WORKING TOWARD A MICROPROPAGATION TECHNIQUE FOR NORTH AMERICAN WHITE OAKS

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by
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ABSTRACT

Oaks have an important role in landscape horticulture and urban forestry. There are numerous species native to a variety of habitats in North America and they readily hybridize between species within a taxonomic section (e.g. the white oak or red oak section). Yet their valuable characteristics have been severely underused due to the difficulty of vegetative propagation. Grafting often results in delayed incompatibility and cuttings do not easily root. Hybrids and unique types of oaks are rarely introduced to the nursery industry. This research investigated the potential for using micropropagation as a technique for the asexual propagation of oaks. Immediate challenges were to obtain responsive explants that produced elongated shoots (shoots ≥ 1 cm), to control contamination during the initiation phase and to subculture shoots to achieve multiplication. Results showed 1 mg l^{-1} BA (6-benzylaminopurine) in Woody Plant Medium to have a significant effect ($P<0.0001$) on obtaining responsive explants from young white oak stock plants. GA_3 (Gibberellic acid) added to the growth media was not beneficial for inducing shoot elongation in slow growing or unresponsive explants. Exogenously applied GA_3 used in conjunction with BA in the media may help shoot elongation. Only one subculture was achieved as the multiplication phase proved problematic with shoots arresting in growth and browning. The concentration of BA (1.0 mg l^{-1} or 0.5 mg l^{-1}) did not have a significant effect on shoot length or number during the multiplication phase. Overall, obtaining responsive explants proved successful and more research is needed to further develop the process.

BIOGRAPHICAL SKETCH

Anne Johnson was born and raised in central Virginia next to the Blue Ridge Mountains. She attended Christopher Newport University in Newport News, Virginia where she earned a Bachelor of Science degree in Biology in 2002. After graduating, she "thru-hiked" the Appalachian Trail and then worked as a field researcher in wildlife biology. After a bit of further traveling and wandering, Anne moved to Ithaca, New York to study horticultural science.

DEDICATION

This thesis is dedicated to Mom, Dad, Amy, Joseph, Caroline and Oliver. To Grace, Isabel, Otis and Snowmouse. It is dedicated to Ithaca, to all my friends in Ithaca and to Josh, Rufus, Hans and Mr. Sebass.

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LIST OF ABBREVIATIONS

WPM……Woody Plant Medium (Lloyd and McCown 1980)

GD……Gresshoff and Doy medium (Gresshoff and Doy, 1972)

BA……6-Benzylaminopurine

GA_3……Gibberellic Acid

IBA……Indole-3-butyric Acid

1. Introduction

The genus *Quercus* L. has over 500 species of trees and shrubs native to regions all over the northern hemisphere. It is difficult to overstate their ecological, economic and cultural value. Oaks provide an essential source of timber for furniture, flooring, interior finishing and veneer. Cork is sourced from oak and the impervious heartwood of oak is used for shipbuilding and wine and whiskey casks. Oaks are prominent in many forest ecosystems and their acorns are a vital food source for wildlife, high in fat and nutrients. In addition, oaks have an important role to play in urban and suburban landscapes. Dirr (1998) describes them as a durable, long-lived and majestic tree. They are an outstanding feature in any city park with a large and spreading growth habit. The grand size, longevity and sturdiness of oaks have made them a familiar symbol in many cultures.

1.1 Genetic diversity of Oaks

Even within a species, oaks exhibit wide variability in phenotypic traits. Furthermore, oaks are found in many different climates throughout the northern hemisphere. They range from the humid, sub-tropical regions of Central America or Asia to the semi-arid terrain of California or the Mediterranean and to the furthest reaches of deciduous forest in the north.

The genus *Quercus* is divided into two subgenera: *Cyclobalanopsis* and *Quercus*. The latter is then subdivided further into 4 sections: *Protobalanus, Cerris, Quercus* (white oaks) and *Rubrae* (red oaks). Oaks readily hybridize between species within their respective section. The number of species counted within the genus *Quercus* contained around 300 in 1862 compared to over 500 species counted in 1998 (Petit et al., 2004). Although interspecific hybrids are not immediately considered a new species, this shows the complexity of delineating species within the genus. Oaks are often discussed in any

studies on plant evolution and speciation because of their ability to intercross (Petit et al., 2004).

Within just the white oak section, species are found growing from Cuba to northern Canada, making the genetic potential of this group appealing. For example, species within this group found in the northern latitudes have cold tolerance while those growing in other regions may have tolerance to drought or poor drainage. Therefore, the wide variety of traits in white oaks, as well as their interspecific hybridization, makes them a desirable group for selection for growth in urban environments.

1.2 Selecting Oaks for the Urban Environment

Trees growing in an urban setting face special challenges such as drought, soil compaction and air pollution. At Cornell University, hybrids between many different species of white oak were created between 2004-2006 to select for tolerance to urban growing conditions. The maternal species were located on the Cornell campus in Ithaca, NY and include several native white oaks: *Quercus bicolor* Willd., *Quercus gambelii* Nutt. x *macrocarpa* Michx., *Quercus macrocarpa, Quercus macrocarpa* 'Ashworth' McArdle and Santamour, *Quercus montana* L., *Quercus muehlenbergii* Engelm., *Quercus* 'Ooti' and *Quercus x warei* 'Long' Cully (Regal Prince®). Pollen from approximately 40 different species from Europe, Asia and North America was used for hybridization. There are around 350 unique hybrid genotypes growing in the field for evaluation.

Specifically, these hybrids are being evaluated for tolerance to soils with high pH. Many native oaks are sensitive to alkaline soils because important nutrients (especially iron and manganese) become less soluble and available for absorption by plant roots at a pH higher than 7.0. Unfortunately, alkalinity is a frequent characteristic of many urban soils (Hollis, 1991). By crossing native, cold hardy oaks with the pollen of a variety of

other white oak species, selection for vigorously growing trees with tolerance to alkaline soils may be possible.

1.3 Asexual Propagation

The genus *Quercus*, however, is notoriously difficult to propagate vegetatively (Dirr, 1998). This characteristic impedes the selection of hybrids, as it is difficult to propagate clones of each genotype for testing in the field. In general, few new varieties of oak are introduced to the nursery industry due to this trait. While selection and propagation by seed is possible, seed storage is difficult (white oak seeds in particular are ready to germinate as soon as they mature in the fall) and propagation by seed continuously introduces variability in traits. Moreover, it can take 30-40 years before a tree reaches maturity and can produce seeds.

Therefore additional research has been focused on developing a method for asexual propagation. Propagation by cuttings has only had limited success due to the recalcitrance of oaks to adventitious rooting (Flemer, 1962). Grafting often results in delayed incompatibility. It can take up to 7 years for the incompatibility to show which includes scion overgrowth of the rootstock, suckering and precocious flowering (Coggeshall, 1996).

Over the past several years, research on asexual propagation by cuttings has shown the importance of shoot etiolation (light exclusion) for the adventitious rooting of several difficult to root woody plants including *Quercus* spp. (Maynard and Bassuk, 1987). Cutting back the stock plant has also been shown to have a significantly positive effect on the rooting of shoots of *Q. bicolor* and *Q. macrocarpa* (Amissah and Bassuk, 2005). It is known that juvenile tissue retains the greatest degree of morphogenetic ability and that juvenile tissue can be found in the stump or at the root-shoot junction (Bonga and Aderkas, 1992).

These findings led to developing a layering and etiolation technique for propagating white oaks, specifically *Q. bicolor* and *Q. macrocarpa* (Amissah and Bassuk, 2004). Dormant 2-6 year old stock plants are cut back to 3-5 cm tall stumps in the spring to force the growth of shoots from the base of the plant. Once buds begin to break, the stumps are covered so that the shoots emerge in an etiolated state. When the shoots are 10-20 cm tall, the base of each shoot is painted with a rooting hormone solution of 8,000 ppm IBA and then surrounded with moist potting mix. Shade is then placed over the entire stump until the etiolated shoots green up and, in the fall, rooted shoots are harvested from the stock plant and potted individually.

This layering technique has been used to provide clones of unique white oak hybrids to be evaluated for high pH tolerance. However, while this technique is successful, it is also slow. A stump produces 3 to 15 shoots suitable for treatment with rooting hormone. Even with high rooting success for a particular genotype, this technique produces a maximum of 10 to 12 rooted shoots per stock plant. Often the result is less than that.

