EFFECTS OF LIGHT REGIME AND IBA CONCENTRATION ON ADVENTITIOUS ROOTING OF AN EASTERN COTTONWOOD *(POPULUS DELTOIDES)* CLONE

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Abstract—Eastern cottonwood (*Populus deltoides*) has received a substantial amount of interest from *in-vitro* studies within the past decade. The ability to efficiently multiply the stock of established clones such as clone 110412 is a valuable asset for forest endeavors. However, a common problem encountered is initiating adventitious rooting in new micropropagation protocols. Stem segments were collected from bud-broken 1 year old clone 110412 cuttings, sterilized, and stimulated to initiate shoots. Developed shoots (~2 cm in height) were excised and placed into one of three rooting media that included indole-3-butyric acid (IBA) concentrations (0.5 mg/L, 1 mg/L, or 2mg/L) in full strength DKW Medium, full strength Gamborg B5 vitamins, 2 percent sucrose, 0.6 percent agar, 10 mg/L AMP, 0.2 ml/L of Fungigone. In addition to IBA concentration, cuttings were randomly assigned to light rack positions to test the effects of wide spectrum fluorescent light (100 µmol m^{-2} s^{-1} photosynthetically active radiation (PAR), 16/8 hour photoperiod) and light emitting diode light (LED; 4:1 red-to-blue diodes, 250 µmol m^{-2} s^{-1} PAR, 16/8 hour photoperiod). After a month of exposure, there was limited rooting exhibited across treatments. However, fluorescents (3.58 ± 1.02) produced significantly better performing microcuttings (judged on morphology, visual vigor, and survival) than LEDs (2.7±0.86) ($p<0.005$). The high light intensity of the LEDs may be prompting weaker performance through unfavorably high transpiration-induced auxin uptake. While LEDs may play a role in future micropropagation protocols, results suggest that wide spectrum florescent lights produce better performing eastern cottonwood 110412 microcuttings.

INTRODUCTION

Eastern cottonwood (*Populus deltoides*), in addition to other *Populus* species and their hybrids, has become an important constituent in woody species biotechnology, *in-vitro* culture, and genetic engineering (Confalonieri and others 2003). The species has been widely cultivated due to its adaptability, growth rate, woody biomass production, wood industry uses (e.g., paper and pulpwood), and has become established as a model system because of its small genome size, short rotation cycle, rapid growth rate, and ease of vegetative propagation (Confalonieri and other 2003, Jansson and Douglas 2007). Over the past 50 years or more, the identification and screening efforts of tree improvement programs have produced a collection of superior clones which could benefit from the application of vegetative multiplication techniques such as micropropagation (Thorpe and others 1991). While each micropropagation process encompasses complex genetic, molecular, and physiological relationships, one step of the process that has received a substantial amount of interest in recent years is the induction and formation of adventitious roots (Costa and others 2013, De Klerk 2002). Two *in-vitro* microenvironmental factors that have been investigated for stimulating favorable adventitious rooting characteristics are auxin concentration and light regime (i.e., quality, intensity, and photoperiod).

While auxin has been well documented as one of the main phytohormones in a number of physiological processes such as apical dominance it has also been documented in a number of adventitious rooting processes such as regulating cell division, cell elongation, and the initiation of root apical meristems (Mironova and others 2010). Recent success of eastern cottonwood micropropagation and *Agrobacterium* transformation studies has been documented with two commonly used auxins, indole-3-acetic acid (IAA) or Indole-3-butyric acid (IBA) (Cavusoglu and others 2011, Chaturvedi and others 2004, Yadav and others 2009). However, a recurrent trend in these experiments is clone-specific responses to identical auxin concentrations, which has prolonged the process of developing new protocols (John and others 2014, Yadav

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and others 2009). In addition, there are still a number of established clones for which a micropropagation, and therefore adventitious rooting, protocol has not been derived. Further research with these clones, such as the 110412, can help determine what auxin concentrations illicit the most favorable adventitious rooting characteristics and how clone-specific responses can be implemented in future protocols. Another benefit to modern micropropagation research is the investigation of new technological advancements such as light-emitting diodes (LEDs) and how they may affect adventitious root formation.

