q. dentata 'Pinnatifida'*
06-1811-3	*Q. bicolor*	*Q. fabri*
06-1812-2	*Q. bicolor*	*Q. fruticosa*
06-1814-2	*Q. bicolor*	*Q. libani*
06-1819-1	*Q. gambelii × Q. macrocarpa*	*Q. lyrata*
06-1821-3	*Q. macrocarpa*	*Q. × comptoniae*
04-564-1-4	*Q. bicolor*	*Q. minima*
05-805-2	*Q. montana*	*Q. geminata*
05-860-2	*Q. muehlenbergii*	*Q. virginiana*

1.3.2. Multiplication

1.3.2.1. Stabilization

After the establishment phase hybrid oak lines were transferred to the multiplication phase. During this phase individual genotypes show the capacity to either stabilized in a continuous multiplication cycle producing an ever-greater number of shoots during each subsequent cycle or decline and perish over time. For most of the hybrid white oak lines, stabilization occurred after a minimum 153 days on average but varied significantly by genotype. Genotypes that can stabilize in the multiplication phase are identified in Table 1.4.

Table 1.4: Stabilized Hybrid Lines in Multiplication Phase

Genotype	**Maternal**	**Paternal**	**Year**
06-1819-1[A,B]	*Q. gambelii* × *macrocarpa*	*Q. lyrata*	2014 & 2017
06-1500-1[A,B]	*Q.* × *warei 'Long' (Regal Prince®)*	Open Pollinated	2014 & 2017
06-1500-6[A,B]	*Q.* × *warei 'Long' (Regal Prince®)*	Open Pollinated	2014 &2017
04-564-1-4[A]	*Q. bicolor*	*Q. minima*	2015
05-805-2[A]	*Q. montana*	*Q. geminata*	2015
05-806-1[A]	*Q. montana*	*Q. lyrata*	2015
05-860-2[A]	*Q. muehlenbergii*	*Q. virginiana*	2015
05-878-1[A]	*Q. muehlenbergii*	*Q. virginiana*	2015
04-568-1	*Q. bicolor*	*Q. turbinella*	2016
05-830-50	*Q. bicolor*	*Q. rugosa*	2016
06-1673-7	*Q. macrocarpa*	Open pollinated	2016
06-1802-1	*Q. bicolor*	*Q. glauca*	2016
06-1804-1	*Q. bicolor*	*Q. vaseyana*	2016
06-1811-3	*Q, bicolor*	*Q. fabri*	2016
06-1812-2	*Q. bicolor*	*Q. fruticosa*	2016
06-1821-3	*Q. macrocarpa*	*Q.* × *comptoniae*	2016
04-566-3	*Q. bicolor*	*Q. muehlenbergii*	2017
06-1810-2	*Q. bicolor*	*Q. dentata*	2017
05-854-15	*Q. muehlenbergii*	*Q. fusiformis*	2017

[A.] Hybrid lines established and stabilized by Bryan Denig using Vieitez et. al. 2009 methods.
[B.] Genotype lines that have been re-established into a continuous multiplication cycle

The stabilization process identified lines that produce an increasing number of shoots during each multiplication cycle. With the capacity for these lines to produce large numbers of shoots,

they were selected for studies that examined modifications to multiplication phase protocols. Table 1.5 shows observational data on how well a series of stabilized hybrid oak lines multiplies in tissue culture. Table 1.7 shows multiplication rates as the number of shoots produced per multiplication cycle for a series of stabilized genotypes. The identification of stabilized lines allowed for additional experimentation to determine if genotypes could be reestablished and stabilized in culture. Hybrid lines that were successfully reestablished included 06-1819-1, 06-1500-1 and 06-1500-6 (Table 1.4).

Table 1.5: Qualitative Observations of Multiplication Rates of Stabilized Hybrid White Oak Genotypes in the Multiplication Phase

Genotype	**Multiplication Rate in culture**
06-1819-1	High
06-1500-1	High
04-566-3	High
06-1500-6	Moderate
06-1821-3	Moderate
05-830-50	Moderate
06-1810-2	Moderate
06-1804-1	Moderate to low
05-854-15	Moderate
06-1673-7	Low

1.3.2.2. ZeatinPVP/50ml and BAP 25ml Media

Modification of multiplication media using PVP was trialed to determine its effects on preventing phenolic oxidation and damage to explants. Inclusion of PVP and zeatin in the establishment media had a statistically significant reduction in the occurrence of phenolic oxidation after two weeks in multiplication media for all genotypes, except 06-1819-1, when compared to the control treatment (Table 1.6). Occurrence of phenolic oxidation was variable by genotype in the control media.

Table 1.6: Presence of Phenolic Oxidation (browning) by Genotype and Media Type

Genotype	Control	n	ZeatinPVP	n	P - value
05-830-50	90%	20	0%	20	<.0001
06-1500-1	51%	61	2%	60	<.0001
06-1500-6	10%	58	0%	58	0.0065
06-1821-3	20%	60	0%	60	0.0003
06-1819-1	20%	20	0%	20	0.106

In addition to observations of phenolic oxidation, the two media types were tested to determine how they affected the number of shoots produced and shoot length in multiplication. Genotype, media type and their combined interaction were statistically shown to effect number of shoots produced and shoot length. For genotypes 06-1500-1 and 06-1821-2 number of shoots produced were equal for the two media types. While genotypes 05-830-50, 06-1500-6 and 06-1819-1 generated significantly more shoots in BAP media compared to ZeatinPVP. All genotypes, aside from 06-1819-1, produced statistically equal shoot lengths regardless of treatment. For genotypes 06-1819-1 BAP media had significantly longer average shoot lengths compared to ZeatinPVP media (Table 1.7).

1.3.2.3. Multiplication cycle treatment 1-5 and 2-2-2:

An experiment was conducted to determine the effect of two different subculturing routines (every two weeks and once after one week) on multiplication rates of hybrid oaks. For all of the genotypes observed, the subculturing frequency treatment had no effect on the number of shoots produced or the shoot length after a period of six weeks (Table 1.8). Significant differences were detected by genotype for both number of shoots produced and shoot length.

1.3.2.4. Shoots and Tips

An experiment was conducted to determine if shoot tips responded differently in the multiplication phase compared to shoots with the terminal bud removed. For this experiment, no

significant differences were detected between number of shoots or shoot length for either treatment (shoot or tip) as seen in Table 1.9. While treatment had no significant effect, there were significant differences between genotypes in terms of number of shoots produced and shoot length.

Table 1.7: Effect of Media Type (BAP vs ZeatinPVP) on Multiplication Rates of Hybrid Oaks

Genotype	Treatment	n	Percent Contaminated	Number of Shoots	95% Conf. Int.	Avg. Shoot Length	95% Conf. Int.
05-830-50	Control	20	10%	0.73 a	± (1.46,0.25)	11.82 a	± (17.12,8.17)
	ZeatinPVP	20	5%	0.02 b	± (0.24,0.04)	6.82 a	± (14.3,3.25)
06-1500-1	Control	61	8%	3.55 a	± (4.34,2.84)	12.06 a	± (14.01,10.49)
	ZeatinPVP	60	13%	2.78 a	± (3.48,2.16)	12.94 a	± (15.03,11.25)
06-1500-6	Control	60	3%	2.27 a	± (2.89,1.72)	10.18 a	± (11.82,8.76)
	ZeatinPVP	54	4%	0.28 b	± (0.55,0.1)	7.46 a	± (9.39,5.93)
06-1819-1	Control	50	16%	1.85 a	± (2.44,1.34)	9.78 a	± (11.36,8.41)
	ZeatinPVP	46	6%	0.72 b	± (1.13,0.4)	6.36 b	± (7.69,5.21)
06-1821-3	Control	54	11%	1.06 a	± (1.43,0.69)	9.39 a	± (11.13,7.92)
	ZeatinPVP	58	3%	1.07 a	± (1.51,0.7)	9.12 a	± (10.7,7.77)
				Prob > ChiSqr		Prob > F	
Genotype (A)				<.0001		<.0001	
Treatment (B)				<.0001		0.0106	
A x B				<.0001		0.0121	

Table 1.8: Effect of Sub Culture Transfer Frequency on Multiplication Rates of Hybrid Oaks

Genotype	Transfer Frequency (wks)	n	Percent Contaminated	Number of Shoots	Shoot Length (mm)	95% Conf. Int.
05-830-	1-5	40	10%	1.4 ± 0.2 a	13.68 a	± (16.57,11.30)
05-830-	2-2-2	40	0%	1.0 ± 0.2 a	13.28 a	± (16.15,10.92
06-1500-	1-5	40	30%	2.3 ± 0.3 a	11.10 a	± (13.01, 9.47)
06-1500-	2-2-2	40	10%	3.4 ± 0.4 a	12.07 a	± (14.11, 10.32)
06-1819-	1-5	40	20%	2.3 ± 0.3 a	10.08 a	± (11.76, 8.64)
06-1819-	2-2-2	40	10%	3.2 ± 0.4 a	12.81 a	± (14.87, 11.03)
06-1821-	1-5	40	0%	1.2 ± 0.2 a	5.42 a	± (6.41, 4.59)
06-1821-	2-2-2	40	0%	1.6 ± 0.2 a	7.27 a	± (8.62, 6.14)
				Prob> ChiSq	Prob > F	
Treatment (A)				0.1366	0.016	
Genotype (B)				<.0001	<.0001	
A x B				0.0537	0.2555	

Table 1.9: Effect of Using Whole Shoots Vs Shoot Tips on Multiplication Rates of Hybrid Oaks

Genotype	Shoot Vs	n	Percent Contaminated	Num. of shoots	Shoot Length	95% Conf. Int.
05-830-50	Shoot	20	0%	1.23 ± 0.23 a	10.11 a	± (12.96, 7.88)
05-830-50	Tip	19	0%	2.31 ± 0.41 a	14.76 a	± (17.96, 12.13
06-1500-1	Shoot	40	0%	5.21 ± 0.41 a	14.05 a	± (15.96, 12.37
06-1500-1	Tip	40	0%	2.99 ± 0.31 b	13.56 a	± (15.37, 11.96
06-1500-6	Shoot	37	3%	0.85 ± 0.18 a	7.56 a	± (8.90, 6.42)
06-1500-6	Tip	37	5%	1.09 ± 0.23 a	7.67 a	± (8.76, 6.71
06-1819-1	Shoot	30	3%	3.98 ± 0.41 a	13.66 a	± (15.80, 11.81)
06-1819-1	Tip	30	0%	2.71 ± 0.37 a	13.61 a	± (15.87, 11.66)
06-1821-3	Shoot	37	0%	0.84 ± 0.18 a	9.72 a	± (11.58, 8.15)
06-1821-3	Tip	37	0%	1.57 ± 0.27 a	8.09 a	± (9.27, 7.05)
				Prob > F	Prob > F	
Genotype (A)				<.0001	<.0001	
Treatment (B)				0.3499	0.5217	
A x B				<.0001	0.0837	

1.3.3. Rooting

Hybrid oak genotypes that were stabilized in a continuous multiplication cycle were trialed for their capacity to root in tissue culture. As with establishment and multiplication experiments, differences in genotype played a significant role in the capacity for shoots to produce and extend roots.

A preliminary experiment was conducted to determine the effeteness of two auxins (IBA vs K-IBA) for root production of two hybrid oaks (06-1500-6 and 06-1821-2). For both genotypes, the number of roots and root length were not significantly different between auxin treatments. Significant differences were observed between genotypes where 06-1500-6 produced significantly more roots compared to 06-1821-3 and no significant interactions were observed between genotype and media. Root length between treatments and genotypes were not significantly different (Table 1.10).

Table 1.10: Effect of Auxin IBA and K-IBA on Rooting Capacity of Two Genotypes of Hybrid Oaks

Genotype	**Auxin**	**n**	**Percent Contam.**	**Number of roots**	**95% Conf. Interval**	**Root Length**	**95% Conf. Interval**
06-1500-6	IBA	15	20%	9.32 a	± (13.00,	11.81 a	± (15.55,8.58)
06-1500-6	K-IBA	15	0%	8.14 a	± (11.21,5.57)	13.83 a	± (17.4,10.66)
06-1821-3	IBA	15	20%	1.83 a	± (3.63,0.64)	10.62 a	± (15.05,6.96)
06-1821-3	K-IBA	15	0%	1.11 a	± (2.40,0.56)	11.30 a	± (15.58,7.71)
				Prob > F		Prob > F	
Genotype (A)				<0.0001		0.329	
Rooting Media Type (B)				0.346		0.480	
A x B				0.851		0.741	

After the IBA vs K-IBA experiment concluded, the remaining genotypes that were stabilized in long term multiplication were trialed for rooting in a second experiment. The percentage of shoots that produced roots varied by genotype from 60-100%. The number of roots produced by

genotypes ranged from 1-3 roots per shoots with the exception of 06-1500-1 that produced 10 on average. Root length varied between genotypes where 06-1500-6 and 06-1821-3 produced significantly longer roots compared to all other genotypes. Statistically significant differences in number of roots produced and root length varied by genotype (Table 1.11).

Table 1.11: Rooting Capacity of Hybrid Oaks Treated with IBA

Genotype	n	Percent(%) Rooting	Num. of Roots	Std Err.	Mean Root Length (mm)	95% Conf. Int.
05-830-50	15	67%	1.67 b	0.77	10.50 b	± (17.94, 6.15)
06-1500-1	15	87%	3.73 b	0.77	11.67 b	± (18.67, 7.30)
06-1500-6	12	100%	10.00 a	0.86	31.06 a	± (50.65, 19.05)
06-1819-1	15	60%	1.87 b	0.77	8.92 b	± (15.69, 5.07)
06-1821-3	12	67%	3.17 b	0.86	26.02 a	± (47.35, 14.29)
			Prob > F		Prob > F	
Genotype			<.0001		0.0029	

1.4. Discussion

1.4.1. Establishment

Contamination rates (Table 1.1) varied between establishment year (2016, 2017, 2018) and by location where shoots used for establishment were harvested (field or growth chamber). Shoots harvested from the growth chamber had a lower contamination rates compared to field grown shoots. The varying of contamination rates by year could be attributed to difference in microbial phenology, depending on the progression of each growing season. Shoots used for force flushing were collected when many plant species were still in winter dormancy and few growing degree days had occurred. In early spring, cold temperatures and limited availability of substrates suitable for microbial development may help explain limited microbial activity and contamination rates from dormant shoots harvested at this time of year (Nedwell, 1999). The same disinfection protocol was used for each growing season leading to the conclusion that differences in microbial communities, activity or abundance could explain differences in

contamination rates. This occurrence of using plant material from field locations compared to protected settings such as greenhouses is reported in the tissue culture literature and shows similar results as our study with higher occurrence of contamination occurring from field derived plants (Niedz and Bausher 2002).

Establishment rates (percent responsive, average shoot number and length) were highly variable and dependent on individual genotypes (Table 1.2). Some genotypes exhibited as much as 100% responsiveness in establishment while others where as low as 0%. Likewise, average shoot length and number of shoots produced from single starting node were also genotype specific. Genotype specific response with some individuals tolerating and performing better than others under tissue culture environments has been found in other oak tissue culture studies during the establishment, multiplication and rooting phases (San-Jose et al. 1988; Vieitez et al. 1993; Vieitez et al. 1994; Vieitez et al. 2009; Herrmann and Buscot 2008; Vengadesan and Pijut 2009) and in oak somatic embryogenesis (Vieitez et al 2012).

Responsiveness to establishment phase was unaffected by individual year in which establishment was trialed. Genotypes that had previously been established in culture and moved to multiplication phase had the capacity to reestablish and stabilize in subsequent years (Table 1.4). This response was similar to the findings of Martinez who in 2008 established oak somatic embryogenesis lines from the same genotypes of trees that Vidal had used for tissue culture in 2003 (Martínez et al. 2012; Vidal et al, 2003). This finding suggests that genotype is a significant factor leading to successfully establishment and multiplication of oaks in tissue culture. While genotype specificity remains a significant factor in successful oak tissue culture, other dynamics

such as phenological development throughout the year (Romano and Loucao 1992), topophysical position (Evers et al. 1993), and age of the stock plant were also significant factors (Romano and Loucao 1992; Evers et al. 1993 Vieitez et al. 1993; Vieitez et al. 1994). The oak tissue culture process in general is challenging and time-consuming with low success rates. Opportunities to establish oaks in tissue culture are limited to the spring of each year. The screening process of moving different hybrid oaks through the establishment phase is labor intensive and limited by both the human capacity of technicians and logistical challenge of managing the harvesting, disinfection and establishment of germplasm from many stock plants.

One of the most significant findings from our study is the identification of specific genotypes that have the capacity for establishment, multiplication, stabilization, shoot growth, elongation, rooting and repeat establishment (Table 1.2, Table 1.4, Table 1.5). While a relatively large number of hybrid oaks show at least some capacity to produce elongated shoots during the establishment phase, (Table 1.2) this does not ensure that these genotypes will be amenable to continued growth during the multiplication phase. Identification of genotypes that have the capacity to tolerate the tissue culture environment represent a significant step towards development of a clonal oak propagation system and release of oak cultivars.

1.4.2. Multiplication

1.4.2.1. Stabilization

As with the establishment phase, not all oak genotypes were amenable to the multiplication process with some hybrid lines perishing over successive multiplication cycles. The number of shoots, shoot length and number of leaves produced varied in multiplication based on specific genotypes (Table 1.7). While some lines declined, others flourished and stabilized over time.

Once hybrid lines reached their stabilization threshold, they appeared to have the capacity to grow in multiplication for an indefinite amount of time. For example, hybrid lines established in 2014 were still actively maintained in multiplication through 2018. In other oak tissue culture studies, the stabilization process of took anywhere from 4 to 12 months (Vieitez et al. 2009; Herrmann and Buscot 2008). For most of the hybrid oak lines under assessment, stabilization was detected in as little as three months and 153 days on average.

After finding stable lines, (Table 1.4) these stabilized genotypes were used run experiments on multiplication phase protocols. Other researchers who have undertaken oak tissue culture work have followed a similar path of identifying highly productive lines and using them for experimentation (Vieitez et al. 2009). While the genotypes highlighted in Table 1.4 proved to be productive and useful for experimentation in the multiplication phase of tissue culture, not all of these hybrid lines are considered top selections for tree introduction. Specifically, genotype 06-1500-6 remains of low interest for introduction because of its *Q.* × *warei* 'Long' (Regal Prince®) x open pollenated parentage. Although these plants exhibit some improved characteristics, they are similar to the other *Q. robur* introductions that already occupy a large portion of the oak cultivar market.

The capacity to reestablish and stabilize individual genotypes into multiplication cycles was a significant finding as it proved that this tissue culture research to be replicable. Table 1.4 show that lines 06-1819-1, 06-1500-1 and 06-1500-6 were established and stabilized in two different years, first in 2014 by Bryan Denig and again in 2017 by the author. This finding importantly indicates that individual genotypes have the capacity to be reestablished. Additionally, the

finding demonstrates that these individual genotypes can be stabilized despite specific physiological or phenological stage of the stock plants at time of establishment.

1.4.2.2. ZeatinPVP/50ml vs BAP 25ml Media

Polyvinylpyrrolidone (PVP) is a water-soluble polymer that can form complexes with polyphenols through hydrogen bonding (Porebski et al. 1997). Oaks are known to contain high quantities of polyphenols such as ellagitannins (hexahydroxydiphenoylesters) and condensed tannins (proanthocyanidins) (Scalbert et al. 1988) which are released as lysate when mechanical damage occurs to cells when cutting stems during the tissue culture process. When these polyphenols are released into tissue culture media, they oxidize turning media dark brown and damages explants. PVP has been used to remove polyphenols during DNA extractions (Porebski et al. 1997). In oak tissue culture, PVP has been added to culture media to eliminate or reduce occurrence of oxidation due to phenolic release. A 1992 oak tissue culture study by Romano and Loucao noted a decline in explant survivability in the establishment phase due to phenolic exudation in *Q. robur*. In the 1992 study, a series of treatments including the use of PVP and a combination of ascorbic and citric acid was experimented with to reduce damage. Treatments, including PVP, proved ineffective at reducing browning and oxidation of media. The investigators resorted to transferring cultures with large amounts of phenolic oxidation within 48 hours after establishment out of the media (Romano and Loucao 1992). A 1994 study on *Q. suber* and *Q. petrea* aimed to determine the effects of pretreating explants with phenolic inhibiting compounds. These included ascorbic acid, citric acid, L-cysteine hydrochloride, and PVP. Their studies showed that PVP prevented browning, but shoot growth was poor. In this same study ascorbic and citric acid prevented the greatest amount of phenolic damage (Toth et

al. 1994). PVP has been used in column chromatography as solid phase with a phosphate buffer as an eluent to extract cytokinins from plant tissue (celery seed). This study observed that pH played a significant factor in the binding affinity of cytokinins to PVP with lower pH's releasing cytokinins with the use of less elution buffer. This finding suggests that pH plays a significant role in the capacity for PVP to bind or release cytokinins into a buffer solution. Regardless of pH, it was observed that different cytokinins were extracted as elution volumes increased. Zeatin released at the lowest elution volumes followed by kinetin and then BAP. This result suggests that different plant hormones bond more or less strongly to PVP depending on their chemical structure, pH and quantities of elution buffer in a solution (Biddington and Thomas 1976). As zeatin requires some of the lowest amounts of elution buffer needed to be extracted from a PVP column, an experiment was designed to learn if its low binding affinity would make this cytokinin plant available in tissue culture media. Browning of media due to phenolic oxidation is most apparent directly after mechanical damage to shoots when cutting nodes during establishment phase or shoots during multiplication phase.

This experiment focused using PVP to reduce damage and attrition during multiplication compared to using the cytokinin BAP alone. The ZeatinPVP media effectively controlled phenolic oxidation in 98% to 100% of the cultures in this treatment. Comparatively, the BAP media showed variable results with phenolic exudation effecting between 10-90% of the cultures based on genotype (Table 1.6). The varied results for the BAP treatment suggest that individual genotypes may produce varying quantities of phenolics and the oxidation may only negatively affect individual lines.

The number of shoots produced were significantly different by genotype, media treatment and their interaction (Table 1.7). For genotypes 05-830-50, 06-1500-6, 06-1819-1, the ZeatinPVP treatment produced significantly fewer shoots compared to BAP alone. Average shoot length was also significantly different by genotype, media treatment and the combination of these factors (Table 1.7). Within individual genotypes both treatments produced equal shoot length for all cultures aside from 06-1819-1 which had shorter shoots in ZeatinPVP media. These results show that the effectiveness of PVP was variable by genotype, but for all the cultures the ZeatinPVP treatment produced fewer, shorter shoots as compared to BAP (Table 1.7). Qualitatively leaves grown in the ZeatinPVP media were a lighter green color, potentially indicating nutrient deficiency compared to the control. This finding suggests that the first two weeks of the multiplication cycle are a critical period in the development and growth of oaks grown in vitro.

While PVP was an effective treatment for reducing phenolic exudation, the negative effects on shoot growth limits the usefulness of this compound. Through observations in the laboratory, it was noted that phenolic oxidation in the multiplication phase was significantly less damaging than in the establishment phase. In establishment, single nodes were used and commonly phenolic browning fully surrounded the cultures. However, during multiplication, the shoots were longer and the primary source of phenolics was from the cut end of the shoot. A longer explant lowers the total overall surface area exposed to phenolic oxidation and shoots can typically still multiply with the browning present. Overall browning and damage due to phenolic exudation is not a major cause of mortality during the multiplication phase.

San-Jose et al. (1988) used zeatin for multiplication and shoot elongation of *Q. robur* cultures. In this study, the researchers moved shoots growing in BAP media for four weeks into media with varying concentrations of zeatin. They noted that for one clone and zeatin concentrations ranging from 0.1 or 1.0 mg/L, shoot length increased when compared to a BAP media. In the same study, another *Q. robur* clone showed no significant differences between the zeatin or BAP treatments (San-Jose et al. 1988). This finding was similar to the result of our study that showed varying results based on genotype.

In a different study, varying concentrations of BAP and zeatin in the multiplication phase were compared in *Q. suber* cultures. The findings showed that explants in BAP media produced a greater number of shoots that were longer compared to a zeatin media (Romano and Loucao 1992). These results agreed with the findings in our study in which the hybrid oaks generally showed a greater number of shoots produced in BAP media. While not significantly different in all cases average shoot length was lower in most hybrids cultured in zeatin media (Table 1.7).

1.4.2.3. Multiplication cycle treatment 1-5 and 2-2-2

An experiment was conducted to determine if changing the transfer frequency of subculturing affected the capacity of hybrid oaks to multiply. If frequency had no effect, then it would be possible to reduce the labor associated with making media and technician time working in the flow hood. Two transfer frequencies were trialed using four genotypes. After forty replications and trialing four genotypes, the number of shoots and shoot length were statistically equal between treatments (Table 1.8). The major differences detected were between genotypes and not due to changes in transfer frequency. This result is similar to the other multiplication experiments that have been conducted with the hybrid oaks, which demonstrated that genotype

was the most variable factor in tissue culture success. No interactions were observed between treatment or treatment x genotype. These results demonstrate that it is possible to reduce labor during the multiplication process by using the 1-5 week transfer frequency.

1.4.2.4. Shoots and tips

An experiment was conducted to determine the effect of using both decapitated shoots and terminal shoot tips during the multiplication phase. The current oak tissue culture system does not have methods that allow oaks to grow indeterminately. Shoots display an episodic growth cycle (Herrmann and Buscot 2008) and after setting a terminal bud, the culture tends to decline due to shoot tip necrosis (Schwarz and Schlarbaum 1993). Previous research determined that placing shoots horizontally in tissue culture media during multiplication promoted the development of lateral nodes that develop of new shoots (San-Jose et al. 1988). Improved efficiency (production of a greater number of shoots) was achieved by removing of apical dome when initiating multiplication phase (Vieitez et al. 1993; Vieitez et al. 2009). Our results using hybrid oaks suggest that apical bud tips can be used in the multiplication phase since they produced an equal number of shoots and shoot length when compared to shoots in the multiplication phase (Table 1.9). Oaks in vitro and under field conditions generated a telescoping growth pattern where internodal length decreased for nodes closer to the apical bud. As a result, when apical buds were harvested along with 3 mm of shoot, a series of lateral buds were also collected. After harvesting tips in the multiplication phase, both the lateral and terminal buds had the capacity to produce multiple new shoot growth. This result demonstrates why both shoots and tips produced an equal number of new shoots during the multiplication phase. The results from our study suggest that apical buds can be used in the multiplication phase of oak tissue culture and provide more explant material which increases overall multiplication efficiency.

1.4.3. Rooting

A preliminary experiment was conducted to determine if there were significant differences between two auxins (IBA vs K-IBA) in inducting root growth of hybrid oaks. Two different genotypes (06-1500-6 and 06-1821-3) were used for the experiment. Significant differences were observed between genotypes with 06-1500-6 producing on average between eight to nine roots per shoot compared to one root per shoot for 06-1821-3 (Table 1.10). This pattern of genotypes performing differently to hormone treatments in a tissue culture system has been consistently observed in both the establishment and multiplication phases. While differences were observed by genotype there was no difference between media type indicating that both forms of IBA are effective at inducing rooting. As a result, IBA was selected for further experimentation on rooting of hybrid oaks.

