LED Light for in Vitro and Ex Vitro Growth of Transgenic American Chestnut (Castanea Dentata)

Tyler Desmarais
SUNY College of Environmental Science and Forestry, tdesmarais7@gmail.com

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LED LIGHT FOR IN VITRO AND EX VITRO GROWTH OF TRANSGENIC

AMERICAN CHESTNUT (CASTANEA DENTATA)

by

Tyler Desmarais

A thesis
Submitted in partial fulfillment
Of the requirements for the
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College of Environmental Science and Forestry
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Department of Environmental and Forest Biology

Approved by:
William A. Powell, Major Professor
Janine DeBaise, Chair, Examining Committee
Melissa Fierke, Department Chair
S. Scott Shannon, Dean, The Graduate School
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Abstract


LED lighting can reduce production costs and improve the quality of micro-cuttings but has not yet been investigated for tissue culture propagation of American chestnut. This study examines the effects of three LED light treatments and one fluorescent control for in vitro shoot elongation and ex vitro rooting of micropropagated, American chestnut. Results showed that all three LED light treatments produced elongated shoots with total leaf surface area and average leaf surface areas equivalent to or greater than those of the fluorescent control. Ex vitro rooting trials showed that TLED treatments produced percent rooting survival and total root length equivalent to the fluorescent control. These findings support the use of broad-spectrum LEDs to replace traditional fluorescent lamps for in vitro elongation and ex vitro rooting of American chestnut and represents an important step in the application of LED lighting technology to the micropropagation of economically significant woody plant species.

Key Words: Micropropagation, Spectral Light, LED, Shoot Elongation, Ex Vitro Rooting, Chestnut

Abbreviations: DRW-Deep Red White, DRWFR- Deep Red White Far-Red, R5B5- 50% DRW and 50% DRWFR, OGH- Old Greenhouse, BAC Biotech Accelerator, TLED- Tissue Culture LED, LED- Light Emitting Diode, MH- Metal Halide, HPS- High Pressure Sodium, SAS- Shade Avoidance Syndrome

T. R. Desmarais
Candidate for the degree of Master of Science, January 2020
William A. Powell, Ph.D.
Department of Environmental Forest Biology
State University of New York College of Environmental Science and Forestry,
Syracuse, New York
History, Traits and Value of American Chestnut (*Castanea dentata*)

The American chestnut (*Castanea dentata*) has often been referred to as a “perfect tree” due to its wide range of qualities that are valuable to both humans and wildlife (Haggas, 2007). It is also a species that is infamous for having been the victim of one of the greatest forest pathogen outbreaks in the history of North America (Anagnostakis, 1978). Prior to the introduction of chestnut blight, the American chestnut was one of the most abundant trees in eastern North America (Tindall et al., 2004), representing about one in every four canopy trees within its range and was a source of significant economic, ecologic and cultural value (Russel, 1987). Due to its many advantages to humans, it is believed that Native Americans had been managing the eastern forest to encourage and favor chestnut trees for thousands of years prior to European colonization (Delcourt and Delcourt, 1997). American chestnut is in the *Fagaceae* family along with oak and beech trees (Kubitzki, 1993). Taking after its large, long lived relatives, American chestnut was sometimes called “the redwood of the east” as old-growth, pre-colonial stands often reached heights of greater than one hundred feet and trunk diameters exceeding six feet across at the base (Burhans and Hebard, 2012). Its life and death and potential rebirth have become an intrinsic part of Appalachian history and the American story (Collins et al., 2018).

Chestnuts are one of many edible nut producing trees native to eastern North America, and their nuts are amongst the most productive, palatable, and consistently bearing (Paillet, 1982). All members of the *Quercus* genus, like chestnuts, produce a nut mast, but these acorns produce biennially and are high in tannins, making them bitter and unpalatable to humans unless they are put through a laborious leaching processing procedure, and even then, they are scarcely palatable (Kubitzki, 1993). In contrast, chestnuts are low in tannins and have a mild, sweet flavor even while still raw and un-processed (Anagnostakis, 1987). The flavor can be further improved through cooking and additional commercial markets can be attained by processing the nuts into value added products such as chestnut flour and beer (Collins et al., 2018). Due to these beneficial nut mast qualities, American chestnut has
the potential to be the most agriculturally significant and commercially viable orchard crop of all native North American nut trees if the effects of chestnut blight could be solved or mitigated (Reed, 1947).

Since most chestnut species readily hybridize with one another, there is great opportunity to utilize the ample genetic material from which plant breeders can develop high yielding, great tasting, commercial, hybrid cultivars (Burnham et al., 1986).

American chestnut wood has several exceptional timber qualities that make it stand out as an ideal species from a timber production standpoint. Like most other members of the Fagaceae family, American chestnut is a tall, straight growing, timber-type tree (Saucier, 1973). In the lumber industry, long, straight lengths of timber are the most valuable as they are more efficiently milled into long, high-value boards and logs than are short, spreading-form trees. Castanea dentata’s Asian cousins Chinese chestnut (C. mollisima) and Japanese chestnut (C. crenata) have more of an orchard-tree form resembling an apple tree more than an oak (Reed, 1947). Asian varieties tend not to grow much taller than forty feet tall and have a more horizontal, spreading form, and often consist of multiple main stems as opposed to a thick central leader (Payne et al., 1983). American chestnut wood is of medium strength (Saucier, 1973). It is not as hard as top-quality wood such as white oak (Quercus alba) and white ash (Fraxanis americana) but is stronger than its weaker relatives such as American beech (Fagus americana) and Asian chestnut species (Kubitzki, 1993).

American chestnut had other beneficial timber qualities in addition to being tall, straight growing and strong. American chestnut was notoriously rot resistant, on levels that rivaled black locust and even pressure treated wood (Haggas, 2007). American chestnut is also a fast-growing species, which is rare for one that is also strong and rot resistant (Saucier, 1973). This fast growth rate combined with the ability to stump sprout after harvest (Tindall et al., 2004) meant that after harvesting a stand of chestnut, foresters would have a much shorter interval before their next cut and harvest, thus improving profitability (Saucier, 1973). Finally, it was abundant and native, so foresters did not need to import
exotic, potentially invasive species for purposes of timber production and did not need to worry about exhausting a rare species (Hepting, 1974). These potential timber and agricultural applications suggest potential justification for the further improvement of asexual chestnut propagation techniques (Haggas, 2007).

**History of Chestnut Blight**

In the late 1800’s, an invasive fungal pathogen (*Cryphonectria parasitica*) was introduced to the United States on Japanese chestnut nursery stock that was being imported to the New York Botanic Garden in New York City (Hepting, 1974). This fungal pathogen had gone undetected until it was discovered in 1904 after being accidentally vectored from its Asian host to infect American chestnuts (Rigling and Prospero, 2018). Asian chestnut varieties such as Japanese chestnut and Chinese chestnut had co-evolved with the blight for thousands of years and had therefore developed natural chestnut blight resistance strategies making blight cankers more of a superficial nuisance than as a serious threat to the fitness of the tree (Berry, 1954). American chestnut, however, evolved in geographic isolation from chestnut blight, and had therefore never developed resistance to the blight, and for this reason, American chestnut was highly susceptible, and as the blight spread, chestnuts began dying by the billions (Anagnostakis, 1987). Chestnut blight attacks the trunks of trees through a process of killing xylem and phloem using the toxin oxalic acid which produces large bark cankers which eventually girdle the tree (Roane et al., 1986). In less than 50 years, American chestnut stands throughout the range of the species from Maine to Georgia had been ravaged by the blight and brought close to extinction (Newhouse, 1990).

Unlike American and European chestnut (*C. sativa*) which show full susceptibility to chestnut blight, Asian chestnut species show degrees of genetic resistance to the pathogen (Berry, 1954). Chestnut blight is native to Asia and has therefore co-evolved with Asian chestnut varieties for
thousands of years (Reed, 1947). This long-term interaction has allowed the Asian chestnut trees and chestnut blight to compete with one another which has, over the course of thousands of years, resulted in the Chinese chestnuts (*C. mollisima*) and Japanese chestnuts (*C. crenata*) developing genetic resistances to the pathogen (Anagnostakis, 1992). This does not mean that Asian varieties are completely immune to the fungus (Newhouse, 1990). Controlled inoculation studies used to measure the resistance of hybrid and Asian species (Anagnostakis, 1992) show that Asian trees still often develop “superficial cankers”, which are minor blight infections which, even though they are rarely virulent enough to girdle the stem, still produce some wounding responses that results in a rough and gnarled bark texture at the site of infection (Burnham, 1981). The two species most discussed with regards to breeding to produce blight resistant chestnut cultivars are Chinese chestnut, and Japanese chestnut (Burnham, 1988).

When trying to develop resistance to newly introduced plant pathogens, a common strategy for plant pathologists is to hybridize the susceptible species with one of its closely related, genetically resistant relatives that are native to the pathogen’s geographic origins (Clapper, 1954) to produce offspring that would have many of the desirable traits of the threatened species combined with the pathogen resistance of its foreign relative (Burnham, 1988). A non-profit organization known as The American Chestnut Foundation began a back-cross breeding program for American chestnut in 1983 with the hopes of producing a B3F3 hybrid of American chestnut and Chinese chestnut that has strong American traits while still retaining Chinese blight resistance (Jaynes and Graves, 1963). While much work has been done over the years and important developments have been made, this strategy for developing a blight resistant American chestnut has several limitations that have slowed down and limited the success of the program (Jaynes and Graves, 1963). American chestnuts have several important genes that code for key American traits that differentiate them from Chinese chestnuts and some of these key genes are ‘diluted out’ in the hybridization process (Clapper, 1954). American trees
have the best tasting nuts of all chestnut species, the best quality timber for woodworking, and are tall and straight forest trees that can compete in a dense forest environment (Haggas, 2007). Chinese Chestnuts on the other hand have less palatable nuts, lower quality timber, and are much shorter and much more prostrate and spreading in their growth form (Payne et al., 1983). In selecting for Chinese blight resistance, many of the genes that code for these beneficial American traits can be bred out during the hybridization process.

Chinese chestnut blight resistance is produced from quantitative genetic resistance (Steiner et al., 2017). This means that there are several different genes that work together to produce blight resistance (Burnham, 1986). If a hybrid does not receive all the resistance genes in a homozygous state, the tree’s resistance to the blight will be reduced (Kubisiak et al., 1997). This makes it difficult to produce B3F3 trees that do not have diluted American traits as well as diluted Chinese blight resistance (Burnham, 1998). While important information has been gained from their years of research, a highly resistant B3F3 is still several years off in the future. However, recent advancements in genetic sequencing and genetic engineering have shown potential to improve the speed and efficacy of this strategy in the future (Steiner et al., 2017).

The American Chestnut Research and Restoration Project at SUNY-ESF used genetic engineering to successfully produce a blight resistant, fully American chestnut (Zhang et al., 2013) without relying on back cross hybridization. The use of biotechnology to mitigate the effects of an introduced pathogen to return a tree species from functional extinction is a novel application of genetic modification in plants and represents an important step forward in the transition of this emerging technology from strictly agricultural purposes to future applications in forestry and threatened species restoration (Maynard et al., 2009). The process used to genetically modify the American chestnut at SUNY-ESF is called Agrobacterium-mediated transformation (Zhang et al., 2013). In this process, a bacterium \( \textit{Agrobacterium tumefaciens} \) is equipped with a plasmid containing the resistance gene which the
bacterium inserts into the plant cell which is then incorporated into genome of the chestnut embryo and undergoes antibiotic marker gene selection and PCR conformation of the transgene (Polin et al., 2006). The single transformed plant cell is then regenerated into shoot cultures which can be multiplied, rooted and acclimatized into several independent plantlets, each of which contain the transgene (Zhang et al., 2013). By changing the genetic make-up of this initial cell and regenerating an entire plant from it, every cell in the plant will contain the transgene and will thus express the blight resistance trait. Further studies have shown that this resistance trait is inherited via sexual reproduction and is present in 50% of the seedlings produced via controlled pollination using transgenic pollen (Newhouse et al., 2014). New emerging gene editing technologies like CRISPR/Cas9 may allow for even more precise genetic engineering techniques for providing genetic resistance to chestnut blight in the future (Belhaj et al., 2015) by increasing expression levels of the transgene, using more efficient promotors or by inserting the transgene adjacent to an existing pathogen defense gene.

Propagating American Chestnut in Tissue Culture

Plant tissue culture is a method of maintaining or growing plant cells and tissue in translucent sterile vessels on plant growth media. This media contains all the essential ingredients required for plant growth and development: water, sugar, macronutrients, micronutrients, vitamins, and plant growth regulators and sometimes gelling agents such as agar or phytagel (Gamborg et al., 1976). The media is then pH amended, heated in an autoclave to thoroughly homogenize the ingredients, dissolve the gelling agents, and sterilize the media so that is not contaminated from incidental exposure to microorganisms which would compete with plantlets for media inside the tissue culture vessel. Even though most of the plant’s energy comes through osmotic imbibition of sucrose in the media, artificial light is often provided to in vitro cultures (Hung et al. 2016). Artificial light is provided to supplement the plant’s energy needs as well as to promote beneficial photomorphogenic growth characteristics (Macedo et al., 2011). Plant tissue culture has many applications ranging from rapid, aseptic, asexual propagation (also
known as micro-propagation), to more involved applications such as genetic transformation (Polin et al. 2006). With regards to general propagation of plants for scientific or horticultural purposes, tissue culture has many benefits and drawbacks that must be considered to determine whether tissue culture is the ideal and most economically viable propagation method to suit your specific need or application (Murashige, 1974). Plant tissue culture is often the most expensive option for plant propagation due to its reliance on skilled labor as well as expensive lab equipment for both in vitro and ex vitro stages of propagation and subsequent acclimatization (Loyd and McCown, 1980). Despite being the most expensive form of plant propagation, growers can sometimes justify the higher cost due to certain opportunities and applications of tissue culture that no other conventional propagation method can achieve. These applications include the establishment of disease-free stock, genetic engineering and long-term preservation of valuable germplasm (Murashige, 1974). Further justifications of employing tissue culture as a plant propagation method for American chestnut are investigated below.

Plant breeders and geneticists are continuously developing new and improved cultivars that have enhanced desirable genetic qualities, and these new cultivars often demand a premium price in the market (Bolar et al., 1998). Orchardists have a strong impetus to plant the most recent and advanced cultivars since their newly established plantings will not come into significant production for several years (De La Rosa et al., 2007). Upon the creation of a new and promising cultivar, the propagation of the new cultivar is often limited by the availability of plant material from which to perform conventional propagation methods such as rooting cuttings, grafting, and layering. Tissue culture provides growers a means of accelerating the rate at which one can propagate new cultivars (Bolar et al., 1998) when tissue availability is limited. Once initiated in vitro, cultures can be rapidly multiplied, turning a single bud into thousands of plantlets in a matter of months (Amin and Jaiswal, 1988). With conventional alternatives, the grower would need to wait several growing seasons for their initial plant to grow large enough to sustainably take cuttings or scion wood without severely impacting the health of the initial stock plant.
Using tissue culture, new cultivars can be rapidly mass produced with the primary limiting factor being the amount of capital which one is willing to invest (Bolar et al., 1998).