1.4 Micropropagtion

Theoretically, micropropagation may rapidly multiply specific genotypes and alleviate any problems associated with the rooting of cuttings. However, oaks are also known to be a recalcitrant group when it comes to micropropagation. *Quercus* spp. in particular exhibit strong episodic and determinate shoot growth making the establishment of stabilized and responsive shoot cultures quite difficult (McCown, 2000). Most oaks produce an initial flush of shoot growth in the spring but then set a terminal, resting bud and will not grow again until the following season after the seasonal chilling requirement has been met (McCown, 2000).

Despite these difficulties, oaks have been successfully micropropagated. A majority of micropropagation research has been done on important European species of white oak: *Q. robur* L. (English Oak), *Q. petarea* Liebl. (Sessile Oak) and *Q. suber* L. (Cork Oak). Chalupa (1988) achieved 81% rooting in more than 12,000 micropropagated shoots of *Q. robur*. In Spain, Vieitez et al. (2012) have developed specific tissue culture protocol for these species. They have also worked to develop techniques for the North American species: *Q. rubra* L. (Red Oak), *Q. alba* L. (White Oak) and *Q. bicolor* (Swamp White Oak) (Vieitez et al., 2009). Ostrolucka et al. (2007) successfully micropropagated *Q. robur, Q. cerris, Q. virgiliana* and *Q. rubra*. Puddephat et al. (1997) in the U.K. have also worked to micropropagate *Q. robur* and Johnson and Walker (1991) successfully achieved shoot proliferation in cultures of *Q. lobata* Nee (Valley Oak) in California.

There are four main stages in the micropropagation process. 1) Initiation: this involves decontamination of the explant, placing the explant in a growth medium and growth of the existing, nodal bud. 2) Stabilization or Multiplication: this is the continuous and uniform shoot growth of buds produced in culture. 3) Rooting: micro-shoots produced in culture are rooted in a growth medium. 4) Acclimatization: rooted micro-shoots are placed in a protected, high humidity environment and slowly exposed to external growth conditions.

Due to the episodic growth character described above, the problematic phase for *Quercus* spp. is the stabilization and multiplication phase. Common problems during this phase are shoot tip necrosis and general decline of shoot vigor (McCown, 2000 and Vieitez et al., 2009). One important approach to help overcome the episodic growth character of *Quercus* is to use juvenile plant tissue for culture (McCown, 2000). This can be accomplished by using somatic or zygotic embryogenesis methods (the meristematic tissue is juvenile by nature) or by manipulating the stock plant directly into rejuvenation. Rejuvenating the stock plant can be achieved by cutting it back to force shoots from the

base of the plant as was done in the field propagation procedure described above. However, this obviously makes the selection of older trees that lack stump sprouts or epicormic shoots difficult (Vieitez et al., 2012). Although, interestingly, research by Veietez et al. (1985) on *Q. robur* used shoots from 3-4 month old seedlings and stump sprouts from the bases of 50-year-old trees that had been cut back to the ground. Both ages were successfully cultured and rooted in vitro. A similar experiment showed the same results for mature chestnut (*Castanea sativa*) (Vieitez et al., 1983).

Another difficulty for micropropagation includes the effect of individual genotypes within species. Due to the genetic variability of oak, individual genotypes of the same species and provenance can have different reactions to culture (Vieitez et al., 2012). This can eventually be overcome by reusing the same donor stock plant that has been proven to perform well in culture to obtain explants. However, if the goal of this micropropagation work is to provide clones of a wide array of specific genotypes for field evaluation, then this attribute could prove problematic.

1.5 Micropropagation of North American Oaks

Vieitez et al. (2009) have done some of the only successful research on micropropagating important North American white oak species. They have been successful in all four stages of the process. For the initiation phase, Vieitez et al. (2009) achieved high rates of explants producing responsive buds— for *Q. bicolor* it was 79-99%. They were also able to achieve stable shoot proliferation by reducing the concentration of the cytokinin 6-benzylaminopurine during the multiplication phase. Vieitez et al. (2009) also overcame the determinate and episodic shoot growth of *Quercus* (which often manifests itself as arrested growth and short shoots) by subculturing shoots in a horizontal position. Finally, using treatments with IBA and activated charcoal in rooting media, they were also able to achieve 70-90% rooting rates for different

genotypes of *Q. alba* and *Q. bicolor*. Johnson and Walker (1991) were able to establish proliferating cultures of *Q. lobata* using BA (0.3 mg l^{-1} or 0.7 mg l^{-1}) in the growth media, but they did not attempt any rooting experiments.

1.6 Research Objective

The objective of the following research was to more fully understand the challenges of micropropagating species of North American white oak and to develop a protocol for the initiation of explants and the stabilization of cultures. The role of BA during initiation and multiplication as well as using gibberllic acid (GA_3) as a means for inducing shoot elongation were investigated. Immediate challenges included controlling contamination while establishing the explants and outlining any particular treatments needed for the stock plant to produce usable explants. The long-term goal of this research is to outline a protocol for producing shoots in vitro that may be rooted and successfully acclimated in large numbers for evaluation in field trials.

2. Materials and Methods

2.1 Plant Material

Stock plants consisted of three different species and two hybrids: *Quercus bicolor, Quercus cerris* L., *Quercus muehlenbergii, Quercus* x *schuettei* Trel., and *Quercus robur* x *bicolor*. Dormant 5-7 year-old stock plants in #3 containers were brought into the greenhouse in February and placed under 24° C day/18° C night temperatures after at least four months of chilling. The chilling requirement was met by placing the potted plants in a refrigerated greenhouse room at 40% humidity and 4° C. The stock plants were cut back to 20-25 cm above the soil upon placing them in the greenhouse.

2.2 Obtaining the Explant

New shoots emerged from the stumps of the cut back stock plants after 2-3 weeks in the greenhouse. As these shoots reached a length with at least 5-7 nodes, but no more than 30 cm in total shoot length, the apical bud was pinched off (shoots longer than 30 cm began to lignify and were difficult to cut with a scalpel while preparing them for culture). Once the axillary buds had begun to swell, but not break (typically around one week after pinching) the shoots were cut from the stock plant at their base and brought into the lab for culture.

Each shoot was stripped of leaves and cut up into nodal segments. Each segment was 2-3 cm long and contained one leaf node with a bud. Occasionally, if two nodes were close together, the segment would contain two nodes. Each nodal segment became an explant.

2.3 Disinfestation

The explants were placed in a Magenta™ GA-7 box (Magenta Corp., Chicago, IL) with tap water and an ounce of Soft-cide®-NA (Thermo-Scientific, Portsmouth NH) hand soap and shaken in soapy water for 1 minute. Afterwards, the explants were poured into a strainer and rinsed with tap water for 1 minute.

The explants were then brought into the laminar flow hood and immersed in 70% ethanol for 1 minute followed immediately by immersion and swirling in a beaker of 300 ml of a 10% bleach solution (0.6% sodium hypochlorite) containing 5 drops of Tween® 20 (Croda International PLC, Snaith, UK) for 5 minutes. They were then rinsed 3 times in succession in beakers with 300 ml of sterile water. After the last rinse, each explant was quickly dipped back into 70% ethanol before being placed on a sterile petri dish to dry. As they dried, the top and bottom ends of each explant (less than 1 mm) were trimmed off with a scalpel in order to remove any tissue damaged by the alcohol or bleach during the decontamination process (disinfestation protocol from C. Weber, personal communication, January 20, 2012).

2.4 Initiation

Explants were placed upright in initiation media in Magenta™ GA-7 boxes or baby food jars, 6 explants per Magenta™ box or 5 explants per jar. They were placed in a growth room under cool white florescent lights (85 W bulbs) with a 16h photoperiod, 21° C temperature and 40% humidity. Each Magenta™ box contained 50 ml of media and each baby food jar contained 33 ml of media. The initiation medium consisted of Woody Plant Medium (Lloyd and McCown 1980) supplemented with 1 mg l^{-1} 6-benzylaminopurine (BA), 30 g l^{-1} sucrose and 7 g l^{-1} Phtyoblend agar (Caisson Labs, North Logan, UT) and 0.9 ml l^{-1} of a 10% solution of Rovral 4F (41.6% iprodione

flowable fungicide) (0.37% final iprodione concentration) (Bayer CropScience, Research Triangle Park, NC). The media was brought to a pH of 5.6-5.7 using 1 M NaOH. All media, Magenta™ boxes and jars were autoclaved at 121° C for 20 minutes prior to use. Control explants were placed in the media described above but with no BA added.