A second rooting study was conducted to determine the capacity of various hybrid oak genotypes to produce roots. Six genotypes were selected and trailed using the standard rooting protocol. Results showed that all genotypes produced roots with an IBA treatment, but the number of shoots that produced roots among genotypes varied between 67% to 100%. Differences between genotypes were again observed with 06-1500-6 producing significantly more roots as compared to other genotypes. Mean root length was greatest for 06-1500-6 and 06-1821-3, which both produced significantly longer roots compared to other genotypes (Table 1.11). Callus development was ubiquitous for all shoots after IBA treatment and growing in PGR-free media. For very short shoots, callus was sometimes as large as the exposed shoot tissue. To avoid callus, future experiments could be conducted where IBA was reduced from 25 mg/l to lower concentrations or using alternative auxins. Roots arising from callus tissue could potentially not

have connected xylem to the main shoots. If this connection did not form, there could be an issue for continued growth during acclimatization. Although differences were observed in both number of roots and root length, the fact that all shoot produced roots demonstrated that the rooting phase of tissue culture would not be the limiting factor for the development of clonally propagated oaks.

One limiting factor affecting cultures during rooting was the occurrence of shoot tip necrosis. Shoot tip necrosis is commonly reported as a limiting factor in oak micropropagation with the terminal bud of a newly developed shoot dying in culture (Schwartz and Schlarbaum 1993; Vieitez et al 1994; Vieitez et al. 2009). While the six-week multiplication cycle is a convenient time scale for oak multiplication, it may be too long for certain genotypes and result in shoot tip necrosis. For certain genotypes, evaluation at four or five weeks should be conducted and shoots could be harvested for continuous multiplication or rooting when they reach their maximum growth point. In our study shoot tip necrosis occurred in the establishment, multiplication and rooting phases of tissue culture. Initial pilots of the rooting method placed shoots in IBA media for one week followed by four weeks in PGR-free media with charcoal. Four weeks in PGR-free media tended to result in shoot tip necrosis occurring in a large number of shoots and therefore the method was modified to reduce this time period to two weeks (1 week IBA, 2 weeks PGR-free).

1.5. Conclusion

The development of clonally propagated oaks has been a long-term goal and dream of many who have studied and propagated oak taxa. The results of this study demonstrate the possibility to develop clonally propagated oaks using tissue culture methods. Genotype specificity remains as

the single largest factor that affects the successful establishment, multiplication and rooting of oaks in tissue culture. As a result, the screening of a large number of hybrid oak genotypes for their capacity to establish, multiply and root in tissue culture presented in this chapter represents a significant finding. These results lay the foundation for large scale production of stress tolerant hybrid oaks for the urban environment and provide a path for successful introduction of cultivars for the Urban Horticulture Institute. Future research should focus on the development of a direct rooting method that bypasses the multiplication phase, which would decrease labor cost and increase production rates.

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2. CHAPTER 2

DIRECT ROOTING METHOD

2.1. Introduction

In 2017 and 2018, experiments were conducted to determine if it would be possible to move oaks in tissue culture directly from the establishment phase to rooting and bypass the multiplication phase. In 2017, a small-scale proof of concept experiment was trialed (Table 2.1). A single genotype known to have the capacity to re-establish and stabilize in culture was selected (06-1500-6). In 2018, a follow-up experiment was conducted to trial the direct to rooting method for an expanded number of hybrid oak genotypes. Eleven hybrid oaks (Table 2.2) were used for the direct to rooting experiment and were selected for their stress tolerance or horticultural characteristics that had been identified through the evaluation process.

In addition to the direct to root experiment, a smaller study was conducted to determine the effect of Plant Preservative Mixture (PPM) on in vitro oak growth and culture contamination. PPM is a broad spectrum, non-selective, fungicidal and biocidal compound that can be added to tissue culture media to suppress contamination growth. It is heat stable and added to media prior to autoclaving. An experiment was conducted where 0.1% PPM was added to establishment media to determine its effectiveness in reducing contamination rates and if it affects shoot growth.

2.2. Methods

For the 2017 proof of concept study shoots for establishment were harvested from both force flush branches in the growth chamber and stool bed stock plants in the field of genotype 06-1500-6. All of the force flushed nodes used for establishment came from a single shoot. Field sourced buds were sourced from three different shoots from the same coppiced clone. Buds were

established using 15 ml of standard establishment media (Woody Plant Media 2.41 g/L, BAP 0.5 mg/L, sucrose 30 g/L, agar 5.5 g/L and pH adjusted to 5.6±0.1) in 25x125mm culture tubes. Nodes in establishment were transferred at two-week intervals and moved to rooting media after a total of six to eight weeks. Rooting protocols followed the standard method (Chapter 1) with elongated shoots being placed in culture tubes with 15 ml of root induction IBA media (Woody Plant Media 2.41 g/L, IBA 25 mg/L, sucrose 30 g/L, agar 5.5 g/L and pH adjusted to 5.6±0.1) for one week before being transferred into 15 ml of PGR free media (Woody Plant Media 2.41 g/L, active charcoal 4 g/L [pH 5.7], sucrose 30 g/L, phytoblend 5.5 g/L, and pH adjusted to 5.6±0.1) for a period of four weeks. Elongating established shoots were assessed at six and eight weeks after establishment and moved to rooting if shoots reached a minimum height of 5 mm.

For the 2018 direct to rooting study eleven hybrid oaks (Table 2.2) were selected to trial in the direct rooting propagation system. During establishment, data collected included starting number of nodes, number and percent of buds swelling, number and percent of buds elongating, number of shoots produced per individual starting node and shoot length. Rooting in PGR free media was first assessed after two weeks for rooting. If no rooting reactivity was observed, shoots were placed back into PGR free media for another two weeks. After four weeks in PGR free rooting media, explants were assessed for rooting capacity. Rooting data included number of shoots moved from establishment to rooting, percentage of shoots developing shoot tip necrosis during rooting, percentage of shoots producing roots, average number of roots per shoot, average root length and percentage of starting shoots moved to acclimatization. A culture was considered to have the capacity to be able to move from rooting to acclimatization if shoots had developed roots, had leaves and there was no presence of shoot tip necrosis. For rooting-establishment data,

number of shoots elongating was square root transformed to achieve data normality and to allow for statistical comparison.

An addition to the direct rooting method an experiment was conducted using Plant Preservative Mixture (PPM) to determine its effectiveness at suppressing growth of contaminates during the establishment phase. All shoots used for the PPM experiment were harvested from the stock block at Blue Grass Lane under field conditions. For statistical analysis, mean number of shoots produced during PPM establishment was square root transformed to achieve normality and shoot length was log transformed.

2.3. Results

In 2017, four shoots of genotype 06-1500-6 from force flushed stems in the growth chamber and five shoots harvested directly from the field were selected for establishment and direct to rooting. All starting nodes from both starting environments (growth chamber and field) produced shoots and roots except for one individual from the growth chamber. The average number of roots produced per shoot ranged from between 1-13 and average root length was between 9-46 mm (Table 2.1).

Table 2.1: 2017 Direct to Root Hybrid Oaks Pilot Study

	Genotype	**n**	**Avg. Shoot Length (mm) Establishment**	**Avg. Num. Roots**	**Avg. Root Length**
Growth Chamber	06-1500-6	4	20.25	2.50	24.25
Field	06-1500-6	5	21.20	6.20	29.51

In 2018, eleven genotypes of hybrid oaks were used to trial the direct rooting method that had been successfully trialed in 2017. In the 2018 study, number of shoots produced per bud and shoot growth (length) during establishment phase all varied significantly between genotypes

(Table 2.2). Number of shoots moved to rooting was determined by the number of shoots that elongated in establishment phase and shoot length. Shoot length across all genotypes was on average 14.49 mm, and all genotypes produced some shoots greater than the 5 mm length required to move shoots to rooting phase. Number of shoots moved to rooting varied by genotype (Table 2.3). Shoots were first assessed after two weeks and no visible root development had occurred. Roots were then assessed at four weeks after being transferred from IBA media and rooting data was obtained. Shoot tip necrosis varied between genotypes from 0%-100% and in some cases eliminated lines that had high instances of damage. The number of individual shoots that produced roots was variable by genotype and typically ranged from 0%-50%, with one genotype (06-1812-2) producing roots for 100% (n=2) of shoots moved to rooting. Number of roots ranged from 2.5-5.0 roots per individual shoot and shoot length ranged from 3.25 mm to 35.33 mm (Table 2.3). The capacity of being able to move rooted shoots to acclimatization was assessed by identifying genotypes that produced roots, had leaves and did not have shoot tip necrosis. Of the starting eleven genotypes moved from establishment to rooting, only three genotypes (05-830-50, 06-1500-6, 06-1819-1) could successfully be moved to acclimatization after the rooting phase (Table 2.3).

Plant preservative mixture was added to standard establishment media to determine its effectiveness at reducing contamination rates and to see if the compound affected shoot growth. PPM reduced contamination rates during establishment for genotypes 05-830-50 and 06-1500-6 and when all genotypes were considered together (Table 2.4). For all other genotypes, PPM had no significant effect on contamination rates (Table 2.4). Although differences in contamination were not significant in all cases, rates were lower in all PPM media compared to standard media.

No significant differences were detected for the number of shoots produced and shoot length between PPM and standard establishment medias (Table 2.5).

Table 2.2: 2018 Direct to Rooting – Establishment Phase

Genotype	n	Num. Bud Swell.	Percent Bud Swell.	Num. Buds Elongating	Percent Bud Elongate	Num. of Shoots	95% Conf. Int.	Shoot Length	Std. Error
04-566-3	27	12	44%	9	33%	1.49	± (2.27,0.88)	19.76	2.84
05-830-50	43	21	49%	19	44%	2.68	± (3.36,2.08)	14.45	1.95
05-854-15	41	14	34%	8	20%	1.00	± (1.7,0.49)	19.13	3.01
05-878-1	37	9	24%	7	19%	1.00	± (1.75,0.46)	16.86	3.22
06-1500-1	18	11	61%	9	50%	2.88	± (3.92,2.00)	9.43	2.84
06-1500-6	18	13	72%	13	72%	1.54	± (2.18,1.01)	24.47	2.36
06-1802-1	40	8	20%	2	5%	2.48	± (4.74,0.95)	8.92	6.01
06-1810-2	43	30	70%	24	56%	1.82	± (2.32,1.38)	13.76	1.74
06-1812-2	41	24	59%	14	34%	1.64	± (2.25,1.12)	9.87	2.20
06-1819-1	44	19	43%	12	27%	2.00	± (2.75,1.36)	11.72	2.46
06-1821-3	33	2	6%	2	6%	1.46	± (3.28,0.37)	11.00	6.01
						Prob > F		Prob > F	
Genotype						0.01		0.0006	

Table 2.3: 2018 Direct to Rooting – Rooting Phase

Genotype	Starting Number of nodes	Num. Moved to Root	Percent Tip Necrosis	Percent Rooting	Avg. Num. Roots	Std. Err	Avg. Root Length	Std. Err	% Survived to Acclimatization Phase
04-566-3	27	4	75%	0%	0.00	0.00	0.00	0.00	0%
05-830-50	43	12	58%	33%	2.42	0.65	11.53	3.72	8.3%
05-854-15	41	4	100%	0%	0.00	0.00	0.00	0.00	0%
05-878-1	37	1	0%	0%	0.00	0.00	0.00	0.00	0%
06-1500-1	18	9	56%	11%	5.00	2.25	23.80	12.89	0%
06-1500-6	18	10	60%	40%	3.80	0.71	10.29	4.08	30%
06-1802-1	40	0	0%	0%	0.00	0.00	0.00	0.00	0%
06-1810-2	43	0	0%	0%	n/a	n/a	n/a	n/a	0%
06-1812-2	41	2	0%	100%	2.00	1.59	35.33	9.11	0%
06-1819-1	44	4	100%	50%	2.50	1.12	34.54	6.44	50%
06-1821-3	33	2	50%	50%	2.50	1.59	3.25	9.11	0%

Table 2.4: PPM vs Standard Establishment Media Contamination Rates

Genotype	PPM	n	Standard	n	Fisher's Exact Two Tailed
05-830-50	6%	18	56%	18	0.0027
06-1500-1	39%	18	50%	18	0.738
06-1500-6	0%	18	28%	18	0.0455
06-1821-3	11%	18	44%	18	0.2285
06-1819-1	44%	18	61%	18	0.5051
All	20%	90	46%	90	0.0004

Table 2.5: PPM vs Standard Establishment Rates

Genotype	Media	Percent Contaminated	n	Mean Num. of Shoots	95% Conf. Int.	Shoot Length	95% Conf. Int.
05-830-50	PPM	6%	18.00	2.06	± (2.66,1.54)	13.09	± (10.04,17.08)
	Reg	56%	18.00	3.68	± (4.63,2.84)	17.39	± (12.62,23.95)
06-1500-1	PPM	39%	18.00	2.48	± (3.27,1.8)	10.09	± (7.32,13.9)
	Reg	50%	18.00	2.87	± (3.82,2.07)	8.41	± (5.90,11.98)
06-1500-6	PPM	0%	18.00	1.13	± (1.64,0.73)	24.88	± (18.53,33.42)
	Reg	28%	18.00	1.53	± (2.12,1.05)	20.41	± (15.2,27.41)
06-1821-3	PPM	11%	18.00	1.22	± (1.96,0.66)	13.60	± (9.10,20.32)
	Reg	44%	18.00	1.87	± (2.94,1.04)	10.91	± (6.79,17.55)
06-1819-1	PPM	44%	18.00	1.22	± (2.23,0.52)	15.87	± (9.33,27.00)
	Reg	61%	18.00	1.00	± (3.15,0.06)	12.00	± (4.15,34.76)
				Prob > F		Prob > F	
Genotype (A)				<.0001		<.0001	
Media Type (B)				0.10		0.44	
A x B				0.44		0.44	

2.4. Discussion and Conclusion

In 2017, a small-scale direct to rooting experiment was attempted as a proof of concept study. Nine shoots of a single genotype (06-1500-6) sourced either from a force flushed growth chamber or field grown shoots were selected from establishing nodes (Table 2.1). In 2017 shoots of 06-1500-6 were maintained in PGR free rooting media for four weeks and produced some very long roots up to 46 mm by the end of the cycle. In instances where long roots grew it made it difficult to transfer them into acclimatization because they were prone to breaking. The 2017 pilot study showed a high success rate of moving oaks from establishment to rooting for a single genotype and as a result the experiment was expanded and repeated in 2018.

In 2018, a follow up experiment was conducted to trial a variety of hybrid oak genotypes with desirable stress tolerance and horticultural characteristics using a direct to root method (Table 2.2 and Table 2.3). As was observed with hybrid oaks in Chapter 1, genotype specificity played a significant role in terms of which lines could develop and elongate shoots during establishment (Table 2.2). The percentage of buds elongating ranged from 6% to 56% depending on genotype. The nodes that did produce shoots had lengths that ranged from 8.92 mm to 24.47 mm. Generally, for rooting, longer shoots are more desirable because they will eventually produce larger starting trees after acclimatization. Variability in the percentage of bud elongation affected the number of shoots that could be moved to rooting. As a result, the number of shoots that made it into the rooting phase was significantly lower than the starting number of nodes.

Similarly, in order to have shoots that would eventually make suitable trees, shoots also had to

have expanded leaves and no shoot tip necrosis. These criteria significantly reduced the number of shoots that could effectively be moved to rooting. Once in the rooting environment, the hybrid oaks were maintained initially for a period of three weeks (one week in IBA media and two weeks in PGR free media). At the end of the three-week period, few roots had formed and explants were again placed in PGR free rooting media for another two weeks. At the end of the five-week cycle (one week in IBA media and four weeks in PGR free media), roots had formed and it was possible to obtain rooting data.

Callus growth was evident on the majority of shoots that produced roots and varied in size from 2-8 mm. In the majority of cases, callus formation was a precursor to root development. An exception to this was observed for two shoots of 06-1819-1 which both had roots that emerged directly from the side of the main shoot. In the rooting phase, shoot tip necrosis became an impediment by killing portions or entire shoots. This significantly reduced the overall number of rooted shoots and partially explains why the percentage of explants that could be moved to acclimatization was so low (Table 2.3). Shoot tip necrosis has been reported as an issue with oaks when maintained for extended periods of time in a tissue culture environment (Schwarz and Schlarbaum 1993; Vieitez et al 1994; Vieitez, et al 2009). The exact reason for this is unknown but may be associated with the fact that oaks have episodic growth cycles under natural and in vitro growing conditions (Herrmann and Buscot 2008).

Cultural practices of removing terminal buds and laying shoots down sideways after a several-weeks growth period allowed for the development of a continuous multiplication cycle for oaks in tissue culture (Vieitez et al. 1994). From observations in the laboratory, the genotypes that

were able to stabilize in tissue culture and be grown in continuous multiplication tended to show fewer signs of shoot tip necrosis at the end of a six-week multiplication cycle. These same cultures, if left for several more weeks after this point, would eventually develop shoot tip necrosis. One possible explanation is that genotypes that can tolerate continuous multiplication cycles tend to not develop shoot tip necrosis until after a six-week multiplication period. This would be in contrast with genotypes that are not tolerant of a continuous multiplication cycle and display shoot tip necrosis within a shorter time period (less than six weeks). Experimentation with silver nitrate ($AgNO_3$) has been utilized in red oak tissue culture leading to some success in reducing instances of shoot tip necrosis (Vieitez et al. 2009). The exact mechanism that allows for some oaks to be maintained in a continuous multiplication cycle has not been fully explained in any detailed study using molecular regulation methods. As oaks go through continuous multiplication phases and stabilize, there are visible changes that occur in their phenotype and growth response compared to establishment phase. Shoots in establishment tend to produce a variety of shoot lengths, including some instances where shoots grow to be long with more leaves that have greater surface area compared to shoots in multiplication. As oaks go through the stabilization process in multiplication, they tend to be shorter and less variable. Genotypes moved to rooting from a continuous multiplication phase tended to show higher rooting rates compared to the direct to rooting method (Table 2.6). The changes that oaks undergo during the stabilization and multiplication process may make them more amenable to rooting phase treatments.

Table 2.6: Number of Shoots Producing Roots in Continuous Multiplication Cycle Compared to a Direct to Root System

	Continuous Multiplication[1]		Direct Root[2]	
Genotype	**n**	**Percent of Shoots Producing Roots**	**n**	**Percent of Shoots Producing Roots**
05-830-50	15	67%	12	33%
06-1500-1	15	87%	9	11%
06-1500-6	12	100%	10	40%
06-1819-1	15	60%	4	50%
06-1821-3	12	67%	2	50%

[1] Data from Chapter 1 Table 1.11
[2] Data from Chapter 2 Table 2.3

In all cases, the shoots in a continuous multiplication cycle had a higher instance of producing roots compared to direct to root methods, although significant differences in numbers of replicates make this comparison difficult to make. This suggests that oaks in a continuous multiplication cycle have a greater capacity to produce roots in the rooting phase. Another explanation is that rooting protocols for continuous multiplication are not as effective for direct to rooting methods and optimization is required to achieve higher rooting success rates.

With the length of time required to stabilize oaks in multiplication, the direct root method is attractive in terms of developing a system that can generate rooted oaks within a single growing season. Another benefit to the direct to root system is the possibility of selecting genotypes that elongate in establishment but don't stabilize in multiplication. For example, in Table 1.2 (Chapter 1) it was shown that many more genotypes have the capacity to elongate shoots in establishment than can be successfully stabilized in multiplication. Selection of shoots that can elongate in establishment and using them in direct to rooting could allow for cloning genotypes that otherwise would fail during multiplication phase. This will allow for a greater number of genotypes of interest to be rooted than otherwise would be possible.

Plant preservative mixture is a fungicidal/biocidal tissue culture additive that was trialed in establishment phase in 2018 to see how it would affect contamination rates and shoot establishment. Contamination rates during establishment were shown to vary significantly by year (Table 1.1, Chapter 1) and location where stock plants were sourced (field vs growth chamber). Field grown shoots had significantly higher contamination rates compared to shoots force flushed in a growth chamber. In an effort to reduce contamination rates, PPM was trialed. For all genotypes, contamination rates were lower in cultures that had PPM included compared to the standard (Table 2.4). While all PPM cultures had lower contamination rates, they were only statistically significantly lower for two genotypes (05-830-50 and 06-1500-6). While this was the case, the fact that contamination rates were lower in all PPM cultures indicated that this compound was effective at suppressing fungal and bacterial growth. For establishment, no significant differences were detected between PPM and standard media for number of shoots produced or shoot length (Table 2.5). As with other establishment studies, significant differences for number of shoots and shoot length were found by genotype. With PPM aiding in reduction of contamination, its use for oak tissue culture can be advised during establishment phase. This study did not continue the examination of PPM use into the multiplication phase so it is unclear what the long-term effects of PPM are on oak shoot development and growth. With PPM acting as a suppressor of bacteria and fungicides, it is recommended that it is continuously used throughout the tissue culture process. This could increase the cost of production for oaks in tissue culture. Considering that it significantly reduces contamination rates it may be worth the cost to add PPM to media during establishment since this phase is one of the most labor intensive and time-consuming steps in the tissue culture process.

For practitioners interested in using a direct to root method, starting with a very large number of nodes during establishment is recommended in order to generate enough shoots for rooting after predicted failures that occur during elongation. More research is required with the direct root method to make it commercially viable with a particular need for doing grid experiments with varying auxins to identify the best concentrations for root production. Additionally, future studies should attempt to move shoots to rooting media during the second or fourth week of the establishment phase (instead of at week six) to see if it would be possible for shoots to continue to elongate and develop roots simultaneously. The direct to root method offers the potential of significantly decreasing production time for developing clonally propagated oaks over using a continuous multiplication cycle and future research should focus on optimization of this protocol.

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3. CHAPTER 3

EFFECT OF SHOOT POSITION ON IN VITRO ESTABLISHMENT, MULTIPLICATION, AND ROOTING OF FOUR OAK SPECIES

3.1. Introduction

An experiment was designed to trial four oak species (*Quercus macrocarpa*, *Quercus bicolor*, *Quercus gambelli* and *Quercus garryana*) in tissue culture. Oak species *Q. bicolor* and *Q. gambelli* have previously been grown in tissue culture (Vieitez, et al. 2009)(Brennan et al. 2017), while this study was the first known time that *Q. garryana* and *Q. macrocarpa* have been trialed. Along with screening these four species for their capacity to grow in vitro the study was also designed to investigate how initial bud position on the stock plant might affect the capacity for establishment in tissue culture. Initial bud/shoot position was investigated using three-year-old trees and harvesting newly grown shoots (1st flush) to use as explant material. These same trees were then cut back to a height of 20 cm from the root flare and were allowed to flush epicormic shoots (2nd flush) from the basal section of the tree. Individual nodes from the 2nd flush shoots were then used to establish the same genotypes as the 1st flush.

Successful establishment of oaks in tissue culture has primarily been achieved using stock plant material that is ontogenetically “juvenile”. Juvenility is a term that has been variably defined by different disciplines, and can refer to plant material that is in its early development life phase (i.e. acorn seedlings, plants of “young” annual age) or arises from a location on a tree that exhibits juvenile characteristics (epicormic and nodal shoots that arise from a position in close proximity to the root/shoot interface). The capacity to establish oaks in tissue culture in a juvenile life phase contrasts with establishment of oaks in a mature phase. Mature phase oaks establish at

rates that vary from poor to impossible for most species (Evers et al. 1993; Vieitez et al. 1994). Mature life phase may refer to trees of a specific chronological annual age, or to shoots harvested from the exterior of a tree's canopy from tissue that has developed reproductive capacity (flowering and fruiting). Experiments have been conducted in attempts to trial establishment of oaks in tissue culture using both mature and juvenile explant source material (Vieitez et al. 1985; San-Jose et al. 1988; Vieitez et al, 1993; Brennan et al. 2017). These studies have generally shown that shoots from juvenile origin tend to have more reactivity in the tissue culture compared to the mature phase, although it is possible at times to establish shoots from mature trees. This effect has been suggested to be a result of oaks in tissue culture maintaining the same development phase state as the location on the stock plant from which it was harvested (Vidal et al. 2003). Molecular mechanisms for controlling phase change and juvenility have been linked to interactions with microRNAs miR156 and miR172 (Poethig, 2013). The relative abundance of these two microRNAs has been shown to correspond with phenotypic and development changes that woody and herbaceous plant species go through during the transition from a juvenile to mature phase (Wang et. al. 2011). Research into juvenility in horticulture has focused on its applicability to propagation practices (Dirr and Heuser 2006) and in relationship to heteroblasty and development studies in plant biology (Poethig, 2010). This study attempted to determine if the initial position of shoots on stock plant affected the success rate of establishing, multiplying and rooting four species of oaks in tissue culture.

In addition to the initial experiment, an establishment study was conducted to determine the effectiveness of a media containing the cytokinin zeatin and the anti-phenolic compound polyvinylpyrrolidone 40 (PVP). Oaks are rich in a variety of phenolic compounds that can be

released due to mechanical damage (Scalbert et al. 1988). Excision of individual nodes in the establishment phase damages cells and causes a release of phenolic compounds into the media. Damage and death of explants due to the release of phenolics and oxidation of the media can reduce establishment effectiveness. Damage due to phenolic oxidation has been observed both in practice in the hybrid oak study (Chapter 1) and in the oak tissue culture literature (Romano and Luocao, 1992; Toth et al. 1994). The combination of zeatin and PVP (Zea-PVP) was used as a substitute for BAP in establishment media to determine the effect of these combined compounds on reducing phenolic damage and promoting plant growth. The occurrence (absence/presence) of phenolic oxidation and shoot growth and development were observed for species *Q. bicolor*, *Q. gambelli* and *Q. garryana*.

3.2. Methods

Four oak species *Quercus macrocarpa*, *Quercus bicolor*, *Quercus gambelli* and *Quercus garryana* in the white oak section (Section *Quercus*) were selected to be trialed in a tissue culture propagation system. Dormant three-year-old bareroot oaks 2-3 feet tall were ordered from Lawyers Nursery in June 2016. Upon arrival, the dormant bareroot oaks were planted in 25-gallon plastic containers in 2.94 ft^3 (83 Liters) of Lamberts LM-111 all-purpose soil-less medium. Shortly after planting, oaks broke dormancy and grew at Cornell's Bluegrass Lane horticulture facility throughout the 2016 growing season in an unheated, polyethylene plastic-covered greenhouse. Oaks were watered on average every two days or as needed when medium became visibly dry. In November 2016 after oaks had gone dormant in the Fall, all containerized trees were transported to Guterman Bioclimatic laboratory facilities and stored in a walk-in refrigerator at 40° F in the dark. Oaks were stored in cold refrigeration for a minimum period of three months. On February 16, 2017, five random individuals from each of the four species (20

trees in total) were removed from the cooler and transported to Dimock Greenhouses where they were placed in a 54 sq. ft. growth chamber. The growth chamber was configured to provide 200-500 µmol/m2/sec of cool white light from T5 fluorescent bulbs for a 16 hour per day photoperiod and eight hours of dark, an average temperature of 70°F and ambient humidity. Irrigation was used in the growth chamber with each container containing six 0.5 gallon per hour drippers. The automatic drip irrigation system was set to run for a two-minute interval, four days a week (Monday, Wed, Friday, Sunday) delivering on average 0.1 gallons (378.5 ml) of water per container during each irrigation cycle. After the oaks broke dormancy, shoot elongation and development was monitored. Shoots were harvested for each species after they reached a minimum length of 10 cm. Each individual species broke dormancy at different times after being removed from refrigerator storage. As a result, each species was established in tissue culture over a period of several weeks (Table 3.1).