All plant species have species-specific physiological and botanical attributes which influence the effectiveness of each conventional propagation method, namely, rooted cuttings, grafting, layering and seed (Vietez et al., 1987). Every species and even every cultivar can have its own degrees of effectiveness for each propagation method. For instance, for in vitro propagation of various Norway Spruce (Picea abies) genotypes, the effects of spectral quality on growth was heavily influenced by cultivar (Latkowska et al., 2000). In general, short-lived, fast growing herbaceous species tend to propagate easily from cuttings while long-lived, slow growing, hardwood species such as walnuts, oaks and chestnuts have low success rates with traditional rooted cuttings (Gonçalves et al., 1998). Hardwood tree species such as walnuts, oaks and chestnuts all propagate readily from seed since their very large seeds contain ample stored energy and nutrients to provide the seedling with vigorous and healthy growth, but this is not a useful propagation characteristic when asexual, clonal propagation methods are needed (Bonga and Aderkas, 1992).

American chestnut is an excellent example of a species which has severe limitations to conventional propagation methods as is difficult to propagate chestnut via rooted cuttings or through layering (Vieitez, et al., 1987). Chestnuts graft well but have the issues of delayed graft incompatibility and the fact that (when grafting with blight resistant scion wood) the rootstock is still susceptible to chestnut blight which would still result in the death of the scion (Craddock and Bassi, 1999). Mature wood is often resistant to rooting, but tissue which is initiated into tissue culture acquires a quality referred to as induced juvenility (Murashige, 1974). This induced juvenility accounts for why tissue culture micro-cuttings root readily while stem cuttings from a mature tree do not (Bonga and Aderkas, 1992). Blight resistant American chestnut does not have any viable conventional propagation methods (Vietetz et al, 1987, Craddock and Bassi, 1999), required genetic engineering (Polin et al., 2006), and has
strong demand for the rapid propagation of a new promising cultivar (Bolar et al., 1998), making it an excellent candidate for tissue culture propagation.

The first generation of transgenic chestnuts are being propagated via plant tissue culture, not only because it was a requirement for initial genetic transformation (Polin et al., 2006), but also because chestnut as a species has proven highly incompatible with conventional asexual propagation methods such as rooted cuttings, layering and grafting (Vieitez et al., 1987). While it is possible to clonally propagate American chestnut via plant tissue culture, this propagation method still has major limitations such as low rooting and acclimatization survival (Oakes et al., 2016). Some species are easier to work with in plant tissue culture than others and like many other hardwood species such as walnut and oak, chestnut has proven to have severe limitations at several stages of in vitro propagation (Kim et al., 1998). Years of propagation methods optimization at SUNY ESF have resulted in a successful in vitro American chestnut production procedure suitable for scientific and research purposes but has yet to reach levels of efficiency that would be considered commercially viable (Oakes et al., 2016).

Tissue culture produced American chestnuts coming out of culture exhibit many classic physiological deficiencies including dysfunctional stomata, lack of leaf cuticle, and absence of root hairs (Aygun and Dumanoglu, 2015). These physiological deficiencies make the plantlets hypersensitive to common plant stresses such as drought stress, waterlogging, and minor pathogen pressure, resulting in high mortality in the greenhouse and field acclimatization stages (Preece and Sutter, 1991). Improving the in vitro propagation efficiency of American chestnut is a critical bottleneck to both the progress of scientific research as well as the eventual restoration of the American chestnut to its native range. Additionally, improvement to propagation efficiency combined with the high potential value and marketability of blight resistant chestnut nursery stock could result in the creation of a new, highly profitable and marketable crop to the commercial nursery, orchard and forestry industries (Oakes et al., 2016).
Background of Horticultural Lighting

Light is made up of electromagnetic radiation in which photons travel as a wave. The “spectrum” of light can be described and categorized by measuring a photon’s wavelength, or the distance between the same points on a periodic wave. The wavelength of a photon is commonly measured in nanometers (nm) and can be used to determine the spectrum or “color” of the light. Less energetic photons have long wavelengths and highly energetic photons have shorter wavelengths. The electromagnetic spectrum can be divided into three main categories of wavelengths which are referred to as a type of light: infrared light (>1000nm), visible light (between 400 and 700 nm), and ultraviolet light (200-300 nm). Photons with wavelengths shorter than that of ultraviolet light (<200 nm) falls into the highly energetic categories of X-rays and Gamma rays, while photons with longer, weaker wavelengths than those of infrared light are microwaves and radio waves. Infrared radiation is not suitable for photosynthesis because it does not contain enough energy for photosynthesis, while ultraviolet radiation contains too much energy and can damage plant tissue. While some work has investigated the role of non-photosynthetically efficient light spectra on secondary metabolite production such as the role of ultraviolet light in cannabinoid production in cannabis (Cannabis sativa) (Zhang and Björn, 2009), the primary spectral quality under consideration for supplemental horticultural lighting are those wavelengths in the visible spectrum. By understanding the nature of light and how it effects plant growth and development, we can manipulate our growing environments to improve overall health, vigor and productivity of our crop.

Different light qualities are used to study the effects of light on plant growth and development and it has been shown that plants exhibit a high degree of physiological, morphological and anatomical changes in response to the spectral quality of their environment (Briggs and Olney, 2001). Plants use light in the visible light spectrum for photosynthesis since it is neither too energetic (UV), nor not energetic enough (infra-red) for powering photosynthesis. For this reason, visible light is also referred to
as photosynthetically active radiation (PAR). White light consists of a balanced combination of all spectra of visible light and can be separated out into its individual colors and wavelengths. Because of this, white light is also referred to as broad-spectrum or composite light (Xu et al., 2019). Each color of light in the visible spectrum has a different degree of photosynthetic efficiency (Van Leperen and Trouwborst, 2012). Plant leaves are commonly green because it is the color of visible light which is least useful for driving photosynthesis and is therefore the spectrum which is least absorbed and most highly reflected (Folta and Maruhnich, 2007). Plants have strongest photomorphological responses and highest photosynthetic efficiency in the red and blue wavelengths of the visible light spectrum (Yorio et al., 2001). The exact ratios of optimal spectra can vary greatly depending on the plant species and depending on the growth phase of the crop (Bellocchi et al., 2001).

Luminous intensity is the measure of the total quantity of light energy striking a surface emitted from a light source. While light spectrum or light quality can be thought of as the “color” of the light, the luminous intensity of a light source can be thought of as it’s “brightness”. It is important to measure and quantify light intensity to provide just the right amount of light for our crop which can vary based on age and propagation phase (Van Lepren and Trouwborst, 2008). There are many units of measure which have been used to measure luminous intensity for horticultural purposes. In the early days of lighting research, brightness was measured in foot-candles, or, the illuminance cast on a surface by a one candle from a source one foot (30.48cm) away. More sophisticated units of measure for photometry have since been created, with the two primary units of measure used in the horticulture industry being Lux and PPFD (Photosynthetic Photon Flux Density). A device used to measure luminous intensity is called photometer and it describes the total amount of light being emitted on a surface per unit of area. Photometers that specifically measure PPFD are called quantum meters. PPFD has become the primary method of measurement used for plant science as it measures the intensity of photosynthetically active radiation (400-700nm) and its unit of measure is μmol/m²/sec.
Light intensity can vary greatly among different environmental conditions. For example, full sunlight at midday has a luminous intensity of around 108,000 Lux or 2000 μmol/m²/sec while sunlight at midday on an overcast day averages only 2,000 Lux or 37 μmol/m²/sec. Each plant species has an optimal luminous intensity depending on their current stage of growth and development or propagation. Chestnuts, for example, are fast growing trees and once they have reached maturity and are well established, grow best in a high light, full sun environment (Haggas, 2007). On the other hand, chestnut seedlings as well as tissue culture propagated chestnuts do not need nearly as much light as mature trees and grow best at lower luminous intensities at these earlier stages of growth and development (Oakes et al. 2016). Other species have specialized as understory plants and prefer to grow in a low-light environment, even when fully mature. They have adapted a slow growth, energy conservation strategy as well as a light spectrum and intensity preferences which make them effective competitors in their specialized, low intensity, reflected green and far-red light niche (Sun et al., 1998).

Sunlight contains photons of light at all electromagnetic radiation energy levels including radio waves, microwaves, infrared radiation, visible light, ultraviolet light, X-rays and gamma rays. When sunlight reaches the earth, it contains approximately 50% infrared light, 40% visible light and 10% ultraviolet light before it is filtered by the atmosphere. Sunlight’s visible spectrum is a highly balanced white light and therefore contains similar levels of violet, blue, green, yellow, orange and red, wave lengths (Fujiwara and Sawada, 2006). Because of its balanced spectrum, plants rarely exhibit light quality-based deficiency symptoms when grown in full sun. Since sunlight is of excellent spectral balance and is available for free in vast quantities, it would seem to be an ideal source of light for horticulture, but sunlight also has detrimental attributes. Sunlight’s limitations as a light source for horticultural and scientific plant growth relates to its highly variable and inconsistent intensity. Sunlight is unreliable, sporadic, difficult to manage and is highly prone to seasonal unavailability. It is also much too high a luminous intensity for tissue culture propagation (Bonga and Aderkas, 1992).
In general, grown outdoors in the presence of full sunlight, plants tend to get more than enough light to grow and survive (Morrow et al., 1989). While a free, full spectrum light source is an important advantage, growing plants outside has disadvantages. Some climates have cold winters with low sunlight which prevents the growth of most crops except during a brief summer growing season. To extend the growing season, or simply to gain better control over the growing environment, horticulturalists have developed ways to insulate plants from temperature extremes by growing crops indoors or in a greenhouse or inside other artificial growth environments. The glazing of these structures shades the plants and induces a light deficit. Supplemental lightning makes up to the difference between, what natural light plants receive in their enclosed growth environment and what they need to thrive (Van Leperen and Trouwborst, 2008).

There are many ways to produce supplemental light, but each have their own limitations with regards to spectral quality, intensity, heat output, bulb and fixture cost, bulb lifespan, electronic control and safety of operation (Morrow, 2008). All supplemental light comes at a cost of electricity, and energy costs can often make or break the profit margin of a greenhouse crop (Bula et al., 1991). Each supplemental lighting strategy not only varies in spectral quality and intensity, but also in efficiency of energy usage (Moe, 1997). The four most common lamp types used to produce supplemental lighting for horticulture are high pressure sodium bulbs (HPS), metal halide bulbs (MH), fluorescents (FL), and light emitting diodes (LEDs). When choosing which type of lighting system to use for a supplemental lighting program, it is important to consider the photosynthetic and photomorphogenic responses your crop will have to the spectrum provided (Morrow, 2008). Each lighting method has advantages and disadvantages and it is important to match your supplemental lighting strategy with both the light quality preferences of your species and the logistical and economic constraints of your plant production business model (Steigerwald et al., 2002).
There are many types of lamps that are used as conventional artificial light sources in the horticulture industry, namely high-pressure sodium, metal halide and fluorescent; each with their own benefits and drawbacks (Van Leperen and Trouwborst, 2008). These differences in suitability to horticultural purposes relate to the lamp’s proficiency in three main categories: spectral quality, electrical efficiency, and cost of the lamp or bulb (Moe, 1997), but also extend to secondary elements such as safety of operation, heat output, suitability to automated control and functional bulb lifespan.

High-pressure sodium (HPS) lamps are a type of high intensity discharge (HID) lamp and are a tried and true means of providing supplemental light in a greenhouse production setting. HPS bulbs output light at a high spectral intensity, but much of the energy used by HPS bulb is given off in the form of heat or as light in the less photosynthetically efficient color spectra of green yellow and orange (Bula et al., 1991). This tendency to produce such high byproduct heat does not lend this technology to vertical or enclosed grow operations such as growth chambers and vertical light racks (Steigerwald et al., 2002). HPS bulbs are low in red and blue light spectra but tend to make up for this deficiency in the sheer quantity of light provided (Morrow et al., 1989). HPS bulbs are more energy efficient than household incandescent bulbs but they are drastically less efficient than LEDs. While they may have high energy costs to operate and short functional bulb lifespans, HPS bulbs are the least expensive bulbs to purchase and have a proven track record for high plant quality in several indoor crops.

Like HPS bulbs, Metal Halides (MH) are another type of high-intensity, gas discharge lamp. The difference is that the quartz arc tube is fortified with additional metal halide compounds such as sodium iodide which improve both the quality of the light and the energy efficiency of the bulb. They are the highest efficiency conventional bulb which is twice as efficient as mercury vapor and five times more efficient than incandescent bulbs (Bula et al., 1991). MH also produces a much more balanced spectrum than that of HPS with higher red and blue light irradiance. The downsides of MH lamps include the inefficiency associated with the fact that they still produce most of their light in the green, yellow,
orange spectra, rendering them far less electrically efficient than LEDs (Heo, et al. 2002). HPS are also among the most expensive bulbs to purchase. Metal halide bulbs also have the added safety drawbacks of being susceptible to exploding towards the end of their lifespan and being comprised of toxic metals (Bula et al., 1991) which become a potential environmental hazard upon disposal.

Fluorescent lights are the current standard option for supplemental lighting for plant tissue culture. Fluorescent lamps run electricity through a mercury vapor tube which causes the metallic compound to fluoresce. Bulbs used for plant growth often contain a phosphor additive which improves the red and blue spectral quality of the bulb. Differing from HPS and MH, fluorescent bulbs are low-intensity discharge and therefore produce light in lower quantity than that of HPS and MH and produce less heat (Moe, 1997). This quality of low byproduct heat allows fluorescent bulbs to be placed in closer proximity to the tissue culture vessels, which allows more shelves per light rack and improves the efficiency of the number of tissue culture plantlets that can be produced per rack in a production room (Van Lepren and Trouwborst, 2008). Tissue culture plants also require much less light than traditional greenhouse crops, so the lower overall spectral intensity of fluorescent bulbs compared to HPS and MH make fluorescent bulbs suitable to indoor vertical growth systems. Fluorescent lamps have a good balance of traits including moderate electrical efficiency, moderate spectral quality with significant irradiance in the red and blue wavelengths. The downsides of fluorescent bulbs include short bulb lifespans, high bulb cost, toxic mercury byproducts upon bulb disposal, and that they produce light of low light intensity and only moderate spectral quality which is not customizable (Moe, 1997).