After one week, to avoid excreted phenolic compounds, which appeared as a dark cloud around the base of each explant, explants were simply moved from one side of the magenta box or jar to the other into fresh portions of media.

After four weeks of initiation culture, the percentage of explants that had produced shoots 1 cm or longer was determined. Shoots 1 cm or more were long enough for sub-culture (Vieitez et al. 2009) and moved into multiplication media. Explants with shoots less than 1 cm were placed into an elongation medium. Each culture was initiated with no less than 25 original explants and, most often, began with at least 30 original explants.

2.5 Elongation

Explants that did not produce shoots long enough to sub-culture after initiation were placed upright into fresh Woody Plant Media with one of the following hormone concentrations: 1 mg l^{-1} GA_3, 0.5 mg l^{-1} GA_3, 1 mg l^{-1} BA or placed into a medium without hormone and treated with an exogenous application of GA_3. The exogenous treatment was a solution of GA_3 dissolved in sterile water with one drop of Tween® 20. The concentration of GA_3 depended on the experiment, but varied between 40 ppm and 500 ppm. Two drops of this solution were applied to the tip of the bud with a sterilized glass pipet. Percentages of explants with shoots greater than 1 cm were noted at 4 week intervals.

2.6 Multiplication

New shoots longer than 1 cm were cut from the original explant and placed upright in fresh Woody Plant Media containing either 1 mg l^{-1} BA or 0.5 mg l^{-1} BA. After 10 weeks, the number and length of new shoots forming at the base of the sub-cultured shoot were counted and measured. Every bud produced was counted as a new shoot even if it was only 1 mm. Shoots were always moved into fresh media every 4 weeks.

2.7 Stock Plant Treatment

Data was collected on whether pinching the tip of the shoot of the stock plant one week before collecting the explants had any effect on the growth of the nodal explants during initiation. The idea being that pinching the apical bud from the shoot will release the axillary buds from apical dominance and improve bud break and shoot development. Also, data was collected on whether obtaining explants from the first or second flush of growth of the stock plant had any effect on explant response during initiation.

2.8 Statistical Analysis

Statistical analyses were performed using JMP® 9.0 (SAS Institute Inc.). After the initiation and elongation stages, the response rate was measured as shoots greater than or equal to 1 cm. Responsive shoots were counted and logistic regression was used (hit = shoot greater than or equal to 1 cm; miss = shoot less than 1cm). Where actual shoot measurements were taken, linear regression was used and when comparing the number of micro shoots produced per shoot during multiplication, analysis of variance was used.

3. Results

Once the nodal explants had been taken and placed in culture, all species released phenolic compounds due to the cutting injury. Typically, after one week, no more phenolics were released unless another cutting injury occurred such as with multiplication. Shoots that were subcultured (cut from the original nodal explant) and placed in multiplication media very often released phenolic compounds once again and had to be transferred to fresh portions of the media after a few days.

Very little basal callusing occurred on the explants that did not receive any hormone in contrast to those that were placed in media with 1 mg l^{-1} BA. Subcultured shoots did not produce as much callus as original explants; subcultured shoots produced adventitious new buds and shoots from the base.

3.1 Initiation

During the 4 week initiation, BA in the media had a significant positive effect on whether or not the axillary bud of the explant produced a shoot 1 cm or longer. Explants placed in media without hormone produced a swollen axillary bud, but little shoot growth occurred (Table 1; Figures 1, 3, 5, 7 and 9).

Table 1:
Percentage of nodal explants that showed axillary shoot growth 1 cm or greater in media with and without 1mg l^{-1} BA.

Species	1 mg l^{-1} BA (n)	No hormone (n)	Prob > chi-square
Q. bicolor	75.6% (74)	12% (25)	<0.0001
Q. schuettei	58.6% (58)	0% (30)	
Q. robur x bicolor	58.1% (56)	15.9% (69)	<0.0001
Q. muehlenbergii	62.3% (130)	0% (58)	
Q. cerris	37.8% (66)	7.5% (67)	<0.0001

Regarding stock plant treatment, pinching the tip of the stock plant shoot to release the axillary buds from apical dominance did not have a significant effect on the response rates of the explants for *Q. bicolor* and *Q. schuettei* (Table 2). Using the second flush of growth from the stock plant may have an effect on explant performance. For *Q. cerris,* explants taken from the first flush of growth performed much better as compared to explants taken from the second flush (Table 2). For *Q. muehlenbergii* and *Q. schuettei,* using the first or second flush of growth did not have an effect on explant performance (Table 2). Whether it is possible to continue harvesting shoots from the stock plant throughout the season or whether the responsiveness of the explant declines with successive flushes of growth still needs to be determined. This may be an attribute heavily dependent on species and even genotype.

Table 2:
Percentage of nodal explants that showed axillary shoot growth 1 cm or greater taken from stock plants with three different treatments (media contained 1 mg l^{-1} BA).

Species	Pinched stock plant shoot tip (n)	Un-pinched stock plant shoot tip (n)	2nd growth flush, shoot tip pinched (n)	Prob > chi-square
Q. bicolor	75.6% (74)	81.25% (32)		0.5301
Q. schuettei	58.6% (58)	52.4 % (42)	77.3% (22)	0.1359
Q. muehlenbergii	62.3 % (130)		61.1% (18)	
Q. cerris	37.8% (66)		0% (41)	

Contamination was controlled during the initiation of explants for the first cultures taken of the season (Table 3). As the season progressed, cultured appeared to have more contamination issues during the initial stages (Table 4).

Table 3:
Overall contamination rates during initiation of new cultures.

Species	Initial explants	Lost to contamination	Rate
Q. bicolor	184	53	28.8%
Q. schuettei	160	49	30.6%
Q. robur x bicolor	138	13	9.4%
Q. muehlenbergii	273	67	24.5%
Q. cerris	220	11	5%

Table 4:
Overall initial contamination rates based on date of culture establishment.

Species	Culture started in April or May	Culture started in June or July
Q. bicolor	13%	36%
Q. schuettei	0%	27%
Q. robur x bicolor	20%	13%
Q. muehlenbergii	20%	46%
Q. cerris	7%	53%

3.2 Elongation

Explants that did not produce axillary shoots greater than 1 cm during initiation were transferred into elongation media. *Q. cerris* had the greatest number of explants available for elongation treatments after initiation. GA_3 in the growth media did not have an effect on shoot elongation. Overall, it was exposure to BA in the growth media that showed any effect on shoot growth (Tables 5 and 6). More experiments are needed to determine whether exogenous applications of GA_3 have a beneficial effect. For *Q. cerris* a 500 ppm application of GA_3 had a significant effect as compared to only 100 ppm application (Table 7, Figure 14).

Table 5:
Percentage of explants that showed shoot growth 1 cm or greater after 8 weeks elongation treatment (following 4 weeks initiation period in media without BA).

Species	1 mg l^{-1} GA_3 (n)	0.5 mg l^{-1} GA_3 (n)	1 mg l^{-1} BA (n)	GA_3 40 ppm (n)	GA_3 500 ppm (n)	Prob > chi-square
Q. bicolor	9% (11)		40% (10)			0.1234
Q. schuettei	0% (15)	0% (15)				
Q. muehlenbergii	0% (12)	0% (12)				
****Q. cerris***	3.7% (27)	8.3% (24)	50% (10)	5.5% (18)	10% (10)	

Table 6:
Percentage of explants that showed shoot growth 1 cm or greater after 8 weeks elongation treatment (following 4 weeks initiation period in media with 1mg/L BA).

Species	1 mg l^{-1} BA (n)	1 mg l^{-1} GA_3 (n)
Q. schuettei	55.5% (9)	
****Q. cerris***	66.6% (12)	37.5% (8)

*For *Q. cerris*, explants that were placed in media with 1 mg l^{-1} BA, whether during the initiation or elongation phase or both, were significantly more likely to produce elongated shoots, $P < 0.0001$ (Chi-square). See Figures 10 and 11.