Table 3.1: 1st and 2nd Flush Dates for Establishment of Four Oak Species

Oak Taxa	1st Flush Harvest Date	2nd Flush – Harvest Date
Quercus gambelii	3/10/17	4/7/17
Quercus garryana	3/20/17	4/18/17
Quercus macrocarpa	3/24/17	4/19/17
Quercus bicolor	3/24/17	4/21/17

For each species, five individual genotypes were selected for establishment experimentation. For establishment in tissue culture, four shoots containing a minimum of five lateral nodes were harvested per individual genotype. Shoots were harvested at two time points for each species. The “1st Flush” represented new growth that had emerged from the naturally developing branch architecture of the four-year-old trees. Shoots were harvested from terminal and lateral buds on the outer portion of the canopy distal from the trunk. After harvesting shoots for establishment, all trees were pruned back to a height of 20 cm from the root flare, removing any lateral branches

if present below this point. After pruning back, the oaks were maintained in the growth chamber under the same initial conditions. After a period of several weeks (Table 3.1) epicormic shoots emerged from the 20 cm stump section. These newly emerged shoots represented the "2nd Flush" treatment and elongated to an average length of 10 cm before harvesting for tissue culture establishment.

3.2.1. Decontamination

Each shoot was placed in an individually labeled 50 ml falcon tube and stored on ice while being transported to the tissue culture laboratory. In the laboratory, 50 ml of ethanol 70% (EtOH) was poured into each falcon tube. Up to eight individual tubes with EtOH solution were placed at a time on an orbital shaker at 200 rpm for one minute. After rotational shaking, all tubes were moved into a laminar flow hood and EtOH was poured off into a waste container. In the flow hood, 50 ml of a Clorox bleach (7.40% sodium hypochlorite) and Tween 80 solution (20% bleach, 80% DI H_2O with 2-3 drops of Tween 80 per 100 ml) was added to each individual falcon tube and capped. Sample tubes were agitated on an orbital shaker for a period of 15 minutes before transferring them back to the flow hood. In the flow hood the bleach/Tween solution was poured off into a waste bucket. All shoots in falcon tubes were then rinsed three times with 50 ml of autoclaved sterile deionized water.

3.2.2. Establishment in culture

Disinfected shoots were cut into one-bud segments 50-100 mm long starting at the end proximal to the cut surface working towards the terminal bud. Individual buds were placed upright into 25 x 150 mm culture tubes filled with 15 ml establishment media. Oak establishment media consisted of Woody Plant Media 2.41 g/L, BAP 0.5 mg/L, sucrose 30 g/ L, agar 5.5 g/L and pH adjusted to 5.6±0.1. After three days, individual buds were moved from one side of the culture

tube to the other to avoid oxidized phenolics that had been released from cut surfaces. If phenolic secretion and oxidation continued after the initial movement, then buds were moved to new test tubes with establishment media. Explants were transferred to fresh media every two weeks. After six weeks, individual buds that had elongated to a length equal to or greater than 5 mm were moved to multiplication phase.

3.2.3. Zea-PVP

An alternative establishment media (Zea-PVP) containing polyvinylpyrrolidone (mol. wt. 40,000) and zeatin was trialed as a treatment to prevent phenolic oxidation. Shoots for the Zea-PVP experiment were harvested from the 2nd flush period and included species *Q. gambelli*, *Q. garryana* and *Q. bicolor*. For each species, three shoots containing a minimum of five nodes were harvested. Five nodes per shoot were established in tissue culture. After a period of three days in establishment media, both the Zea-PVP media and the standard BAP media were assessed for phenolic oxidation. Phenolic oxidation was assessed by assigning either an absence or presence designation for cultures that showed visible browning in the media. For cultures that exhibited phenolic browning, nodes were either transferred to another area in the test tube free of oxidized phenolics or transferred to a test tube with fresh media. Phenolic transfers were done for both BAP media and Zea-PVP media if phenolic browning was present in large quantities of the test tube media. Fourteen days after the initial establishment, the Zea-PVP and BAP cultures were transferred to the standard BAP media. Subculturing for both treatments occurred at two-week intervals for both media treatments. All cultures were assessed six weeks after establishment to determine the number of shoots that emerged and shoot elongation length. Monitoring for contamination was conducted on a weekly basis and cultures were removed from the experiment if detected. After the establishment phase the Zea-PVP experiment was

concluded.

3.2.4. Multiplication

At the end of the six-week establishment cycle, shoots that elongated to a length greater than 5 mm were moved to multiplication phase. When moving shoots to multiplication, all leaves were removed from each shoot along with the terminal bud and the directly-adjacent 2 mm of stem. Removal of the terminal bud forces lateral bud development and formation of new shoots which can then be used in future multiplication cycles. Shoots were placed horizontally in 111 ml baby food jars with 25 ml of multiplication media. Shoots were subcultured (transferred to fresh media) every two weeks and were maintained for a period of six weeks. After six weeks shoots were harvested and the multiplication process was repeated. Oaks were maintained in multiplication media containing Woody Plant Media 2.41g/L, BAP 0.2 or 0.1 mg/L, sucrose 30 g/ L, agar 5.5 g/L and pH adjusted to 5.6 ± 0.1. For the first two weeks of the multiplication cycle, BAP 0.2 mg/L was used in the multiplication media followed by BAP 0.1 mg/L for weeks three through six. Four shoots were placed in each individual jar. All oaks were maintained for three multiplication cycles before being moved to a rooting media. At the end of each multiplication cycle the number of shoots and shoot lengths were measured. Observation of contamination was conducted on a weekly basis and cultures were removed if infection was detected.

3.2.5. Rooting

Shoots greater than 5 mm in height with living terminal buds and a minimum of three leaves were selected and moved to rooting. Total shoot length and total number of leaves were recorded prior to being moved to rooting media. Indole-3-butric acid (IBA) rooting media was used for the rooting induction phase. IBA root induction media was comprised of Woody Plant Media 2.41

g/L, IBA 25 mg/L, sucrose 30 g/L, phytoblend 5.5 g/L, and pH adjusted to 5.6 ± 0.1. Explants were maintained for a period of seven days in IBA root induction media before being transferred to plant growth regulator (PGR) free media for a period of two weeks. PGR free media was comprised of Woody Plant Media 2.41 g/L, active charcoal 4 g/L(pH 5.7), sucrose 30 g/L, phytoblend 5.5 g/L, and pH adjusted to 5.6 ± 0.1. Single shoots from the multiplication phase were placed individually in 25x150 mm culture tubes containing 15 ml of rooting media. At the end of the rooting cycle data was collected on absence/presence of shoot tip necrosis, callus size, development of primary roots, and number of roots produced.

3.2.6. Statistical Analysis

Statistical analysis was conducted using JMP Pro v.14. Non-normally distributed data was transformed to reach normal distributions. For establishment phases 1st vs 2nd flush data on number of shoots was log transformed to achieve normality. Zea-PVP shoot length was square root transformed and the number of shoots was log transformed. All other data had normal distribution of residuals without transformation.

3.3. Results

3.3.1. First and Second Flush

For establishment phase, two factors were analyzed, genotype and flush period (1st vs 2nd), to determine if they had an effect on the establishment success of four individual genotypes of oak species (*Q. bicolor*, *Q. gambelli*, *Q. garryana* and *Q. macrocarpa*). Bud swelling (Table 3.2), a measure of the capacity for an individual node in establishment media to remain alive and show signs of expansion, was first considered after the initial six-week establishment period. The effect test for bud swelling showed a significant difference between genotypes and interaction factors of genotype by 1st vs 2nd flush, while no significant differences were detected for factor

1st vs 2nd flush alone (Table 3.3). Percentage of shoots elongating was not affected by 1st vs 2nd flush while statistically significant differences were detected on both the individual genotype level and the interaction of factors genotype by 1st vs 2nd flush (Table 3.3). For the factor "number of shoots" that emerged from initial starting node, there was a significant difference for genotypes and 1st vs 2nd flush but not for the interactions of genotype by 1st vs 2nd flush (Table 3.3). Average number of shoots across all genotypes and 1st and 2nd flush was 1.42 ± 0.79 shoots per node. Average number of shoots produced for all species and flush periods were generally statistically overlapping and indicated similar mean values. Figure 3.1 indicates this with the Tukey HSD test showing overlapping mean values with matching letters. For individual genotypes, the number of shoots produced were generally higher in the 2nd flush compared to 1st flush, although there was significant overlap in confidence intervals between the two flush periods (Figure 3.1).

Average shoot length showed significant differences between the factors genotype, 1st vs 2nd flush and their interaction (Table 3.3). Average shoot length across all genotypes and 1st vs 2nd flush was 9.83mm ± 4.49mm. Although statistically significant differences were detected between genotype and 1st vs 2nd flush the majority of shoot lengths were overlapping with Tukey HSD matching letter analysis. Exceptions to this included QGAM 5 and QMAC 5, each of which had produced significantly longer shoots during the 2nd flush period (Figure 3.2)

Table 3.2: Establishment - Four Oak Species In Vitro Establishment During First and Second Flush periods

Genotype	1st vs 2nd	n	Contaminated	% Swelling	% elongating	Shoot Length (mm)	Std Err.	# of shoots	CI ± 95%
QBIC 1	1ST	20	0%	100%	55%	11.46	± 1.74	1.00	± (1.37, 0.74)
	2ND	20	0%	75%	50%	9.5	± 1.83	1.23	± (1.71, 0.89)
QBIC 2	1ST	20	15%	85%	60%	10.34	± 1.67	1.84	± (2.48, 1.38)
	2ND	20	0%	80%	70%	9.36	± 1.54	3.84	± (5.06, 2.92)
QBIC 3	1ST*	18	0%	100%	77%	16.00	± 7.75	2	± (2.35, 0.79)
	2ND*	0	n/a	n/a	n/a	n/a	n/a	n/a	n/a
QBIC 4	1ST*	19	11%	42%	0%	0	± 0	0	± 0
	2ND*	20	0%	30%	0%	0	± 0	0	± 0
QBIC 5	1ST	19	0%	89%	26%	11.8	± 2.58	1.82	± (2.89, 1.16)
	2ND	20	0%	100%	90%	10.24	± 1.4	2.88	± (3.67, 2.26)
QGAM1	1ST	20	0%	70%	30%	7.5	± 2.36	1.00	± (1.53, 0.66)
	2ND	25	0%	48%	20%	6.25	± 2.88	1.00	± (1.68, 0.6)
QGAM2	1ST	20	5%	90%	50%	10.45	± 1.92	1.08	± (1.53, 0.77)
	2ND	20	0%	75%	65%	12	± 1.6	1.45	± (1.94, 1.1)
QGAM 3	1ST	20	0%	70%	55%	11	± 1.74	1.13	± (1.55, 0.84)
	2ND	20	0%	80%	70%	12.84	± 1.67	2.39	± (3.14, 1.82)
QGAM 4	1ST	20	10%	85%	80%	8.5	± 1.44	1.17	± (1.51, 0.91)
	2ND	15	0%	100%	73%	10.64	± 1.74	1.51	± (2.07, 1.12)
QGAM 5	1ST	17	6%	71%	41%	5.58	± 2.18	1.22	± (1.8, 0.83)
	2ND	19	0%	95%	84%	24.4	± 1.49	1.30	± (1.68, 1.01)
QGAR 1	1ST	18	6%	50%	39%	9	± 2.18	1.10	± (1.63, 0.75)
	2ND	20	10%	90%	75%	9.14	± 1.49	1.13	± (1.47, 0.87)
QGAR 2	1ST	18	6%	39%	6%	13	± 5.76	1.00	± (2.8, 0.36)
	2ND	20	0%	75%	70%	9.5	± 1.54	1.43	± (1.88, 1.09)
QGAR 3	1ST	19	16%	47%	32%	13.67	± 2.36	1.12	± (1.71, 0.74)
	2ND	18	0%	33%	28%	13.8	± 2.58	1.32	± (2.09, 0.84)
QGAR 4	1ST	18	6%	17%	11%	8.5	± 4.08	1.00	± (2.07, 0.49)
	2ND	20	0%	50%	50%	13.1	± 1.83	1.23	± (1.71, 0.89)
QMAC 1	1ST*	20	0%	85%	0%	0	± 0	0.00	± 0
	2ND	20	10%	70%	45%	9.67	± 1.9	2.03	± (3.18, 1.3)
QMAC 2	1ST	19	5%	58%	21%	9.75	± 2.88	2.91	± (4.87, 1.75)
	2ND	20	0%	90%	65%	14.24	± 1.6	1.90	± (2.54, 1.44)
QMAC 3	1ST	20	0%	95%	50%	8.1	± 1.83	1.20	± (1.66, 0.87)
	2ND	20	0%	70%	45%	9.34	± 1.92	1.49	± (2.1, 1.06)
QMAC 4	1ST	19	11%	84%	47%	10	± 1.92	1.00	± (1.41, 0.72)
	2ND	20	0%	95%	60%	11.59	± 1.67	1.38	± (1.86, 1.03)
QMAC 5	1ST	19	0%	74%	32%	4.17	± 2.36	1.20	± (1.83, 0.79)
	2ND	20	0%	100%	80%	15.38	± 1.44	2.89	±(3.74,2.24)

* Indicates genotypes excluded from statistical analysis. QBIC 3 was excluded because there was no 2nd flush period shoots for statistical comparison and QBIC 4 because 0% of shoots elongated for both 1st and 2nd flush treatments.

Table 3.3: Establishment - Effect Test for First and Second flush Establishment of Four Species (Table 3.2)

	Percent (%) Bud Swelling	**Percent (%) Shoot Elongation**	**Shoot Length**	**Number of shoots**
Statistical Test	Prob>ChiSq	Prob>ChiSq	Prob > F	Prob > F
Genotype (A)	<.0001	<.0001	0.0495	<.0001
1st vs 2nd flush (B)	0.9973	1	0.0029	0.0001
A x B	<.0001	0.0228	<.0001	0.0705

*Two-way ANOVA excludes genotypes QBIC 4 and QMAC 1. Both genotypes did not have 1st and 2nd establishment period data required for comparison.

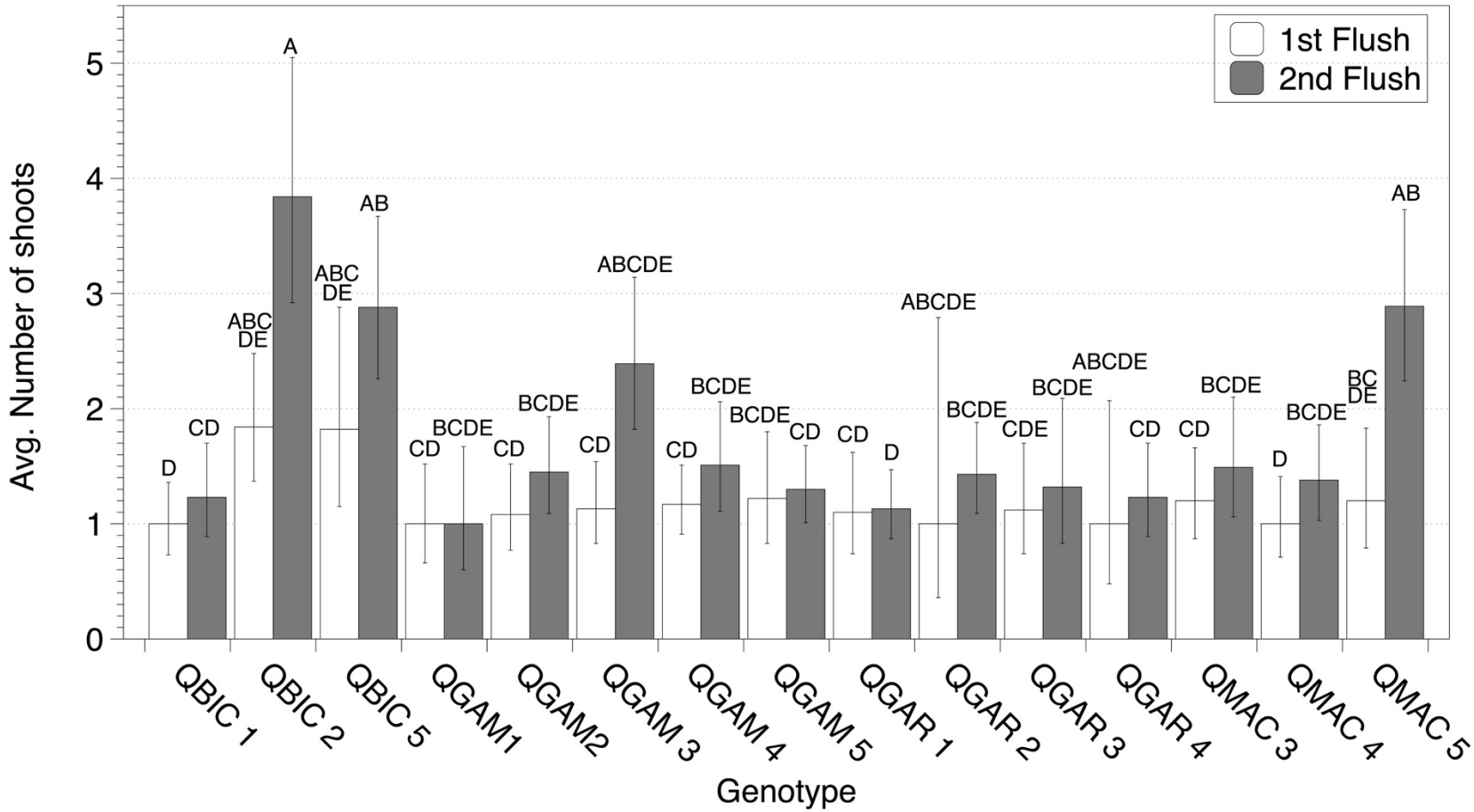

Figure 3.1 Establishment - Average Number of Shoots Produced In Vitro per Genotype by First and Second Flush

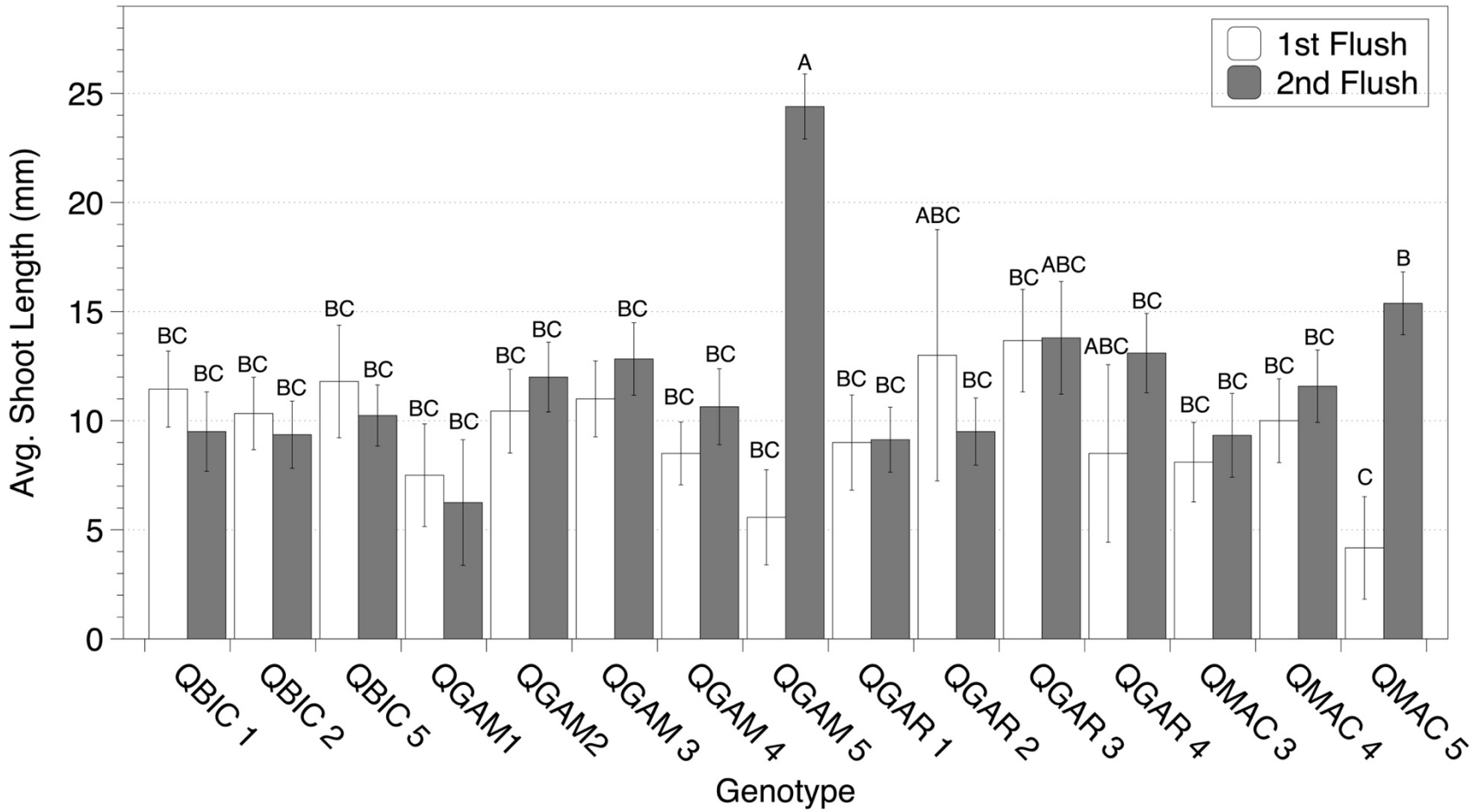

Figure 3.2: Average Shoot Length In Vitro by Genotype First and Second Flush

3.3.2. Zea-PVP

During the 2nd flush period, three oak species (*Q. bicolor, Q. gambelii and Q. garyana*) were selected and used to trial establishment effectiveness using the standard cytokinin BAP compared to the combination of cytokinin zeatin and the anti-phenolic agent PVP40 (Zea-PVP). The species *Q. macrocarpa* was excluded due to lack of available plant material. Phenolic exudation and damage were noted by the presence or absence of oxidized phenolics in the establishment media. Phenolic exudation was observed by the visual inspection of the media and determination if any browning had occurred. Presence of phenolics was observable in both BAP and Zea-PVP medias but varied significantly between genotypes and media types. BAP media showed phenolic oxidation in 90%-100% of cultures while Zea-PVP media varied significantly from 10%-93% of cultures (Table 3.4, Figure 3.3). The effect test indicates that the presence of phenolic oxidation was significantly different between genotypes and establishment media but not their interaction (Table 3.5).

Bud swelling and shoot elongation both showed significant differences between genotypes and establishment media (Table 3.5). Differences between treatments (Zea-PVP vs BAP) did not show a consistent pattern in terms of promoting bud swelling or elongation. Difference in bud swelling and elongation varied by individual genotypes (Table 3.4). Number of shoots produced from initial starting node varied significantly by genotype but not by media type or genotype x media type (Table 3.5). On average across all genotypes and treatments, number of shoots produced was 1.75 ± 0.98. Most genotypes produced an equal number of shoots or had statistically overlapping mean values for both media types (Figure 3.4).

Shoot length was significantly different by genotype and interaction factor genotype by media type but not for media type alone (Table 3.5). Average shoot length across all treatments and genotypes was on average 10.34 mm ± 4.25 mm. Shoot length varied by genotype and ranged from 4.98mm to 22.73mm. Media treatment had no significant effect on individual genotype shoot length with some individuals having either longer or shorter shoots with the BAP or Zea-PVP treatment (Figure 3.5).

Table 3.4: Establishment - Three Oak Species in Tissue Culture Media Containing Either Zea-PVP or BAP

Genotype	Treatment	n	Contam. %	Phenolic Present	% Bud swelling	% Elongating	Shoot Length	CI ± 95%	Num. of Shoots	CI ± 95%
QBIC 1	BAP	15	0%	93%	73%	53%	7.76	± (4.33, 12.17)	1.19	± (1.77, 0.81)
	Zea-PVP	15	0%	33%	80%	73%	10.71	± (7.14, 15)	1.44	± (2.02, 1.03)
QBIC 2	BAP	10	0%	90%	80%	80%	8.86	± (5.17, 13.54)	3.86	± (5.73, 2.61)
	Zea-PVP	10	0%	10%	80%	60%	6.9	± (3.29, 11.82)	4.18	± (6.58, 2.65)
QBIC 5	BAP	15	7%	93%	100%	87%	8.37	± (5.48, 11.86)	3.39	± (4.61, 2.49)
	Zea-PVP	15	13%	80%	47%	33%	11.9	± (6.55, 18.83)	2.41	± (3.96, 1.47)
QGAM 1	BAP	10	10%	90%	50%	30%	4.98	± (1.17, 11.42)	1	± (1.91, 0.53)
	Zea-PVP	9	33%	67%	56%	22%	7.85	± (1.95, 17.72)	1.42	± (3.11, 0.65)
QGAM 2	BAP	15	7%	100%	73%	73%	10.76	± (7.18, 15.05)	1.29	± (1.8, 0.92)
	Zea-PVP	15	7%	60%	67%	67%	11.08	± (7.29, 15.67)	1.57	± (2.24, 1.11)
QGAM 3	BAP	15	7%	100%	100%	87%	10.02	± (6.93, 13.66)	2.19	± (3.02, 1.59)
	Zea-PVP	14	0%	64%	79%	79%	18.74	± (13.69, 24.59)	2.03	± (2.83, 1.45)
QGAM 5	BAP	14	0%	100%	93%	93%	22.73	± (17.76, 28.29)	1.13	± (1.55, 0.82)
	Zea-PVP	13	0%	77%	77%	62%	11.1	± (6.9, 16.28)	1	± (1.49, 0.68)
QGAR 1	BAP	15	20%	100%	87%	87%	8.2	± (5.34, 11.65)	1.15	± (1.57, 0.85)
	Zea-PVP	15	7%	80%	60%	60%	4.63	± (2.1, 8.15)	1.09	± (1.57, 0.75)
QGAR 2	BAP	15	7%	100%	87%	80%	8.7	± (5.64, 12.41)	1.39	± (1.91, 1.01)
	Zea-PVP	15	0%	93%	73%	67%	10.29	± (6.47, 14.98)	1.24	± (1.76, 0.87)
QGAR 3	BAP	14	7%	100%	36%	29%	14.14	± (7.64, 22.61)	1	± (1.91, 0.53)
	Zea-PVP	12	0%	83%	8%	8%	9	± (1.02, 24.9)	1	± (3.05, 0.33)

Table 3.5: Effect Test for Comparison of Two Establishment Medias Containing Zea-PVP or BAP

	Phenolic Present[a]	**% Bud Swelling**[a]	**% Elongating**[a]	**Shoot Length**[b]	**# of Shoots**[b]
Genotype (A)	0.0395	<.0001	<.0001	0.0001	<.0001
Establishment Media Type (B)	<.0001	0.0001	0.0012	0.9328	0.9284
A x B	0.7073	0.0579	0.2184	0.0065	0.9339

a. Statistical analysis was conducted using a ChiSquare test
b. Statistical analysis was conducted using a Two-way ANOVA F-test