**Light Emitting Diodes**

Light Emitting Diodes (LEDs) are the most recent technological development in horticultural lighting and produce light in a different electrical process than any of its conventional alternatives (Morrow, 2008). Instead of producing light via heat-based incandescence (HPS), metallic gas-based
fluorescence (fluorescents), or high-intensity gas discharge (MH), LEDs produce light by running electricity through a solid-state semiconductor diode. Light produced by a solid-state process is known as electroluminescence (Steigerwald et al., 2002). This method of producing light has several advantages over its conventional alternatives (Morrow, 2008).

The greatest potential advantage of LEDs over conventional alternatives is in its cost savings through electrical efficiency (Bula et al., 1991). Compared to LEDs, conventional light sources have high electricity consumption and low light output efficiency, and often the light that is produced falls within the less photosynthetically efficient spectra (Heo et al., 2002). LEDs can be customized to reduce the quantity of light produced in the green, yellow, and orange spectra that are less photosynthetically active; thereby reducing unnecessary energy expenses (Sager et al., 1982). By manipulating the spectra of your light source, growers can utilize photomorphogenesis to produce plant growth morphology that suit their production needs (Dougher and Bugbee, 2001). Electroluminescence has the additionally advantageous physical property of producing little byproduct heat compared to conventional alternatives (Morrow et al., 1989). This reduces the heat stress on the plant and allows growers the ability to increase the proximity of plants to the light source allowing more shelves per light rack, and further improving electrical efficiency. Solid-state semiconductors are also very stable compared to gaseous and incandescent light sources which results in LED fixtures having a much longer lifespan than that of its conventional alternatives (Kim et al., 2007). LEDs also offer increased safety to technicians and the environment since they do not produce the toxic metal byproducts associated with fluorescents and do not have the potential to shatter and explode towards the end of their lifespan like metal halides. LEDs are also unique in that they can be connected to electrical control systems allowing growers to dim the brightness of the lamp down to the ideal spectral intensity for the particular growth phase of the crop without adjusting the height of the fixture (Fujiwara and Sawada, 2006). Due to its potential to drastically reduce operating electrical costs, reduce long-term hardware costs, improve control over
spectral quality and intensity, improve safety and even produce superior plant morphology, LED lights represent an important supplemental light source for tissue culture and greenhouse crops (Morrow, 2008).

As with all new and emerging technologies, advancement can take time and often new technologies will not make a substantial break into commercial markets until research and development has progressed to the point where the product breaches a certain economic threshold of effectiveness. Up until recently, the scientific consensus was that LEDs simply had not yet reached this threshold (Heo et al., 2002). Either the long-term energy savings did not justify the short-term high cost of the LED light fixtures or the LED’s limited spectrum produced an inferior quality plant (Morrow, 2008). In recent years, the prices of LED lamps and fixtures have fallen while performance has increased dramatically (Steigwald et al., 2002) with newer fixtures being able to produce at high intensity and at a full spectrum. With its many advantages over conventional alternatives including low operating costs, high control over spectral quality and quantity, low heat-output, long bulb life-span and many improved safety considerations as well as its dwindling historical limitations (high hardware costs and low performance), LEDs show strong promise towards being the optimal choice for supplemental lighting for future horticultural production systems (Moe, 1997), especially for vertical light racks and enclosed growth chambers (Morrow, 2008).

**Light Quality, Photoreceptors and Photomorphogenesis**

Tropisms are the ways by which plants alter their growth patterns in an adaptive response to their environmental conditions (Briggs and Olney, 2001). Gravitropism, for example, is a plant’s natural tendency to grow away from the direction of greatest gravitational force (Chen et al., 1999). Phototropism is the plant growth response to the quality and intensity and even the direction of the source of light. Phototropism is responsible for many clever plant adaptations such as trees ability to change the angle of their leaf orientation over the course of a day to maximize light interception.
A classic example of phototropism is the plant growth phenomena known as etiolation, a plant growth pattern which is induced by a lack of light and results in the plant tissue becoming white and brittle and as the plant changes its growth form by prioritizing rapid growth and cell elongation at the expense of a sturdy physiological structure. Phototropism plays a key role in the physiological development of optimal plant material in an *in vitro* plant production system (Rossi et al., 1993).

Plants rely on photoreceptors to sense their environment to maximize photosynthetic efficiency, maintain their circadian rhythms and respond to competition for light from other plants. These photoreceptors fall into four main families, phytochromes, cryptochromes, phototropin and superchrome (Briggs and Olney, 2001). These photoreceptors respond to environmental conditions to work, sometimes in conjunction and sometimes independently, to produce a cascade of gene transcription and hormonal signals which result in plant growth patterns meant to maximize photosynthetic productivity based on the quantity, quality, duration and direction of light (Stephano et al., 2003). This response pattern to minimize shade competition is referred to as shade avoidance syndrome (SAS) (Keller et al., 2011). Competition from other plants results in red light being filtered out while the far-red light reflects through the canopy producing a low red to far-red ratio in the understory. Photoreceptors sense this change in light quality with rapid photomorphogenic responses favoring shoot elongation, an increase in apical dominance resulting in suppression of axillary buds and reduced leaf expansion to elongate and overcome nearby vegetative competition (Morelli and Ruberti, 2000). Other photoreceptors sense horizontal blue light and trigger genetic expression to direct growth towards gaps that open in the canopy (Ballare, 1999).

The main photoreceptors associated with the SAS response is a family of five chromoproteins called phytochromes (Briggs and Olney, 2001). Of all the members of this group, phytochrome B (phyB) plays the most significant role in photomorphogenesis. PhyB directly controls the amount of two key proteins within the cell, phytochrome-interacting factor 4 and 5 (PIF4 and PIF5). PIF4 and PIF5 influence
gene expression resulting in the production of endogenous auxin as well as signaling components (Hornitschek et al., 2012). Phytochromes can “fold” or change their isometric forms depending on the quality of light they are receiving. PhyB has two forms, the Pr form has optimal photosynthetic efficiency in the red-light spectrum while the Pfr form has optimal photosynthetic efficiency in the far-red light spectrum. The folding or ‘kinking’ of these proteins is a signaling response and is reversible in that phyB proteins will quickly change back and forth between Pr and Pfr forms based on the ratio of red to far-red light the cell receives from its environment. The Pr form of Phytochrome B is sometimes known as the ‘active’ form while the Pfr is called the in-active form. This process is a key biochemical initiation factor to the following cascade of photomorphological responses generated by phyB (Smith, 1995).

Accumulation of Pfr in response to far-red light triggers an increase in production of endogenous auxins in young leaves and in the apical bud. This increased apical dominance suppresses axillary bud break by inducing abscisic acid production in axillary buds. Suppression of axillary bud break has been successful in tomato by applying a 5-minute treatment of far-red light once a day following a 16-hour white light photoperiod (Tucker, 1976). Pfr accumulation can increase the cell elongation rate by up to four times the normal rate and will quickly return to normal elongation rates in the presence of red rich light by conversion of phyB from Pfr back to Pr (Morelli and Ruberti, 2000). An example of this process in action can be shown from a study on the effects of light quality on the morphogenesis of in vitro azorina (Azorina vidalii) where shoots grown under high red, low far-red light ratios produced high axillary bud break and branching (Moreira and Debergh, 1997). This suggests that high red, low far-red light would be useful in improving multiplication rates.

While phyB is the primary photoreceptor in this process, there are many different types of phytochromes, each with their own physical properties and biochemical characteristics and each phytochrome has different roles in photomorphogenesis due to the differences in their resulting genetic expression (Whitelam and Halliday, 1999). Genetic evidence from Arabidopsis suggests that each
member of the phytochrome family have biochemically different signaling pathways (Quail, 1998). While the phytochrome family contains five members ranging from phytochrome A (phyA) to phytochrome E (phyE), phyB and phyA have by far the most significant effects in overall photomorphogenesis (Hiltbrunner et al., 2006). While phyB plays a key role in the classical red/far-red reversible response, phyA is the critical photoreceptor for signaling in the far-red high-irradiance response (Smith 1995). PhyA modulates gene expression by a mechanism called nuclear accumulation where the photoreceptor is translocated from the cytoplasm into the nucleus (Fankhauser and Chen, 2008). In addition to its role in sensing red/far-red ratios, PhyA has also been found to play a role in blue-light responses (Lin, 2000).

Phytochromes interact, not only with light signals, but also with plant hormones and endogenous growth regulators (Franklin, 2008). For instance, phyB has been shown to be kept active by the interaction between red light and a cytokinin receptor known as ARR4 (Fankhauser, 2002). Shade avoidance also relies on interactions between ethylene and phytochrome to produce gibberellin triggered elongation responses. Ethylene de-activates DELLA repressor proteins allowing gibberellic acid to induce cell elongation (Pierik et al., 2004). The effects of photoreceptors on photomorphological responses can also be affected by and sometimes overcome by exogenous plant growth regulators in tissue culture media. In vitro propagation of rhododendron (Rhododendron spp.) grown in cytokinin limiting media produced results in which red light produced longer shoots than those cultured under white and blue light, but when grown on media where cytokinin was not limiting, there was no significant difference between the red, white and blue light treatments (Norton et al., 1988). Additionally, in a study of the photomorphogenic effects of light spectra on in vitro rooting of Prunus sp., shoots were rooted more rapidly and without rooting hormone under red light, while those rooted under white, blue or far-red rooted more slowly and required a 0.5μM NAA treatment to achieve similar in vitro rooting success (Rossi et al., 1993).
Cryptochromes are blue and UV-A light photoreceptor chromoproteins that functionally act as sensors of light quantity and affect several aspects of photomorphotropism (Briggs and Olney, 2001). Cryptochromes are activated by blue light similarly to how Pr and Pfr are activated and deactivated by red and far-red light respectively. The two main cryptochromes are cryptochrome 1 (CRY1) and cryptochrome 2 (Cry2). Another photoreceptor called phototropin is blue light activated and plays a role in activating cryptochromes further down the signal transduction pathway and mediates phototropism from blue light (Lin, 2000). Photomorphological effects of cryptochrome and phototropin activation include opening and closing of stomata, elongation responses and initiating gene transcription normally resulting in growth towards a light source (Briggs and Huala, 1999). In a study of soybean (Glycine max) showed that plants grown under a blue-light deficient spectrum exhibited similar physiological characteristics as the treatment that was grown under heavy shade (Britz and Sager, 1990). In in vitro cultured grape (Vitis sp.), shoot length was increased under blue-light treatments (Chée, 1986). Cryptochromes also play a critical role in maintaining circadian rhythms (Cashmore et al., 1999).

By creating Cry1 and Cry2 overexpressing and Cry1 and Cry2 deleted mutants, the primary effects of each cryptochrome protein was identified. Cry1 was found to play essential roles in initiating cell elongation responses such as hypocotyl elongation, cotyledon expansion, petiole elongation, flower stem elongation, leaf expansion and blue light suppression of stem growth. Cry2 also plays a role in blue light suppression of stem growth, and hypocotyl elongation and cotyledon opening, but is most notable for its role in photoperiodic timing and maintenance of circadian rhythms (Briggs and Huala, 1999). Cryptochromes and phototropins also act as critical photoreceptors for the opening and closing of stomata. Stomates open and close based on the amount of K⁺ salts in the stomatal guard cells. Blue-light activates the H⁺ pump mechanisms within the cell needed for this K⁺ accumulation (Kinoshita and Shimazaki, 1999). In broad bean (Vicia faba), a 30 second blue-light pulse was found to open stomates while a 30 second green light pulse treatment closed them (Frechillia et al., 2000).
Green light has historically been associated with the photomorphological response of plants slowing down or stopping plant growth due to its deactivation of cryptochromes, shifting them from their semireduced active form to their fully reduced, inactive form (Sellaro et al., 2010). Green light also falls within one of the lowest categories of photosynthetic efficiency as chlorophyll has very limited green light absorption (Folta and Maruhnich, 2007) and has been considered of low importance to incorporate into LED light systems, especially considering the low electrical efficiency of producing green light using LEDs compared to red and blue light (Snowden et al. 2016). Green light has also been found to produce shade avoidance symptoms such as orienting leaves upward and inducing petiole elongation (Zhang et al., 2011). These two assumptions have been reconsidered as the discovery of a green light photoreceptor called phycoerythrobilin was found to re-direct its green light excitation energy to chlorophyll molecules functionally turning green light into red light (Sun et al. 1998). When leaves become saturated with red and blue light in the upper layers of cells, green light filters through and causes excitation in the lower cells further driving the photosynthetic process (Lin et al., 2013). Green light’s tendency to filter through the canopy and drive secondary photosynthesis in lower light conditions could make it an important spectrum of light for plants in the forest understory (Folta and Maruhnich, 2007).

Effects of Spectral Quality on In Vitro Plant Growth

Photomorphogenic responses to light quality can vary significantly between species as well as among different genotypes within a species. Embryos of several cultivars of Norway spruce (Picea abies) were cultured under the same light spectra but yielded significantly different somatic embryo proliferation depending on genotype (Latkowska et al., 2000). Because of this, in addition to applying general patterns presented by the function of photoreceptors, it is important to investigate the specific effects of photomorphological responses to light spectra on individual species. One of the most effective means of testing the effects of different light spectra on individual species is to provide artificial light.
sources with colored cellulose acetate filters to remove a spectrum and assess the resulting photomorphogenic response (Noè et al., 1998). In *in vitro* growth of Dianthus (*Dianthus sp.*), use of colored cellulose light filters showed that red light encouraged growth and extension of axillary buds while blue light produced significant reductions in chlorophyll levels as well as shoot elongation (Marks and Simpson, 1999). Another study of *in vitro* propagation of blueberry (*Vaccinium corymbosum*) showed that filtering out red light produced a marked reduction in multiplication rates while filtering out blue light produced significant leaf and shoot elongation (Noè et al., 1998). Other than using colored cellulose light filters to remove certain light spectra, another means of observing and quantifying the photomorphotropic responses of certain light spectra is to use ‘superbright’ red and blue LEDs. *Cymbidium* plantlets cultured *in vitro* under superbright red light produced leaves with higher total surface area but lower total chlorophyll content than those produced under superbright blue LEDs (Tanaka et al., 1998). In a separate study of *in vitro* culture of blueberry, 4 different narrow spectrum light treatments and one broad spectrum white light treatment (100% R, 80% R + 20% B, 50% R + 50% B, 100% B and a cool-white fluorescent control) were applied to two different blueberry genotypes. In both cultivars tested, the highest average leaf areas produced were from the 100% red light treatments. These two examples show the important role red light plays in inducing axillary branching and leaf expansion in blueberry (Hung et al., 2016).

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Chapter II

LED light for *in vitro* and *ex vitro* growth of American chestnut
Introduction and Hypothesis

LED lights for plant growth offer many potential advantages over conventional lighting alternatives, mainly in cost savings from lower electricity costs as well as the ability to control the light’s spectral quality to direct plant growth to achieve certain photomorphogenic responses (Bula et al., 1991). However, being a relatively new technology, LEDs have not always produced plants of equivalent quality to those cultured under conventional supplemental light sources (Morrow, 2008). As with many emerging technologies, advancements in LED technology have been exponential over the past several years, and LED technology may have reached a threshold where it can produce a comparable, if not superior plant at a fraction of the energy cost (Bula et al., 1991). Considering the increasing volume of tissue culture propagated American chestnut for research purposes as well as the future commercial propagation of this ecologically and economically valuable species, investigation of the economic viability of LED alternatives to conventional lighting sources is warranted (Oakes et al., 2016).