Table 7:
Percentage of explants with shoot growth 1 cm or greater 4 weeks after exogenous GA_3 treatment (following 4 weeks initiation in 1 mg l^{-1} BA)

	100 ppm GA_3 (n)	**500 ppm GA_3 (n)**	**Prob > chi-square**
Q. cerris	6.6% (15)	69.23% (13)	0.0007

3.3 Multiplication

During the multiplication phase, the effect of the concentration of BA on shoot length and multiplication rate was examined. Lowering the concentration of BA by half did not have a significant effect on shoot length for *Q. bicolor* or *Q. muehlenbergii* (Table 8). With a larger sample size, decreasing the concentration of BA may have a beneficial effect on the shoot length of *Q. schuettei* and for the multiplication rate of *Q. muehlenbergii* (Tables 8 and 9). The average shoot length achieved for *Q. bicolor* and *Q. schuettei* of 1 cm or greater is satisfactory for further subculture or for transfer to rooting media (Figures 2 and 4).

Q. muehlenbergii had the highest rates of multiplication (Table 9, Figure 8) but the average shoot length was so short that shoots would have been difficult to subculture and may not have survived transfer to rooting media. Lowering the concentration of BA by more than half should be investigated for the multiplication phase.

Table 8:
Effect of BA concentration on shoot length of subcultured shoots after 10-12 weeks multiplication.

Species (n)	BA concentration (mg l^{-1})	Mean shoot length (cm)	± SD (cm)	Prob > F
Q. bicolor				
(16)	0.5	1.04	0.47	NS
(19)	1.0	1.107	0.53	
Q. schuettei				
(12)	0.5	1.25	0.7	0.1915
(12)	1.0	0.97	0.61	
Q. muehlenbergii				
*(16)	1.0	0.68	0.39	
(24)	0.5	0.5	0.51	NS
(23)	1.0	0.53	0.5	

Table 9:
Effect of BA concentration on mean number of shoots produced per subcultured shoot after 10-12 weeks multiplication.

Species (n)	BA concentration (mg l^{-1})	Mean # shoots	Prob > F
Q. bicolor			
(16)	0.5	2.8125	NS
(19)	1.0	2.7368	
Q. schuettei			
(12)	0.5	1.25	
(12)	1.0	2.33	0.3552
Q. robur x bicolor			
(17)	1.0	2.05	
Q. muehlenbergii			
*(16)	1.0	4.25	
(24)	0.5	4.71	0.1252
(23)	1.0	3.58	

*Culture initiated on an earlier date, not included in the statistical analysis. See Figure 8.

4. Discussion

4.1 Initiation and Elongation

Overall, the initiation phases were successful. In media supplemented with 1 mg l^{-1} BA, the number of explants producing elongated shoots was comparable to the results of Vieitez et al. (2009). Vieitez et al. (2009) achieved positive response rates (explants producing shoots ≥ 1 cm) of more than 50% for four genotypes of *Q. bicolor*, for two genotypes of *Q. rubra* and one genotype of *Q. alba*. The response rates for the four genotypes of *Q. bicolor* were 76.2%, 54.1%, 83.1% and 93.8%. Our response rates were 75.6%, 58.6%, 58.1%, 62.3% and 37.8% for *Q. bicolor, Q. schuettei, Q. robur* x *bicolor, Q. muehlenbergii* and *Q. cerris* respectively (Table 1). Their results were achieved on GD medium (Gresshoff and Doy, 1972) supplemented with 0.5 mg l^{-1} BA.

It is clear that across all species, BA is essential for shoot formation and growth. BA had a significant effect on shoot growth for all five species as compared to growth media without hormone (Table 1). Explants placed in media without hormone, typically produced swollen buds at the node that remained green and alive but produced little shoot growth. Some species (*Q. bicolor, Q.* x *schuettei and Q. muehlenbergii*) produced more than one shoot from the node of the explant during the initiation phase (Figures 1 and 7).

Using GA_3 in the media in an attempt to induce shoots to elongate did not produce any beneficial results (Table 5). Further experiments are needed to understand whether exogenous applications of GA_3 have potential to aid in shoot elongation during initiation or multiplication phases. Our results indicate, that along with BA in the media, exogenous GA_3 significantly affected shoot elongation in *Q. cerris*. However, the quality

of the shoot produced for further subculture may be reduced (Figure 14). Amissah and Bassuk (2004) used GA_{4+7} to increase bud break and induce shoot growth on stock plants of *Q. bicolor* and *Q. macrocarpa*. However, in light of the successful initial response rates with BA, the use of GA_3 may not be necessary except in the case of especially slow growing species or genotypes.

4.2 Stabilization and Multiplication

Previous research has typically reduced the concentration of BA for the multiplication stage, as BA is needed for shoot induction but can inhibit shoot elongation (Vieitez et al. 1985, 2009, San Jose 1988). Johnson and Walker found that 0.7 mg l^{-1} BA produced the longest shoots (in contrast to 1.5 mg l^{-1} BA) with acceptable multiplication rates for *Q. lobata*. However, our results showed no significant difference between media with 0.5 mg l^{-1} BA or 1.0 mg l^{-1} BA for shoot length or number of shoots produced during the multiplication phase (Tables 8 and 9). With larger sample sizes and a steeper reduction in BA concentration, significant results may occur. Although Puddephat et al. (1997) did not find any differences in shoot length based on BA concentration after 4 weeks initiation for *Q. robur*. At this point, since the difference in length and quantity of shoots was not significant during the multiplication stage, it would be better to multiply shoots with lower concentrations of BA, as cytokinins can inhibit rooting at later stages of the micropropagation process (Bonga and Aderkas 1992).

As with previous research, the stabilization or multiplication phase was problematic. When shoots from the original explant were subcultured upright in multiplication media, the tip often died and the shoot did not grow further or produce any leaves (Figures 4, 6, 8 and 13). New buds and shoots multiplied and grew from the base of the subcultured shoot, but these too would often arrest in growth and not produce expanded leaves. Even during the initiation stage, new shoots emerging from the explant

would sometimes exhibit tip browning. Therefore, only one subculture and one round of multiplication was achieved.

This is evidence of the episodic and determinate growth characteristic of oaks described in the introduction. Vieitez et al. (2009) also experienced this when shoots were cultured upright. Even with frequent transfers into fresh media, shoot growth arrested and was followed by browning and death. Vengadesan and Pujit (2009) also observed a general decline in shoot vigor when culturing seedling shoots of *Q. rubra*.

Vieitez et al. (2009) attribute their success in overcoming this determinate shoot growth and establishing stable proliferation of shoots with the horizontal orientation of subcultured shoots. Shoots greater than 1 cm from the initiation phase were tipped, stripped of leaves and subcultured in a horizontal position. In this stressful position, vigorous axillary shoots were produced. Interestingly, the apical half of the mother shoot produced more shoots than the basal half and the basal half produced callus. According to Vieitez et al. (2009), the bi-weekly transfer of shoots to fresh media was also beneficial in keeping shoots vigorous (plantlets must absorb nutrients in their immediate vicinity, as they do not yet have roots). Also, removing necrotic parts of the plant tissue may be beneficial, as it will keep the dying tissue from harming the healthy cells with toxic phenolics (Bonga and Aderkas, 1992). Removing the tip of shoots as described above for subculture would accomplish this if any shoot tip necrosis occurred during initiation.

Fully expanded leaves are another measure of shoot health that could have been counted during the initiation and multiplication phases. Although *Q. cerris* had the poorest response rate in terms of shoot length, it produced fully expanded, healthy leaves (Figures 9-13). From the start of the culture in mid-April, this line has appeared to be the most vigorous. The other four species only produced small or scale-like leaves (Figures 1, 3, 5 and 7) and there was an observably greater loss of vigor as these cultures progressed. Vieitez et al. (2009) had similar findings: “when the only cytokinin in the media is BA, *Q. alba* and *Q. bicolor* tended to form elongated shoots with small or scale

like leaves." When the natural cytokinin, zeatin, was used at part of the shoot proliferation medium, shoots were "vigorous with a normal appearance."