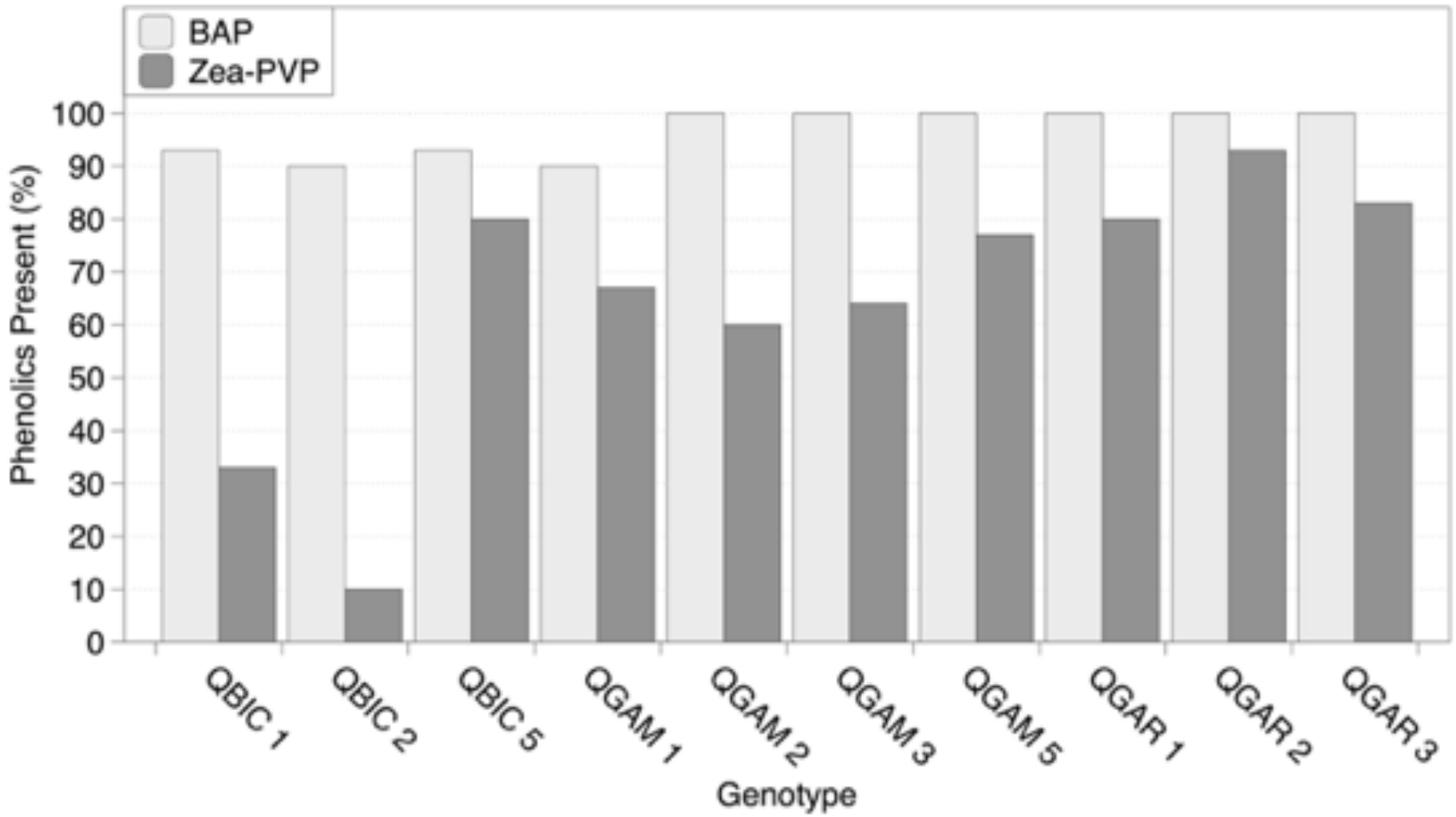

Figure 3.3: Establishment - Percentage (%) of Tissue cultures With Observed Phenolic Oxidation in Establishment Media After Two Weeks in Culture

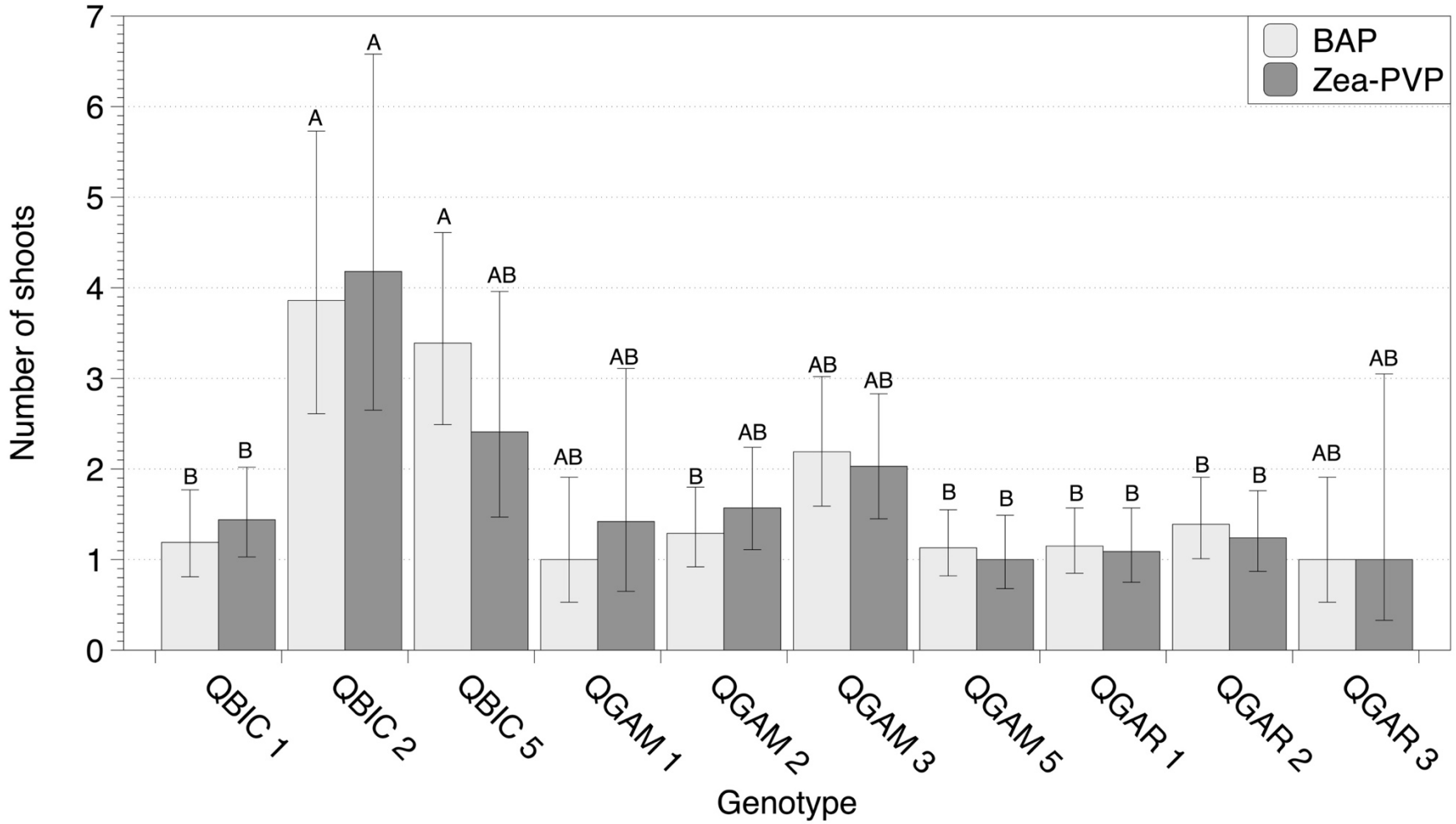

Figure 3.4: Establishment – Zea-PVP vs BAP Number of Shoots Produced In Vitro During Establishment Phase

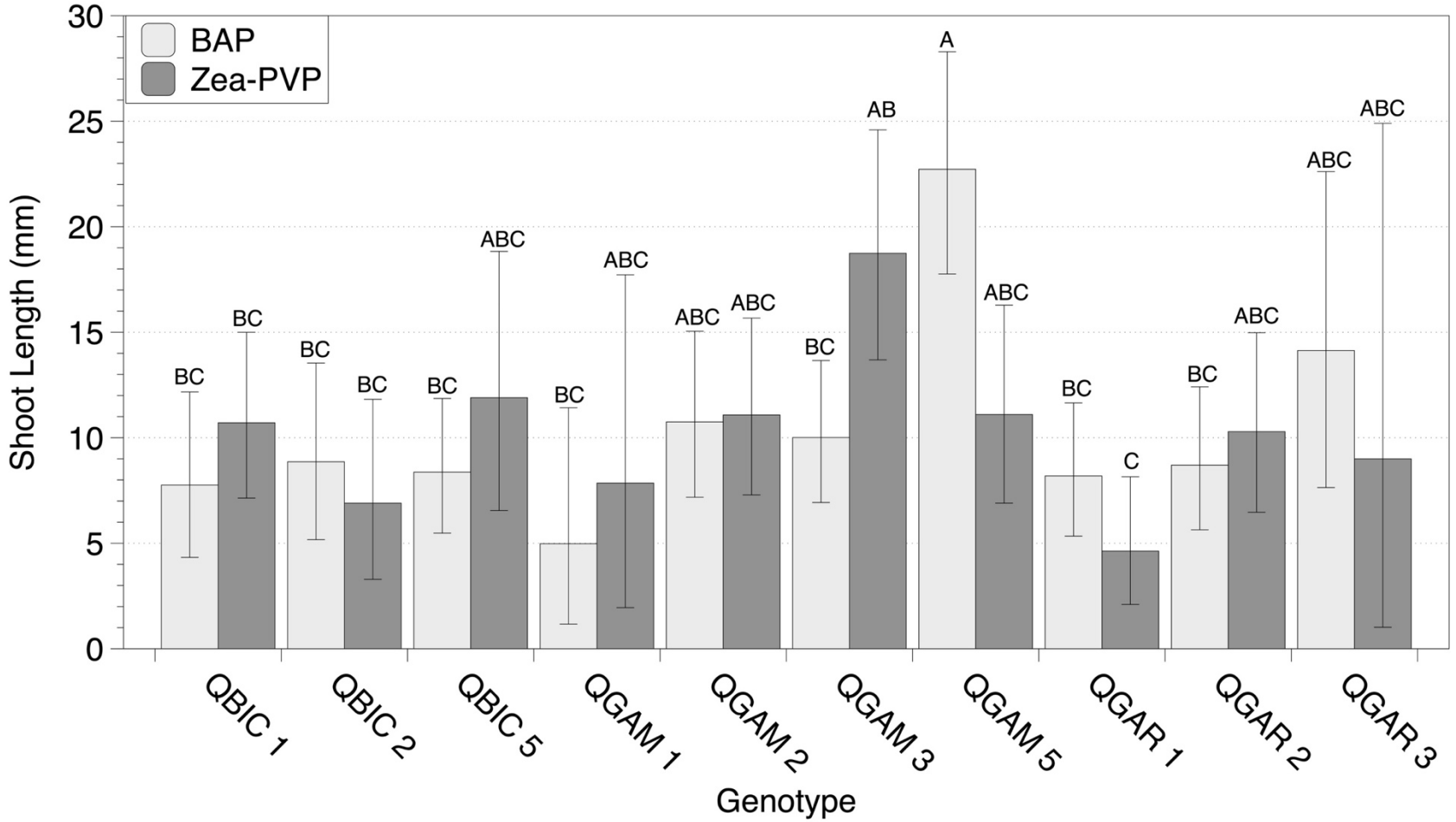

Figure 3.5: Establishment – Zea-PVP vs BAP Shoot Length In Vitro

3.4. Multiplication

Total number of shoots produced and shoot lengths by genotype were averaged across all three multiplication cycles (Table 3.6). Average shoot length across all genotypes and flush periods was 7.46 mm ± 2.03 mm. Different genotypes had varying capacities to grow in the multiplication phase and as a result some individual genotype lines were able to reproduce in culture while others declined. Figure 3.6 and Figure 3.7 show the dynamics of number of shoots produced per multiplication cycle for individual genotype by species. In Figure 3.6 and Figure 3.7, Round 0 is the starting number of nodes during establishment. Rounds 1-3 show number of shoots produced during three multiplication cycles. Round 4 is the number of shoots moved from multiplication to rooting that were greater than 5 mm and had a minimum of three leaves per shoot. Genotype had a significant effect on shoot length and total number of shoots produced over the three multiplication cycles (Table 3.6).

3.5. Rooting

The number of shoots that moved to rooting varied by genotype, 1st vs 2nd flush (Table 3.7 and Table 3.8) and number of shoots produced during the multiplication cycle (Table 3.6, Figure 3.6 and Figure 3.7). The criteria for moving shoots to rooting required shoots to be 5 mm long with a minimum of three lateral leaves and no shoot tip necrosis. Once in the rooting phase, shoot tip necrosis affected 17-100% of shoots after the three-week rooting cycle (Table 3.7 and Table 3.8). Callus formation occurred for 50%-100% of all rooting genotypes. All genotypes that produced roots first had callus formation. Shoots producing roots varied by genotype from 0%-100%. Number of primary roots ranged from 1 to 15 by genotype as did average root length varying from 8.53 mm to 23.53 mm.

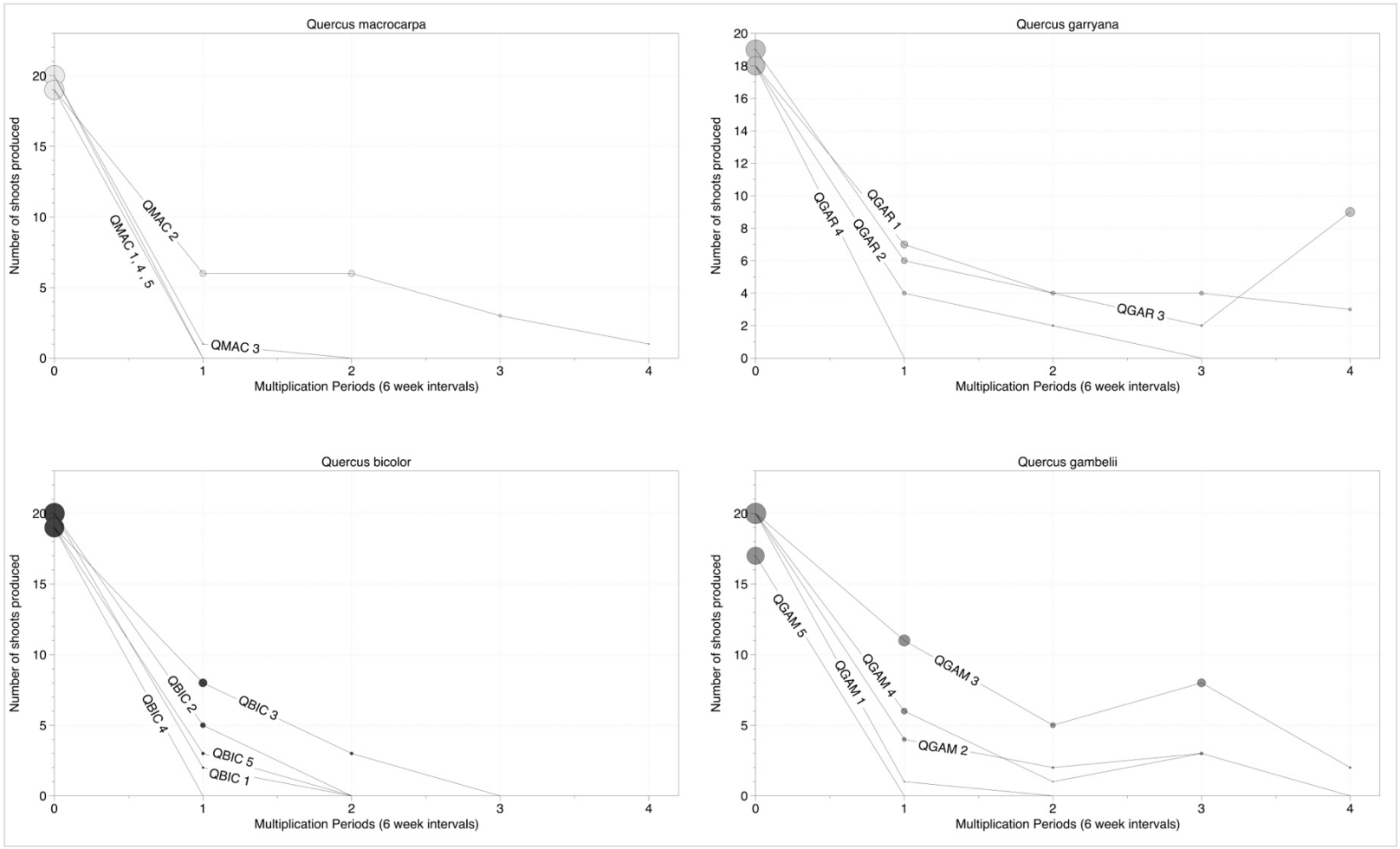

*Figure 3.6: Multiplication 1st Flush - Number of Shoots Produced Over the Course of Three Multiplication Cycles (Periods 1-3) and Number of Shoots Greater Than 5 mm Moved to Rooting (Period 4) by Species and Individual Genotypes. * Size of circle corresponds to number of shoots produced (y-axis). **Round 0 = multiplication, Rounds 1-3 = multiplication, Round 4 = shoots moved to rooting phase*

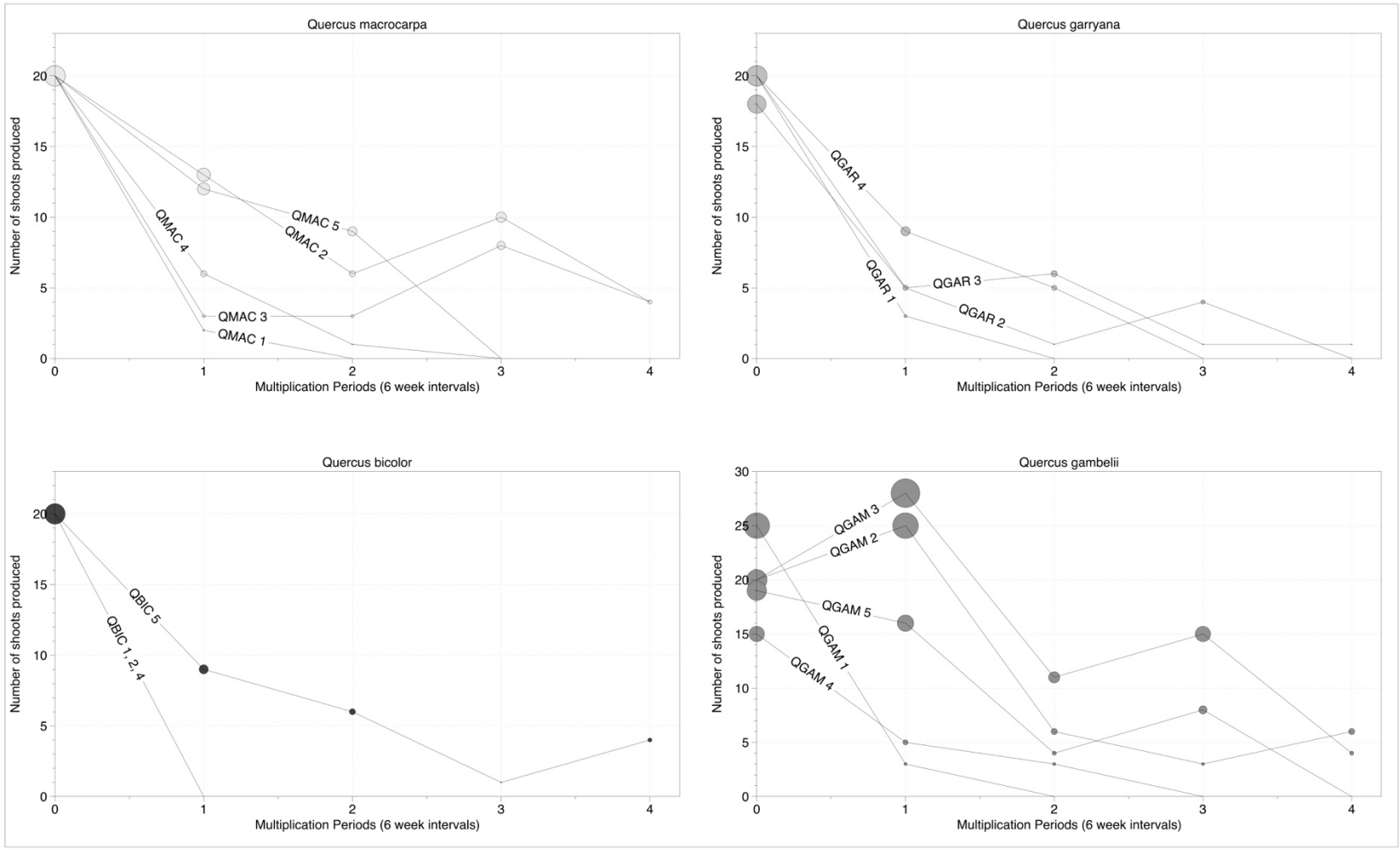

*Figure 3.7: Multiplication 2nd Flush – Number of Shoots Produced Over the Course of Three Multiplication Cycles (Periods 1-3) and Number of Shoots Greater Than 5 mm Moved to Rooting (Period 4) by Species and Individual Genotypes. * Size of circle corresponds to number of shoots produced (y-axis). **Round 0 = multiplication, Rounds 1-3 = multiplication, Round 4 = shoots moved to rooting phase.*

Table 3.6: Multiplication - Average Shoot Length by Genotype Over The Course of Three Multiplication Rounds

GENOTYPE	FLUSH	n	AVG. SHOOT	STD. ERR
QBIC 1	1	2	5	3.51
QBIC 1	2	0	0	0
QBIC 2	1	5	6.6	2.22
QBIC 2	2	0	0	0
QBIC 3	1	11	8.05	2.3
QBIC 3	2	0	0	0
QBIC 5	1	3	5.34	2.86
QBIC 5	2	16	14.01	2.91
QGAM 1	1	1	6	4.96
QGAM 1	2	3	7.34	3.05
QGAM 2	1	9	10.37	2.96
QGAM 2	2	34	8.86	1.99
QGAM 3	1	24	9.68	1.83
QGAM 3	2	54	10.85	1.27
QGAM 4	1	10	12.78	3.29
QGAM 4	2	8	10.77	2.67
QGAR 1	1	15	8.91	2.29
QGAR 1	2	3	8.00	3.05
QGAR 2	1	6	16.59	3.18
QGAR 2	2	10	9.85	3.32
QGAR 3	1	12	6.87	2.25
QGAR 3	2	12	12.59	3.11
QGAR 4	1	0	0	0
QGAR 4	2	14	9.33	2.04
QMAC 1	1	0	0	0
QMAC 1	2	2	11.00	3.73
QMAC 2	1	15	10.12	2.32
QMAC 2	2	29	12.00	1.70
QMAC 3	1	1	6	4.96
QMAC 3	2	14	9.67	2.58
QMAC 4	1	0	0	0
QMAC 4	2	7	6.59	3.66
QMAC 5	1	0	0	0
QMAC 5	2	21	10.32	1.62

Table 3.7: Rooting - 1st Flush

Genotype	Num. shoots moved to root	Flush	Shoot tip necrosis (%)	Callus Present (%)	Avg. Callus Size (mm)	Std. Err.	# of shoots producing roots	% Producing roots	# of primary roots	Average Root Length (mm)
QGAM 3	2	1	50%	100%	5.5	1.46	1	50%	7	14.43
QGAR 1	3	1	100%	100%	7.33	1.19	1	33%	3	10
QGAR 3	9	1	67%	89%	4.78	0.68	n/a	n/a	n/a	n/a
QMAC 2	1	1	100%	100%	11.00	2.06	1	100%	1	23

Table 3.8: Rooting – 2nd Flush

Genotype	Num. shoots moved to root	Flush	Shoot tip necrosis (%)	Callus Present (%)	Avg. Callus Size (mm)	Std. Err.	# of shoots producing roots	% Producing roots	# of primary roots	Average Root Length (mm)
QBIC 5	2	2	25%	100%	3.50	1.83	1	50%	15	8.53
QGAM 2	6	2	17%	0%	0	0	0	0%	n/a	n/a
QGAM 3	4	2	25%	75%	6.67	2.11	2	50%	4.5	23.54
QGAR 3	1	2	100%	100%	6	3.66	0	0%	n/a	n/a
QMAC 2	4	2	0%	50%	5.25	1.83	2	50%	5.5	19.47
QMAC 3	4	2	25%	100%	6.25	1.83	4	100%	3.75	16.52

3.6. Discussion

Oak species *Q. bicolor* (Vieitez et al. 2009) and *Q. gambelli* (Brennan et al. 2017) have previously been grown experimentally in tissue culture for research. For *Q. macrocarpa* and *Q. garryana*, this study represents the first experimental use and successful propagation of these species using a tissue culture system. This study shows that it is possible to grow all four of these species in a tissue culture. While establishment, multiplication and rooting rates were all highly variable based on genotype this research suggests that more than 60 individuals starting buds are required to effectively screen a species to determine if it is amenable to the tissue culture environment.

3.6.1. First and Second Flush

Four oak species (*Q. bicolor*, *Q. gambelli*, *Q. garryana* and *Q. macrocarpa*) were assessed to determine if initial bud and shoot position had an effect on bud swelling, shoot elongation, shoot length and number of shoots emerging in the establishment phases of tissue culture. Shoots and nodes used for establishing explants were derived from the outer canopy of four-year-old trees and epicormic shoots that emerged from within 20 cm of the root flare. Oaks in the establishment phase of the tissue culture were assessed based on a series of successive developmental measures. The first developmental stage assessed was number of initial buds that swelled after six weeks in establishment media. A bud was considered to be swelling if it remained alive (visibly green) and if the apical dome started to expand in culture. Secondly these buds were then assessed to determine what percentage started to elongate past 1 mm of swelling. Bud swelling and apical dome expansion occurred in a greater number of individuals than shoot expansion. Many buds remained alive but were not able to develop past this initial swelling (Table 3.2).

On average when interaction effect of genotype and flush (1st vs 2nd) were considered together, no difference was detected in the number of shoots that emerged during the establishment phase. Across almost all genotypes either an equal or greater number of shoots were produced in the 2nd flush. QBIC 2, QBIC 5, QGAM 3 and QMAC 5 produced a significantly greater number of shoots during the 2nd flush (Figure 3.1). Having more than one-shoot emerge during establishment phase was not anticipated since all cultures were initiated with a single lateral bud. It appears that the tissue culture environment promoted single buds to differentiate and produce multiple shoots from a single nodal point. While this was observed for the above noted genotypes it was not consistent. On average across all genotypes the average number of shoots that emerged during establishment was closer to one.

Shoot length across all genotypes and flush periods had overlapping mean values as were indicated by matching letters using Tukey HSD assessment (Figure 3.2). Exceptions to this were observed with QGAM 5 and QMAC 5 which both produced significantly longer shoots in the 2nd flush period compared to the 1st flush period (Figure 3.2). For the majority of other genotypes, no difference in shoot length was detected between 1st vs 2nd flush. Neither the 1st nor 2nd flush showed a consistent pattern of developing longer shoots. In some cases, 1st flush shoots were longer compared to 2nd flush and vice versa. Although significant differences were detected using a two-way ANOVA for genotype, 1st vs 2nd flush and their interaction the differences were minimal in real world application. With a desired outcome of generating shoots longer than 5 mm in order to move them from establishment to multiplication, the majority of shoots reached this length threshold.

Shoot elongation between genotypes and 1st vs 2nd flush was highly variable and no clear pattern emerged in terms of what factor played a dominant role in shoot development (Table 3.2). A two-way ANOVA effect test did not detect significant differences between 1st vs 2nd flush in percentage of shoots elongating thus indicating that flush period was not a significant factor. This points out that individual genotypes are likely the most important factor to consider in terms of a shoot's ability to elongate and tolerate the tissue culture environment. In the continuous multiplication phase, genotype seemed to play the largest single factor in which individuals could adapt to the tissue culture environment or not (Figure 3.6 and Figure 3.7). What the establishment data shows is that genotypes that do not necessarily multiply well can still produce elongated shoots during the establishment phase.