Tissue culture plants get most of their energy, not through photosynthesis, but from osmotic absorption of sugars from the tissue culture media (Murashige, 1974). Therefore, the purpose of this study is less focused on the light spectrum’s influence on photosynthetic efficiency and more towards the tissue’s physiological, photomorphogenic responses to the light spectra to which they are exposed. For example, some ideal plantlet qualities that predict successful rooting and acclimatization include thick, long stems with large and broad leaves (Hung et al., 2016). Certain light spectra can be used to affect in vitro photomorphotropism to produce ideal micro-cutting characteristics such as high chlorophyll content and enhanced leaf surface area (Sæbø et al., 1995). Additionally, different stages of tissue culture propagation may call for differing light spectrums and light intensities. For example, the spectral quality that promotes ideal plant growth responses in the multiplication phase may not also be optimal for plants in the elongation or rooting phase (Rossi et al., 1993).
Prior to the development of TLED lights, all LED lights systems have worked with electrical ballasts and fixtures that differed so significantly from common, legacy lighting equipment that plant propagators would be required to purchase entirely new systems of light fixtures and ballasts in order to convert from conventional to LED based lighting systems (Morrow, 2008). A recent technology known as TLED light bulbs were introduced to the horticulture industry which use LED technology in a bulb that is designed to be inserted directly into existing T8 fluorescent light fixtures. This technology has the potential to allow plant propagators to transition from less electricity efficient fluorescent bulbs to LED bulbs with a significantly reduced overhead hardware cost.

Even if LED light treatments produce plants which are not significantly higher or lower quality than those produced using legacy methods, transitioning to LEDs could still be justified as reduction in electricity costs would eventually cover the initial hardware cost associated with purchasing the TLED bulbs (Bula et al., 1991). Ideally, the fact that LEDs give growers the ability to provide specific spectral qualities and intensities tailored to each phase of production helps maintain optimal growth conditions for all phases of production resulting in plants with improved photomorphological characteristics while simultaneously reducing plant production costs (Morrow, 2008). This is the first known publication of the investing LED light spectral quality and intensity for optimizing in vitro elongation and ex vitro rooting of American chestnut. In this study, we will determine how well American chestnut grows under three different LED spectra as compared to a standard cool-white fluorescent control by testing the following hypothesis:

My hypothesis for the spectral quality elongation experiment is that all TLED treatments will produce increased leaf surface area compared to the fluorescent control due to their higher output of red, blue and green light. Therefore, if apical shoot tips of American chestnut are incubated in vitro under three different full spectrum TLED light treatments DRWFR (High far-red), ½ DRW and ½ DRWFR (medium far-red), DRW (No far-red) and a cool-white fluorescent control (Figure 14), all three TLED
treatments will produce shoots with estimated total leaf surface area and average leaf surface area greater than or equal to that of the fluorescent control. Due to the pronounced effect that high far-red : low red light ratio has on antagonism to leaf expansion (Morelli and Ruberti, 2000), I also hypothesize that the highest total leaf surface area and average leaf size amongst all treatments will be the no far-red light, DRW TLED treatment.

Since the use of full-spectrum LEDs have been shown to promote increased plant growth and photosynthetic efficiency compared to fluorescent bulbs for ex vitro rooting of micro-cuttings in the past (Xu et al., 2019), my hypothesis for the spectral quality rooting experiment is that all TLED treatments will produce micro-cuttings with improved root quality compared to the fluorescent control. Therefore, if ex vitro rooted American chestnut micro-cuttings are incubated under three different full spectrum TLED light treatments DRWFR (High far-red), ½ DRW and ½ DRWFR (medium far-red), DRW (No far-red) and a cool-white fluorescent control (Figure 14), all three TLED treatments will produce shoots with % survival and total root length greater than or equal to that of the fluorescent control. I also hypothesize that of all the TLED treatments, the DRW (low far-red) treatment will have the lowest % survival and total root length while the DRWFR should have the highest % survival and total root length because of the strong apical dominance induced by the treatment’s high far-red : red light ratio and its subsequent increase in endogenous auxins (Morelli and Ruberti, 2000).

In addition to spectral quality, spectral intensity strongly influences plant growth and development in tissue culture multiplication and elongation (Alverenga et al., 2015). Spectral intensities of between 30 and 50 μmol/m²/sec have been found to be successful for the in vitro incubation of woody species (Bonga and Aderkas, 1992) while higher tissue culture light intensities between 80 and 100 μmol/m²/sec have been shown to be less effective for incubating woody species and more suitable for herbaceous species (Wang et al., 2009). Due to increased tissue culture growth rates of woody species incubated under low light intensities, I hypothesize for the LED light intensity experiment that
lower light intensity treatments will produce American chestnut micro-cuttings with greater leaf surface area than higher light intensity treatments. Therefore, if apical shoot tips of American chestnut are incubated in vitro under three different full spectrum TLED light intensities, a 2 bulb Arize™ treatment, 3 bulb Arize™ treatment, a 4 bulb Arize™ treatment, and a 2 bulb cool-white fluorescent control (Figure 15), the 2 bulb Arize™ treatment and the 2 bulb fluorescent treatment will produce shoots with the total leaf surface area and average leaf surface area greater than that of the 3 bulb Arize™ and 4 bulb Arize™ treatments. I also hypothesize that the 2 bulb Arize™ treatment will produce micro-cuttings with total leaf surface area and average leaf surface area equal to or greater than the fluorescent control.

**Plant Materials, Culture Media and Vessels**

The plant tissue used in the following experiments was acquired from previously initiated and established shoot cultures of the SUNY ESF American Chestnut Research & Restoration Project lab. These original cultures (described below) were multiplied and sub-cultured to produce a large volume of highly uniform vigorous tissue to conduct the following experiments. Incubation of all plant cultures prior to the experiment were grown for six-week sub-culture cycles on the light racks of the plant tissue culture laboratory in Marshall Hall at the State University of New York college of Environmental Science and Forestry (SUNY-ESF) and were grown in American Chestnut Multiplication (ACM) tissue culture media (described in Appendix II). Large quantities of apical shoot tips and elongated shoots were produced for each repetition of the elongation and rooting experiments to reduce the effect of tissue quality on experimental results. With abundant, high quality tissue available for each experiment, tissue selection could be highly selective ensuring a high degree of tissue quality and uniformity going into each experiment (Figure 1).
**Figure 1.** Examples of apical shoot tips of high quality and uniformity (Left) and elongated micro-cuttings ready for *ex vitro* rooting (Right).

Triple node stem segments approximately 1 cm in length and 1mm in stem diameter from *in vitro* shoot cultures of *C. dentata* underwent one subculture cycle with a duration of six weeks on American Chestnut Multiplication media (Appendix II). Stem segments were cultured on ACM media to initiate axillary bud break to produce new apical shoot tips from each of the stem’s axillary buds. After one, six-week subculture cycle, 1 cm long apical shoot tips were harvested and transferred to elongation media (Oakes et al., 2016). All transfers were conducted in sterile laminar flow hoods in the Marshall Hall tissue culture lab.

Apical shoot tips harvested from the cubes of ACM media and were transferred to the second subculture cycle on Chinese Chestnut Pre-Rooting (CCPR) media (Appendix II) for shoot elongation. After one subculture cycle in CCPR, apical shoots had fully elongated to a length of between 7 and 8 cm. Shoots were then harvested for *ex vitro* rooting into Jiffy Pellets™ or were recycled for further
multiplication into 1 cm axillary bud stem segments in ACM media. All transfers were conducted in sterile laminar flow hoods in the Marshall Hall tissue culture lab.

The transformed genotypes or ‘events’ used for the LED lighting experiments were LM-B4SX58 and LM-B4SX54. Ellis #1 is the cultivar name of a clonal line of pure American chestnut established in somatic embryo tissue culture from zygotic embryos of American chestnut seed (Polin et al., 2006) and is the un-transformed base of the LM-B4SX58, LM-B4SX54 events. Embryos from Ellis #1 were multiplied to produce many identical somatic embryos, and these embryos were transformed via Agrobacterium-mediated transformation to contain an oxalate oxidase gene, providing the resulting events with genetic resistance to chestnut blight (Zhang et al., 2013). The two lead events produced from this transformation were LM-B4SX58 and LM-B4SX54. These events both contained only one insertion of the gene of interest and showed similar, high levels of expression. The first replication of the LED light spectrum experiment for both rooting and elongation utilized all tissue from LM-B4SX58. The second replications used an even split between LM-B4SX54 and Ellis #1 and the third replications used exclusively Ellis #1 tissue. Tissue used for the LED Light intensity experiment used exclusively Ellis #1 tissue. All three events are clonally identical except for the gene of interest inserted exhibit similar growth rates and morphology *in vitro* (Oakes et al., 2016).

The culture vessels used for the following experiments were standard cube shaped plant tissue culture multiplication vessels known by the trade name Magenta™ cubes. These Magenta™ cube vessels come in two sizes, tall and short. Tall vessels are 9.5 cm tall and have a total volume of 300 mL while short magenta cubes have a height of 7.5 cm and a total volume of 250 mL. For the following experiments, the tall, 300 mL Magenta™ cube vessels were used for both multiplication and elongation *in vitro* production stages (Figure 2). Approximately 80 mL of media was added to each vessel and chestnut tissue spent six weeks in each sub-culture cycle. The lid of each vessel contains a 1 cm
diameter, round micro-filter air ventilation disk which facilitates passive sterile gas exchange primarily for allowing ethylene to exit the vessel (Biddington, 1992).

Rooted shoots were incubated in tall, cylindrical, translucent rooting vessels 12 cm in height and 10 cm in diameter. These containers did not have vented lids and were fully sealed to retain 100% relative humidity within the vessel to minimize desiccation during rooting and acclimatization (Figure 2). Containers were large enough to contain 3 Jiffy Pellets™ with each pellet containing one micro-cutting. These vessels were chosen over our traditional, larger rooting vessels used for general plant production (which can enclose 32 Jiffy Pellets™ per vessel) to increase the number of experimental units to which each light treatment was applied.

**Figure 2.** Examples of multiplication and rooting vessels used. Tall Magenta™ cube vessels used for multiplication and elongation (left) and tall cylindrical, translucent rooting vessels (right).

Both chestnut tissue culture media types, ACM and CCPR (Appendix II), contain the same quantities of macronutrients, micronutrients, vitamins, sugar and agar. Their recipes only differ in the
amount of plant growth regulators added. ACM media, used primarily for tissue multiplication, contains high BA to IBA ratios which promotes axillary bud break resulting in large quantities of new apical shoot tips (Oakes et al., 2016). These apical shoot tips were then harvested and transferred to fresh elongation media. CCPR elongation media has a balanced BA to IBA ratio to encourage stem and leaf elongation, both of which are important factors in producing high quality micro-cuttings (Hung et al., 2016). Both media use Woody Plant Medium (WPM) as the macronutrient source and are supplemented with Nitch and Nitch (N&N) vitamins. Each liter of ACM is supplemented with 0.5 mL of 1 millimolar concentration 6-Benzylaminopurine (BA), a cytokinin plant growth regulator and 5 µL of 1 millimolar concentration of Indole-3-butyric acid (IBA), an auxin plant growth regulator. Each liter of CCPR media is supplemented with 0.25 mL of 1 millimolar concentration BA and 0.25 mL of 1 millimolar concentration of IBA. Both medias were adjusted to a pH of 5.5. 30 g/L of lab-grade sucrose was added as well as 7 g/L of agar gelling agent and was then sterilized by autoclaving at 121°C for 20 minutes at 105 kPa.

Description of Incubation Environments

For both multiplication and elongation steps required prior to initiation of each experiment, shoot cultures were incubated on standard plant tissue culture light racks for 6 weeks per subculture cycle at 22°C under cool-white fluorescent bulbs with a 16-hour photoperiod. Distance from light fixture to plant tissue culture vessel height can strongly influence the light intensity at the level the plants are exposed to it. The distance from light fixture to plant tissue culture vessel of each shelf were uniformized to eliminate this as a variable (Figure 3). The bulbs within the light fixtures are hung 15 cm above the lid of each tissue culture vessel and 24 cm above the height of the media within the vessel.

To ensure that the results of each light shelf reflect only the spectrum and intensity of the bulbs on its same treatment shelf, it was important to minimize and quantify the spillover light from one shelf to another and from external environmental sources. The greatest potential external influence that
could have skewed the results of the light quality would be the ambient light of the light rack room, especially diffuse sunlight from windows as it has such high intensity and such a broad spectrum. Direct sunlight measures 1500 μmol m⁻² s⁻¹ so with a full white spectrum so even diffuse sunlight exposure could produce a significant effect. Average intensities of standard, non-plant growth related indoor light fixtures are 10 μmol m⁻² s⁻¹ which is low, but not insignificant. To prevent the intrusion of these external environmental sources of light, both locations used for the spectral quality and spectral intensity experiments were conducted in rooms which were isolated from external light sources.

Figure 3. A photograph taken at the Biotech Accelerator Lab showing uniform shelf dimensions across all light treatments and relative distance from light fixture to vessels.
The other potential source of un-intended environmental light sources comes from spillover from the experimental light fixtures themselves. Light could potentially bounce off walls and surroundings around the light rack insulation and effect the treatments below them, albeit in minute quantities. In order to reduce this spillover effect, the base of each shelf beneath the tissue culture vessels is covered with a sheet of metallic insulation that both insulates the shelf from the heat of the light fixture beneath it and is also highly reflective, preventing light fixtures from effecting plants on other shelves above or below them. The only way to prevent horizontal light from bouncing off of walls and effecting other shelves would have been to enclose each shelf with side barriers to produce a nearly sealed chamber as opposed to an open sided shelf, but this would have trapped in heat and raised the temperature of the incubation environment to temperatures greater than that which is optimal for TC plant growth.

Since sealing the sides of the light rack would leave it prone to overheating, it was hypothesized that the negative effects of overheating outweighed the negative effects of minor light spillover. For this reason, open sided light racks were used, and spillover was acknowledged and quantified. All three alternate shelves (without their shelf’s light turned on) were tested at tissue culture media height with only the light on a single shelf on in the light room. All other shelves measured a light intensity of between 0 and 1 µmol m\(^{-2}\)s\(^{-1}\) at media height. This is not to say that there is no spillover, but rather that it is simply occurring at such low intensities that are unlikely to have a significant effect.