Light quality (i.e., color), intensity, and photoperiod can each be manipulated within an *in-vitro* system to produce a unique light regime with the intent of stimulating specific physiological and morphological changes, such as adventitious rooting, in microcuttings. Studies performed within the past 20 years have documented adequate *in-vitro Populus* adventitious rooting using low intensity (~50-100 µmol $m^{-2}s^{-1}$ PAR) white fluorescents on a 16 hour light / 8 hour dark (i.e., long-day) photoperiod (Cavusoglu and others 2011, Gozukirmizi and others 1998, Kang and others 2009, Noël and others 2002, Phan and others 2004, Thakur and Srivastava 2006, Yadav and others 2009). However, there have been a number of studies, conducted on both woody species and other crop species, that have documented the potential role of far-red light (Kraepiel and others 2001), red light (Li and others 2010), blue light (Pinker and others 1989), and the implications of potential auxin-light quality interactions (Britz and Sager 1990, Chée 1986). In addition, recent technological advancements such as LEDs have provided researchers new resources to implement within *in-vitro* light quality research.

LED's compact engineering, solid-state construction, and especially their specific light spectrum capabilities has caught the attention of a number of plant tissue culture and horticultural researchers (Bula and others 1991, Morrow 2008). While there has been documented success, in terms of favorable adventitious rooting characteristics, in a number of annual crops such as Akihime strawberry (*Fragria* x *ananassa* Akihime) (Nhut and others 2003) and upland cotton (*Gossypium hirsutum* L.) (Li and others 2010) there is currently a lack of woody species, and specifically eastern cottonwood, *in-vitro* research utilizing LEDs. Given the lack of information on micropropagation adventitious rooting protocols for eastern cottonwood clone 110412, especially in response to auxin concentration and light regime, the objective of this study was to investigate the effects of traditional fluorescent and LED light regimes and three IBA concentrations (0.5 mg/L, 1 mg/L, 2mg/L) on adventitious rooting.

METHODS

Plant Material

The eastern cottonwood 110412 clone was originally selected for propagation by the USDA Forest Service Center for Bottomland Hardwood Research due to its excellent rooting, good form, and above average growth on various sites during initial screening trials (Personal communication, Dr. Randy Rousseau). The 110412 clone has also been cited for its phytoremediation abilities (Cardellino 2001, Minogue and others 2012, Rockwood and others 2006).Approximately 61 cm long hardwood cuttings of clone 110412 were collected from 1-year old, coppiced trees located on the campus of Mississippi State University (Starkville, MS) during late winter 2013. Cuttings were taken out of cold storage (4°C) during late fall 2014, cut to 20 cm in length, and soaked in room-temperature water for one week. After soaking, the base of each cutting was dipped in GreenLight© Rooting Hormone (San Antonio, TX) and placed (dipped end down) into 950 ml Mini-Treepots (Stuewe & Sons, Inc., Tangent, OR) containing EarthGro topsoil until only ~25 percent of the cutting remained aboveground. The Mini-Treepots were then placed into 3.28 L growing trays, six pots per tray. The growing trays were placed underneath four Sylvania T12 40W wide spectrum fluorescent tube light bulbs emitting 102.3 ± 6.8 µmol $m^{-2}s^{-1}$ photosynthetically active radiation (PAR) on a 16/8 (i.e., long day) photoperiod until initial bud break. All hardwood cutting establishment took place in a lab at the School of Forest Resources, University of Arkansas at Monticello campus.

Stem Collection and Shoot Initiation

Internode and node segments were collected from shoots developed from hardwood cuttings in early January, 2015. These were cut into 3 cm segments and then surface sterilized using a 25 minute agitated wash in 15mL of 30 percent bleach-ddH_20 solution supplemented with ~1 mL Tween® 20 and overhead ultraviolent light exposure (fig. 1). After the initial wash, stem segments were gently rocked ten times with sterilized ddH_20, drained, and gently rocked an additional ten times with fresh sterilized ddH_20. Each stem segment was then pressed into a BioWorld™ Extra Deep Mono Petri Dishes containing ~36 mL of shoot initiation media (SIM) to stimulate shoot development. The SIM consisted of full strength McCown Woody Plant Medium (McCown and Lloyd 1981), 3 percent sucrose, full strength Gamborg B5 vitamins, 1 percent agar, 0.7 mg/L 6-Benzylaminopurine (BAP), 0.01 mg/L Indole-3-butyric acid (IBA), 0.3 mg/mL Ampicillin Trihydrate (AMP), and 0.5 ml/L of 100x stock solution Fungigone. SIM pH was adjusted to 5.8 using 1 M NaOH. All tissue transfers took place under a sterile hood and all plates were wrapped with Parafilm before removal from the hood. SIM plates were then randomly