3.6.2. Zea-PVP vs BAP

A primary research objective of this study was to determine if the combination of cytokinin zeatin and PVP was able to reduce oxidation of phenolic compounds released into the tissue culture media. Oaks are known to contain high quantities of polyphenols such as ellagitannins (hexahydroxydiphenoylesters) and condensed tannins (proanthocyanidins) which are released as lysate when mechanical damage occurs to cells when cutting stems during the tissue culture process (Scalbert et al. 1988). When these polyphenols are released into tissue culture media, they oxidize turning media dark brown, damaging and sometimes killing explants (Romano et al. 1992). The common practice in tissue culture laboratories when dealing with plants that release phenolic compounds is to monitor cultures. If phenolic exudation is observed, the explants are moved to a phenolic free media, either on the opposite side of the original vessel or by placement in a new vessel (Romano et al. 1992). While this process is simple it is also time consuming and

requires using extra vessels and media which adds to production costs.

In this study the effect of the Zea-PVP media on reducing phenolic oxidation varied by individual oak genotypes and media types. Presence of phenolic oxidation was observed in 90%-100% of BAP cultures three days after establishment. Comparatively, media containing Zea-PVP showed variability in its capacity to reduce phenolic browning and ranged from 10% to 93% (Table 3.4). The effect test indicated that the presence of phenolic oxidation was significantly different between genotypes and establishment media but not their interaction (Table 3.5). With significant amounts of phenolic oxidation being observed in all BAP cultures this suggests that all genotypes, regardless of individual species or taxa, release phenolic compounds into media. Some Zea-PVP cultures such as QBIC 1 and QBIC 2, effectively reduced oxidation compared to BAP alone. This suggests that PVP can be a suitable compound for reducing phenolic browning. Other researchers have had mixed effects with PVP, reporting that it was both effective (Toth et al. 1994) and ineffective (Romano et al. 1992) at reducing browning and oxidation.

Variable effectiveness of the Zea-PVP media in reducing phenolic oxidation across genotypes suggests that optimizing exact PVP concentrations may be required to fully reduce phenolic damage. Individual physiology of genotypes could also play a role in the amount of phenolic compounds produced and released into tissue culture media. Since this experiment only observed absence or presence of phenolic browning, it was not possible to determine the quantities or composition of phenolic compounds.

Number of shoots produced in the establishment phase was most significantly affected by

individual genotype and not media treatment. The majority of genotypes produced a statistically equal number of shoots by treatment with the exception of QBIC 5 and QGAM 2, although for both these genotypes differences were overlapping and were within standard error (Figure 3.4). Most genotypes produced a single shoot per individual starting node with the exception of QBIC 2, QBIC 5 and QGAM 3, each having produced on average between 2-4 shoots per node. This indicates that during growth phase the initial buds were able to differentiate into multiple new shoots.

Shoot length varied most significantly by individual genotype and media treatments did not significantly affect shoot length (Figure 3.5). Some genotypes grew longer in either BAP or Zea-PVP media and demonstrated that differences on the genotype level may be more significant than media treatment. Tukey HSD analysis showed that most treatments and genotypes were statically overlapping. While shoot length varied by genotype and treatment, almost all shoots were longer than the 5 m threshold required to move shoots to the multiplication phase.

Zea-PVP was only used in the first two weeks of the multiplication cycle and explants were moved over to the standard BAP media for the remainder of the six weeks in establishment. For shoot elongation, growth, and number of shoots produced, genotype was the most significant factor effecting these measures. This suggest that identification of individual genotypes that establish well in the tissue culture environment is a key step in order to develop a clonal propagation system for oak species. With Zea-PVP not having a strong effect compared to BAP on either number of shoots produced or shoot length during establishment, the data suggests that it can effectively be used to control phenolic oxidation for some genotypes. While PVP didn't

reduce phenolic oxidation in all genotypes (Figure 3.3) it does suggest that it is effective for some genotypes. Individual genotypes should be tested with PVP to determine its effective concentration range and general efficacy.

3.6.3. Multiplication

Average shoot length and number of shoots produced varied significantly between genotypes and multiplication cycles (Table 3.6, Figure 3.6 and Figure 3.7). Over successive multiplication cycles the majority of genotypes, regardless of species, steadily declined with individual lines eventually dying out. Figure 3.6 and Figure 3.7 show this effect with most genotypes starting in the establishment phase (Round 0) with between 15 to 25 nodes and throughout the successive multiplication phase (Rounds 1-3) reducing down to zero shoots. While the majority of genotypes dropped to zero during the multiplication phase, a few genotypes were tolerant of the multiplication environment and were able to survive for successive cycles. Examples of this include 1st flush QGAM 3 and QGAR 3 (Figure 3.6) and 2nd flush QMAC 2, QMAC 3, QGAM 3 and QGAM 5 (Figure 3.7). This pattern of individual genotypes responding positively in culture with other lines dying out over time was commonly observed in the hybrid oak study in Chapter 1. The typical pattern observed with hybrid oaks is that the lines that are capable of successfully multiplying will stabilize over consecutive multiplication cycles and eventually be able to be maintained indefinitely. In this study the stabilization point was not reached within three multiplication cycles although some shoots started to show a positive response to the multiplication environment. Researchers who have studied oaks in tissue culture have notated that stabilization can require between 4 to 12 months (Herrmann and Buscot 2008; Vieitez et al. 2009). While the stabilization period was not met for these four species of oaks, this study demonstrates the overall dynamics experienced when establishing oaks in tissue culture. It

shows that it is possible to establish and multiply all four of these species and be able to move a series of them to rooting phase. For future researchers, or propagators, who wish to use these methods this study suggests that trialing a large number of individual genotypes is required in order to determine which individuals will be responsive to the tissue culture environment.

Genotype specificity has commonly been cited in the literature with some genotypes responding to tissue cultures and others failing (Vieitez et al. 1993; Vieitez et al. 1994; Herrmann and Buscot 2008; Vieitez et al. 2009; Vengadesan and Pijut 2009; Vieitez et al. 2012). Genotype specificity remains as one of the single largest factors affecting the prevention of the wide-scale adoption of the use of oak tissue culture methods. The hybrid oak research showed that it is possible to re-establish individual genotypes back into a tissue culture system (Chapter 1) suggesting that genotype is more important than physiological state of nodes when harvested for establishment. This makes current tissue culture methods appropriate for selection of individual oaks with desirable horticulture characteristics and development of cultivars. This study did not find a significant difference between the 1st vs 2nd flush periods. With trees only being four years old and not showing signs of developmental maturity (catkin or acorn development) they likely were still in a juvenile developmental phase. Use of juvenile plant stock material has been shown to increase successful multiplication and rooting in tissue culture (Evers and Eeden 1993; Chalupa 1993). This poses a challenge in identifying horticulturally desirable oaks while still in a juvenile state. The coppice system developed by the Urban Horticulture Institute provides an effective means of producing large numbers of juvenile shoots for use in tissue culture.

The average shoot length across all genotypes in multiplication was shorter on average compared

to shoot length during the establishment phase (est. average shoot length = 9.83 mm ± 4.49 mm vs multiplication 7.46 mm ± 2.22 mm). Oaks in tissue culture tended to develop shoot tip necrosis when maintained in the multiplication cycle for an extended period of time. The specific number of weeks that shoot tip necrosis occurred after the start of the multiplication cycle varied with genotypes. After the six-week multiplication period, shoots tended to reach their maximum height and shoot tip necrosis often occurred shortly thereafter. Oaks have an episodic growth pattern under natural and in vitro grown conditions (Herrmann and Buscot 2008). The determinate growth pattern of oaks in tissue culture could be a result of this episodic growth pattern. Moving shoots from the multiplication to rooting phase requires long shoots with a series of developed leaves. The tissue culture protocol used in this study set the shoot length standard to 5 mm or greater and at least three developed leaves. Period #4 in Figure 3.6 and Figure 3.7 shows the number of shoots moved to rooting per genotype with relatively few shoots reaching this 5 mm criteria. In the hybrid oak studies, continuous multiplication and stabilization were achieved with a number of specific genotypes. Stabilized genotypes can be multiplied indefinitely in tissue culture and as result there tends to be a greater number of shoots available for rooting and more opportunity to harvest shoots greater than 5 mm. It is unclear what the specific mechanism is that allows for stabilization to occur. The fact that individual genotypes can be re-established (Chapter 1) into a continuous multiplication cycle suggests that it is not due to somatic mutation but instead the genetic expression by the individual genotype.

Statistical comparison of the effect of the initial 1st vs 2nd flush period was not possible in the multiplication phase due to the variable number of shoots produced per individual genotype (Table 3.6). This was due to the fact that some genotype lines declined during successive multiplication cycles. Table 3.6 demonstrates the genotype effect with the varying number of

shoots produced per genotype across all three multiplication cycles ranging from 0 to 54. While statistical comparison was not possible this data shows that individual genotypes vary significantly in their capacity to survive and grow in the multiplication phase (Table 3.6, Figure 3.6 and Figure 3.7).

3.6.4. Rooting

Rooting in vitro varied by genotype, 1st vs 2nd flush period, and number of shoots produced during the multiplication phase. In order to move shoots from multiplication to rooting, shoots were required to be 5 mm in length and have a minimum of three leaves. As a result, the number of shoots moving from multiplication to rooting was dependent on shoot capacity to grow in the multiplication phase. Shoot tip necrosis emerged as an issue in the rooting phase and affected from 0% - 100% of individual shoots by genotype (Table 3.7 and Table 3.8). Tip necrosis was damaging to explants and occurred when individual shoots reached their maximum growth point and were maintained in culture for extended periods of time. For some genotypes this can occur within the standard 6-week multiplication phase. A potential cultural practice to avoid shoot tip necrosis could be alteration of subculture timing. Shoots could be moved to a rooting phase after four weeks in the multiplication cycle when the shoots are still actively elongating. This could potentially allow for the shoot to continue its shoot growth phase and root growth phase simultaneously. Shoot tip necrosis has been reported as an issue for others in oak tissue culture (Schwarz and Schlarbaum 1993; Vieitez et al 1994; Vieitez et al. 2009). Vieitez's 2009 study found that the addition of silver nitrate ($AgNO_3$) to tissue culture media reduced occurrence of shoot tip necrosis in *Q. rubra*. Silver nitrate was considered for use in this study but was rejected due to concerns about safe disposal of the compound in tissue culture media after use due to its potential as an environmental hazard for aquatic life.

Callus formation occurred in the majority of genotypes (50%-100%) and varied in size (3.5 mm – 11.00 mm) (Table 3.7 and Table 3.8). Callus formation was a prerequisite for root production and but did not guarantee that roots would grow. Only a fraction of shoots that were moved to the rooting phase were capable of producing roots. When rooting did occur, it typically resulted in the formation of more than one root with the number of roots ranging from 1-15. Root length was robust with averages ranging from 8.53 mm to 23.54 mm. With shoot growth on average 7.46 mm long, root length oftentimes was much longer than shoot length. Genotype specificity played a role in which shoots developed root systems and identification of genotypes that root well is required to use these methods.

3.7. Conclusion

This study found that genotype was the single largest factor affecting in vitro establishment and multiplication and rooting for four oak species (*Q. bicolor*, *Q. garryana*, *Q. gambelli* and *Q. macrocarpa*). This experiment was the first time *Q. macrocarpa* and *Q. garryana* were successfully grown in a tissue culture environment. Additions of Zea-PVP to establishment media reduced occurrence of phenolic oxidation in establishment media and produced approximately equal number of shoots and shoot length compared to using the standard cytokinin BAP. Effectiveness of Zea-PVP for reducing phenolic oxidation varied by genotype. No significant differences were found between cultures derived from 1st vs 2nd flush period in terms of how they responded in establishment, multiplication or rooting. In order to effectively stabilize oaks in multiplication cycle more than three multiplication phases are required. This study contributes to the development of a tissue culture system that can be used to clonally propagate a variety of oak species and genotypes.

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4. CHAPTER 4

OSMOTIC ADJUSTMENT AND GAS EXCHANGE RESPONSE DURING DROUGHT FOR TWO TREE SPECIES (*Quercus bicolor* & *Betula pendula*) GROWN IN CONTAINERS WITH LIMITED SOIL VOLUME

4.1. Introduction

Street trees have been recognized for the environmental, social and economic value that they provide to urban neighborhoods (Mullaney et al. 2015). Trees growing in urbanized environments face a wide variety of stresses that limit their life span and capacity to grow into their genetically determined potential as mature specimens (Hirons and Percival 2012). Urban soils have distinct characteristics that differentiate them from naturally occurring soils such as high bulk density (compaction), low porosity, low available water holding capacity (AWHC), reduced organic matter, reduced microbial activity and high pH (Jim 1998, Scharenbroch et al. 2005, Sax et al. 2017). In the urban environment, poor tree growth and canopy development have been associated with degraded soils (Layman et al. 2016) and inadequate soil volumes (Day and Amateis 2011).

With urban populations projected to grow, landscape architects and planners are increasingly reliant on landscapes to provide ecosystem services. Ecosystem services aid in creating functioning landscape ecology that provides benefits for people living in cities (Bolund and Hunhammer 1999). Ecosystem services have been shown to provide both economic and non-monetary benefits to city inhabitants (Elmqvist et al. 2015). One such benefit street trees providing is localized cooling by shading and the reduction of the urban heat island effect (Hardin et al. 2007, Pataki et al. 2011). Additional services include the interception of rain,

which reduces runoff (Gómez-Baggethun and Barton 2013) and carbon sequestration and storage (Nowak and Crane 2002). Tree size affects how ecosystem services are delivered. For example, large trees provide greater carbon storage and shading compared to smaller trees. The lifespan and survivability of urban trees are affected by the tree's age, the species selected and the land use type where its planted. Small diameter trees planted in commercial, industrial and transportation land use types have higher mortality rates as compared to similar sized trees planted in other locations (Nowak et al. 2004). These mortality rates may be associated with soil compaction and low water holding capacity due to degraded soil quality associated with urbanized sites (Nowak et al. 2004). For urban trees to grow into mature specimens that provide maximum ecosystem services, species that can grow in soils with low water holding capacity and that have physiological strategies to tolerate high stress environments must be identified.

When designing sites for planting trees in the urban environment, consideration of both soil quality and soil volume are critical to ensure maximum tree growth and associated ecosystem services. Soil volume calculation methods are useful tools for landscape architects and city planners to determine tree growth and canopy cover based on tree pit volumes (DeGaetano and Hudson 2000, Trowbridge and Bassuk 2004, Haege and Leake 2014). While generic soil volume recommendations are useful for approximate volume estimations, they do not consider localized environmental factors. There are soil volume calculations that integrate environmental parameters (e.g. rainfall frequency, evaporative demanded, soil available water holding capacity) and tree physiological characteristics (e.g. leaf area index) in order to determine tree growth based on local site conditions (Lindsey 1991, Lindsey and Bassuk 1992). The consideration of the AWHC of the soil and its effect on tree transpiration rates is of paramount importance in this

integrated model. Soils with low AWHC can result in premature decline and death of young urban tree plantings (Gilbertson and Bradshaw 1990).

Limited soil moisture affecting tree growth performance is not only an issue in the urbanized environment but is also encountered in container nursery production of trees (Ray and Sinclair 1998). Peat based nursery substrates, with or without the additions of amendments, can have significantly different volumetric water content and water holding capacity (Sax and Scharenbroch 2017). Varying water holding capacity in greenhouse growing mediums can result in the need for frequent irrigation and cause physiological changes in nursery stock when responding to water deficit conditions. Water deficit irrigation can be used intentionally at the end a plant's production cycle to prepare nursery stock for growing in the landscape without supplemental irrigation (Banon et al. 2003). Plants that are tolerant of drought conditions have morphological and physiological characteristics that allow them to tolerate a low AWHC environments (Franco et al. 2006).

Osmotic adjustment is a mechanism that some tree species utilize to tolerate growing under low soil water conditions. Osmotic adjustment is the capacity for a plant to lower its turgor loss point over the growing season by accumulating or synthesizing osmotically active solutes into the vacuole and cytosol of its cells (Sanders and Arndt 2012). Increasing solutes within cells allows trees to maintain cell turgor under increasingly negative water potentials. Trees that can osmotically adjust can also maintain gas exchange rates (photosynthesis, stomatal conductance and transpiration) under drought conditions (Sanders and Arndt 2012).

Models have been developed for temperate tree species that use a vapor pressure osmometer (VPO) to quantify solute concentrations in leaves and convert them to leaf water potential at turgor loss point (Sjöman et al. 2018b). Comparing changes in turgor loss point over the growing season allows for the quantification of osmotic adjustment and provides a measure of stress tolerance. Identification of trees that display tolerance over the avoidance mechanism allows for the selection and development of trees suitable for dry climates and for sites with limited soil volume.

Studies using these models focused on screening large numbers of tree species growing in the landscape for their capacity to osmotically adjust (Sjöman et al 2015, Sjöman et al.2018a , Sjöman et al. 2018b). While this has provided a broad overview of osmotic adjustment across many species, less attention has focused on using controlled water deficit conditions to study osmotic adjustment jointly with gas exchange measurements.

This study was designed to investigate how water deficit conditions effect physiological changes in trees growing in limited soil volume. Using the recently developed method of determining turgor loss point for temperate tree species, this study aimed to develop a deeper understanding of how gas exchange (photosynthesis, transpiration, stomatal conductance) is affected in relation to osmotic adjustment for trees under drought conditions.

4.2. Methods

Three-year-old bareroot *Quercus bicolor* and *Betula pendula* were purchased from Lawyers Nursery Inc. in February 2014 and grown in 25-gallon plastic containers in 2.94 ft^3 (83 Liters) of Lamberts LM-111 all-purpose soilless medium. Trees were grown at Cornell's Bluegrass Lane

facility for a period of four years in an unheated polyethylene plastic covered greenhouse (poly-house). Annually, 77 grams of 3-4 month 15-9-12 NPK Osmocote plus fertilizer were applied to each tree in addition to 50 grams of Micromax micronutrient fertilizer. Fertilizer applications were made in May each year at the start of the growing season. From 2014-2017 trees were hand watered every other day throughout the course of the growing season or when the medium was visibly dry. At the start of the 2017 growing season, trees were pruned to standardize branch length and foliage cover for above ground growth. Tree placement was randomized by species and irrigation treatment in the poly-house. In the 2018 growing season, three drip irrigation emitters with four gallon per hour capacity were installed in each container. From May 1st until June 21st, drip irrigation was applied every other day and left running until soils reached field capacity. After June 21st, five *Q. bicolor* and *B. pendula* trees were selected at random from the original ten trees of each species to undergo a water deficit (drought) treatment. The selected individuals were placed on three bricks to elevate the pots from the floor of the poly-house to avoid absorbing excess water from irrigation flooding. From June 22nd – August 31st the growing medium for water deficit treatment trees (the selected individuals) were measured daily at the top of the growing medium with a three-pronged TDR Theta Probe (ThetaProbe ML2, Delta-T Devices, London, England). When medium moisture reached between 5% to 10% (0.05- 0.1 m^3/m^3) volumetric water content, irrigation was turned on and containers were watered until they reached field capacity. The irrigation frequency for drought treatments was once per week on average.

Observations of turgor loss point and gas exchange for both well-watered and water deficient trees occurred three times over the course of the 2018 growing season. The first sampling period

occurred during the week of June 18-21 and was denoted as “spring measurements.” The second “mid-summer” period was July 23-26 and the third “late summer” measurement period was August 28-31.

4.2.1. Turgor Loss Point Assessment

Methods developed by Sjoman et al. (2018b) were used to measure leaf osmolality and conversion to turgor loss point. Three sun exposed branches extending 3 m above the top of the growing medium with no pest or disease symptoms present were selected from each of the study trees. During the evening before measurements were taken, these branches were excised from the tree and transported to the laboratory within 30 minutes. In the laboratory, the cut surfaces of the branches were submerged in a bucket of water. The distal end of the branches was cut under water using hand pruners at approximately 3cm above the previous cut surface without exposing the ends to air. Branches were covered with a black cloth and kept in a dark laboratory to allow shoots to reach full turgor overnight. On the day of measurement, one leaf from each of the three branches was excised. Harvested leaves were at least three nodes back from the terminal bud and, regardless of the sampling period, collected from the season’s first flush of growth. One leaf disk per leaf was taken using an 8mm cork borer from the mid-lamina region avoiding any primary first or second order veins. Leaf disks were wrapped in aluminon foil, labeled and placed into liquid nitrogen for a minimum of two minutes. Once leaf disks were removed from liquid nitrogen, they were pierced fifteen times using sharp forceps. Leaf disks were placed into a Vapro 5600 vapor pressure osmometer (Elitech Group, Puteaux France) in a standard 10 µl chamber and allowed to equilibrate for a period of ten minutes before taking the first solute concentration (Kovacs et al 2013.) measurement in mmol Kg^{-1}. After the first measurement was taken, another two more measurements were immediately conducted for a total of three readings

per individual leaf sample. All three readings were recorded per leaf sample and averaged with all other readings from the same plant. Room temperature was recorded on an hourly basis. Solute concentration c_s was converted to osmotic potential (Ψ_π) using Van't Hoff relation (Eq. 1):

$$\Psi\pi = - RTc_s \quad (1)$$

Where R is the gas constant, T is temperature in Kelvin, and c_s is solute concentration. Since branch and leaf samples were allowed to equilibrate in water over night prior to measurement, osmotic potential is considered to be at full turgor ($\Psi_{\pi 100}$). Sjoman et al. (2018) developed a modified equation for temperate tree species that can estimate water potential at turgor loss point (Ψ_{P0}) from osmotic potential at full turgor ($\Psi_{\pi 100}$) (Eq. 2):

$$\Psi_{P0} = - 0.2554 + 1.1243 \times \Psi_{\pi 100} \quad (2)$$

Osmotic adjustment ($\Delta\Psi_{\pi 100}$) can be calculated by determining the difference between early season measurements of Ψ_{P0} and late season measurements. This difference shows a plant's ability to change turgor loss point over the growing season.

4.2.2. Gas Exchange

Gas exchange measurements were taken between 3pm and 5pm on the same days as VPO readings and on three different days over the course of the growing season. Gas exchange was measured using a LI-COR LI-6400 XT portable gas exchange system (Li-Cor Inc, Lincoln, Nebraska) and included photosynthetic carbon assimilation rates (μmol CO_2 $m^{-2}S^{-1}$), stomatal conductance (mol H_20 $m^{-2}S^{-1}$), transpiration (mmol H_20 $m^{-2}S^{-1}$), and vapor pressure deficit (kPa).

The LI-6400 XT was set to 725 PAR (red=500, blue=1000) light, 400 μmol mol $^{-1}$ CO_2, flow rate of 400, 6 cm^2 leaf area, stomatal ratio of 0.5, with ambient temperature and humidity. Gas exchange measurements were taken on random leaves across the canopy that were at least three nodes from the terminal bud and represented the first flush of growth. A total of five leaf readings were taken per individual tree. A three-pronged TDR Theta Probe (Theta Probe ML2, Delta-T Devices, London, England) was inserted vertically into the soil to measure medium moisture at three locations in the container during gas exchange measurements.

4.3. Results

4.3.1. Turgor Loss Point

Osmotic potential at full turgor ($\Psi_{\pi 100}$) was significantly different for *Q. bicolor* by treatment (well-watered versus water deficient), sampling period (spring, mid-summer, late summer) and their interaction (treatment x period) as seen in Table 4.1. For *Q. bicolor*, both the well-watered and water deficit treatment trees turgor loss point decreased as the growing season continued (Figure 4.1). The water deficit treatment resulted in a larger increase in turgor lost point compared to well-watered specimen (Figure 4.1). Osmotic adjustment ($\Delta\Psi_{\pi 100}$) for water deficient *Q. bicolor* were significantly greater than well-watered oaks (Table 4.2).

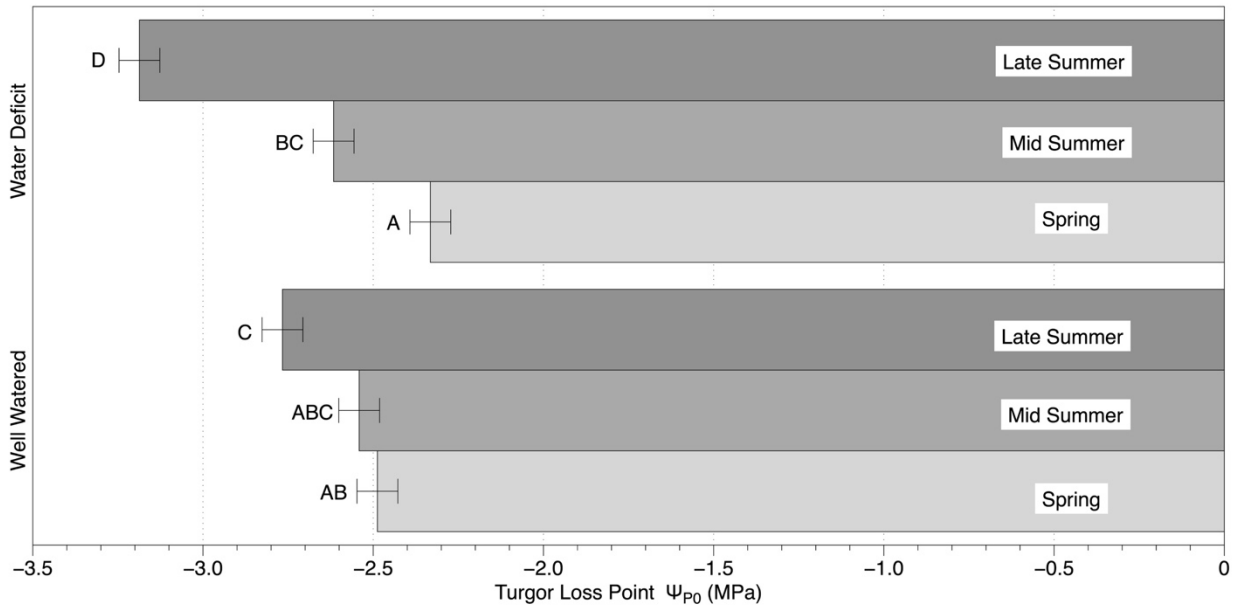

Figure 4.1: Quercus bicolor - Changes in Turgor Loss Point Over Course of 2018 Growing Season

Table 4.1:Two-way ANOVA Effect Test for Factors Treatment (Well-watered vs Water Deficit), Period (Spring, Mid-summer, Late Summer) and Treatment x Period for Quercus bicolor

Effect Test	**Prob > F**
Treatment (A)	0.0215
Period (B)	<.0001
A x B	<.0001

Table 4.2: Osmotic Adjustment ($\Delta\Psi\pi100$) for Quercus bicolor and Betula pendula by Treatment Over the Course of the Growing Season

Species	**Well-Watered (MPa)**	**Water Deficit (MPa)**	**Std Error**
Q. bicolor	-0.279 B	-0.855 C	0.083
B. pendula	0.151 A	-0.025 AB	0.103
Effect Test			**Prob > F**
Species			<0.0001
Treatment			<0.0001
Species x Treatment			0.0342

For *B. pendula* osmotic potential at full turgor ($\Psi_{\pi 100}$) was significantly different by treatment and period, but not for their interaction (treatment x period) (Table 4.3). Mean values for all treatments and sampling periods were statistically equal with the exception of the mid-summer

water deficit measurement, which was lower (less negative) than any other sampling periods or treatments (Figure 4.2). Osmotic adjustment for *B. pendula* was not statistically different between the well-watered and water deficit treatments with overlapping standard error (Table 4.2).