Differences in the inherent physical characteristics of the experimental environments where tissue culture experiments are conducted such as temperature and season can affect plant growth (Vijaya and Padmaja, 2002). The two locations used for these experiments were the isolated fume-hood room in the American Chestnut Rooting and Acclimatization Lab at the Upstate Medical Biotech Accelerator Lab (BAC) and the old growth chamber room in the basement of the Old Greenhouse building (OGH) at SUNY-ESF. Both rooms were chosen for their isolation from external lighting sources.
as well as their generally cool and stable temperatures which are ideal for tissue culture plantlets.

However, upon measuring average daily temperatures of both environments (Table 1) the BAC’s active HVAC cooling systems maintained a stable temperature of 24°C while the OGH growth chamber room which relies on passive cooling via thermal mass insulation maintained a hotter temperature of 32°C.

Table 1. This table provides the location, duration and average daily temperature as recorded by an incremental temperature logger of each of the incubation environments in which the LED experiments were conducted.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Location of Experiment</th>
<th>Start Date of Experiment</th>
<th>End Date of Experiment</th>
<th>Average Temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>1st Rep. LED Light Spectrum (Elongation)</td>
<td>Biotech Accelerator Lab</td>
<td>12/24/2016</td>
<td>2/5/2017</td>
<td>24°C</td>
</tr>
<tr>
<td>1st Rep. LED Light Spectrum (Rooting)</td>
<td>Biotech Accelerator Lab</td>
<td>1/15/2017</td>
<td>2/5/2017</td>
<td>24°C</td>
</tr>
<tr>
<td>2nd Rep. LED Light Spectrum (Rooting)</td>
<td>Old Greenhouse Basement</td>
<td>7/7/2017</td>
<td>7/28/2017</td>
<td>32°C</td>
</tr>
<tr>
<td>3rd Rep. LED Light Spectrum (Elongation)</td>
<td>Biotech Accelerator Lab</td>
<td>9/19/2018</td>
<td>10/31/2018</td>
<td>24°C</td>
</tr>
<tr>
<td>3rd Rep. LED Light Spectrum (Rooting)</td>
<td>Biotech Accelerator Lab</td>
<td>10/1/2018</td>
<td>10/22/2018</td>
<td>24°C</td>
</tr>
<tr>
<td>LED Light Intensity Experiment (Elongation)</td>
<td>Old Greenhouse Basement</td>
<td>9/19/2018</td>
<td>10/31/2018</td>
<td>27°C</td>
</tr>
</tbody>
</table>

**LED Lighting Options for Tissue Culture (TLEDs vs Production Modules)**

Part of the inspiration for this tissue culture lighting experiment came from our discovery of a new product from Philips Lighting Solutions known as the Tissue Culture LED Tube bulb or TLED. This is a T8 size light bulb that fits into a standard T8 fluorescent light fixtures but produces light via electroluminescence rather than fluorescence. The TLED bulbs produced by Philips Lighting Solutions and are offered in two different “balanced blends” of light spectral quality; Deep-Red White (DRW), Deep-Red White Far Red (DRWFR). Philips marketed the two bulb types as DRW containing higher levels of “violet and blue light” (within the 450-550 nm spectral range) while DRWFR contained higher levels of far-red light (within the 700-750 nm spectral range). However, when the spectral quality of the bulbs was measured at SUNY-ESF, it was discovered that the only difference was in the levels of far-red light.

All spectra other than far-red were nearly identical between DRW and DRWFR (Figure 14). Upon
discontinuation of the DRW and DRWFR bulbs, the company offered Arize™ TLED bulbs that were nearly identical in spectral quality as the DRW bulbs (Figure 15). These Arize™ bulbs were used for the spectral intensity experiment to uniformize spectral quality as a variable.

Prior to purchasing the bulbs for the experiment, customer service representatives from Philips lighting solutions constructed a light rack that mimicked the exact dimensions we used at the SUNY ESF tissue culture lab with light fixtures hanging 15 cm above the lid of each tissue culture vessel and 24 cm above the height of the media within the vessel. The customer service representative then used a spectral radiometer to measure the spectral quality and intensity produced by the DRW and DRWFR bulbs (Figures 4 and 5) and a cool-white fluorescent bulb (Figure 6). These spectral quality readings strongly resemble the spectral quality readings that we recorded when we independently verified the spectral quality of the bulbs using SUNY ESF’s spectral radiometer excepting a few key differences.

These manufacturer-provided spectral quality measurements differed from those taken in the following experiment in that there was no difference between the DRW Bulbs and the DRWFR bulbs in the amount of violet, blue and green light (Figures 4 and 5). Thus, the only functional difference between the bulbs was the quantity of far-red light with DRWFR bulbs having significant spikes in the far-red spectrum and the DRW bulb having functionally no far-red light (Figure 14). Differences between spectral radiometer measurements provided by manufacturer and those taken during this experiment are discussed further below.
Figure 4. Philip's Lighting Solutions spectral radiograph of spectral intensities of TLED- DRW\textsuperscript{TM} bulb.

Figure 5. Philip’s Lighting Solutions spectral radiograph of spectral intensities of TLED- DRWF\textsuperscript{TM} bulb.
Prior to the invention of TLEDs, when considering transitioning a production system from conventional lighting to LED lighting, LED light bars (discussed further below) were the only LED option available. This new TLED product offered potential advantages and cost savings over LED light bars, at the cost of a slight reduction in energy efficiency compared to LED light bars. TLEDs have a lower light intensity output than LED light bars, which is more suitable to tissue culture growth (Steigerwald et al., 2002). Because the TLED fixtures consist of 4 bulbs in a single fixture, this gives the grower the ability to adjust light intensity by adding or removing bulbs as needed. The greatest potential advantage of TLEDs over LED production modules consists of the lower initial hardware cost since customers only needing to purchase new bulbs as opposed to purchasing entirely new LED light fixtures and ballasts.

There are two main ways of providing LED lighting in vertical growth systems. The most common way is to use conventional LED production modules also known as “light bars” which consist of an
internal ballast connected to a solid bar of LEDs which do not contain conventional “bulbs” but rather a series of diodes (Bula et al., 1991). These types of LED light fixtures can produce light at high intensities suitable for mature greenhouse crop production, but at intensities greater than that which is ideal for tissue culture production. One example of this style of LED Production Module fixture is the “GreenPower™ LED Light Bar” sold by Hort Americas. Assuming the chestnut project’s current shelf dimensions and using two Philips GreenPower™ Light bars per shelf, the crop would receive light at an average intensity of 87 μmol/m²/sec. Using three GreenPower™ Light bars per shelf, the crop would receive light at an average intensity of 131 μmol/m²/sec (Figures 7 and 8). These LED light bars would produce light at intensities are much greater than what would be necessary for tissue culture incubation and would require a much higher initial hardware cost to transition from fluorescent to an LED based lighting system (Van Lepere and Trouwborst, 2008).

**Figure 7.** Isoline diagram of SUNY shelf with two GreenPower™ Light Bars (average of 87 μmol/m²/sec).

**Figure 8.** Isoline diagram of SUNY shelf with three GreenPower™ Light Bars (average of 131 μmol/m²/sec).
In addition to having lower initial hardware costs, TLEDs produce LED light at spectral intensities much more suited to the needs of tissue culture plants. Assuming the chestnut project’s current shelf dimensions and using a standard T8 Light fixture with 4 TLED DRW bulbs, light intensities would be produced at an average of 50 μmol/m²/sec, much closer to intensities recommended for micropropagation of tree species (Bonga and Aderkas, 1992). Light intensities under 4 DRWFR bulbs would be produced at an average of 58 μmol/m²/sec. These spectral distributions of these intensities are visually represented in the following isoline diagrams (Figures 9 and 10).

**Figure 9.** Isoline diagram of light intensity for SUNY ESF shelf dimensions with four DRW Philips TLEDs (average of 50 μmol/m²/sec)

**Figure 10.** Isoline diagram of SUNY ESF shelf with four DRWFR Philips TLEDs (average of 58 μmol/m²/sec)

Since both DRW and DRWFR bulbs produce light in intensities suitable for tissue culture propagation of American chestnut without the need of further dimmer hardware, and do not require
the purchase of additional light fixtures and ballasts, this transitional LED technology is suitable for production needs for the American chestnut project and warrants further investigation of their viability as a light source.

**Background on Light Spectrum Irradiance Measurements**

A spectrophotometer is an instrument that measures light intensity as a function of wavelength. The spectrophotometer uses a fiber optic cable to diffraction graft the white light, separating it into its various wavelengths. It then uses a detector to quantify how many photons are striking it and at what wavelength and the photons are converted to electrons and stored. The electrons measured by the spectrophotometer are then converted to a voltage and given a ‘digital number’ which represents a quantification of total electrons collected. This digital number functions as a unitless measurement for quantifying the relative intensity of reflectance and irradiance across the visible light spectrum. Irradiance measurements were taken with the fiber optic cable pointed directly upward at the light source as opposed to reflectance measurements where the fiber optic cable was directed downward at the surface of a leaf. The SUNY ESF Forestry department’s spectrophotometer was used to take irradiance measurements of the light spectrum of each of the four light treatments. Measurements of relative spectral distribution were taken for experiments conducted at the BAC as well as the irradiance measurements for each of the four light treatments of the LED light intensity experiment conducted in the basement of the Old Greenhouse. The spectrophotometer has many attachments which can be added to the fiber optic cable to more consistently or efficiently measure reflectance from leaves. Since this experiment did not measure reflectance, but instead measured irradiance directly from the light source, the bare fiber optic cable was used with no additional attachments.

Fiber optic cables are fragile and if they are bent at too sharp of an angle the glass tubes within the cable can shatter and reduce the accuracy of the spectrophotometer’s measurements. To prevent
excessive bending of the cable while still taking the measurements at the height of the media, a hole was punched through the light shelf’s foam insulation so that the fiber optic cable could be inserted from below at an angle perfectly perpendicular to the light rack shelf (Figure 1).  

**Figure 1.** This photograph shows the spectroradiometer set-up when taking irradiance measurements. The instrument itself is connected to a laptop for data collection via ethernet cable and the fiberoptic cable runs from the spectroradiometer to the shelf and is inserted vertically through the base of the shelf in order to prevent bending the fragile fiber optic cable. It is held at media height and perpendicular to the light source by a ceramic sensor holder.

It is important that the sensor at the end of the fiber optic cable be at a uniform height and be facing directly at the light source to get an accurate and consistent measurement of irradiance. Even a
few degrees deviation from a direct angle at the light fixtures could skew intensity measurements. The
distance from the light source to the end of the fiber optic cable can also greatly influence irradiance
measurements. In order to maintain the consistency at which both the height and angle irradiance
measurements were taken, a ceramic sensor holder was used, consisting of a vertical shaft hollowed out
through the center of a 2 cm tall ceramic button that was used to hold the fiber optic cable perfectly
upright and at a uniform height while the measurements were taken (Figure 1). To maintain
consistency of methods, all spectrophotometer and light intensity measurements were taken at the
height of the media (approx. 2 cm above the surface of the shelf) using this ceramic sensor holder. The
cable was inserted through the bottom of the light rack, through a hole in the insulation and through the
ceramic holder until the whole tip of the cable emerged from the sensor holder. Each of the six
measurements per treatment were taken with the ceramic button holding the fiber optic cable in place.
**Figure 12.** This photograph shows the ceramic sensor holder used to hold the fiber optic cable at a precise height and angle. The sensor holder is shown next to a magenta cube showing that the measurements were taken at media height.

The DN of each irradiance measurement will be different depending not only on the distance from the light source and angle of the fiber optic cable, but also based on where the measurement is taken beneath the light fixture. The closer the sensor is to the center of the shelf, the higher the irradiance measurement’s DN will be and the farther you move away from the center, the lower the DN’s will be. In order to get an accurate and representative measurement for the entire shelf of each treatment, six separate irradiance measurements were taken for each light treatment and were averaged together to get the average irradiance levels for each light shelf (Figure 13). Each of the six measurements were taken at the height of the media within the 8 cm x 64 cm rectangle centered by both length and width on the light rack which is where the plants were incubated during the experiment.
Figure 13. This figure shows the spectral intensity of each of the six individual measurements (blue lines) taken of the DRW light treatment in the LED spectral distribution experiments. These measurements were averaged in order to establish an accurate and representative mean irradiance measurement (red line) for each treatment.

LED Spectral Quality Experiment Light Treatments

Each TLED bulb treatment had a slightly different spectral distribution and therefore produced slightly different average light intensity outputs so these average intensities will be provided below alongside the corresponding treatment (Table 2). All light intensity measurements taken were recorded with photosynthetic photon flux density units (µmol m⁻²s⁻¹) and were measured using a quantum handheld photometer. The standard light intensity (measured at the height of the media) under the control treatment of two cool-white fluorescent bulbs was 41 µmol m⁻²s⁻¹. This 41 µmol m⁻²s⁻¹ light intensity falls within the center of the average light intensity range (30 - 50 µmol m⁻²s⁻¹) for tissue culture incubation of woody species (Oakes et al., 2016). When designing our LED light treatment shelves, we attempted to achieve a similar overall light intensity while retaining the existing light rack/shelf dimensions so that this factor is uniform throughout all four treatments. Average light intensities produced by each light treatment vary depending on the bulb type and combination of bulbs used and range from an intensity of 41 µmol m⁻²s⁻¹ (fluorescent) to 60 µmol m⁻²s⁻¹ (DRW). Average shelf intensities of each spectral quality light treatment are shown in Table 2.

Table 2. The table shows average light intensity per light quality treatment. Twelve light intensity measurements were made in a randomized pattern on each shelf at the height of the media and were used to produce the average light intensity of each shelf.
The fluorescent bulbs used as the control treatment (treatment D) are standard cool-white fluorescent, T8 plant growth lamps from the brand name Sunlite™. The bulbs used for LED light for treatments A, B and C are industry oriented T8 LED bulbs known as a ‘Philips Greenpower’ Tissue Culture LEDs (TLED). The major differences in the treatments for the LED light quality experiments turned out to be subtler than previously anticipated from the irradiance measurements provided from the Hort Americas customer service representative. From measurements provided from the bulb manufacturer, it appeared that the DRW bulbs had higher spikes in the blue (450 nm) and green (500-600 nm) spectra, but the measurements I made at the site of my experiment yielded differing results. In the industry-provided measurements, DRW bulbs had 200% greater output of green and blue wavelengths compared to the DRWFR bulbs, but in my measurements, there was no difference in blue and green light between DRW and DRWFR bulbs (Figures 4, 5 and 14). The only significant difference between the DRW and DRWFR bulbs was within the far-red spectrum (700 – 770 nm) with the DRWFR bulbs achieving a light intensity DN of 3000 while the DRW bulb only achieved a DN of 100 (Figure 14).