Modifying the nutrient content of the basal media may also have a beneficial effect for vigorous shoot development and expanded leaves. In their research on *Q. robur*, Vieitez et al. (1985) tested several different basal media and reported that the macronutrient formula of Heller (1953) in combination with the micronutrients of Murashige and Skoog (1962) with the addition of 1mM $(NH_4)_2SO_4$, as well as Gresshoff and Doy (1972) media, produced cultures with "healthy-looking, large, well formed leaves which exhibited neither flaccid nor necrotic shoots." However, in 2009, Vieitez et al. reported that there was no difference in the quantity or quality of shoots produced on WPM or GD media for *Q. alba* or *Q. bicolor*. At that time, it was the addition of zeatin that was most important for healthy leaves and shoots.

There may be some benefit to trimming callus on shoots. This was tried with *Q. cerris* explants that had been in culture for eight weeks or more and had accumulated a large ball of callus at their base and were not progressing (Figure 12). Once the callus was trimmed with a scalpel, the shoots continued growing (although, because of the cutting injury, phenolics were released and explants had to be transferred out of the phenolics after a few days). It appeared that large amounts of callus were inhibiting the uptake of nutrients and hormones; therefore trimming it may have helped the shoot. This finding is based only on anecdotal evidence.

4.3 Contamination Problems

Contamination was controlled during the initiation phase, but occasionally contamination would appear in a culture after several weeks had passed. This was probably due to contaminants being introduced during a transfer or subculture. But there were occasions where bacterial contamination appeared around the plantlet seemingly out

of nowhere, as if the plant's defenses were no longer able to keep it suppressed. Viss et al. (1991) devised a method for screening explants for bacterial infection, which they have used regularly for micropropagation of *Juglans, Malus* and *Prunus*. The bases of explants are rubbed across a petri dish with bacterial growth medium as shoots are transferred to multiplication media. After a few days incubation time, shoots excised from explants that caused bacterial growth are discarded. This could be beneficial, as bacterial contamination did not necessarily appear directly after initiation. It often appeared many weeks into culture. Sometimes it was possible to "keep ahead" of the bacteria by continuously transferring the explant or shoot to fresh media, but this was time consuming and eventually the shoots were lost anyway.

The first explants taken of the season were the cleanest and fewer were lost to initial contamination (Table 4). Contamination increased as the season progressed. Even though stock plants remained in the cooler until they were needed for culture, they were immediately exposed in the greenhouse to insect, fungal and bacterial populations that increased throughout the spring and summer. Aphids, thrips and fungus gnats were also present in the greenhouse during the summer. Successive flushes of growth from the stock plant may be more susceptible to contamination for this reason. It is imperative to use healthy, vigorous and clean, greenhouse-grown stock plants to aid in controlling contamination.

4.4 Conclusion

Based on previous research, cutting back the stock plant to rejuvenate the shoots is an essential step in preparing the stock plant for obtaining explants. Pinching the tip of the shoot prior to taking explants did not have any effect on initiation. More research is needed to understand whether using the second flush of growth from the stock plant has an effect on explant performance. Explants from second flushes may be harder to

disinfest and not as vigorous. It will be useful to know if shoots can continue to be harvested as explants throughout the growing season.

BA is essential for shoot growth during initiation and multiplication for all five species used in this research. It was also beneficial for inducing elongation in *Q. cerris* and *Q.* x *schuettei*. The length and quantity of shoots produced during multiplication may be positively affected by lowering the concentration of BA and more experiments are needed to determine a beneficial level. GA_3 did not have any effect on shoot growth when added to the media, while exogenous applications of GA_3 may have been helpful when used in conjunction with BA. Overall, *Q. bicolor* had the best initial response rates and the best multiplication rates were achieved with *Q. bicolor* and *Q. muehlenbergii*. Although *Q. cerris* was slow growing, it produced the most leaves and maintained the healthiest looking cultures (Figure 13).

4.5 Future Research

Young (seedling to 5 year old) and healthy stock plants are needed for future micropropagation work. Using only greenhouse grown stock plants is also a huge benefit as they are generally easier to disinfest.

For further research, two different projects could begin to address the problems so far encountered. The first would be to skip the difficult multiplication and stabilization phase all together. Instead, since the initiation phase has been successful, initiating shoots in culture and then subculturing them directly onto rooting media would be worth attempting. Experimenting with beneficial levels of IBA, length of exposure to IBA, using activated charcoal in the rooting media and using etiolation to induce rooting are all treatments to investigate.

This would basically be rooting micro-cuttings in vitro and has the potential to be more successful than the field layering propagation technique. For example, if 50

explants could be taken from the first growth flush of one donor plant of *Q. bicolor* with a response rate of 75%, there would be 37 shoots available for transfer to rooting media. If even only half of these rooted, there would still be 19 clones of one mother plant. The work can be accomplished by one person and would take perhaps 8-12 weeks as opposed to an entire year.

The second project would be to continue with the traditional method of micropropagation. Subculturing shoots in a horizontal position versus a vertical position and adding zeatin to the multiplication media (and perhaps even the initiation media) should be tried. The drawback of zeatin is that it is expensive: $9.50 for 5 grams of BA compared to $795.00 for 1 gram of zeatin (caissonlabs.com, 11/11/12). Taking data on the percentage of shoots with at least 2 fully expanded leaves will also be important as a measure of shoot health. If latent bacterial infection is a continuing problem, then bacterial screening should be incorporated into the protocol.

Overall, using tissue culture as a means for asexually propagating North American white oaks shows promise and warrants further investigation specifically of rooting shoots directly after the initiation phase and tackling the challenges of the multiplication phase.

APPENDIX

WPM Formula (mg/L) as described by Lloyd and McCown (1980)

Ammonium Nitrate	400
Boric Acid	6.2
Calcium Chloride, Anhydrous	72.5
Calcium Nitrate	386
Cupric Sulfate•$5H_2O$	0.25
Na_2 EDTA•$2H_2O$	37.3
Ferrous Sulfate•$7H_2O$	27.85
Magnesium Sulfate, Anhydrous	180.7
Manganese Sulfate•H_2O	22.3
Molybdic Acid (Sodium Salt)•$2H_2O$	0.25
Potassium Phosphate, Monobasic	170
Potassium Sulfate	990
Zinc Sulfate•$7H_2O$	8.6

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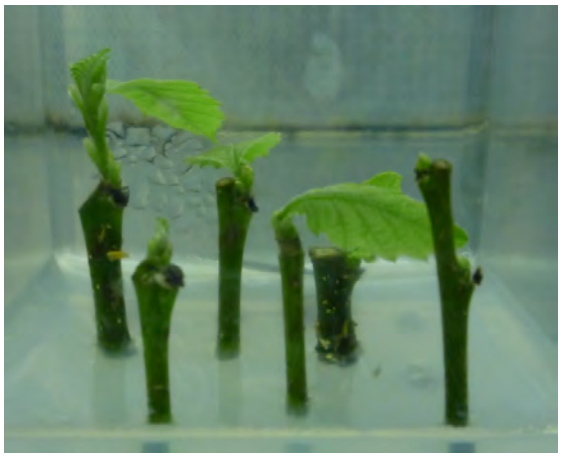
Figure 1.

A) *Q. bicolor* explants after 4 weeks initiation in WPM with 1 mg l^{-1} BA. **B)** *Q. bicolor* in WPM without hormone.

A)  **B)** 

C) *Q. bicolor* culture started July 9 after 4 weeks in WPM with 1 mg l^{-1} BA.

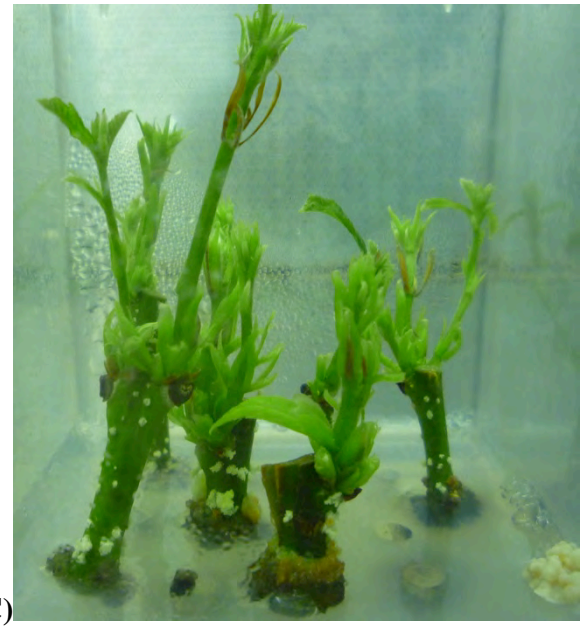
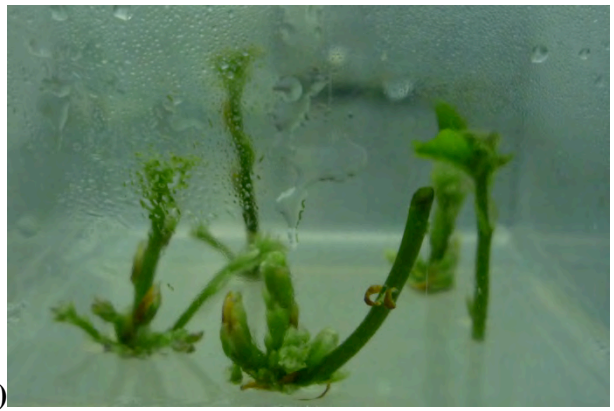
C) 

Figure 2.