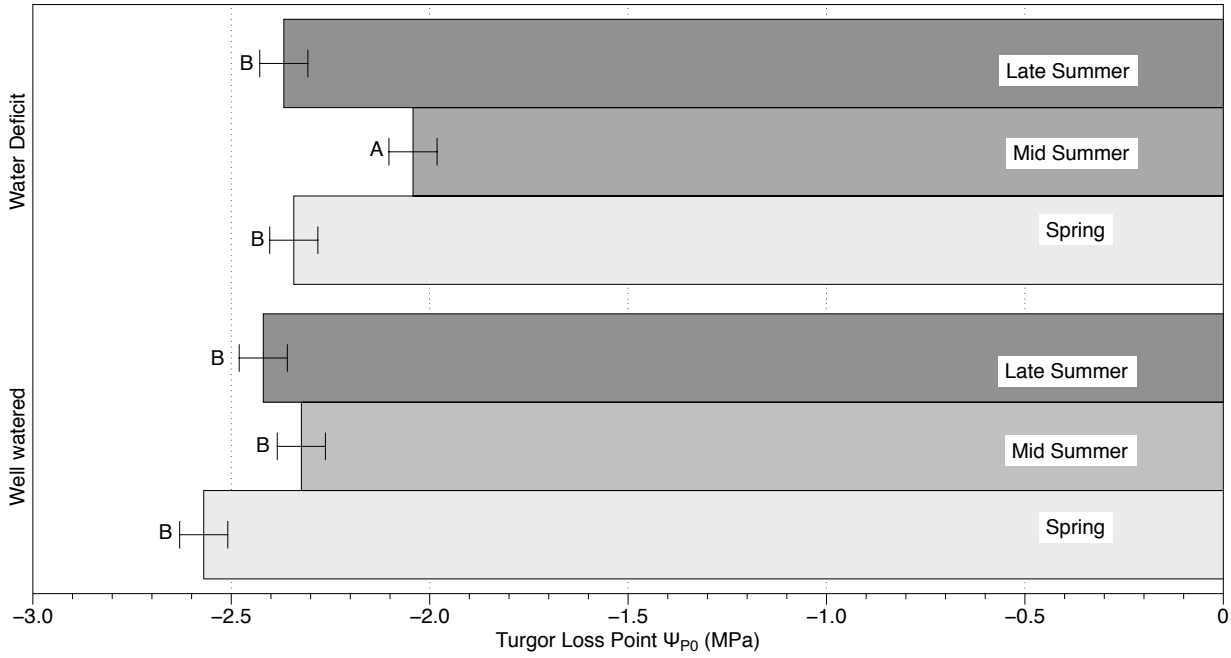

Figure 4.2: Betula pendula – Changes in Turgor Loss Point Over Course of 2018 Growing Season

Table 4.3: Two-way ANOVA Effect Test for Factors Treatment (Well-watered vs Water Deficit), Period (Spring, Mid-summer, Late Summer) and Treatment x Period for Betula pendula

Effect Test	**Prob > F**
Treatment (A)	0.0002
Period (B)	<.0001
A x B	0.1435

4.3.2. Gas Exchange

Gas exchange rates and responses varied between tree species and treatments. For *Q. bicolor*, photosynthetic carbon assimilation rates remained at statistically equal levels through spring and mid-summer for both well-watered and water deficit treatments. In late summer, the water deficit treatment remained equal to the previous time periods while the well-watered treatment had significantly increased photosynthesis rates (Figure 4.3). Stomatal conductance for both

treatments were statistically equal during both the spring and mid-summer periods. By contrast, in the late summer period, the well-watered trees increased stomatal conductance to the highest rates observed all year while water deficient trees dropped to its lowest rates (Figure 4.3). Transpiration rates showed a pattern similar to photosynthesis by treatment and time period. Transpiration remained at statistically equal levels through the spring and mid-summer for both well-watered and water deficit treatments. In late summer, water deficit treatment remained equal to the previous time periods while the well-watered treatment significantly increased transpiration (Figure 4.3). Vapor pressure deficit (VPD) for water deficient trees started higher than well-watered during the spring period. During mid-summer, the VPD for both treatments were equal while later during the summer, the water deficient trees had significantly larger VPD values compared to well-watered trees (Figure 4.3). All gas exchange measurements (photosynthetic carbon assimilation, stomatal conductance, transpiration and VPD) were statistically significant by treatment, period and for the interaction effect of factors treatment x period with Prob. > F =<0.0001.

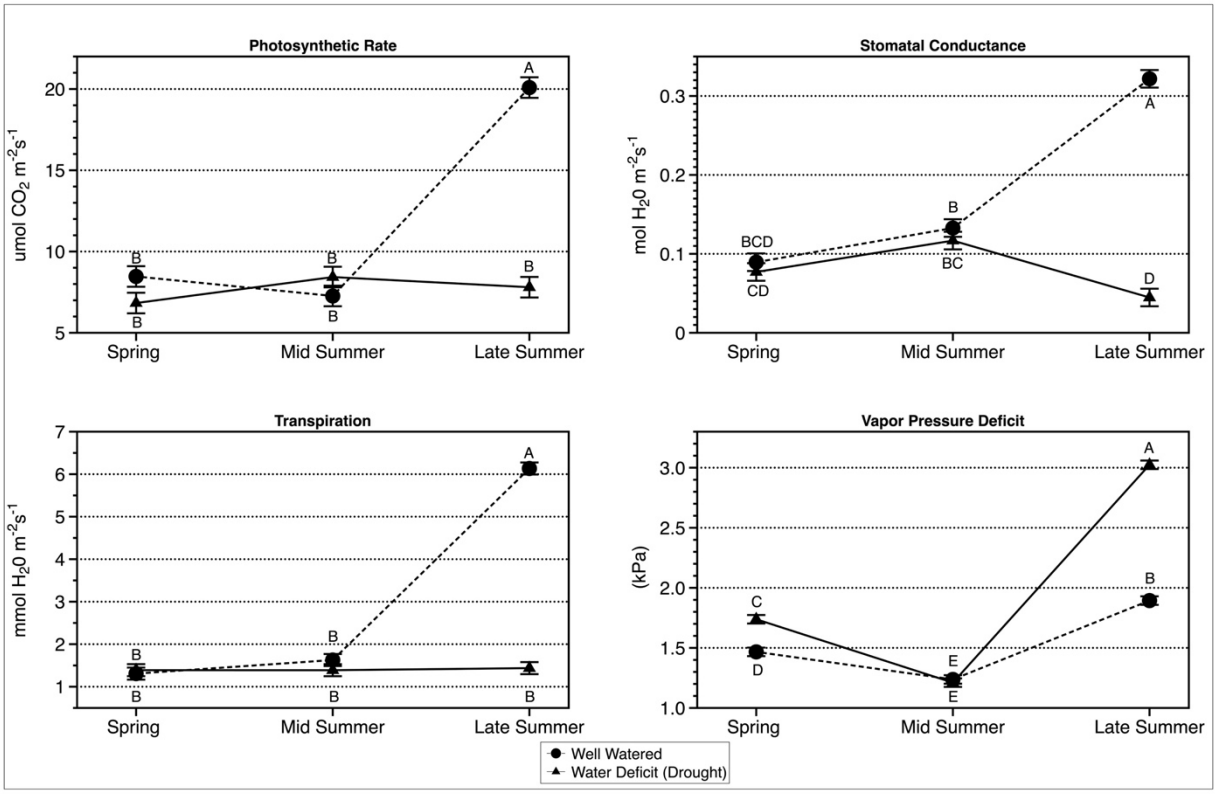

Figure 4.3: Quercus bicolor Compiled Gas Exchange Rates (photosynthetic rate, stomatal conductance, transpiration, vapor pressure deficit) by Well-Watered and Water Deficit (Drought) Irrigation Treatments

Gas exchange for *B. pendula* varied significantly between treatments and sampling periods. Photosynthetic rates in the spring measurement period were slightly higher for water deficient trees than well-watered but were statistically overlapping, indicating equal values. For water deficient trees, photosynthetic rates climbed to high values in mid-summer before dropping to their lowest rates in late summer. The well-watered treatment started at moderate photosynthetic rates in spring and maintained these values through the mid-summer and late summer sampling periods (Figure 4.4). Stomatal conductance for well-watered trees started off at low levels in the spring before climbing to high levels in mid-summer and in late summer returning to low levels equal to spring measurements. For water deficient trees, stomatal conductance started with high levels in the spring and declined in both summer periods. By late summer, both treatments maintained statistically equal stomatal conductance rates (Figure 4.4). For transpiration water deficient trees started off the spring with high transpiration rates that were maintained through mid-summer before steeply declining in late summer. Well-watered trees by contrast started with moderate transpiration rates in the spring and raised slightly mid-summer before lowering in the

late summer to equal rates as spring (Figure 4.4). Vapor pressure deficit for both water treatments started with equal values in spring. For water deficit treatment, VPD increased significantly in mid-summer before lowering during late summer to rates equal to the start of the growing season. Well-watered trees had an inverse response with decreasing VPD in mid-summer before raising to high values in late summer (Figure 4.4). All gas exchange measurements (photosynthetic carbon assimilation, stomatal conductance, transpiration and vapor pressure deficit) were statistically significant by treatment, period and for their interaction (treatment x period) with Prob. > F value of <0.0001. For *B. pendula*, the only exception was for the treatment factor for stomatal conductance which had a non-significant prob. > F value of 0.6812.

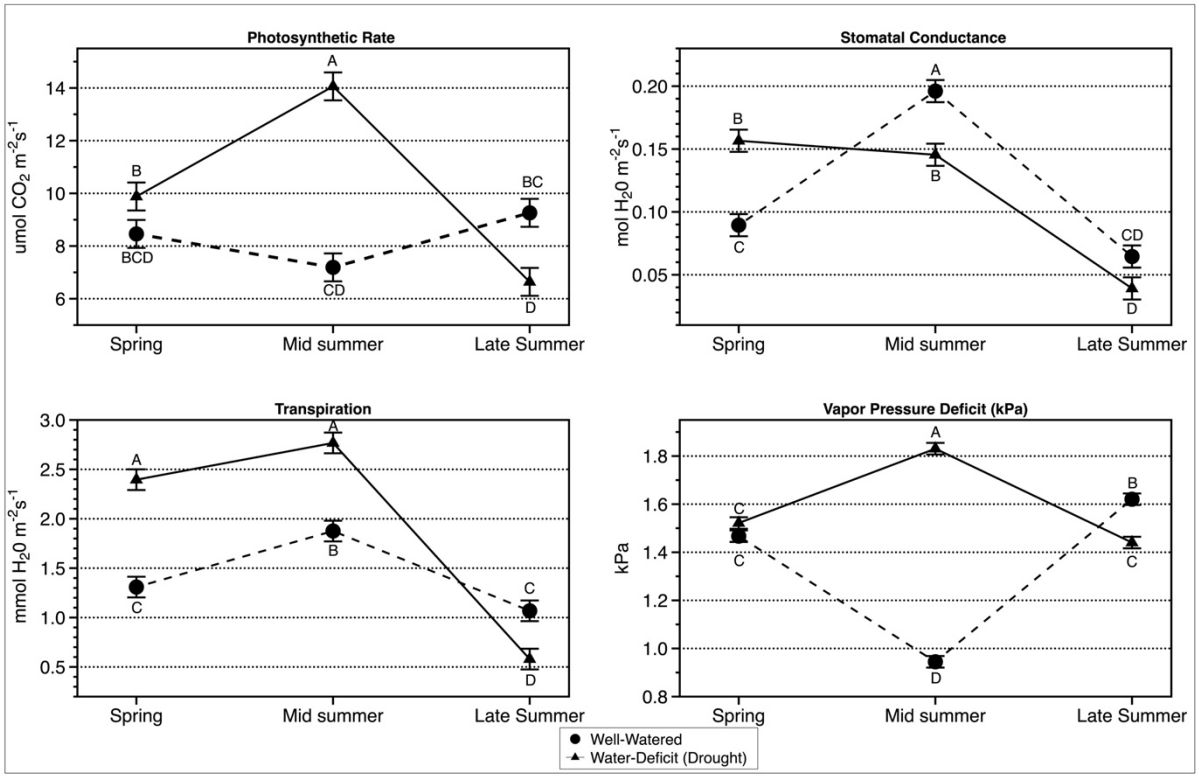

Figure 4.4: Betula pendula Compiled Gas Exchange Rates (photosynthetic rate, stomatal conductance, transpiration, vapor pressure deficit) by Well-Watered and Water Deficit (Drought) Irrigation Treatments

4.3.3. Leaf Drop and Field Observations

Over the course of the water deficit experiment, both tree species had differing responses to drought conditions. *Quercus bicolor* tolerated the drought conditions and maintained canopy

cover without the losing foliage. In contrast, *Betula pendula* exhibited an avoidance response by shedding leaves in response to water deficit conditions that started mid-summer.

4.4. Discussion

This study was designed to observe how two tree species (*Quercus bicolor* & *Betula pendula*) growing in limited volume would respond to water deficit conditions. This research has applications to optimize watering regimens for trees grown in limited soil volume / containers as well as to develop a better understanding of how the osmotic adjustment mechanisms affects the physiology of these two-tree species during drought conditions. This data is useful for determining stress tolerance thresholds, screening trees for avoidance and tolerance mechanism and provides insights into the ecosystem services these species could provide.

The *Quercus bicolor* response to growing in limited soil volume during a period of water deficit shows a tolerance mechanism to drought conditions. Water deficient oaks progressively decreased their turgor loss point over the course of the growing season in response to drought conditions (Figure 4.1). Osmotic adjustment was also observed in the well-watered treatment with a progressively decreased turgor loss point over the growing season. This osmotic adjustment response in non-water limited oaks has been observed in *Q.ilex* (Villar-Salvador and Planelles 2004) and in a series of North American species (Abrams 1990). While this was true, the total osmotic adjustment was significantly larger for the deficit treatment compared to the well-watered treatment (Table 4.2). This comparison indicates that osmotic adjustment is a primary strategy that *Quercus bicolor* employs under both well-watered and water deficit conditions. Mediterranean oak species (*Q. ilex, Q. coccifera* and *Q. faginea*) have been shown to be in a similar range of turgor loss points as *Q. bicolor* in this study and use this mechanism as a strategy of coping with drought stress (Castro-Díez and Navarro, 2007). Likewise, oak species

Q. acutissima, Q. frainetto, Q. muehlenbergii have all shown the capacity to osmotically adjust throughout the growing season when grown under field conditions (Sjöman et al. 2018b) in a similar capacity as *Q. bicolor*.

Gas exchange measurements (photosynthetic rate, stomatal conductance and transpiration rates) for both the well-watered and water deficient oaks showed similar responses during the spring and mid-summer measurement periods (Figure 4.3). Turgor loss point was also statistically equal for both treatments during these two time periods (Figure 4.1). During the late summer, the well-watered trees showed a large and significant increase in photosynthetic carbon assimilation, stomatal conductance and transpiration as compared to both the water deficit treatment and the well-watered treatment in the spring and mid-summer sampling periods (Figure 4.3). This large change in gas exchange for well-watered oaks in late summer could potentially be a strategy that *Q. bicolor* uses to increase carbohydrate storage rates prior to dormancy. By contrast, the water deficit treatment did not show a similar increase but did maintain gas exchange at equal rates to mid-summer and spring measurements (Figure 4.3). The increasing of solutes in cells allows for the decrease in turgor loss point and maintained gas exchange when soil moisture is limited. Additionally, the oaks showed no signs of premature leaf drop and were able to maintain their leaf cover for the extent of the growing season. The maintained gas exchange and osmotic adjustment for water deficient trees shows a clear tolerance pattern for *Q. bicolor*.

In contrast, *Betula pendula* when challenged with drought displayed an avoidance strategy by dropping its leaves, which reduced total canopy foliage cover as the growing season continued. Changes in turgor loss point for *B. pendula* had minor variations between both treatments and

time periods but their interaction (treatment x period) was not significant (Table 4.3). This result indicates that the combined effects of treatment x sampling period did not significantly change turgor loss point. This conclusion is corroborated with evidence demonstrating that turgor loss points were statistically overlapping in standard error for both treatments during all sample periods, with the exception of water deficit mid-summer (Figure 4.2) and that osmotic adjustment between treatments were equal (Table 4.2). A related species (*Betula nigra*) grown under field conditions did not show a significant difference in turgor loss point over the growing season (Sjöman et al. 2018b). This suggest that for both birches (*B. pendula* & *B. nigra*), changes in turgor loss point and subsequent osmotic adjustment is not a significant strategy employed by these species.

While no treatment x period effect was detected, both well-watered and water deficient *B. pendula* trees had decreased their turgor loss point mid-summer, as compared to spring or late summer (Figure 4.2). Direct measurement methods of solute potential, such as the vapor pressure method used in this study, can have error associated with them when symplast water dilutes apoplast water were solutes are accumulated (Sanders and Arndt 2012). This dilution of low solute symplast water into apoplast water can result in solute concentration measurements having decreased (less negative) solute potentials. For *B. pendula* under both well-watered and water deficit conditions, transpiration rates were highest in mid-summer (Figure 4.4). A potential explanation for the decrease in turgor loss point mid-season for both treatments could be that leaf-water potentials were higher at this time due to increased transpiration rates, signifying a greater amount of water transported to the leaves. The increase of water in the leaves via transpiration could then dilute solute concentrations when using the VPO method. Alternatively,

when leaf disks were collected, major leaf veins could have been included in leaf disks potentially increasing water content in the samples. Future research using this study system should be undertaken to develop a deeper understanding how water potentials are affected by drought conditions for *B. pendula* and other tree species studied using gas exchange VPO methods.

Gas exchange measurements for *B. pendula* varied between both treatments and sampling periods. Well-watered birches maintained stable photosynthetic carbon assimilation rates throughout the growing season with minimal variation at each sampling period (Fig 4.). Photosynthesis for water deficient birches was far more variable beginning with moderate rates in the spring (similar to the well-watered treatment), then significantly increasing to very high rates mid-summer before decreasing to very low rates in the late summer. Transpiration and vapor pressure deficit for water deficient *B. pendula* showed a similar pattern of increase and decline over the growing season as did the photosynthetic rates. Vapor pressure deficit increased driving force in *B. pendula* increasing water loss through transpiration (Figure 4.4). This pattern is consistent with other plant species that have observed increases in VPD also with increasing transpiration rates (Franks and Farquhar 1999, Sinclair et al. 2017). Stomatal conductance in water deficient birch showed a decline throughout the growing season with consecutively lower rates from spring to late summer. Stomatal conductance had a similar pattern of decline as transpiration with less water being released to the atmosphere as soil water moisture declined and VPD increased.

Leaf shedding by *B. pendula* was apparent by the mid-summer sampling period and continued to

increase into late summer with all of the water deficient birches losing substantial amounts of their canopy. For these same trees, an increase in photosynthesis, transpiration and VPD all occurred mid-summer as these birches were challenged with drought (Figure 4.4). The large increase in photosynthetic rate could be a consequence of water deficit conditions with greater amounts of carbon being fixed while water was being lost via transpiration. The shedding of leaves could therefore be a strategy of decreasing overall leaf surface area and subsequent water loss through transpiration. By the late summer sampling period, the birches were losing fewer leaves and had adjusted to the low soil water levels by significantly decreasing photosynthetic rates, stomatal conductance, and transpiration. This result was similar to the findings of Ranny et al. (1991) who saw a decrease in net photosynthesis and stomatal conductance for Birches under drought conditions (Ranney et al. 1991).

Well-watered birches showed an opposite response as water deficient trees with a small non-statistically significant decrease in photosynthesis mid-summer and a significant increase in stomatal conductance and transpiration (Figure 4.4). In late summer, well-watered birches maintained the same photosynthetic rate as the two previous sampling periods while stomatal conductance and transpiration declined. This result shows that *B. pendula* over the growing season became more efficient at fixing carbon via photosynthesis by losing less water through stomatal conductance and transpiration under well-watered conditions. Overall for *B. pendula* it was shown that soil water status plays a major role in how this tree responds to drought conditions as they display an avoidance strategy of shedding leaves.

Nowak et al. (2004) demonstrated that street tree mortality rates can be species-specific. This

association supports the concept that tree selection is key to the long-term survival of street trees. Therefore, selecting species with known stress tolerance should be used in challenging environments to maximize the potential ecosystem services an individual tree can provide. Under drought conditions in this study, *B. pendula* drops its leaves as part of an avoidance strategy while *Q. bicolor* displayed a tolerance strategy resulting in leaf retention. When considering the cooling microclimate ecosystem service through shading, *Q. bicolor* would be a better alternative to *B. pendula* in its capacity to maintain canopy cover. This study therefore demonstrates the importance of evaluating not only which trees have tolerance or avoidance mechanisms, but also specifically how much stress is required for these contrasting strategies to be expressed. Determining the thresholds of tolerance and avoidance strategies would aid in the understanding of the capacity of different tree species to deliver specific ecosystem services under a variety of environmental conditions.

4.5. Conclusion

This study combines measurements of osmotic adjustment and gas exchange rates in an effort to understand the effects of drought conditions on trees grown in limited soil volume. *Quercus bicolor* and *Betula pendula* growing in limited soil volume showed contrasting responses of tolerance (maintain leaves) and avoidance (leaf drop) in limited soil water deficit conditions. *Q. bicolor* increased solute concentration and effectively osmotically adjusted under drought and, to a lesser extent, well-watered conditions. Gas exchange rates for water deficient *Q. bicolor* that had osmotically adjusted were maintained at equal rates to spring well-watered oaks. When compared to the well-watered treatments, *B. pendula* under water deficit conditions did not osmotically adjust and demonstrated significantly lower gas exchange rates by the end of the growing season. The findings from this study identify the changes in physiology that occur for

these two tree species when grown in limited soil volume and are applicable to practitioners in nursery production and urban forestry. Future studies utilizing these methods will help to determine stress tolerance thresholds for species under drought conditions and aid in the selection of street trees that can provide optimal ecosystem services in adverse environments.

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5. APPENDIX

5.1. Recommended Genotypes for Cultivar Introduction

Based on the performance of hybrid oaks in tissue culture propagation (Chapter 1 and 2) and evaluation (Appendix 5.6-5.10) a number of individual genotypes are recommended for plant introduction. The following descriptions are evaluation notes for each of these selected genotypes.

Genotype: 06-1819-1

Hybrid Cross: (*Quercus gambelii* × *Quercus macrocarpa*) *x Quercus lyrate*

Evaluation Notes: Genotype 06-1819-1 is the most widely planted and evaluated genotype of hybrid white oak from the UHI and could be its first introduction into the nursery industry. This genotype multiplies well in tissue culture having the capacity to stabilize in multiplication. It has been re-established in tissue culture in 2014, 2017 and trialed for direct to rooting in 2018. With 06-1819-1 being a good multiplier this genotype was used for many of the tissue culture experiments in chapters 1 and 2. This genotype has been planted out in a variety of locations (F.R. Newman Arboretum, Cornell campus outside Plant Science Building and Schictels nursery) and in all of these sites this tree has developed a strong central leader, good form and shows a high degree of pest and disease resistance.

Figure 5.1: Pictures of Genotype 06-1819-1; Growing on the Cornell campus South West of Plant Science Building, planted in 2017 (Upper left); foliage (Lower left); Schichtels nursery trialing field site in Springville, NY (Upper right

Genotype:06-1810-2

Hybrid Cross: *Quercus bicolor* x *Quercus dentata* 'Pinnatifida'

Evaluation Notes: Genotype 06-1810-2 is hybrid line that has the capacity to stabilized in culture and showed up to 51% responsiveness to establishment in 2017 and 2018. In multiplication phase this genotype is a moderate multiplier. In the field plantings at Schichtels Nursery this tree showed exceptional form with a strong central leader, dark green foliage, no signs of pest and disease with the exception of a small amount of powdery mildew in 2017.

Figure 5.2: Pictures of Genotype 06-1810-2; foliage (left) and tree growing at Schichtels Nursery in Springville NY (right).

Genotype: 06-1500-1

Hybrid Cross: *Quercus Warei* 'Long' (Regal Prince) x Open

Evaluation Notes: Genotype 06-1500-1 had the capacity to stabilize in culture and showed high multiplication rates (Chapter 1, Table 1.7, 1.8 and 1.9). During establishment in 2018 this genotype showed relatively high rates of shoot elongation (48%). This line displayed a high rooting percentage (87%) (Chapter 1, Table 1.11). In the Bluegrass lane plantings, no major pest or disease issues were observed. In the Schichtels planting this genotype was reported as slow growing while in Bluegrass lane it was one of the tallest trees in the evaluation block. Foliage has no resemblance to *Q. robur* and it does not display typical upright fastigiate form of Regal Prince. This hybrid has shown the capacity to grow in alkaline substrates without a large degree of chlorosis. When grown in pH 8 soils this clone maintained green leaves and had a SPAD values of 31.1 (Denig et. al 2014).

Figure 5.3: Pictures of Genotype 06-1500-1; tree growing in evaluation nursery at Cornell's Bluegrass Lane (upper left); leaf form (lower left) and foliage (upper right).

Genotype:04-566-3

Hybrid Cross: *Quercus bicolor* x *Quercus fusiformis*

Evaluation Notes: Genotype 04-566-3 had the capacity to stabilized in continuous multiplication phase in tissue culture with moderate multiplication rates. When this genotype was challenged with drought it expressed a large change in turgor loss point over the growing season, showing its capacity to osmotically adjustment (Figure 5.1). This could indicate this genotypes capacity to grow under drought conditions. Minor amounts of powdery mildew were observed on this clone in 2015 in the stock block at bluegrass lane and Schichtels nursery in 2017. No major pest issues were observed. A strong central leader developed in Schictels trees with a mix of both long and short branches giving it an asymmetrical form. Further evaluation is required for this genotype prior to introduction but it shows some desirable characteristics.

Figure 5.4: Pictures of Genotype 04-566-3; Two field grown trees at Schictels Nursery (upper left and right) and foliage (lower middle)

Genotype: 05-830-50

Hybrid Cross: *Quercus bicolor* x *Quercus rugosa*

Evaluation Notes: Genotype 05-830-50 has the capacity to stabilize in tissue culture and had a moderate rate of multiplication. In the Schichtels planting one of the trees formed a central leader with a tall tight form while another was somewhat asymmetrical. Bright yellow petioles are ornamentally attractive in this genotype. In the Schichtels plantings the leaves had signs of hole shot likely from pest damage early in the season. Leaves had yellow dappling and it was unclear if this was a result of pest damage or nutrient deficiency. This line requires more evaluation and would benefit from evaluation at multiple locations.

Figure 5.5 Pictures of genotype 05-830-50; tree growing at Blue Grass Lane showing the tree form (upper left); Foliage (lower left), ornamental yellow petioles (upper right)

5.2. Acclimatization Protocols

Shoots after moving through the rooting phases were moved to acclimation phase. Shoots that were to be moved to acclimatization were required to be living with no signs of shoot tip necrosis and minimum of a single root. Cone-tainers (25x100mm) (Ray Leach RLC3) were filled with Lambert 111 and watered to field capacity. Rooted shoots were moved with forceps and placed into the growing media. Low density polyethylene bags are wrapped around the entirety of the cone-tainer and shoot before being secured with a rubber band. Plastic bag covered containers were maintained in the growth chamber and environmental conditions were the same as for tissue culture plants. After a period of three weeks a hole was pierced into the plastic bag to reduce humidity levels and help leaf cuticle development. On a weekly basis new holes were cut in the bag. When media become visible dry it was watered until field capacity was reached. Acclimatizing plants were monitored and when they started to show signs of new shoot and leaf growth plastic bags were fully removed.

5.3. Gibberellic Acid (GA_3 & GA_{4+7}) Multiplication Grid Experiment

An experiment was conducted trialing the use of two different gibberellic acids (GA_3 and GA_{4+7}) for their effect on multiplication of hybrid oaks. A grid experiment was set up that trialed four different concentrations of the two Gibberellic acids at 0, 0.2, 1, 2 mg/L concentrations. Media was the standard BAP multiplication media from chapter 1 with the addition of the two GAs at different concentrations. Four shoots per jar were used for the experiment. For the gibberellic acid experiment hybrid oaks that been multiplying in a standard BAP media were used. Over successive multiplication cycles both forms of GA caused hybrid lines to decline and die out. As a result of these declining multiplication rates the investigation with GA_3 and GA_{4+7} ended.