In the irradiance measurements provided to me by Hort Americas, within the blue light spectrum (450 nm) the DRW spiked at a DN of 25,000 while the DRWFR bulb spiked at a DN of 12,000 which would suggest that the DRWFR bulb only output 48% of the blue light intensity produced by the DRW bulb. However, during spectrophotometer irradiance measurements conducted at SUNY ESF, the DRW spiked at a DN of 4,300 while the DRWFR bulb spiked at a DN of 4,000 which would suggest that the DRWFR bulb output 93% of the blue light intensity compared to that of the DRW bulb.
Figure 14. The average irradiance distributions of each treatment in the LED spectral quality experiment were overlaid in the figure to show the relative differences in the spectral distribution of each light treatment: DRW, DRWFR, R5B5 and cool-white fluorescent control.

Information provided to me from the manufacturer suggested that the DRWFR bulbs produced 48% as much blue light output as the DRW bulbs, while my measurements suggested that DRWFR bulbs output 93% as much blue light as the DRW bulbs. This is a major discrepancy and is a good example of why it is important to measure the specific conditions of one’s experimental environment rather than relying on product information sheets and generalized product information provided from manufacturers.

Since the on-site light treatment measurements showed a much less significant difference between DRW and DRWFR bulbs in the blue and green wavelength than originally anticipated, and since
both bulbs also have very similar DNs in the red spectrum (both had an intensity of approximately 12,000 DN), the only significant difference in the TLED light quality experiment treatments is their relative intensity in the far-red wavelengths (700 – 770 nm). Within the far-red spectrum, the DRW bulb peaked at a DN of 100, the DRWFR bulb treatment peaked at a DN of 3,000 and the ½ DRW, ½ DRWFR treatment peaked at 1,500 just as expected. This shows that the true functional difference between the light quality of each treatment is that the DRW bulbs represent “low-to-no far-red”, the DRWFR bulbs represent a “high far-red” and the ½ DRW, ½ DRWFR represent a “medium far-red” treatment.

**LED Light Intensity Experiment Light Treatments**

In addition to determining what spectral quality tissue culture American chestnut prefers, it was also important to determine what LED light intensity produces the highest quality micro-cuttings. For the light intensity experiment, the brand of TLED bulbs used were Arize™ TLED bulbs to keep the light spectral quality of the light treatment uniform (excepting the fluorescent 2 bulb control). These Arize™ bulbs have a balanced light spectrum like the DRW TLED from the spectral quality experiment. The Arize™ bulbs, being a different brand from the Philips TLED Greenpower™ bulbs used in the light spectrum experiments, produced different and unique light intensities (Table 3) and light quality (Figure 15) from those produced by the DRW and DRWFR bulbs. As anticipated, all Arize™ treatments showed the same patterns of spectral quality. All intensities peaked at similar wavelengths with the intensity of the peak corresponding to the number of bulbs in the treatment with a greater number of bulbs correlating with an increased spectral intensity (Figure 15). Arize™ bulbs were purchased for this experiment because DRW and DRWFR bulbs had been discontinued by the time the spectral intensity experiment was suggested. We did not use existing DRW and DRWFR bulbs for this experiment as we did not have enough bulbs to keep spectral quality uniform throughout the experiment. Arize™ bulbs have the additional drawback of requiring extensive re-wiring of the T8 Light fixture that they are inserted into to function properly, thus nullifying the convenience of TLEDs.
Table 3. Twelve light intensity measurements were made on each shelf at the height of the media using a standard light intensity photometer. These 12 individual measurements per treatment were used to produce the average light intensity of each shelf.

<table>
<thead>
<tr>
<th>Trt. Letter</th>
<th>Shelf Light Treatment</th>
<th># of Bulbs</th>
<th>Average Light Intensity of Shelf</th>
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<tbody>
<tr>
<td>A</td>
<td>Arize™ TLED Tubes</td>
<td>2 Arize</td>
<td>51 μmol m⁻²s⁻¹</td>
</tr>
<tr>
<td>B</td>
<td>Arize™ TLED Tubes</td>
<td>3 Arize</td>
<td>68 μmol m⁻²s⁻¹</td>
</tr>
<tr>
<td>C</td>
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</tr>
<tr>
<td>D</td>
<td>Cool-white Fluorescent- Sunlite</td>
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</tbody>
</table>

Figure 15. The figure shows the spectral distributions recorded with a spectroradiometer of the four light treatments used in the light intensity experiment. The three Arize™ LED Light treatments all show a similar pattern to their distributions with the only difference being their intensity rather than their distribution across the visible light spectrum. Note the similarity in spectral quality of the Arize™ bulbs to the DRW bulbs (Figure 14) in that both have balanced blue, green and red, but no far-red light.
**Ex Vitro Rooting Procedure**

Jiffy pellets™ (a peat-based soilless rooting medium) were hydrated with water raised to an EC of 1.0. EC using Miracid™ 30-10-10 (N-P-K) and was then adjusted to a pH of 5.5. 1 mm holes were dibbled into each pellet prior to sticking the cutting into the pellet to prevent bending the stem during insertion. Fully elongated chestnut micro-cuttings were harvested from shoot cultures which spent 6 weeks in CCPR media. Cuttings were removed from the Magenta™ cubes and shoots were excised from the basal callus balls with a cut at a 45° angle to increase surface area of exposed vascular tissue. Leaves and axillary buds were removed from the bottom 1 cm of the micro-cutting and the cutting was then dipped into Clonex Rooting Gel™ (HydroDynamics International, Lansing, MI) to a depth of 1 cm and immediately inserted into the dibbled holes in the Jiffy pellets (Oakes et al., 2016). Shoots and pellets were then placed into the sealed rooting vessels and incubated under their respective light treatments under a 16-hour photoperiod for three weeks.

**Data Collection and Statistical Analysis**

The variables measured for shoot elongation experiments were number of leaves, stem caliper, stem length, total leaf surface area and average leaf surface area. Stem thickness was measured using a micro caliper. These variables have been used as a means of quantifying micro-cutting quality in previous tissue culture lighting experiments (Hung et al., 2016). The micro-cuttings were dissected and leaf dimensions (length and width) were measured by hand with a ruler (Figure 16). Then the length of each leaf was multiplied by width of each leaf and then by 0.7 (an approximation of the percentage of the area of a rectangle occupied by an ellipse) to generate a measurement of the estimated surface area of each leaf. Knowing the surface areas of each individual leaf and the total number of leaves per shoot allowed me to determine average leaf surface area and total leaf surface area of each shoot. These measurements provide important information because simply looking at total leaf surface area can be
misleading in assessing micro-cutting quality. Generally, plants that have the highest chance of successful rooting and acclimatization are those with many large leaves per shoot (Oakes et al., 2016). If a cutting has a high number of small leaves with low average leaf area, it can still produce a high total leaf surface area. By providing both total leaf surface area and average leaf surface area, we can provide a more complete picture of the quality of the micro-cuttings produced as well as getting a better understanding of the spectral quality’s effect on leaf expansion.

For the ex vitro rooting experiments, the variables measured were percentage of overall survival of the rooting process (% survival), number of roots per plant, and total root length of each plantlet. After 3 weeks of rooting, the soilless rooting media (Jiffy Pellets™) were carefully removed to expose roots allowing for counting and precise hand measurement of the length of each root (Figure 16).

**Figure 16.** Micro-cutting quality assessment: Leaves were removed from the stem. Leaves and stem were placed under a sheet of glass to flatten them for individual hand measurements (Left). Root quality assessment: Prior to measurement, the soilless media was carefully removed without damaging the root system (Right). Both images are of the same micro-cutting before and after media was removal.
The shoot elongation experiment was conducted three times in completely randomized designs with 10 vessels per treatment and each vessel contained 4 micro-cuttings. The ex vitro rooting experiment was conducted twice in a completely randomized design with 8 vessels per treatment and each vessel containing 3 rooted cuttings. All data were statistically analyzed by one-way ANOVA using Minitab statistics software. Tukeys Pairwise Comparison was used for mean separation when significant differences among treatment means were detected by one-way ANOVA. Means were presented with standard errors, and treatment differences or interactions were regarded as significant at p < 0.05.

Due to economic and logistical constraints, we were unable to replicate each lighting treatment as it would have required purchasing 10 times as many bulbs (raising the cost of the experiment from $500 to $5,000) and would require 10 times as much light rack space for incubation environments which were isolated from external light sources, which was already difficult to find for one single light rack with 4 fixtures. Ideal experimental design would have called for 10 light fixtures of DRWFR TLEDs, 10 light fixtures of DRW TLEDs, 10 light fixtures of R5B5, and 10 fluorescent fixtures with one cube of tissue under each since the light fixture itself was the experimental unit. This would have, if one or more of the light bulbs proved to be faulty, reduced the impact of defective bulbs from producing false results, however this optimal experimental design was economically and logistically unfeasible for this study, so we settled for this form of pseudoreplication. Pseudoreplication has been used for other horticultural experiments when logistical barriers to true replication were unfeasible (Zheng et al., 2002) such as in a study measuring the phytotoxic effects of horticultural oils on azaleas (Rhododendron spp.).

Results

In the first repetition of the spectral quality shoot elongation experiment, the DRW (no-far red) TLED light treatment, DRWFR (high far-red) TLED light treatment and the R5B5 (medium far-red) TLED treatments all produced shoots with estimated total leaf surface areas that were not significantly
different from one another, however the DRWFR, DRW and Fluorescent treatments were also not significantly different from one another with respect to estimated total leaf surface area. There was however a significantly higher estimated total leaf surface area from the R5B5 treatment over the fluorescent treatment (Figure 17).

Figure 17. This figure shows the estimated total leaf surface area results of the three repetitions of the light quality elongation experiment. The three graphs show the results of the first repetition of the experiment conducted in the BAC (left), the second repetition conducted at the OGH (center) and the third repetition conducted at the BAC (right).

In the second repetition of the spectral quality shoot elongation experiment, DRW, DRWFR and R5B5 treatments all produced shoots with estimated total leaf surface area that did not differ from one another but all TLED treatments had total leaf surface area that were significantly less than that of the fluorescent treatments (Figure 17). In the third repetition of the spectral quality shoot elongation experiment, the DRW and DRWFR TLED light treatments produced total leaf surface areas significantly
greater than those produced by the fluorescent treatment while the R5B5 treatment produced total leaf surface areas that were not significantly greater than those produced by the fluorescent control (Figure 17).

In the first repetition of the spectral quality elongation experiment, all four treatments, DRW, DRWFR, R5B5 and the fluorescent control yielded average leaf sizes that did not differ (Figure 18). In the second repetition of the spectral quality elongation experiment, the DRW, R5B5 and fluorescent control treatments did not differ significantly from one another. The DRW, DRWFR and R5B5 treatments also did not yield statistically significant differences in estimated average leaf size. However, there was a difference between DRWFR and the fluorescent control with the DRWFR having significantly lower average leaf size than the fluorescent treatment. In the third repetition, the DRW, R5B5 and fluorescent treatments did not produce statistically significant differences in average leaf size, but the DRWFR treatment produced leaves with average leaf size greater than all other treatments.

Figure 18. This figure shows the results of the average leaf size of the three repetitions of the spectral quality elongation experiment. The three graphs show the results of the first repetition of the
experiment conducted in the BAC (left), the second repetition conducted at the OGH (center) and the third repetition conducted at the BAC (right).

Additional variables to quantify micro-cutting quality were measured during spectral quality shoot elongation experiments including stem micro-caliper, length of micro-cutting and number of leaves per shoot. In the first repetition of the experiment, variables other than total leaf surface area and average leaf size did not yield any results with statistically significant differences amongst treatments (Table 4). In the second repetition of the experiment, the fluorescent treatment produced statistically higher stem micro-caliper, length of micro-cutting and number of leaves per shoot than all TLED treatments (Table 5). In the third repetition of the experiment, secondary variables yielded several significant differences (Table 6). The DRW and DRWFR TLED treatments produced the highest number of leaves per shoot that were significantly greater than that of R5B5 and the fluorescent control. Additionally, the fluorescent treatment produced stem micro-caliper greater than that of the DRWFR and R5B5 treatments but did not differ significantly from the DRW treatment.

**Table 4.** Summarized results of all statistics collected on all variables measured for the first repetition of the light spectrum elongation experiment. Treatments that share a letter with one another did not differ. *

<table>
<thead>
<tr>
<th>Rep #</th>
<th>Variable Measured</th>
<th>DRW</th>
<th>DRWFR</th>
<th>R5B5</th>
<th>Fluorescent</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Average Leaf Surface Area</td>
<td>A</td>
<td>A</td>
<td>A</td>
<td>A</td>
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<tr>
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<td>A</td>
<td>B</td>
</tr>
<tr>
<td>1</td>
<td>Stem Micro-Caliper</td>
<td>A</td>
<td>A</td>
<td>A</td>
<td>A</td>
</tr>
<tr>
<td>1</td>
<td>Stem Length of Micro-Cutting</td>
<td>A</td>
<td>A</td>
<td>A</td>
<td>A</td>
</tr>
<tr>
<td>1</td>
<td>Number of Leaves per Shoot</td>
<td>A</td>
<td>A</td>
<td>A</td>
<td>A</td>
</tr>
</tbody>
</table>
Table 5. Summarized results of all statistics collected on all variables measured for the second repetition of the light spectrum shoot quality experiment. Treatments that share a letter with one another did not differ. *

<table>
<thead>
<tr>
<th>Rep #</th>
<th>Variable Measured</th>
<th>DRW</th>
<th>DRWFR</th>
<th>R5B5</th>
<th>Fluorescent</th>
</tr>
</thead>
<tbody>
<tr>
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<td>Average Leaf Surface Area</td>
<td>AB</td>
<td>B</td>
<td>AB</td>
<td>A</td>
</tr>
<tr>
<td>2</td>
<td>Total Leaf Surface Area</td>
<td>B</td>
<td>B</td>
<td>B</td>
<td>A</td>
</tr>
<tr>
<td>2</td>
<td>Stem Micro-Caliper</td>
<td>B</td>
<td>B</td>
<td>B</td>
<td>A</td>
</tr>
<tr>
<td>2</td>
<td>Stem Length of Micro-Cutting</td>
<td>B</td>
<td>B</td>
<td>B</td>
<td>A</td>
</tr>
<tr>
<td>2</td>
<td>Number of Leaves per Shoot</td>
<td>B</td>
<td>B</td>
<td>B</td>
<td>A</td>
</tr>
</tbody>
</table>

Table 6. Summarized results of all statistics collected on all variables measured for the third repetition of the light spectrum shoot quality experiment. Treatments that share a letter with one another do not differ. *

<table>
<thead>
<tr>
<th>Rep #</th>
<th>Variable Measured</th>
<th>DRW</th>
<th>DRWFR</th>
<th>R5B5</th>
<th>Fluorescent</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>Average Leaf Surface Area</td>
<td>B</td>
<td>A</td>
<td>B</td>
<td>B</td>
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<tr>
<td>3</td>
<td>Total Leaf Surface Area</td>
<td>A</td>
<td>A</td>
<td>B</td>
<td>B</td>
</tr>
<tr>
<td>3</td>
<td>Stem Micro-Caliper</td>
<td>AB</td>
<td>B</td>
<td>B</td>
<td>A</td>
</tr>
<tr>
<td>3</td>
<td>Stem Length of Micro-Cutting</td>
<td>A</td>
<td>B</td>
<td>B</td>
<td>B</td>
</tr>
<tr>
<td>3</td>
<td>Number of Leaves per Shoot</td>
<td>A</td>
<td>AB</td>
<td>C</td>
<td>BC</td>
</tr>
</tbody>
</table>

In the first repetition of the spectral quality rooting experiment conducted at the BAC, all four treatments DRW, DRWFR, R5B5 and the fluorescent control produced total rooting survival, number of roots and total root lengths that did not significantly differ from one another (Figure 19 and 20). Number of roots per shoot also did not differ in the first repetition of the experiment (Table 7).