A), B) and **C)** *Q. bicolor* shoots subcultured for 6 weeks in multiplication media with 1.0 or 0.5 mg l^{-1} BA.

A)



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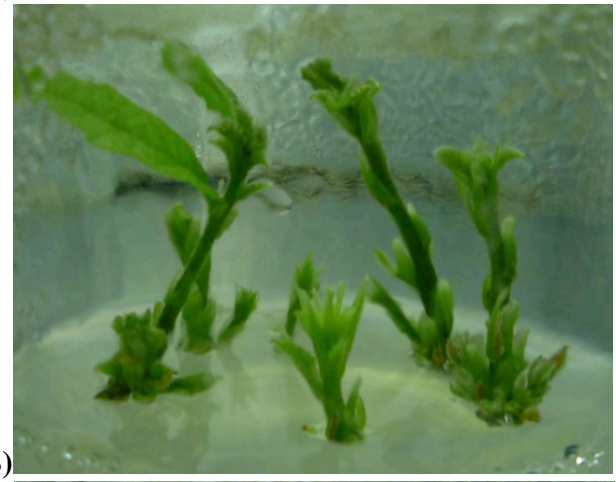


C)

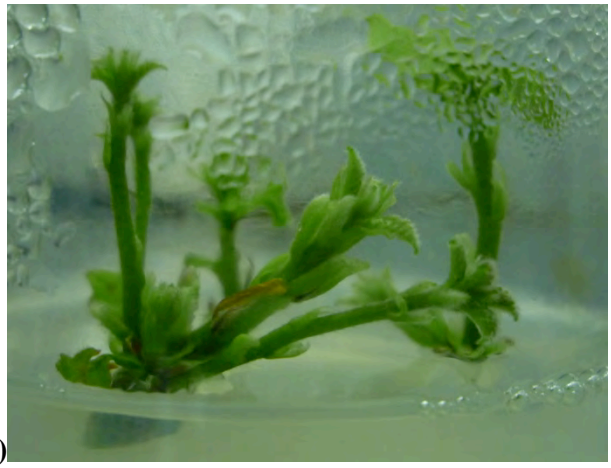


Figure 3.

A) *Q*. x *schuettei* explants after 4 weeks initiation in WPM with 1 mg l^{-1} BA.

A) 

B) *Q*. x *schuettei* explants after 8 weeks in WPM without hormone.

B) 

Figure 4.

A) *Q*. x *schuettei* shoots subcultured for 3 weeks on WPM with 1 mg l^{-1} BA.

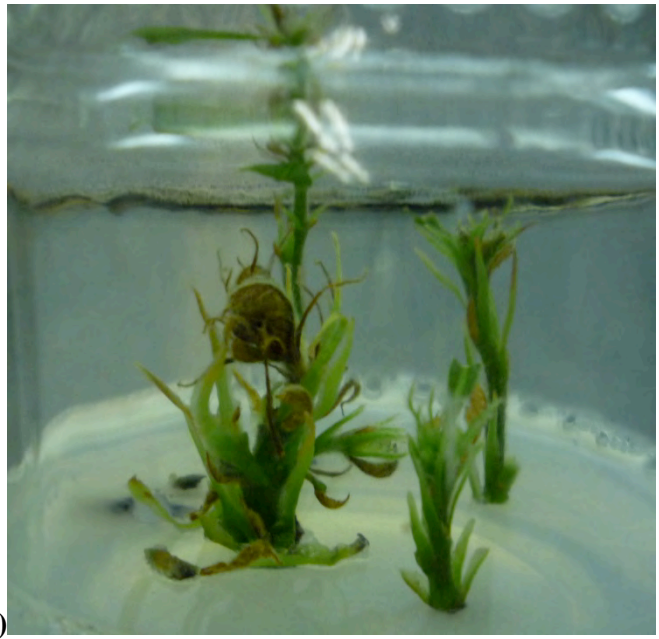
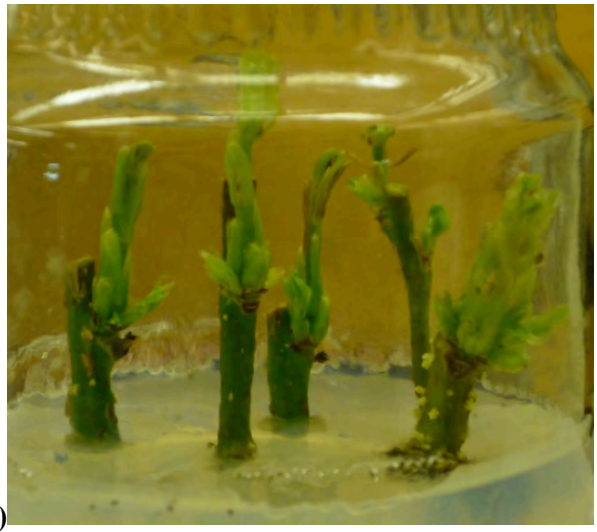
A) 

Figure 5.

A) *Q. robur* x *bicolor* explants after 4 weeks initiation in WPM with 1mg l^{-1} BA. **B)** *Q. robur* x *bicolor* explants in WPM without hormone.

A)



B)

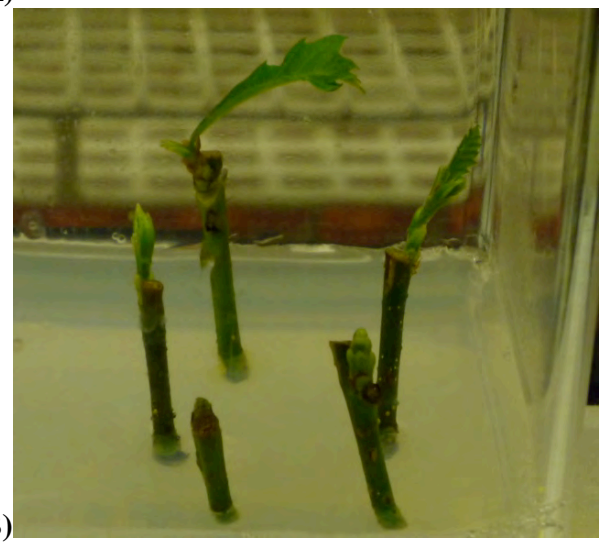
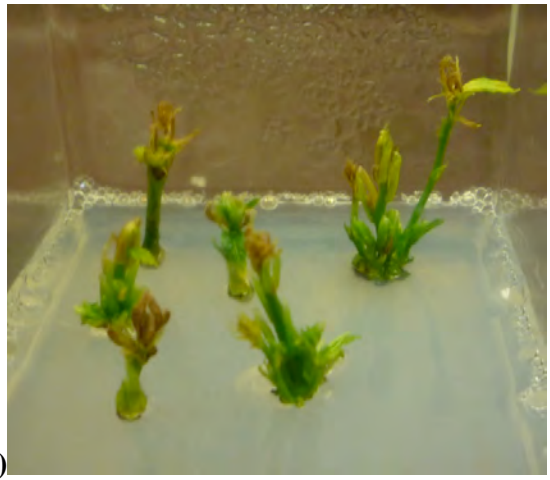
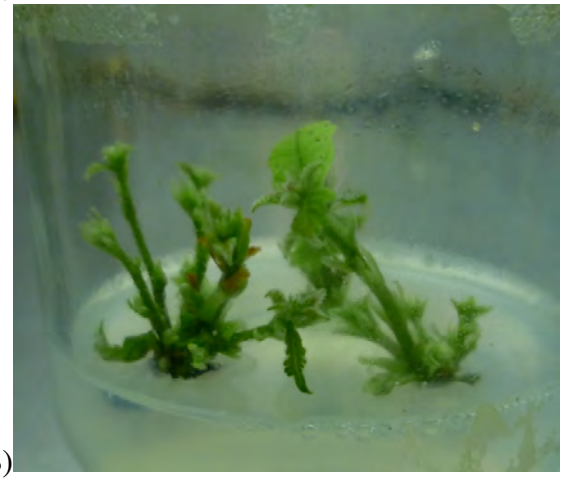
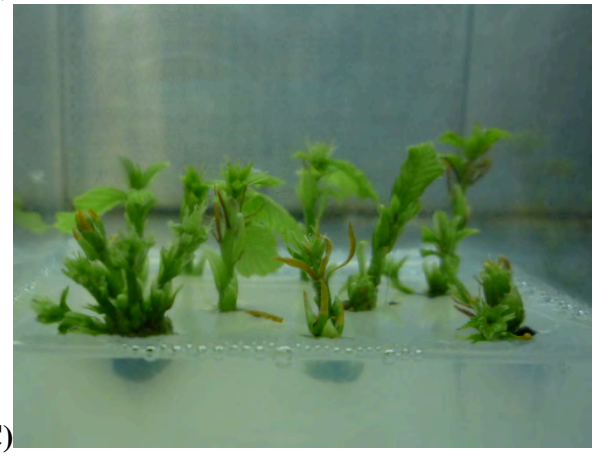