Table 5.1. GA_3 - Grid Trial

Genotype	GA_3 Concentration (mg/l)	n	Shoot Length	Num. of shoots	± Conf. Int.
06-1819-1	0	10	22.15 a	0.12 a	± (0.5013, 0.0006)
06-1819-1	0.2	10	9.7 b	0.34 ab	± (0.9014, 0.0468)
06-1819-1	1	10	6.4 b	0.25 ab	± (0.7510, 0.0178)
06-1819-1	2	10	2.5 b	1.32 b	± (2.2934, 0.6104)
06-1500-1	0	10	10.55 a	1.40 a	± (2.4341, 0.3659)
06-1500-1	0.2	10	9.0 a	1.20 a	± (2.2341, 0.1659)
06-1500-1	1	10	12.28 a	2.00 a	± (3.0341, 0.9659)
06-1500-1	2	10	14.08a	2.20 a	± (3.2341, 1.1659)
			Prob > F	**Prob > F**	
GA_3 (A)			0.0034	0.0681	
Genotype(B)			0.5829	0.0029	
A x B			0.1078	0.614	

Table 5.2. GA 4+7 - Grid Trial

Genotype	GA_{4+7} Concentration	n	Percent Contaminated	Shoot Length	Number of shoots
06-1819-1	0	9	40%	13.50 abc	1.00 ± 0.316 b
06-1819-1	0.2	6	60%	9.50 c	1.36 ± 0.369 b
06-1819-1	1	15	0%	19.17 abc	1.29 ± 0.327 b
06-1819-1	2	15	0%	18.65 abc	0.79 ± 0.327 b
06-1500-1	0	15	0%	11.67 bc	3.00 ± 0.408 a
06-1500-1	0.2	11	27%	12.50 abc	1.00 ± 0.499 b
06-1500-1	1	14	7%	14.83 abc	0.93 ± 0.316 b
06-1500-1	2	14	7%	13.67 abc	1.33 ± 0.316 b
				Prob > F	**Prob > F**
GA_{4+7} (A)				0.0925	0.0302
Genotype (B)				0.2897	0.0791
A x B				0.5254	0.0046

5.4. +/- GR24 Grid Multiplication Experiment

GR24 a synthetic analog to strigolactone was trialed in multiplication phase with genotype 06-1500-6 to determine its effect in tissue culture multiplication. This hormone has been noted to reduce side branching in ex-plants and it was trialed to see if it would affect shoot elongation for

oak tissue cultures. Stock solution was made using 1 mg of GR24 dissolved in 1 ml acetone and 9ml of Millipore H_20. This made a final solution that was 10 ml in volume with a GR24 concentration of 1mg/10ml. The stock solution was sterile filtered and pipetted into individual 2 ml aliquots. GR24 was added to the standard multiplication media (Chapter 1) at concentrations of 0, 0.1, 0.5 and 1 mg/L. Ten shoots and tips of genotype 06-1500-6 were grown for a single multiplication phase and number of shoots and shoot length were determined at the end of the cycle. No significant differences were detected in terms of shoot length or number of shoots with increasing concentrations of GR24. The experiment was terminated after this single experiment

Table 5.3. +/- GR24 Grid Multiplication Experiment

GR24 Concentration (mg/L)	Shoot Vs Tip	n	Num. of shoots	Shoot Length (mm)	95% Conf. Int.
0	Shoot	10	1.9 ± 0.41 a	5.28 a	± (7.92, 3.51)
0	Tip	10	0.6 ± 0.41 b	7.41 a	± (11.53,4.77)
0.1	Shoot	10	1.2 ± 0.41 a	7.11 b	± (12.34, 4.10)
0.1	Tip	10	0.9 ± 0.41 a	3.34 a	± (4.77,2.34)
0.5	Shoot	10	1.1 ± 0.41 a	6.61 b	± (12.43, 3.51)
0.5	Tip	10	0.6 ± 0.41 a	2.57 a	± (3.98,1.66)
1	Shoot	10	0.4 ± 0.41 a	4.84 a	± (9.14,2.56)
1	Tip	10	0.7 ± 0.41 a	3.64 a	± (5.43,2.43)
			Prob>F	Prob>F	
Concentration (A)			0.3529	0.322	
Shoot Vs Tip (B)			0.1351	0.0196	
A x B			0.3028	0.0352	

5.5. Hybrid Oak Genotypes Stabilized in Continuous Multiplication Cycle By Bryan Denig from 2014-2017

Table 5.4 shows a series of hybrid oak genotypes that have the capacity to stabilize in a continuous multiplication cycle using standard methods described in chapter 1. These hybrid

lines were established by Bryan Denig between 2014-2017 and have not been formally reported aside from personal communications with the author of this dissertation.

Table 5.4: Hybrid Oak Genotypes Stabilized in Continuous Multiplication Cycle by Bryan Denig from 2014-2017

Genotype	Maternal	Paternal	Multiplication Rate	Stabilized Established
05-805-2	*Q. montana*	*Q. geminata*	good	Stabilized
05-806-1	*Q. montana*	*Q. lyrata*	fair	Stabilized
06-1819-1	*Q. gambelii* × *macrocarpa*	*Q. lyrata*	good	Stabilized
04-564-1-4	*Q. bicolor*	*Q. minima*	good	Stabilized
04-566-3	*Q. bicolor*	*Q. muehlenbergii*	fair	Stabilized
05-878-1	*Q. muehlenbergii*	*Q. virginiana*	good	Stabilized
05-860-2	*Q. muehlenbergii*	*Q. virginiana*	fair	Stabilized
06-1811-3	*Q. bicolor*	*Q. fabri*	poor	Established
05-854-5	*Q. muehlenbergii*	*Q. fusiformis*	poor	Established
06-1802-1	*Q. bicolor*	*Q. glauca*	poor	Established

5.6. Osmotic Adjustment and Drought Tolerance of Hybrid Oaks

5.6.1. Introduction and Methods

A selection of hybrid white oak genotypes was evaluated for their capacity osmotically adjust when grown under field grown conditions. Osmotic adjustment is a mechanism used in plants to tolerate water deficit (drought) conditions (See chapter 4 for further discussion on osmotic adjustment). Evaluation of a selection of hybrid oaks was carried out during the 2017 growing season. One year old shoots of select hybrid white oaks with a fully developed first flush of leaves were harvested in the evening of June 12th, 2017 for the first round of measurements and on August 14th for a second round. Shoots were harvested from the genotypes 06-1810-1, 06-1821-3, 06-1814-2, 05-905-3, 05-922-1, 06-1819-1, 05-905-1, 06-1812-2. Excised branches were taken to the laboratory within 20 minutes. At the laboratory, branches were recut under water at least two nodes distal to the original cut and placed in a plastic bucket of water without exposing the cut surface to the air. Branches were rehydrated overnight in a dark chamber. On June 13th,

2017 (the following day) three leaf discs (one per leaf) were taken from fully expanded leaves using a 7 mm cork borer from the mid-lamina region between the mid-rib and leaf margin. To minimize potential sources of error, no leaf discs were taken from lamina regions with first and second order veins. All discs were tightly wrapped in foil to limit condensation or frost after freezing. Foil-wrapped leaf discs were then submerged in liquid nitrogen for 2 min to fracture the cell membrane and walls. Upon removal from the liquid nitrogen, leaf discs were punctured 10–15 times with sharp tipped forceps before sealing the leaf disc in the vapor pressure osmometer (VPO) (Vapro 5600, Wescor, Logan, UT, USA). Puncturing of leaf discs is done to facilitate evaporation through the leaf cuticle and decrease equilibration time in the chamber. Leaf discs were sealed in the VPO using a standard 10 µl chamber. Initial solute concentration (mmol kg^{-1}) readings were taken after 10 min equilibration time. Measurements were repeated a total number of three times after equilibration. All three readings were recorded per leaf sample and averaged with all other readings from the same individual plant. Room temperature was recorded on an hourly basis. Solute concentration c_s was converted to osmotic potential (Ψ_π) using Van't Hoff relation (Eq. 1):

$$\Psi\pi = - RTc_s \quad (1)$$

Where R is the gas constant, T is temperature in Kelvin, and c_s is solute concentration. Since branch and leaf samples were allowed to equilibrate in water over night prior to measurement, osmotic potential was considered to be at full turgor ($\Psi_{\pi 100}$). Sjoman et al. (2018) developed a modified equation for temperate tree species that can estimate water potential at turgor loss point (Ψ_{P0}) from osmotic potential at full turgor ($\Psi_{\pi 100}$) (Eq. 2):

$$\Psi_{P0} = - 0.2554 + 1.1243 \times \Psi_{\pi 100} \quad (2)$$

Osmotic adjustment ($\Delta\Psi_{\pi 100}$) can be calculated by determining the difference between early

season measurements of Ψ_{P0} and late season measurements. This difference shows a plant's ability to change turgor loss point over the growing season.

5.6.2. Results

Osmotic Adjustment:

Eleven hybrid oak genotypes turgor loss points were assessed at the start and end of the 2017 growing season in order to determine their capacity for osmotic adjustment. Average turgor loss point across all genotypes was -2.06 MPa in the spring and -2.41 MPa in the summer. All genotypes showed a decrease in turgor loss point over the course of the growing season (Figure 5.6). Significant differences were detected in turgor loss point for genotype (A), early vs late (B) sample period and their interaction (A x B). Osmotic adjustment varied by genotype with some hybrids having large changes in turgor loss point over the growing such as 06-1733-17, 04-566-3, 06-1802-1, 05-922-1, 05-905-3. All other genotypes showed small changes in turgor loss points (Figure 5.6).

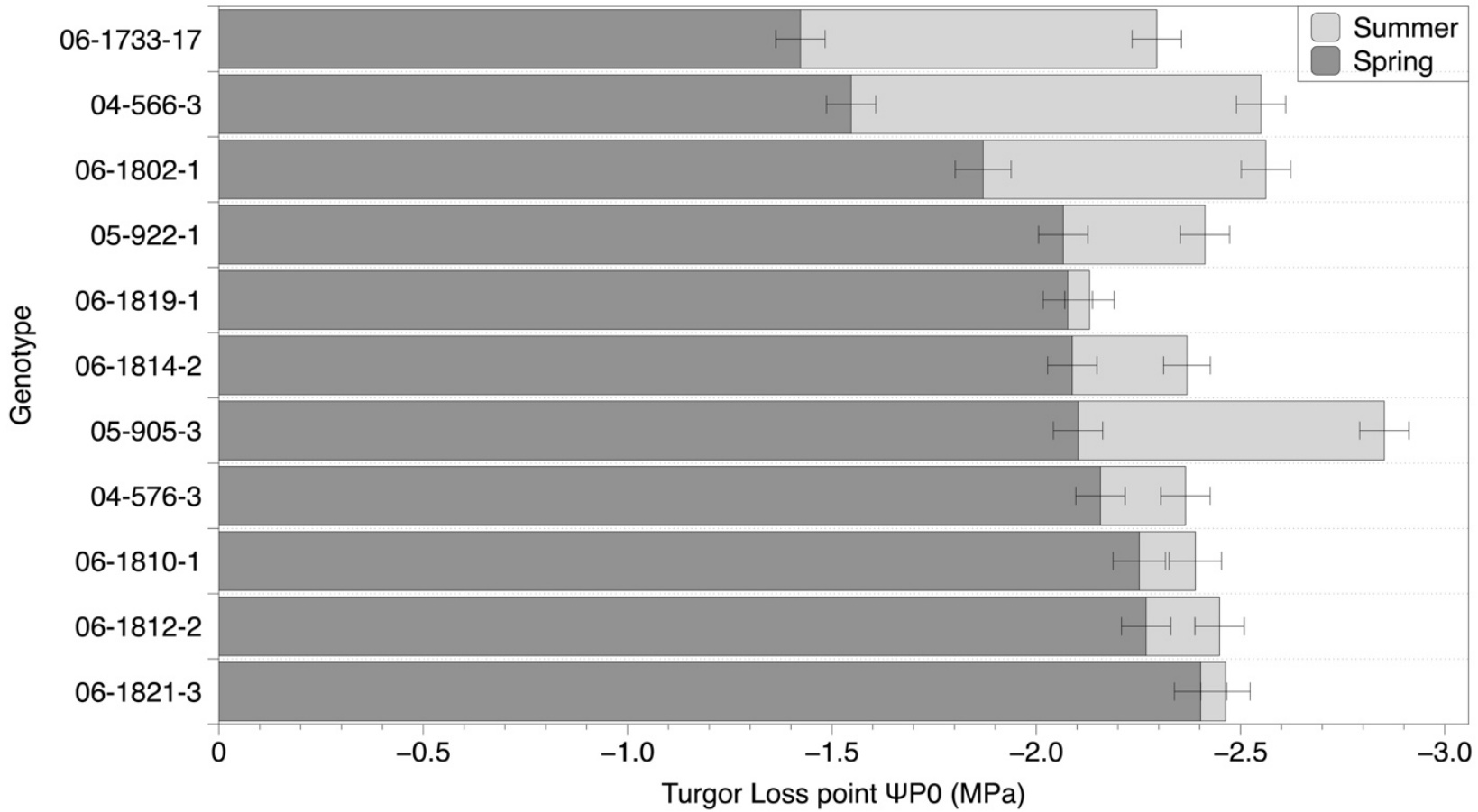

Figure 5.6: Changes in Turgor Loss Point of Eleven Hybrid Oaks from Spring to Late Summer in 2017

Table 5.5: Effect test For Figure 5.6- Genotype and Sample Period Effect on Osmotic Adjustment for Hybrid Oaks

Source	**Prob > F**
Genotype (A)	<.0001
Sample period (B)	<.0001
A x B	<.0001

5.6.3. Discussion

In 2017 turgor loss point was assessed for eleven genotypes of hybrid oaks to investigate osmotic adjustment as a drought tolerance mechanism. Decreasing turgor loss point over a growing season is facilitated by accumulation or synthesis of solutes within leaf cells (Sanders and Arndt 2012). The increasing of solutes into cells allows leaves to maintain cell turgor and gas exchange under increasingly negative leaf water potentials. Changes in turgor loss points over a growing season shows a plants capacity to adjust to soil environments with limited available water. Studies have investigated osmotic adjustment in Maples (*Acer*) (Sjöman et al 2015), Magnolia (Sjöman et al 2018a), and a series of landscape trees including oaks (*Quercus*)(Sjöman et al 2018b) using the same method as this paper. Osmotic adjustment under non drought conditions has been shown to occur in a series of norther American species (Abrams 1990) and in *Q.ilex* (Villar-Salvador and Planelles 2004). The hybrid oaks turgor loss point was assed under field conditions in 2017 meaning that there was no control of irrigation or rainfall. All hybrid oak genotypes showed a change in turgor loss point in late season compared to early season (Figure 5.6) although the differences varied by genotype. Genotypes 06-1733-17, 04-566-3, 06-1802-1, 05-922-1 and 05-905-3 showed large changes in osmotic adjustment while changes in all other genotypes were small. Changes in turgor loss point were within a similar to range to what was observed in *Q. bicolor* (Chapter #4) and *Q. acutissima, Q. frainetto, Q. muehlenbergii* (Sjöman et al. 2018b). Over the course of all seasons that the author worked with hybrid oaks (2014-2018) none of the stock plants showed signs of drought induced leaf shedding. Using the vapor pressure osmometer to determine changes in turgor loss point provides quantitative set of data to determine hybrid oaks capacity to tolerate drought conditions. Observation of turgor loss point under field conditions provides a general sense of how the hybrid oaks perform in the landscape.

Yearly variation in rainfall and temperature may significantly affect osmotic adjustment in oaks and as result the turgor loss points reported here should be considered as a general assessment under field conditions. In the future tissue culture or stool bed propagated hybrid oaks can be used to conduct controlled drought experiments in containers to develop a more robust understanding of the osmotic adjustment mechanism.

5.7. 2015 Notes on Pest and Disease – Field Grown Trees (Block B)

In 2015 a qualitative examination of pest and disease presence/absence and pressure was conducted of trees growing in northern blue grass lane stock block (Group B – Bryan Denig Masters Thesis). These trees were propagated using the modified stool bed method and were planted in the Group B block in 2013 (GPS: 42.460894, -76.463253). Visual examination of trees was conducted on October 6, 2015. Powdery mildew and anthracnose were the primary diseases scouted for during this assessment. Disease's evaluated included powdery mildew on quantitative scale increasing from 0 to 4. A score of 0 meant no evidence of powdery mildew and score of 4 meant at least one single leaf was more than 75% covered in mildew. Oak anthracnose was assessed as either present or absent based. Pests assessed included scale, Japanese beetle herbivory and aphids. Any other abiotic or leaf abnormalities were also noted such as galls or potential herbicide drift damage.

Table 5.6: 2015 Pest and Disease Notes – Field Grown Trees (Block B)

Genotype	Powdery Mildew	Anthracnose	Scale	Pest	Abiotic/ Others
06-1746-9	1	n	1	Scale	Herbivore damage holes
06-1742-2	2	n	1	Japanese beetle, Scale	Herbivore damage
06-1730-8	3	n	1	Japanese beetle, Scale	
04-570-2	0	y	1	Japanese beetle, Scale	Black leaf spots
04-565-1-1	3	n	1	Japanese beetle, Scale	
06-1735-5	3	n	1	Japanese beetle, Scale	Black leaf spot, tip die back
06-1733-4	0		3	Japanese beetle	
04-575-2	0	n	0		Herbivore damage dappled chlorosis
06-1800-1	1	n	1	Japanese beetle, Scale	Severe leaf spot
04-568-2-2	0	y	1	Japanese beetle	
06-1811-4	2	n	1	Japanese beetle	Herbivore damage
06-1800-2	1	n	1	Japanese beetle, Aphid	
06-1728-3	4	n	1	Japanese beetle, Aphid	
06-1745-5	4	n	1	Japanese beetle, Aphid	Tip necrosis, black leaf spot
06-1726-1	2	n	1		
06-1673-4	3	n	1	Japanese beetle, Aphid	Early browning of leaves
06-1500-1	0	n	1	Japanese beetle	
06-1812-2	0	n	1		Herbivore damage, leaf spot, leaves browning early
06-1819-1	0	n	1		Clean foliage, good looking tree
06-1800-1-1	1	n	0	Japanese beetle	
06-1804-1	0	n	2		Herbivore damage, relatively clean leaves aside from scale
06-1800-7	1	n	0	Japanese beetle	Powdery mildew on new growth only

06-1800-8	1	n	1	Japanese beetle, Aphid	Powdery mildew on new growth only
06-1813-3	0	n	1	Japanese beetle	Clean foliage aside from beetles
06-1812-1	0	n	0	Gal	Herbivore damage, gall on stems
06-1500-7	0	n	1		Brown leaf spot, leaves browning, foliage not very clean
06-1800-7	1	n	2	Japanese beetle	Herbivore damage
06-1744-6	1	n	0		Leaf spot, herbivore
06-1741-6	3	n	0		minor leaf spot, herbivore damage
06-1808-1	3	n	1	Japanese beetle	
06-1673-6	0	n	1		Black leaf spot on leaf margins, leaf spot, herbivore damage
06-1730-1	1	n	0	Aphid	Herbivore damage
06-1734-1	0	n	1	Japanese beetle	
04-566-4	1	n		Japanese beetle	
06-1800-10	0	n	1	Japanese beetle	Brown leaf spot, early leaf browning
06-1821-3	0	n			Pesticide deformation/damage, herbivore, shoot tip die back
06-1742-1	4	n	2	Japanese beetle	
06-1800-9	4	n			
04-576-3	0	n	0	Japanese beetle	Leaves look terrible, brown many leaf spots, poor quality
06-1811-3	0	n	1		Herbivore damage, leaf spot, leaf browning slight
06-1800-3	1	n	1	Japanese beetle	Herbivore damage, minor leaf spot
06-1800-16	3	n	0	Japanese beetle	Leaf yellowing
06-1639-1	4	n	1	Japanese beetle	
04-560-5-1	2	n	0		Herbivore damage
06-1500-6	0	n	1		Few brown leaves. ok quality for Q. x warei
06-1819-1	0	n	1		Yellow leaf splotch, pretty clean

06-1802-1	0	n	1		Pesticide drift deformation and damage, brown leaf spot and yellow, new growth clean
06-1748-2	3	n	1	Japanese beetle	Leaf spot yellow
04-574-3	0	n	0		Brown leaf spot, all over, poo condition
06-1800-5	1	n	1	Japanese beetle	
06-1733-2	1	n	1	Japanese beetle	Strange leaf disease with small fungal cups, leaf browning
05-1811-1	0	n	1		Leaf spot and browning, herbivore damage
05-906-3	0	n	3	Japanese beetle	
04-566-3	1	n	0		Leaf spot minor, minor herbivore damage
04-568-1	0	n	0	Japanese beetle, gal	clean aside from gals
04-562-1	1	n	1		Leaf spot, minor herbivore damage
05-905-1	0	n	0		Minor leaf spot
06-1743-3	3	n	2		Herbivore damage
06-1673-7	2	n	1		Severe browning and leaf spot
06-1800-4	1	n	1	Aphid	Brown leaf spot

5.8. Hybrid Oak Stock Block Disease Evaluation 2015

The hybrid oaks were assessed for pest and disease damage on October 6th, 2015 in the primary stock block at Blue Grass Lane (42.460638, -76.463208). All hybrids oaks had been coppiced in the spring and allowed to grow for the season before evaluation. Hybrid oak stock plants were only noted in the evaluation if no pest or disease damage was observed.

Table 5.7: 2015 Hybrid Oak Genotypes Showing No Pest or Disease Damage in the Blue Grass Lane Stock Block in 2015

04-570-2
04-577-4
04-577-1
05-874-3
04-569-1-3
04-568-2-1
05-857-2
05-805-2
05-811-1
06-1813-1
06-1813-4
05-812-1
06-1812-1
05-860-2
05-806-1
06-1804-1
05-853-1
05-899-1
06-1500-2
06-1500-3
05-854-22
05-854-21
05-854-15
05-854-1
05-854-6
06-1500-7
06-1733-11
06-1804-1

05-860-2
05-874-3
05-905-3
06-1819-1

5.9. 2016 Stock Block Disease Evaluation

On October 25th, 2016 hybrid oaks in the primary stock block at Blue Grass Lane were observed for pest and disease. Hybrids were noted if they had no signs of pest or disease damage.

In addition, a series of genotypes that had stabilized in continuous multiplication phase were observed for pest and disease prevalence. 2016 was a very dry year so it could be considered a test year for powdery mildew.

Table 5.8: 2016 Notes on Pest and Disease Prevalence of Hybrid Oaks in Stock Block

Genotype	**Observations**
04-577-5	Leaf hole shot, no disease
04-571-1	Clean, no disease
04-577-3	Clean, minor leaf hole shot
04-576-3	Clean, no disease
04-572-1	Minor leaf spot
05-874-3	clean, disease free
05-905-1	clean with minor leaf shot
05-905-3	Minor mildew, leaves very dark and lustrous
04-566-4-2	clean leaves, minor leaf hole shot
05-922-1	clean leaves
06-1811-3	clean leaves
05-857-3	clean leaves
05-857-2	clean leaves, vigorous growth
05-805-2	Minor gal and leaf spot
05-879-1	Clean leaves, disease free
06-1612-1	Minor leaf spot
06-1819-1	Minor mildew, splotchy yellow pattern on leaves
05-860-2	Minor leaf spot and leaf hole shot

05-860-3	Clean leaves
05-806-1	Clean leaves
06-1500-7	Galls on leaves, Japanese beetle damage
05-854-15	Clean, vigorous
05-854-5	Clean, vigorous
05-872-1	Clean leaves
05-854-22	Clean leaves
05-854-18	Clean leaves

5.10. 2016 Field Notes of Hybrid Oaks Field Grown at Schichtel's Nursery

Field grown oaks at Schichtels nursery in Springville NY were observed to determine general condition and pest and disease tolerance of the hybrid oaks growing at this site in September 2016.