In the second repetition of the spectral quality rooting experiment conducted in the OGH growth chamber room, all four treatments DRW, DRWFR, R5B5 and the fluorescent control also produced total rooting survival and total root lengths that did not significantly differ from one another
(Figure 19 and 20). Number of roots per shoot also did not differ in the second repetition of the experiment (Table 8).

**Figure 19.** This figure shows the results of percent rooting survival of the two repetitions of the spectral quality *ex vitro* rooting experiments. Results of the first repetition are shown on the left while results from the second repetition are shown in the figure on the right.

**Figure 20.** This figure shows the total root length results of the two repetitions of the spectral quality *ex vitro* rooting experiments. Results of the first repetition are shown on the left while results from the second repetition are shown in the figure on the right.
Table 7. Summarized results of all statistics collected on all variables measured for the first repetition of the light spectrum root quality experiment. Treatments that share a letter with one another do not differ. *

<table>
<thead>
<tr>
<th>Rep #</th>
<th>Variable Measured</th>
<th>DRW</th>
<th>DRWFR</th>
<th>R5B5</th>
<th>Fluorescent</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>% Survival</td>
<td>A</td>
<td>A</td>
<td>A</td>
<td>A</td>
</tr>
<tr>
<td>1</td>
<td>Number of Roots per Shoot</td>
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<td>A</td>
<td>A</td>
<td>A</td>
</tr>
<tr>
<td>1</td>
<td>Total Root Length per Shoot</td>
<td>A</td>
<td>A</td>
<td>A</td>
<td>A</td>
</tr>
</tbody>
</table>

Table 8. Summarized results of all statistics collected on all variables measured for the second repetition of the light spectrum root quality experiment. Treatments that share a letter with one another do not differ. *

<table>
<thead>
<tr>
<th>Rep #</th>
<th>Variable Measured</th>
<th>DRW</th>
<th>DRWFR</th>
<th>R5B5</th>
<th>Fluorescent</th>
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<tbody>
<tr>
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<td>% Survival</td>
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<td>A</td>
<td>A</td>
<td>A</td>
</tr>
<tr>
<td>2</td>
<td>Number of Roots per Shoot</td>
<td>A</td>
<td>A</td>
<td>A</td>
<td>A</td>
</tr>
<tr>
<td>2</td>
<td>Total Root Length per Shoot</td>
<td>A</td>
<td>A</td>
<td>A</td>
<td>A</td>
</tr>
</tbody>
</table>

In the LED light intensity shoot elongation experiment, the 2 bulb Arize™ TLED treatment produced estimated total leaf area that did not differ significantly from the fluorescent control, while the fluorescent treatment produced estimated total leaf area that was greater than that of the 3 bulb Arize™ TLED treatment and the 4 bulb Arize™ TLED treatment (Figure 21). The 2 bulb Arize™ TLED treatment, the 4 bulb Arize™ TLED treatment and the fluorescent control all produced average leaf sizes that did not differ significantly from one another, but the fluorescent bulb produced average leaf sizes that were greater than that of the 3 bulb Arize™ TLED treatment (Figure 22).
**Figure 21.** This figure shows the estimated total leaf surface area results of the TLED Arize™ bulb light intensity elongation experiment. This experiment was conducted in the OGH growth chamber room.

**Figure 22.** This figure shows the average leaf size results of the TLED Arize™ bulb light intensity elongation experiment. This experiment was conducted in the OGH growth chamber room.
The light intensity experiment yielded no significant differences amongst all four treatments for stem micro-caliper and stem length of micro-cutting but produced more varied results with the number of leaves per shoot. The 2 Bulb Arize™ TLED treatment yielded number of leaves per shoot that did not differ significantly from the fluorescent control (Table 9). The fluorescent treatment produced more leaves per shoot than the 3 bulb and 4 bulb Arize™ TLED treatments. The 2 bulb Arize™ TLED treatment also produced more leaves per shoot than the 3 bulb Arize™ TLED treatment.

Table 9. Summarized results of all statistics collected on all variables measured for the light intensity experiment. Treatments that share a letter with one another do not differ. *

<table>
<thead>
<tr>
<th>Rep #</th>
<th>Variable Measured</th>
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<th>3 Bulb</th>
<th>4 Bulb</th>
<th>Fluorescent</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Average Leaf Surface Area</td>
<td>AB</td>
<td>B</td>
<td>AB</td>
<td>A</td>
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<tr>
<td>1</td>
<td>Total Leaf Surface Area</td>
<td>AB</td>
<td>B</td>
<td>B</td>
<td>A</td>
</tr>
<tr>
<td>1</td>
<td>Stem Micro-Caliper</td>
<td>A</td>
<td>A</td>
<td>A</td>
<td>A</td>
</tr>
<tr>
<td>1</td>
<td>Stem Length of Micro-Cutting</td>
<td>A</td>
<td>A</td>
<td>A</td>
<td>A</td>
</tr>
<tr>
<td>1</td>
<td>Number of Leaves per Shoot</td>
<td>AB</td>
<td>C</td>
<td>BC</td>
<td>A</td>
</tr>
</tbody>
</table>

Discussion

The results of the experiments testing the effects of spectral quality on shoot elongation of American chestnut provided enough evidence to support the hypothesis that full spectrum LED light is a viable alternative for the in vitro elongation of American chestnut when compared to cool-white fluorescent alternatives. The first and third repetitions of the shoot elongation study provided strong evidence that all three TLED treatments produced micro-cuttings with total leaf area and average leaf size greater than or equal to that of the fluorescent control (Figure 17 and 18). Total leaf surface area and average leaf size are key morphological traits which have previously been used to quantify the quality of micro-cuttings in rabbiteye blueberry (Vaccinium ashei Reade) (Hung et al., 2016). The third repetition of the spectral quality shoot elongation study provided evidence to suggest that far-red light improves average leaf size of chestnut micro-cuttings during the elongation phase (Figure 18). This
increase in average leaf size under the DRWFR light treatment may have been caused by the photomorphological response of the shoots to far-red light which suppressed axillary buds and encouraged apical dominance. The suppression of axillary bud growth may have resulted in the shoots wasting less resources on axillary bud development allowing for further investment in leaf expansion (Tucker, 1976). In the third repetition of the experiment, there was no statistical difference between the DRW (no far-red light) and DRWFR (high red-light) treatments in terms of total leaf area (Figure 17) while the R5B5 treatment (½ DRW, ½ DRWFR, medium far-red light) produced lower total leaf area than DRW and DRWFR (Figure 17). However, since DRW and DRWFR yielded the same total leaf area and R5B5 is the midpoint between the high far-red and no far-red treatments, these results do not provide constructive evidence to determine the influence of far-red light on leaf expansion.

With regards to estimated average leaf size, results yielded similar trends to that to that of estimated total leaf surface area with a few key differences (Figure 18). While the first repetition of the spectral quality elongation experiment yielded no statistical differences in average leaf size between any TLED treatment (Figure 18), the third repetition of the spectral quality elongation experiment showed that the DRWFR (high far-red light) treatment produced average leaf size that was statistically greater than the DRW or R5B5 treatments as well as the fluorescent control (Figure 18). Because far-red light was not shown to increase average leaf size in any other repetition of the spectral quality experiment, this study only presents slight evidence that far-red light increases average leaf size in micro-propagated chestnut. These results support rejecting the hypothesis that far-red light promotes elongation growth response which would increase stem length at the expense of leaf expansion. Increased average leaf size observed in the DRWFR treatment during the third repetition of the spectral quality elongation experiment may have been the result of Pfr accumulation resulting from exposure to far-red light suppressing axillary bud break resulting in more resources being allocated to leaf expansion as opposed to axillary growth (Morrow, 2008).
Spectrophotometer measurements showed that there were no significant differences between the three TLED light treatments’ spectral quality other than in their amount of far-red light (Figure 14) with DRW containing low far-red light, DRWFR containing high far-red light and R5B5 containing medium levels of far-red light. The lower than expected effects of far-red light on in vitro shoot elongation could be due to the relatively less significant impact of far-red light compared to the overpowering effects of the TLED treatment’s improved spectral quality in the red, green and blue spectrums (Sun et al. 1998). Green light may have played a key role in the success of the TLED treatments since the TLED treatments have greater quality and intensity of green light than the fluorescent control (Figure 14). Green light has been shown to play an important role in photosynthesis under low light conditions (Kim et al., 2004) by re-directing the excitation energy of the photoreceptor phycoerythrobilin to chlorophyll cells resulting in indirect improvement to overall photosynthesis in the chlorophyll molecule (Sun et al. 1998). Since red and blue light also play such critical roles in stimulating photosynthesis and overall plant development, the increased spectral quality and intensity in the red and blue wavelengths of the TLED light treatments were likely the primary catalyst for the increased total leaf surface area and average leaf size produced by the TLED treatments (Morrow, 2008).

Photomorphogenic responses to blue light include increased leaf chlorophyll levels and an increase in epidermal cell area (Sæbø et al., 1995) and beneficial photomorphogenic responses to red light include increased leaf expansion as well as increased photosynthetic efficiency (Hung et al., 2016), both of which could have contributed to overall leaf expansion of TLED treatments. Evidence of these trends has been demonstrated in the in vitro propagation of blueberry by Hung et al. where 4 different narrow spectrum light treatments (100% red, 80% red + 20% blue, 50% red + 50% blue, 100% blue) and a cool-white fluorescent control were applied to blueberry cultures. Highest leaf surface area was produced from the 100% red light treatment and the highest chlorophyll content was produced by the 100% blue light treatment (Hung et al., 2016). Studies like this used narrow-band LEDs, only applying red and/or
blue light to each treatment group without any green, yellow, orange light. This represents a major difference from this TLED chestnut study which applied a full spectrum light treatment with differences in only a single wavelength (far-red) (Figure 14).

More pronounced photomorphological effects of individual wavelengths could have been produced in this experiment if the light treatments had consisted of narrow band, single spectrum light treatments like those used by Hung et al. However, since the purpose of this experiment was to test the efficacy of TLED bulbs which were only offered in broad spectrum light blends, narrow wavelength treatments were not an available option for this experiment. The main point of comparison of this experiment was that of full spectrum LEDs compared to cool-white fluorescent controls with the effects of far-red light intensity being a secondary consideration. For future investigations to better quantify the photomorphogenic responses of tissue culture American chestnut to specific wavelengths, there are three ways to overcome this issue of broad spectrum light treatments diluting the effects of individual wavelengths such as were observed in this experiment: narrow band light treatments (Hung et al., 2016), ceramic light filters for removal of individual wavelengths (Marks and Simpson, 1999) and superbright wavelength treatments (Tanaka et al., 1998).

Of the four elongation experiments conducted, the three repetitions of spectral quality for shoot elongation and the single repetition of the light intensity elongation study, two distinct patterns were observed; one in which the TLED treatments tended to out-perform the fluorescent control in terms of total leaf surface area and average leaf size and one in which the fluorescent control outperformed the TLED treatments for total leaf surface area and average leaf size (Figures 17, 18, 21 and 22). These trends appeared to be more highly correlated with the experimental incubation environment than they were the light treatments themselves. TLED treatments out-performed the fluorescent controls for total leaf surface area and average leaf size when incubated in the climate-controlled BAC incubation room while the fluorescent controls outperformed the TLED treatments when
conducted in the OGH incubation room (Figures 23 and 24). This suggests that the higher environmental temperature of the OGH incubation environment may have been unsuitable for *in vitro* plant growth (Table 1). The negative effects of sub-optimal environmental conditions may have overpowered the effects of the light treatments and resulted in the difference between the results trends in the second repetition of the spectral quality elongation experiment and the intensity experiment (both of which were conducted in the OGH) and the results produced in the 1st and 3rd reps of the spectral quality elongation experiment (both of which took place at the BAC) (Table 1). This explanation of the different results trends of the second repetition of the spectral quality elongation experiment suggest that the first and third repetitions are more representative of the light treatment effects as they were conducted under optimal tissue culture incubation conditions.

![Graphs showing total leaf surface area](image)

**Figure 23.** These graphs show the total leaf surface area of all four shoot elongation experiments set to the same y axis scale. The first repetition (left) and third repetition (center left) which were both conducted at the BAC are displayed next to one another while the second repetition (center right) and
the light intensity experiment (right) which were both conducted at the OGH are displayed next to one another.

**Figure 24.** These graphs show the average leaf size of all four shoot elongation experiments set to the same y axis scale. The first repetition (left) and third repetition (center left) which were both conducted at the BAC are displayed next to one another while the second repetition (center right) and the light intensity experiment (right) which were both conducted at the OGH are displayed next to one another.

The results of the experiments testing the effects of spectral quality on shoot elongation provided enough evidence to support the hypothesis that full spectrum LED light is a viable alternative for the *in vitro* elongation of American chestnut when compared to common, cool-white fluorescent alternatives and can do so at a fraction of the electricity cost (Steigerwald et al., 2002). This study presents slight evidence that far-red light may increase average leaf size in *in vitro* elongation of chestnut micro-cuttings. It also shows the importance of maintaining a single incubation environment for all repetitions of an experiment as well as the importance of choosing an incubation environment
that has adequate environmental controls that allow the experiment to be conducted at temperatures that are optimal for tissue culture growth.