Figure 6.

A), B), C) and **D)** *Q. robur* x *bicolor* shoots subcultured on WPM with 1 mg l^{-1} BA.

A) 

B) 

C) 

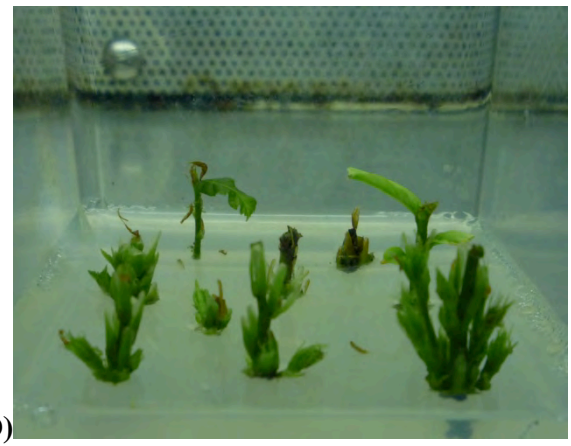
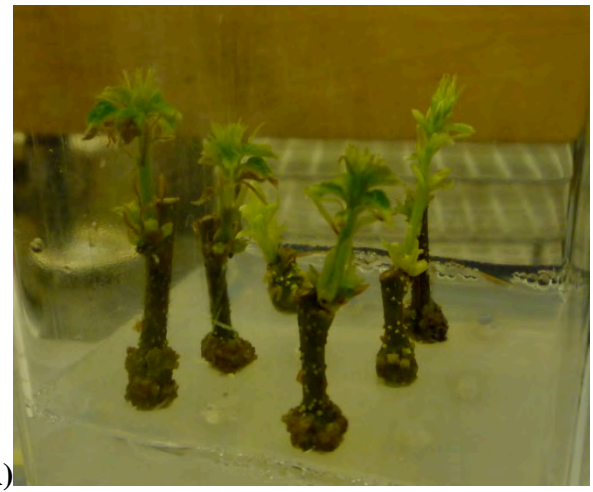
D) 

Figure 7.

A) *Q. muehlenbergii* after 4 weeks initiation on WPM with 1 mg l^{-1} BA, culture started April 27; **B)** culture started May 26

A) 

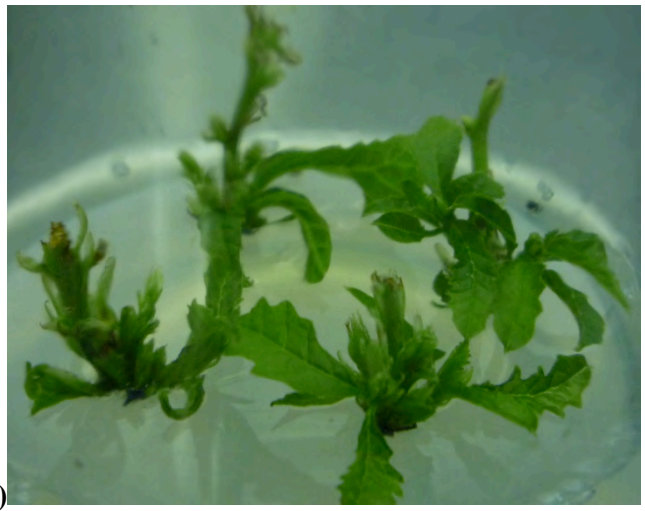
B) 

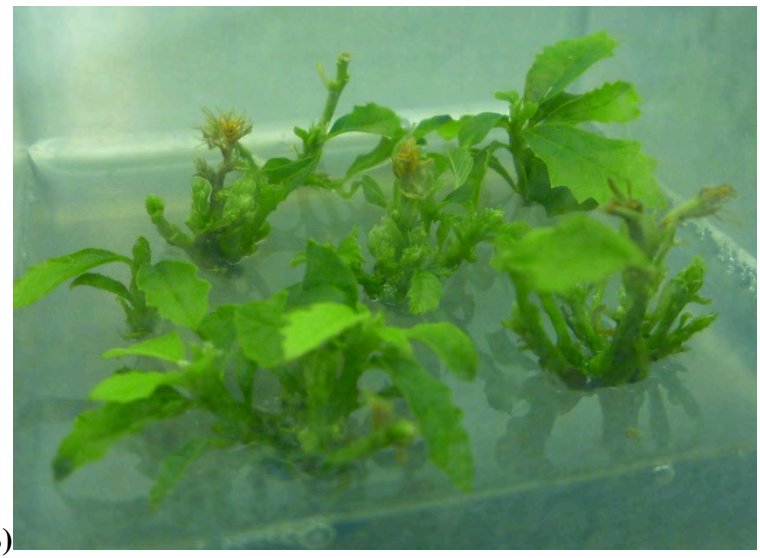
C) *Q. muehlenbergii* after 4 weeks initiation on WPM without hormone.

C) 

Figure 8.

A) and **B)** *Q. muehlenbergii* shoots subcultured on multiplication media after 6 weeks (from culture started April 27).

A) 

B) 

C) *Q. muehlenbergii* shoots subcultured on multiplication media after 6 weeks (from culture started May 26).

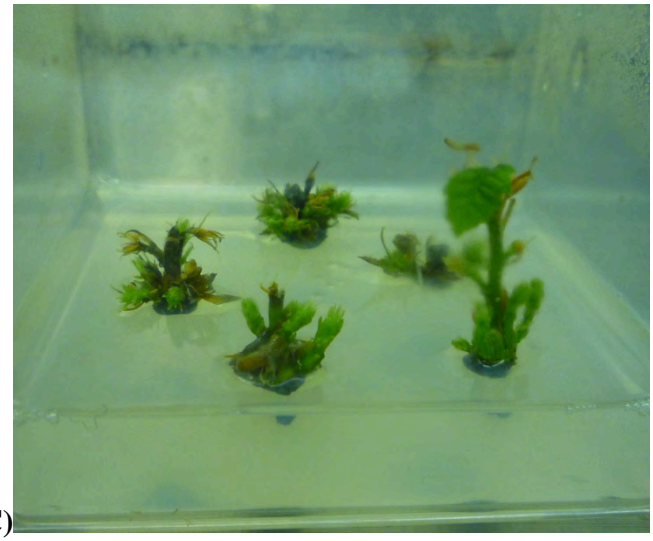
C) 

Figure 9.

A) *Q. cerris* explants in WPM without hormone after 4 weeks initiation; **B)** *Q. cerris* explants in WPM with 1 mg l^{-1} BA after 4 weeks initiation.

A)  **B)** 

Figure 10.

A) Elongation: *Q. cerris* explants 4 weeks after exogenous treatment with 40 ppm GA_3 (after 4 weeks initiation in 1 mg l^{-1} BA); **B)** explants after 4 weeks in media with 1 mg l^{-1} BA (after initiation in 1 mg l^{-1} BA).