Table 5.9: 2016 Field Notes of Hybrid Oaks Field Grown at Schichtel's Nursery 2016

Genotype	**Field Observations**
06-1673-4	Powdery mildew present
06-1812-2	Clean leaves, ok form
06-1746-9	Clean leaves
06-1746-9	
04-566-3	
06-1500-7	Amazing growth and form.
05-899-2	Very slow growing, clean and interesting leaves
04-565-1-4	Clean leaves, good form
05-830-50	
05-830-50	Tall, tightly branched, central leader
04-576-3	Slow growing, leaf spot
04-561-1	Glossy leaves, good form
05-805-1	
06-1673-2	Slow growing, awful plant
05-906-3	Slight lean to one direction, weird form
06-1673-4	Dead
06-1818-1	
04-576-3	Dead

06-1673-4	Curvy, wacky form
06-1500-8	Leaf spot
06-1500-4	Tall, ok form, no mildew
04-566-3	
06-1800-4	
04-567-2	
04-560-5	Clean leaves, tall, ok form, Japanese beetle damage
06-1733-17	Spreading pyramidal form, Japanese beetle damage
06-1818-1	Good form, leaves dropped earlier than other oaks in block
06-1800-13	Powdery mildew, Japanese beetle damage, ok form
06-1800-3	Leaf spot
05-805-1	Powdery mildew
06-1800-13	Powdery mildew
06-1800-3	Tree in bad health
05-805-1	Powdery mildew, good caliper, central leader, spreading form
06-1750-1	
06-1737-1	Leaning, weird form, leaf spot
04-576-3	Leaf spot, ok form
06-1814-2	Some powdery mildew, good form
05-906-3	Leaning, awful form
05-830-50	Exfoliating bark, some leaf spot
04-566-3	Might have good fall color?
06-1500-1	Slow growing
06-1810-2	Clean leaves, tall, good caliper, central leader
06-1819-1	Dead
06-1813-4	
06-1819-1	Disease free, hole shot, open form with central leader
04-565-1-4	Disease free, dark green leaves, spreading form
04-566-3	Dead
06-1800-13	Disease free, dark green leaves, Japanese beetle damage, bad form- maybe fixable with pruning
04-576-3	Leaf spots bad form
04-560-3	
05-806-1	
04-563-4	Minor leaf spot, central leader
06-1800-9	Some mildew, moderate leaf spot, central leader
06-1633-1	Leaf spot, slow growing
04-568-2	Dead

06-1812-2	Wide spreading
06-1800-1	Narrow form, lead spot
06-1724-2	Disease free, hole shot, good form
05-904-2	Disease free, goofy leaves, some mildew
06-1500-4	Powdery mildew
04-574-3	Powdery mildew, slow growing
06-1743-6	Slow growing
04-569-1	Slow growing
06-1673-3	Dark green leaves, some mildew
06-1800-6	Tons of mildew
04-576-1	Some disease pressure, tall, strong central leader
06-1801-1	Powdery mildew, leaning
06-744-4 (06-1744-4?)	Powdery mildew, slow growing
04-571-2	Very slow growing, corky bark, terrible form
06-1750-1	Powdery Mildew
06-1800-17	Very diseased
05-906-3	Bad form
06-1813-3	Dead

5.11. 2017 Field Notes of Hybrid Oaks Field Grown at Schichtel's Nursery

Table 5.10: 2017 Field Notes of Hybrid Oaks Field Grown at Schichtel's Nursery

Genotype	**Observations**
04-576-1	Weird, branching, wonky off center, interesting cruciform foliage with thin waste like *Q. macrocarpa*, a few acorns have developed, some Japanese beetle damage,
04-574-3	Leaf tip necrosis
06-1500-4	Forms pretty good, mostly dark green leaves, small amount of mildew
05-904-2	Sparse branching, somewhat leaning, leaf damage, looks generally off
06-1724-2	Japanese beetle damage moderate, some mildew, good form, central leader, full and open branching
06-1800-1	fastigiate, a little wonky, yellow stippling on leaves
06-1800-9	Strong central leader, powdery mildew, moderate Japanese beetle, branches spaced out making it feel like the foliage is not very full
04-563-4	Leaves chlorotic, central leader present, large leaves, some leaf damage
04-574-3	Interesting cruciform leaves are cool, little wonky in growth forum, dense foliage
04-565-1-4	Extremely wide, not very tall, no leader on top although, very full tree, some mildew, moderate Japanese beetle damage
06-1819-1	Tall, small amount of leaf hole shot, good branch angles up to 45 degrees, good growth, overall a good looking specimen.
05-830-50	Some shoot tip damage, exfoliating bark is ornamentally interesting, large leaves, some hole shot, moderate Japanese beetle damage, small amount of chlorosis.
04-561-1	Large dark green leaves, full foliage, some Japanese beetle damage, a central leader, growing straight, some sooty spot from aphids
06-1818-1	Large tree, leaves spaced out, fairly evenly branched, very straight, petioles nice orange-red, upright branching form. Could be a great tree with branches spaced out.
06-1500-4	Large, central leader, some leaf tip damage,
04-566-3	Wide branching in the bottom, fairly central leader, acorns present, mix of long and short branches form exfoliating bark,

04-567-2	Cool exfoliating bark, many branches out at 90-degree angle or dipping down, Japanese beetle damage
04-560-5	Powdery mildew and sooty spots from aphid, large leaves, new sprout dieback,
06-1733-17	Wonky, form, 90-degree branch angles, some Japanese beetle damage
06-1818-1	Defoliated possible from early leaf drop, large tree, looks bad for no apparent reason.
06-1800-13	Japanese beetle damage, acorns present, some mildew. A tall and wide tree.
04-576-3	*Q. macrocarpa* / cruciform leaves, wonky branching that kind of twists, cool leaf morph., some brown leaf spot
06-1814-2	Very full, upright straight tree, pretty good form, acorns present, some powdery mildew, moderate Japanese beetle damage, very dense foliage.
05-830-50	Large leaves, some Japanese beetle damage and yellowing leaf spot, kind of asymmetrical but could be from shading from neighboring tree, wider branch angle
06-1810-2	Dark lustrous leaves, some mildew, very full canopy, many acorns, form somewhat asymmetrical, branch angle between 90 and 45 degrees.
04-565-1-4	wonky form, branches in all directions, acorns present, some mildew, tree titled, moderate Japanese beetle damage.
06-1500-7	Very tall, tallest tree in the block, doesn't look very much like *Q. warei*, half as wide as tall. Branches curve up, *Q. macrocarpa* type leaves, looks very different than siblings, clean, should be attempted in culture again if it hasn't been used in the past
06-1746-9	Large leaves, lustrous green, some Japanese beetle damage, central leader, little slower growing, oval shaped form, small amount of mildew and soot from aphids,
06-1746-9	Smaller than sibling next to it, Japanese beetle damage, dark green leaves, less soot and mildew then previous one, slow grower, somewhat oval in shaped
06-1812-2	Looks like this tree lost its central leader, twisting branches, leaves are spaced out giving it a not very dense feel, Japanese beetle damage, not in love with this tree
06-1673-4	Upright form, Japanese beetle damage, looks like regal prince but not as symmetrical or tight, mildew and aphid soot present.

5.12. F.R. Newman Arboretum SPAD Evaluation of Hybrid Oaks:

Hybrid oaks that had been propagated using the modified stool bed technique were planted in the urban tree collection at the F.R. Newman Arboretum in The Cornell Botanic Garden in 2013. The urban tree collection is planted in berms of soils composed of dredge from restoration of the arboretum ponds. These soils were reported to have poor structure and high pH giving them common characteristics of urban soils. Hybrid oaks were planted in these soils to see how they would respond to a high pH soil environment. SPAD evaluations were taken in the fall of 2015, 2016 and 2017 to determine if these high pH soils were causing chlorosis. Five leaf samples were scanned with a SPAD meter on the third leaf from the terminal bud on branches randomly distributed around the canopy. SPAD evaluation and visual inspection did not show signs of chlorosis over the three years these trees were observed. The data presented here is raw SPAD values and is available if researchers would like to refer to these numbers or evaluate SPAD for these trees in the future.

Table 5.11: 2015 F.R. Arboretum Hybrid Oak SPAD Evaluation

			SPAD Readings						
Genotype	**Year Evaluated**	**Date Data Collected**	**1**	**2**	**3**	**4**	**5**	**Average**	**Std. Dev.**
06-1821-3	2015	9/15/15	32	30	32.4	32.5	38.6	33.1	3.24
06-1811-3	2015	9/15/15	25.4	25.6	26.1	17.8	23.8	23.74	3.43
06-1800-16	2015	9/15/15	45.7	39.7	39.9	33.3	41.7	40.06	4.48
06-1800-4	2015	9/15/15	39.8	39.7	43.5	33.6	37.8	38.88	3.60
06-1800-9	2015	9/15/15	43.5	45.9	52.1	39	34.8	43.06	6.61
04-572-1	2015	9/15/15	40.9	45.9	43.1	38.9	47	43.16	3.37
06-1800-17	2015	9/15/15	41.4	36.7	43.6	37.4	39.6	39.74	2.85
05-903-1	2015	9/15/15	28.9	25.3	28.1	26.1	24.1	26.5	1.98
05-905-2	2015	9/15/15	34.4	36.7	34.1	36	33.9	35.02	1.25
06-1741-3	2015	9/15/15	32.2	30.1	30.4	28.7	36.3	31.54	2.94
05-906-3	2015	9/15/15	31.1	33.2	38.6	42.2	40.3	37.08	4.74
06-1724-1	2015	9/15/15	31	32.2	34.2	32.3	31.8	32.3	1.18
06-1800-5	2015	9/15/15	41.2	33.9	28.9	31.6	41.2	35.36	5.62
04- 575-1	2015	9/15/15	39.7	41.6	39.7	39.1	37.8	39.58	1.37
06-1801-10	2015	9/15/15	36.8	35.2	30	34.6	29.9	33.3	3.16
06-1800-1	2015	9/15/15	33.7	30.4	35.8	34.1	39.6	34.72	3.36
06-1741-6	2015	9/15/15	26.9	23.7	28.3	24.7	23.5	25.42	2.10
05-905-1	2015	9/15/15	25.9	28.8	24.8	22.2	27	25.74	2.47
06-1800-7	2015	9/15/15	31.7	22.2	30.5	23.4	25.5	26.66	4.24
06-1802-1	2015	9/15/15	33.1	35.8	30.1	36.1	28.5	32.72	3.38
04-576-3	2015	9/15/15	39.9	37.5	39.6	38.8	36.8	38.52	1.34
04-565-2-1	2015	9/15/15	30.4	30.3	30.3	35.6	29.9	31.3	2.41
04-574-3-1	2015	9/15/15							

06-1744-6	2015	9/15/15							
04-572-2	2015	9/15/15	37.9	42.1	39.1	43.3	41.3	40.74	2.21
06-1733-5	2015	9/15/15	25.8	26.9	26.8	29	14.5	24.6	5.76
06-1800-5	2015	9/15/15	44.7	40	32.3	30	32	35.8	6.27
06-1800-4	2015	9/15/15	37.4	29.5	38.2	31.7	37.2	34.8	3.93
06-1744-4	2015	9/15/15	31.3	26.8	25.4	28.8	22.9	27.04	3.21
05-906-3	2015	9/15/15	36.4	37.8	38.2	32.2	36.3	36.18	2.38
06-1744-6	2015	9/15/15	31	31.5	33	30.5	29.9	31.18	1.18
05-922-1	2015	9/15/15	39.6	31.2	36.6	31.1	39.5	35.6	4.24
04-576-3	2015	9/15/15	37.2	35.2	40	30.9	34	35.46	3.41
06-1821-3	2015	9/15/15	27.3	22.5	27	24.4	25.5	25.34	1.97
04-575-2	2015	9/15/15	36.9	34.2	38.9	38.6	34.6	36.64	2.19
04-574-3	2015	9/15/15	36.7	38.4	38.2	39.3	40.2	38.56	1.31
06-1733-5	2015	9/15/15	32.6	30	31.6	37.8	29.6	32.32	3.29
05-922-1	2015	9/15/15	28.5	27.6	30	39.2	42	33.46	6.65
06-1725-1	2015	9/15/15	36.3	34.1	35.1	31.4	26.4	32.66	3.94
06-1808-1	2015	9/15/15	39.3	38	34.6	39.7	41.1	38.54	2.46
06-1747-5	2015	9/15/15	48.7	37.7	41.6	39.2	37.7	40.98	4.60
06-1800-10	2015	9/15/15	28.9	32.2	29	27.8	29	29.38	1.66
06-1819-1	2015	9/15/15	33.3	34.8	31.6	31.9	30	32.32	1.82
06-1744-4	2015	9/15/15	37.8	39.1	35.4	36.5	32.8	36.32	2.41
06-1808-1	2015	9/15/15	39.7	39.3	43.2	36.7	25.5	36.88	6.77
06-1800-9	2015	9/15/15	39.6	31.3	37	40.5	37	37.08	3.59
04-564-1-4	2015	9/15/15	37.4	43.9	34.6	36.9	41.1	38.78	3.69
06-1800-7	2015	9/15/15	37.2	32.4	34	38.7	30.5	34.56	3.38
06-1742-1	2015	9/15/15	45.7	36.2	39	51.6	36.7	41.84	6.64

Table 5.12: 2016 F.R. Arboretum Hybrid Oak SPAD Evaluation

			SPAD Readings						
Genotype	**Year Evaluated**	**Date Data Collected**	**1**	**2**	**3**	**4**	**5**	**Average**	**Std. Dev.**
06-1811-3	2016	9/15/16	39.8	40.5	41.5	39.9	37.5	39.84	1.47
06-1800-16	2016	9/15/16	41.5	36.6	41.3	42.2	42.9	40.9	2.48
06-1800-4	2016	9/15/16	41.4	39.1	39.4	38.2	37.4	39.1	1.51
06-1800-9	2016	9/15/16	41.5	37.6	43.2	47.6	47.2	43.42	4.16
04-572-1	2016	9/15/16	45.6	40	45.7	45	43.1	43.88	2.41
06-1800-7	2016	9/15/16	38.7	43.9	43.3	45	43.1	42.8	2.41
05-903-1	2016	9/15/16	32.4	29.6	31.4	32.7	30.9	31.4	1.24
05-905-2	2016	9/15/16	28.3	36.1	37.6	35.1	32.6	33.94	3.64
06-1741-3	2016	9/15/16	38.6	39.8	34.6	33.9	33.3	36.04	2.95
05-906-3	2016	9/15/16	31.8	29.3	31.5	30.3	32.1	31	1.17
06-1724-1	2016	9/15/16	31.4	34.2	38.5	37.3	33	34.88	2.96
06-1800-5	2016	9/15/16	42.1	39.4	43.4	64.7	65.9	51.1	13.05
04-575-1	2016	9/15/16	40.5	41.9	39.4	42.4	41.3	41.1	1.19
06-1801-10	2016	9/15/16	31.9	24.2	26.3	23.6	26.4	26.48	3.28
06-1800-1	2016	9/15/16	29.1	27.3	33.7	41.2	34.5	33.16	5.42
06-1741-6	2016	9/15/16	n/a	n/a	n/a	n/a	n/a	n/a	n/a
05-905-1	2016	9/15/16	29.6	37.1	42.2	41.9	41.3	38.42	5.34
06-1800-7	2016	9/15/16	24.5	21.9	30	26.6	21	24.8	3.65
06-1802-1	2016	9/15/16	40.9	41.2	40.5	40.4	41.2	40.84	0.38
04-576-3	2016	9/15/16	41.3	32.1	38.8	31.2	3.6	29.4	15.05
04-565-2-1	2016	9/15/16	40.5	42.7	35.9	40.7	42.6	40.48	2.76
04-575-3-1	2016	9/15/16	n/a	n/a	n/a	n/a	n/a	n/a	n/a
06-1744-6	2016	9/15/16	n/a	n/a	n/a	n/a	n/a	n/a	n/a

04-575-2	2016	9/15/16	42	42.4	39.9	44	44.6	42.58	1.85
06-1744-6	2016	9/15/16	n/a	n/a	n/a	n/a	n/a	n/a	n/a
06-1800-5	2016	9/15/16	34.6	45.6	36.7	40.9	46.3	40.82	5.21
06-1733-5	2016	9/15/16	37.2	41.1	42.1	40.9	45.9	41.44	3.11
06-1800-4	2016	9/15/16	41.5	44.7	43.6	44.6	40.5	42.98	1.89
06-1744-4	2016	9/15/16	38.6	38.5	40.5	45.9	46.1	41.92	3.81
05-906-3	2016	9/15/16	35	35.6	35.9	27	19.3	30.56	7.3
04-574-3	2016	9/15/16	44.6	41.9	36.2	41.6	42.6	41.38	3.12
04-575-2	2016	9/15/16	n/a	n/a	n/a	n/a	n/a	n/a	n/a
06-1821-3	2016	9/15/16	39	40.4	39.5	37.3	36.8	38.6	1.51
04-576-3	2016	9/15/16	46.2	46.1	48	46.6	45.9	46.56	0.84
05-922-1	2016	9/15/16	48.4	52.1	50.6	50.5	44.2	49.16	3.07
06-1744-6	2016	9/15/16	37.4	39	36.9	38.8	36.8	37.78	1.05
06-1733-5	2016	9/15/16	38.4	46.1	31.6	28.1	31.8	35.2	7.14
05-922-1	2016	9/15/16	46.2	54.2	46.8	48.7	47.7	48.72	3.21
06-1725-1	2016	9/15/16	49.4	38.3	44.5	42.3	46.1	44.12	4.16
06-1808-1	2016	9/15/16	38.1	43	38.2	40.5	38.9	39.74	2.06
06-1747-5	2016	9/15/16	27.4	44.8	41.4	47.7	50	42.26	8.91
06-1800-10	2016	9/15/16	31	27.3	33.3	28.4	28.9	29.78	2.38
06-1819-1	2016	9/15/16	37.6	38	34	36.2	39.4	37.04	2.05
06-1744-4	2016	9/15/16	41.1	40.5	37.3	43.1	40.8	40.56	2.09
04-574-3	2016	9/15/16	41.7	39.6	40.9	43.8	38.6	40.92	2
06-1808-1	2016	9/15/16	38.5	37.8	37.2	37.4	39.5	38.08	0.94
06-1800-9	2016	9/15/16	44.1	38.8	40.9	41.7	39.6	41.02	2.06
06-1800-7	2016	9/15/16	43.7	48	48.9	49.5	38.5	45.72	4.63
04-564-1-4	2016	9/15/16	30.8	31.6	29.1	29.7	28.9	30.02	1.15
06-1742-1	2016	9/15/16	46.5	46.6	47.7	43.1	51.5	47.08	3.01

Table 5.13: 2017 F.R. Arboretum Hybrid Oak SPAD Evaluation

			SPAD Readings						
Genotype	**Year Planted**	**Date Data Collected**	**1**	**2**	**3**	**4**	**5**	**Average**	**Std. Dev.**
06-1800-6	2017	10/16/17	32.9	34.4	31.9	40.2	29.9	33.86	3.90
06-1800-4	2017	10/16/17	29.3	27.3	24	24.7	23.8	25.82	2.40
06-1800-9	2017	10/16/17	23.9	26	21.3	27.5	27.1	25.16	2.57
04-572-1	2017	10/16/17	45	35.3	45.5	48.7	47.9	44.48	5.36
06-1800-17	2017	10/16/17	39.6	35.5	37.7	44.9	40.9	39.72	3.54
05-903-1	2017	10/16/17	25.1	23.3	22.8	22	21.9	23.02	1.30
05-905-2	2017	10/16/17	27.5	34.2	24.3	26.9	27.1	28	3.69
06-1741-3	2017	10/16/17	34.6	34.1	30.8	33.6	29.5	32.52	2.24
05-906-3	2017	10/16/17	38.3	33.5	34.5	39.8	49.5	39.12	6.36
06-1724-1	2017	10/16/17	n/a	n/a	n/a	n/a	n/a	n/a	n/a
06-1800-5	2017	10/16/17	31.8	35.5	28.4	30.2	33	31.78	2.70
04-575-1	2017	10/16/17	n/a	n/a	n/a	n/a	n/a	n/a	n/a
06-1801-10	2017	10/16/17	24.6	23.4	22.7	27.6	31.1	25.88	3.47
06-1800-17	2017	10/16/17	21.9	23.1	21.3	31	30.8	25.62	4.86
06-1711-6	2017	10/16/17	n/a	n/a	n/a	n/a	n/a	n/a	n/a
05-905-1	2017	10/16/17	31.2	28.6	27.3	23.5	25.8	27.28	2.90
06-1800-7	2017	10/16/17	18.6	30	24	28.1	28.9	25.92	4.68
06-1802-1	2017	10/16/17	25.4	26.5	27.5	29.7	23.2	26.46	2.42
04-576-3	2017	10/16/17	n/a	n/a	n/a	n/a	n/a	n/a	n/a
04-565-2-1	2017	10/16/17	37.1	35.3	31	38.2	33.1	34.94	2.93

04-574-3-1	2017	10/16/17	31	29.5	29	30.1	27.4	29.4	1.34
06-1744-6	2017	10/16/17	n/a	n/a	n/a	n/a	n/a	n/a	n/a
04-575-2	2017	10/16/17	25.7	26.9	22.3	25.3	27	25.44	1.90
06-1800-5	2017	10/16/17	36.1	43.4	30.4	36.9	42	37.76	5.18
06-1733-5	2017	10/16/17	29.2	21.9	23.5	26.2	23.8	24.92	2.84
06-1800-4	2017	10/16/17	41.7	47.2	38.9	39.6	41.2	41.72	3.27
06-1744-4	2017	10/16/17	39.7	43	37.9	39.1	33.6	38.66	3.40
05-906-3	2017	10/16/17	32.2	28.6	27.6	32.8	31	30.44	2.26
04-574-3	2017	10/16/17	34.2	31.9	23.5	37.6	35.4	32.52	5.45
04-575-2	2017	10/16/17	n/a	n/a	n/a	n/a	n/a	n/a	n/a
06-1821-3	2017	10/16/17	n/a	n/a	n/a	n/a	n/a	n/a	n/a
04-576-3	2017	10/16/17	n/a	n/a	n/a	n/a	n/a	n/a	n/a
05-922-1	2017	10/16/17	33.1	36.9	33.5	40.1	31.8	35.08	3.38
06-1744-6	2017	10/16/17	24.8	22.1	24.3	29.7	29.2	26.02	3.30
06-1733-5	2017	10/16/17	40.3	33.9	48.7	44	35	40.38	6.19
05-922-1	2017	10/16/17	46.1	41.6	38.3	38.2	37.4	40.32	3.61
06-1725-1	2017	10/16/17	43.7	43	42	40	44	42.54	1.61
06-1801-1	2017	10/16/17	34.2	39.7	36.5	40.9	37.1	37.68	2.66
06-1800-1	2017	10/16/17	32.1	30.7	26.9	23.9	28.1	28.34	3.22
06-1819-1	2017	10/16/17	25.6	29.7	27.4	29.5	28	28.04	1.68
04-574-3	2017	10/16/17	30.9	28	36.3	37.5	34.4	33.42	3.93
06-1808-1	2017	10/16/17	39.5	36.3	38.4	33.8	33.9	36.38	2.58
06-1800-9	2017	10/16/17	32.8	32	28.1	24.8	25.4	28.62	3.68
06-1800-7	2017	10/16/17	38.9	40.1	42.9	36.8	35.6	38.86	2.86
04-564-1-4	2017	10/16/17	21.4	20.1	23	20.7	18.7	20.78	1.59
06-1742-1	2017	10/16/17	42.7	42.2	38.6	39.7	45.1	41.66	2.57

5.13. Citation Summary of Species, Media and Hormones Used In Oak Tissue Culture

Table 5.14: Species Used in Oak Tissue Culture in the literature

Species	**Number of References**	**Citation**
Q. acerifolia	1	(Kramer and Pence 2012)
Q. arkansana	1	(Brennan et al. 2017)
Q. alba	2	(Schwarz and Schlarbaum, 1993) (Vieitez et al. 2009)
Q. bicolor	1	Vieitez et al. 2009)
Q. boyntonii	1	(Brennan et al. 2017)
Q. canbyi	1	(Brennan et al. 2017)
Q. chrysolepis	1	(Brennan et al. 2017)
Q. dummosa	1	(Brennan et al. 2017)
Q. engelmannii	1	(Brennan et al. 2017)
Q. euboica	3	(Kartsonas and Papafotiou 2007a), (Kartsonas and Papafotiou 2007b)(Kartsonas and Papafotiou 2008)
Q. gambelii	1	(Brennan et al. 2017)
Q. georgiana	1	(Kramer and Pence 2012)
Q. graciliformis	1	(Brennan et al. 2017)
Q. palmeri	1	(Brennan et al. 2017)
Q. petraea	2	(Toth et al 1994) (Chalupa, 1993)
Q. robur	8	(Toth et al, 1994) (Romano et al 1995), (Evers et al 1993) (Chalupa, 1993)(Vieitez et al 1994) (Vidal et al 2003), (Herrmann and Buscot 2008), (Martínez et al 2011)
Q. rubra	5	(Seckinger et al 1979), (Schwarz and Schlarbaum, 1993), (Vieitez et al. 1993), (Vieitez et al. 2009) (Vengadesan and Pijut 2009)
Q. semecarpifolia	1	(Tamta et al. 2008)
Q. serrata	1	(Pandey and Tamta 2014)
Q. shumardii	1	(Benett 1986)
Q. suber	3	(Romano and Martins-Loução 1992)(Romano et al 1992),

		(Lebtahi and Bouguedoura 2015)
Q. texana	1	(Brennan et al. 2017)
Q. tomentella	1	(Brennan et al. 2017)
Q. vacciniifolia	1	(Brennan et al. 2017)

Table 5.15: Media Used For Oak Tissue Culture

Media	**Number of references**	**citations**
Murashige & Skoog (MS)	4	(Seckinger et al 1979) (Romano and Martins-Loução 1992) (Toth et al 1994), (Tamta et al. 2008)
Murashige & Skoog (MS) (½ strength)	3	(Herrmann and Buscot 2008), (Vengadesan and Pijut 2009), (Lebtahi and Bouguedoura 2015),
Gresshoff & Doy	10	(Vieitez et al. 1985), (San-Jose et al, 1988)(Romano and Martins-Loução 1992)(Romano et al 1992), (Romano et al 1995), (Chalupa 1993), (Vieitez et al. 1994), (Vieitez et al. 2009), (Martínez et al. 2011), (Brennan et al. 2017)
Heller medium	1	(Vieitez et al. 1985)
Wood Plant Media	13	(Benett, 1986), (Chalupa 1988), (Romano and Martins-Loucao, 1992), (Toth et al 1994), (Chalupa, 1993), (Vieitez et al 1993), (Vieitez et al 2009), (Vengadesan and Pijut 2009), (Pandey and Tamta 2014), (Brennan et al. 2017), (Kartsonas and Papafotiou 2007b) (Kartsonas

		and Papafotiou 2007a), (Tamta et al. 2008)
BTM: Broad Leaf Tree Medium	2	(Chalupa 1988)(Chalupa, 1993)
HS: Schenk & Hilderbrand	1	(Toth et al 1994)
VSV: Vieitez et al 1985)	2	(Schwarz and Schlarbaum, 1993) (Vieitez et al 1985)

Table 5.16: Hormones and Additives Used For Oak Tissue Culture

Hormone / Additive	**# of Refs.**	**Type of Additive**	**Citations**
BAP: (6-Benzylaminopurine)	21	Cytokinin	(Seckinger et al 1979)(Vieitez et al. 1985) (Benett, 1986)(Chalupa 1988), (San-Jose et al, 1988), (Romano and Martins-Loução 1992) (Romano et al. 1992), (Toth et al 1994), (Romano and Martins-Loução 1995), (Schwarz and Schlarbaum, 1993), , (Vieitez et al. 1993), (Vieitez et al. 1994), (Vieitez et al 2009), (Herrmann and Buscot 2008), (Vengadesan and Pijut 2009), (Martínez et al. 2011), (Pandey and Tamta 2014), (Lebtahi and Bouguedoura 2015), (Kartsonas and Papafotiou 2007b)

			(Kartsonas and Papafotiou 2007a), (Tamta et al. 2008)
NAA: (1-Naphthylacetic acid	10	Auxin	(Seckinger et al 1979)(Chalupa 1988) (Romano et al. 1992), (Toth et al 1994), (Schwarz and Schlarbaum, 1993), , (Chalupa, 1993), (Vieitez et al. 1993), (Pandey and Tamta 2014), (Lebtahi and Bouguedoura 2015), (Kartsonas and Papafotiou 2007a)
2iP 6-(gamma,gamma-dimethylallylamino) purine	4	Cytokinin	(Benett, 1986), (Vengadesan and Pijut 2009), (Pandey and Tamta 2014), (Kartsonas and Papafotiou 2007a)
IBA Indoole-3-butyric acid	15	Auxin	(Benett, 1986), (Chalupa 1988)(San-Jose et al, 1988), (Romano et al. 1992), (Toth et al 1994), (Chalupa, 1993), (Vieitez et al. 1993), (Vieitez et al. 1994), (Vidal et al 2003), (Vieitez et al 2009), (Herrmann and Buscot 2008), (Martínez et al. 2011), (Lebtahi and Bouguedoura 2015), (Kartsonas and Papafotiou 2007a), (Tamta et al. 2008)
GA Gibberellic Acid	2	GA	(Benett, 1986), (Vengadesan and Pijut 2009)
Thidiazuron	4	Cytokinin	(Chalupa 1988) (Vengadesan and Pijut 2009), (Pandey and Tamta 2014), (Kartsonas and Papafotiou 2007a)
Kinetin	3	Cytokinin	(Chalupa 1988)(Toth et al 1994), (Pandey and Tamta 2014)

Zeatin	3	Cytokinin	(San-Jose et al, 1988), Romano et al. 1992, (Vieitez et al 2009)
IAA: (Indole-3-acetic acid)	6	auxin	(Romano and Martins-Loução 1992), Romano et al. 1992, (Toth et al 1994), (Romano and Martins-Loução 1995), (Vieitez et al. 1993), (Pandey and Tamta 2014)
BPA (N-benzyl-9-(2-tetrahydropyranyl) adenine	1	cytokinin	(Chalupa, 1993)
BAP + NAA	2	Auxin + cytokinin	(Schwarz and Schlarbaum, 1993)(Kartsonas and Papafotiou 2007a)
$AgNO_3$ (Silver Nitrate)	2	Additive	(Vieitez et al 2009), (Pandey and Tamta 2014)
Active charcoal	1	Additive	(Vieitez et al 2009)
Casein hydrolysate	2	Additive	(Vengadesan and Pijut 2009), (Pandey and Tamta 2014)
Myoinositol	1	Additive	(Kartsonas and Papafotiou 2007b)
Thiamine	1	Additive	(Kartsonas and Papafotiou 2007)
Pyridoxine:	1	Additive	(Kartsonas and Papafotiou 2007b)
Nicotinic acid	1	Additive	(Kartsonas and Papafotiou 2007b)

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