The results of the experiment testing the effects of spectral quality on *ex vitro* rooting provided enough evidence to support the hypothesis that full spectrum TLED light is a viable alternative for the *ex vitro* rooting of American chestnut micro-cuttings when compared to common fluorescent alternatives as in both repetitions of the experiment all TLED treatment produced % survival and total root length equivalent to the fluorescent control (Figures 19 and 20). However, because there were no differences between any of the TLED experiments in both repetitions of the *ex vitro* rooting experiments, there is not enough evidence to accept the hypothesis that increased far-red light will improve the rooting of chestnut micro-cuttings. It was hypothesized that because of the strong apical dominance associated with high far-red light and its subsequent increase in endogenous auxins that the cuttings rooted under the high far-red, DRWFR treatment would have higher % survival and total root length than the no far-red DRW treatment (Morelli and Ruberti, 2000). This hypothesis was not supported by the results of this experiment as there were no significant differences in either repetitions among any variables measured (Figure 19 and 20). A potential explanation for the lack of significant impact of far-red light on rooting survival and total root length could be the overpowering effect of the enhanced quality and quantity of red, blue, and green light in the TLED treatments. Previous investigations into the effects of composite LED light on root growth have shown that broad spectrum LED light treatments produced significant increases in rooting rate and total root length compared to narrow band red and blue light as well as fluorescent controls (Xu et al., 2019). Red light has been found to promote rooting without the need of exogenous auxins while red light deficient treatments did not root without the aid of exogenous auxins (Rossi et al., 1993). Far-red light may have affecting rooting quality but it’s treatment effect may have been reduced to unobservable levels by the increased rooting survival and total root length produced by increased quantity and quality of red, blue, and green light (Lin et al., 2013).
The results of the spectral intensity experiments provided enough evidence to support the hypothesis that 2 Arize™ TLED bulbs per fixture treatment will produce optimal light intensity for elongating American chestnut micro-cuttings under broad composite LED light. The 2 bulb treatment produced shoots with total leaf surface area and average leaf surface area equivalent to that of the fluorescent control while the 3 bulb and 4 bulb treatments produced total leaf surface area as well as average leaf size less than that of the fluorescent control (Figures 21 and 22). The 2 bulb treatment likely performed well because, of all the TLED treatments, the 2 bulb light treatment produced an intensity that was the closest to the standard intensity for in vitro culture of woody plants; 50 μmol/m²/sec (Bonga & Aderkas, 1992) (Table 3). The 3 bulb (68 μmol/m²/sec) and 4 bulb (83 μmol/m²/sec) treatments produced the total leaf surface areas and average leaf size lower than those of the fluorescent control, likely because their spectral intensity exceeds the recommended intensity of 31 μmol/m²/sec which has proven successful for in vitro elongation of American chestnut in the past (Oakes et al., 2016). The target spectral intensity recommended for in vitro incubation of plants ranges from 50 μmol/m²/sec for woody species (Bonga and Aderkas, 1992) to 100 μmol/m²/sec for vigorous herbaceous species like hemp (Cannabis sativa) (Wang et al., 2009). The results of this study show that under balanced TLED light spectra, the optimal spectral intensity for the elongation of chestnut micro-cuttings to produce maximum leaf expansion is 51 μmol/m²/sec which can be achieved with 2 Arize™ TLED light bulbs inserted into a standard fluorescent light fixture. Apical shoot tips elongated at this intensity under composite LED light will produce micro-cuttings of equivalent quality to those incubated under cool-white fluorescent bulbs at a fraction of the electricity cost (Bula et al., 1991).

**References**


Biddington, N. L. “The Influence of Ethylene in Plant Tissue Culture.” *Plant Growth Regulation*, vol. 11, no. 2, 1992


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Conclusions

This study represents the first investigation into the use of composite LED lights for in vitro elongation and ex vitro rooting of transgenic American chestnut (Castanea dentata). Three different full spectrum TLED light treatments; DRW, DRWFR, 50% DRW 50% DRWFR (R5B5), and a cool-white fluorescent control were applied to American chestnut during in vitro elongation and ex vitro rooting. Spectrophotometer measurements of the light treatments revealed that the spectral quality of the LED light treatments were similar to one another with each containing significant amounts of red, blue and green light, all of which play critical roles in plant growth and development (Lin et al., 2013). The only differences between treatments was the amount of far-red light they contained with DRW representing a no-far-red treatment, DRWFR representing a high far-red treatment and R5B5 representing a medium far-red treatment (Figure 14). The results of the experiments testing the effects of spectral quality on shoot elongation provided enough evidence to support the hypothesis that full spectrum LED light is a viable alternative for the in vitro elongation of American chestnut micro-cuttings when compared to common, cool-white fluorescent alternatives (Figure 17 and 18). This study also presents some evidence that far-red light may increase average leaf size in in vitro elongation of chestnut micro-cuttings (Figure 18) due far-red light increasing apical dominance which suppresses axillary bud break (Tucker, 1976).

Studies have found full spectrum LED light sources to improve growth, morphology and photosynthesis in ex vitro rooting of woody species (Xu et al., 2019). The results of the experiment testing the effects of spectral quality on ex vitro rooting provided enough evidence to support the hypothesis that full spectrum TLED light is a viable alternative for the ex vitro rooting of American chestnut when compared to common fluorescent alternatives as in both repetitions of the experiment all TLED treatment produced % survival and total root length equivalent to the fluorescent control (Figures 19 and 20). However, because there were no differences between any of the TLED experiments in both repetitions of the ex vitro rooting experiments, there is not enough evidence to accept the
hypothesis that the increased far-red light will improve the ex vitro rooting of chestnut micro-cuttings as was suggested by previous studies of the effects of spectral quality on rooting tissue culture micro-cuttings (Morelli and Ruberti, 2000). For future investigations to better quantify the photomorphogenic responses of tissue culture American chestnut to specific wavelengths, there are three ways to overcome the issue of broad spectrum light treatments diluting the effects of individual wavelengths such as were observed in this experiment: narrow band light treatments (Hung et al., 2016), ceramic light filters for removal of individual wavelengths (Marks and Simpson, 1999) and superbright wavelength treatments (Tanaka et al., 1998).

This study also conducted experiments to determine the optimal LED light intensity for the elongation of in vitro American Chestnut using broad spectrum TLED Arize™ bulbs. Three different LED light intensity treatments; 2 Arize™ TLED bulbs per shelf (51 μmol/m²/sec), 3 Arize™ TLED bulbs per shelf (68 μmol/m²/sec) and 4 Arize™ TLED bulbs per shelf (83 μmol/m²/sec) were tested as well as a fluorescent control (41 μmol/m²/sec) were applied to micro-propagated American chestnut during the elongation phase. Treatments did not differ in their spectral quality, and only differed in their light intensity (Figure 15). The results of the spectral intensity experiments provided enough evidence to support the hypothesis that 2 Arize™ TLED bulbs per fixture will produce optimal light intensity for producing high-quality micro-cuttings incubated under broad spectrum TLEDs (Figures 21 and 22). These findings agree with other studies which have found that the intensity of the 2-bulb treatment (51 μmol/m²/sec) has been successful for the micro-propagation of other woody tree species (Bonga and Aderkas, 1992).

The American Chestnut Research and Restoration Project grows and maintains several incubation light racks of chestnut cultures and therefore consumes large amounts of energy operating its dozens of fluorescent light fixtures. Part of the purpose of this experiment was to test out TLED bulbs to see if they were a quality product that justified the American Chestnut Research and Restoration
Project making a large-scale investment to transitioning TLED technology. While TLED bulbs are a novel technology that allow growers to gain many of the benefits of an LED based light source without the additional overhead cost of re-purchasing ballasts and fixtures, they are a transitionary technology between T8 fluorescent fixtures and LED light bars which present their own set of limitations and are rapidly falling out of favor for both academic and production-oriented growers. TLED bulbs served their purpose for this experiment as they allowed an economical opportunity to conduct small-scale tests to establish proof of concept for the viability of using broad spectrum LED lights for the micro-propagation of American chestnut, but they rapidly become a sub-optimal choice compared to LED light bars and production modules for growers looking to scale up their production systems (Moe, 1997). TLED bulbs are only offered in a few prefabricated light spectrum combinations which may not suit every grower’s production needs. They also produce light in low intensities that are not suitable for any production other than propagation phases that require low light intensities. Also, while they do save growers who already own T8 fluorescent light fixtures the cost of having to purchase new ballasts and fixtures, the bulbs themselves are expensive, they are less electrically efficient than LED production modules and they have already begun to be discontinued by several professional horticulture suppliers (Steigerwald et al., 2002). While DRW and DRWFR bulbs offer convenient and efficient transition from Fluorescent to LED light, these bulbs are now discontinued and the TLED Arize™ bulbs require extensive re-wiring of each T8 light fixture ballast prior to bulb installation which minimizes the convenience of transition that TLED bulbs were intended to provide.

LED light bars offer several advantages over TLED bulbs. Light bars are highly customizable. Customers can choose exactly what color diodes they would like their light bars to contain (Van Lepren and Trouwborst, 2008) instead of being tied to the one or two spectral combinations that are offered for these prefabricated TLED products. This allows growers to tailor their light quality to the needs of their specific crop and phase of production (Bula et al., 1991). From a scientific perspective, this control over
diode color composition would also allow researcher to design their own spectra allowing them to produce more pronounced photomorphological responses (Fujiwara and Sawada, 2006), and to more accurately study the effects of specific wavelengths without having to use ceramic light filters or having their treatment effects diluted by non-treatment wavelengths (Morrow, 2008). LED light bars can produce much higher light intensities than TLEDs and are more suited to electrically controlled dimmers which allow the grower to reduce and increase spectral intensities as needed (Liu et al., 2014). While the TLED treatment in this experiment with the highest spectral intensity was 83 μmol/m²/sec, LED light bars can easily reach intensities of 1200 μmol/m²/sec if necessary and can also be dimmed down to propagation irradiance levels as low as 50 μmol/m²/sec providing growers high versatility. LED light bar technology is continuously improving. LED light bar electrical efficiency continues to improve while the cost of ballasts and fixtures continues to fall, thus nullifying the primary disadvantage of light bars being their higher initial hardware cost (Bula et al., 1991). For any future investigations into the application of LED light for the in vitro and ex vitro propagation of American chestnut, LED production modules should be used over TLED bulbs to produce broad-spectrum LED light.

References


Appendix I: Pesticide Phytotoxicity Assessment

In addition to light spectrum and intensity studies, a pesticide phytotoxicity experiment was conducted to determine whether any commonly used commercial pesticides used on transgenic and non-transgenic plantlets and seedlings (post-acclimatization) had any detrimental phytotoxic effects on the plants. Plants used in the study consisted of four of each of the following genotypes per-treatment group: American Chestnut Seedlings, Chinese Chestnut plants, LM-B4SX54 and LM-B4SX58. The pesticides included in the experiment are fungicides, miticides and insecticides commonly used in standard nursery stock production. Eagle 20 EW and Affirm are fungicides intended to treat powdery mildew. Judo and Sultan are dedicated miticides and Avid is an insecticide/miticide. All products have partial to full systemic qualities.

Having consulted industry specialists regarding potential phytotoxicity and recommended application rates (Table 10) as well as having sprayed the plants with these products several times and seen the results, I hypothesized that all pesticide applications would yield no phytotoxicity and would exhibit % healthy leaves remaining 2 weeks after application to be equivalent to that of the un-treated controls.

Table 10. Pesticide Application Rates and Recommended Application Intervals

<table>
<thead>
<tr>
<th>Pesticide</th>
<th>Pesticide Type</th>
<th>Application Rate/Gal (mL)</th>
<th>Recommended Application Interval (Days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eagle 20 EW</td>
<td>Fungicide</td>
<td>3.75</td>
<td>10</td>
</tr>
<tr>
<td>Affirm</td>
<td>Fungicide</td>
<td>5</td>
<td>7</td>
</tr>
<tr>
<td>Judo</td>
<td>Miticide</td>
<td>1.25</td>
<td>Do Not Re-Apply</td>
</tr>
<tr>
<td>Avid</td>
<td>Insecticide/Miticide</td>
<td>2.5</td>
<td>7</td>
</tr>
<tr>
<td>Sultan</td>
<td>Miticide</td>
<td>3.75</td>
<td>14</td>
</tr>
<tr>
<td>Kontos</td>
<td>Miticide</td>
<td>1</td>
<td>14</td>
</tr>
<tr>
<td>Capsule</td>
<td>Surfactant</td>
<td>2.5</td>
<td>0</td>
</tr>
</tbody>
</table>

Plants were thinned to only lush, healthy leaves prior to pesticide application and were kept outdoors in shade tents in 9” nursery tubes for the duration of the experiment. Each group was treated
with the corresponding pesticide treatment twice, once immediately after leaf thinning to healthy leaves only, and again 2 weeks later with the same product (note that this lies outside any product’s Recommended Application Interval). Observations of phytotoxicity/ % Healthy leaves intact post-application were collected 2 weeks after the last application. None of the treatments showed significant increases in leaf phytotoxicity compared to the un-treated control (Figure 25) suggesting that each of these pesticides should be considered safe for application to American Chestnut Seedlings, and American and Chinese chestnuts produced from tissue culture.

![Pesticide Phytotoxicity Experiment](image.png)

**Figure 25.** Results of the Pesticide Phytotoxicity Experiment show no significant difference between any of the pesticide treated chestnuts compared to the un-treated control.
Appendix II: Tissue Culture Media

Table 11. Media Components Recipe for 1 L of Chinese Chestnut Pre-Rooting Media (CCPR)

<table>
<thead>
<tr>
<th>Media Component</th>
<th>Stock Solution</th>
<th>Amount per 1 Liter</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>1 L</td>
<td></td>
</tr>
<tr>
<td>WPM</td>
<td>2.3 g</td>
<td></td>
</tr>
<tr>
<td>N + N</td>
<td>109 mg</td>
<td></td>
</tr>
<tr>
<td>Sucrose</td>
<td>30 g</td>
<td></td>
</tr>
<tr>
<td>Agar</td>
<td>7 g</td>
<td></td>
</tr>
<tr>
<td>BA</td>
<td>1 mM</td>
<td>.25 mL</td>
</tr>
<tr>
<td>IBA</td>
<td>1 mM</td>
<td>.25 mL</td>
</tr>
</tbody>
</table>

Table 12. Media Components Recipe for 1 L of American Chestnut Multiplication Media (ACM)

<table>
<thead>
<tr>
<th>Media Component</th>
<th>Stock Solution</th>
<th>Amount per 1 Liter</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>1 L</td>
<td></td>
</tr>
<tr>
<td>WPM</td>
<td>2.3 g</td>
<td></td>
</tr>
<tr>
<td>N + N</td>
<td>109 mg</td>
<td></td>
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<tr>
<td>Sucrose</td>
<td>30 g</td>
<td></td>
</tr>
<tr>
<td>Agar</td>
<td>7 g</td>
<td></td>
</tr>
<tr>
<td>BA</td>
<td>1 mM</td>
<td>0.5 mL</td>
</tr>
<tr>
<td>IBA</td>
<td>1 mM</td>
<td>5 μL</td>
</tr>
</tbody>
</table>
Resume

Tyler R. Desmarais
500 Mendon Road, Unit. 403 Cumberland, RI 02864
401-300-2473
Tdesmarais7@gmail.com

Education

State University of New York
MS, Plant Science and Biotechnology
January 2020

College of Environmental Science and Forestry (SUNY-ESF)

University of Rhode Island
BS, Environmental Horticulture and Sustainable Agriculture
May 2012

Lincoln University
Applied Horticulture
December 2010

Research Experience

SUNY-ESF
Research Assistant
2014-2018

Publications