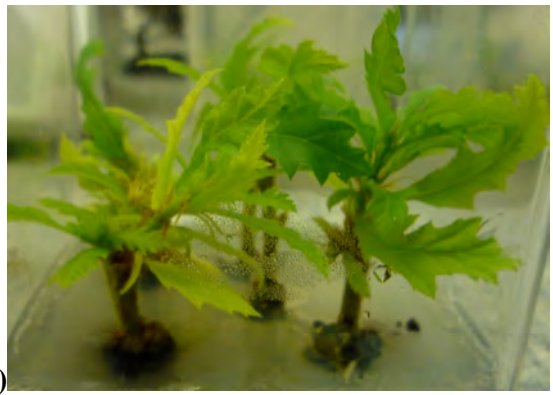
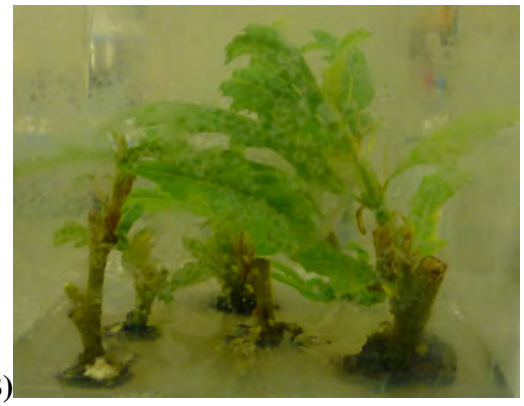
A)  **B)** 

Figure 11.

A) Elongation: *Q. cerris* explants 4 weeks after exogenous treatment with 40 ppm GA_3 (after 4 weeks initiation without hormone); **B)** *Q. cerris* explants after 4 weeks in WPM without hormone (after 4 weeks initiation without hormone).

A) 

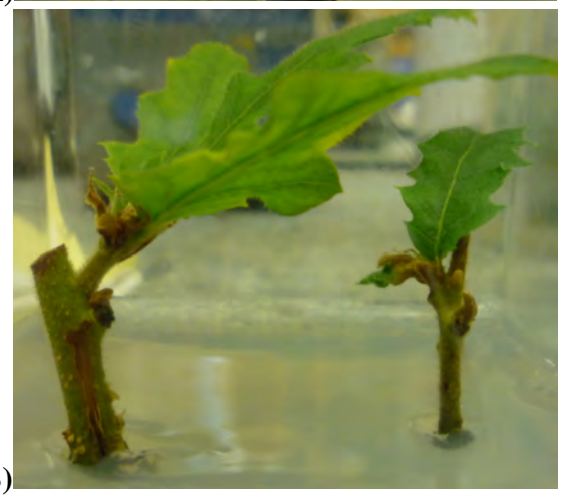
B) 

Figure 12.

A) Callus on *Q. cerris* explants after over 8 weeks in culture.

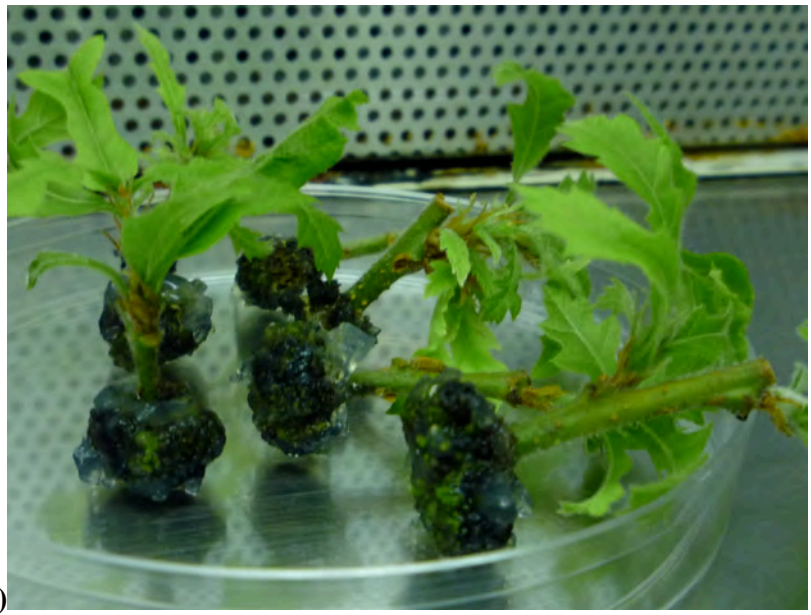
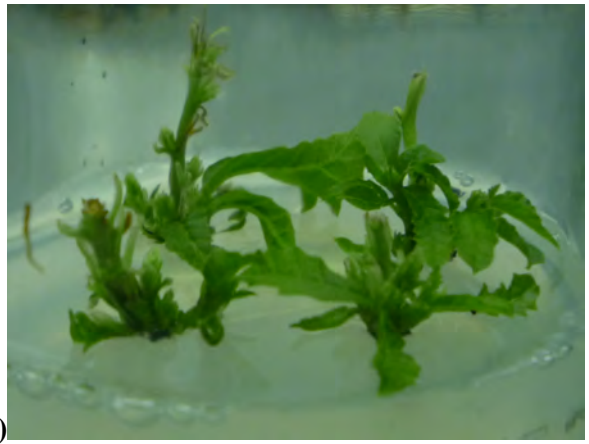
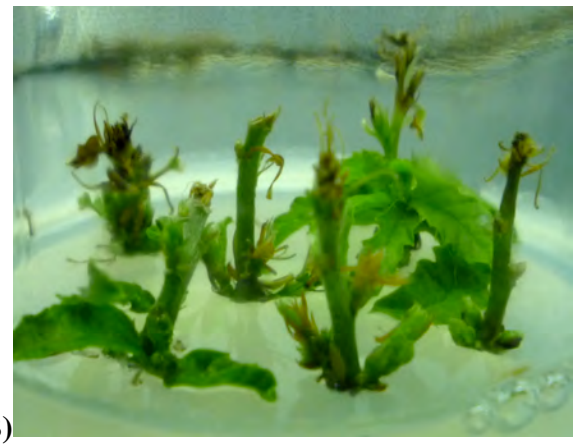
A) 

Figure 13.

A) and **B)** *Q. cerris* shoots 8 weeks after subculturing onto multiplication media; **C)** *Q. cerris* shoots 16 weeks after subculturing onto multiplication media.

A) 

B) 

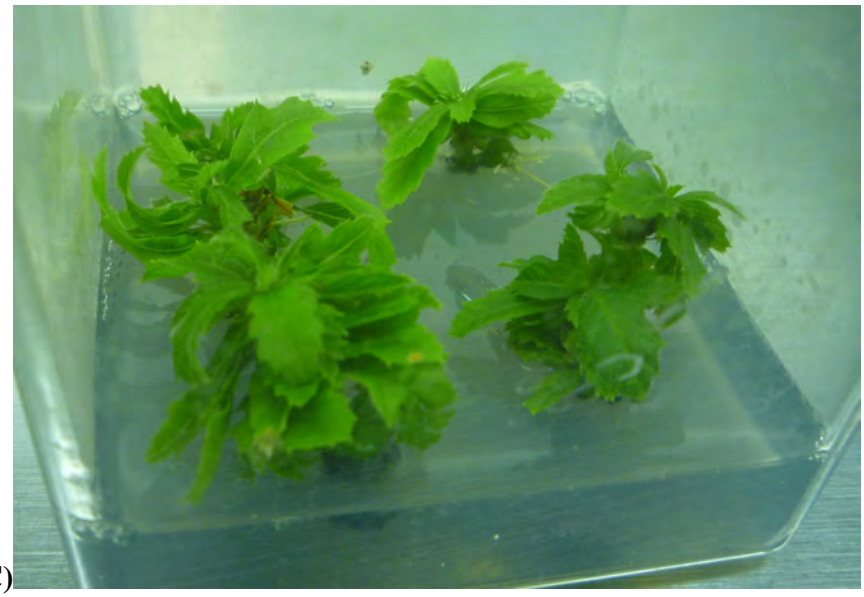
C) 

Figure 14.

A) *Q. cerris* explants 4 weeks after exogenous 500 ppm GA_3 treatment (after 4 weeks initiation in 1 mg/L BA).