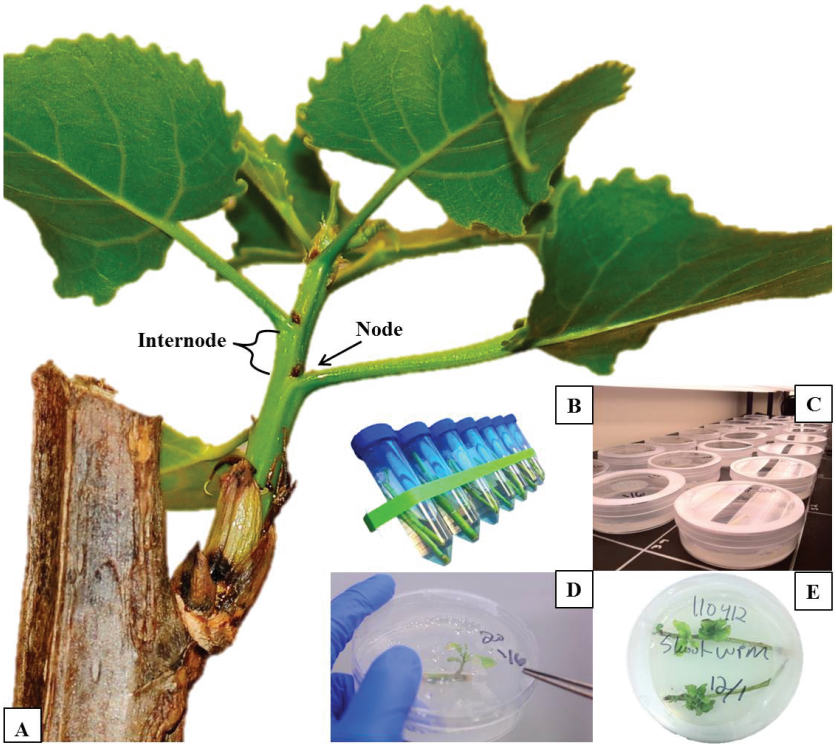

Figure 1—Stem Collection and Sterilization Process. The aboveground biomass, including node and internode segments, of eastern cottonwood (Populus deltoides) clone 110412 cuttings was excised in early January, 2015 (A). All non-stem material (i.e., leaf material) was excised from the stem segments before they were washed in 15 mL of a 30% bleach, ddH20 solution supplemented with approximately 1mL of Tween 20 for 25 minutes (B). The sterilized stem segments were cut into 3 cm segments, placed in SIM plates, and randomly distributed within the SIM light rack (C). After 2-3 weeks shoots were adequately initiated (D, E).

distributed across a SIM light rack (table 1). Stem segments were immediately transferred to a fresh SIM plate upon any sign of contamination.

Shoot Excision, Microcutting Collection, and Experimental Rooting Conditions

After a shoot reached approximately 2 cm in height in the SIM plates, the shoot was excised from its respective stem segment at the lowest feasible point of connection (e.g., microcutting). Each microcutting was vertically positioned in a PhytoTechnology Laboratories® 177 mL culture vessel enclosed by vented PhytoCaps™ containing ~42 mL of rooting media (RM). Three RM were tested, all identical except for different concentrations of IBA. Each RM consisted of full strength DKW medium with vitamins (Driver and Kuniyuki 1984, McGranahan and others 1987), full strength Gamborg B5 vitamins, 2 percent sucrose, 0.6 percent agar, 10 mg/L AMP, 0.2 ml/L of 100x stock solution Fungigone, and either 0.5 mg/L, 1 mg/L , or 2mg/L of IBA. Each RMs' pH was adjusted to 5.6 using 1 M NaOH.

Once microcuttings were randomly assigned to one of the three IBA concentrations, they were randomly placed on light racks where they were exposed to either fluorescent or LED light: 4 culture vessels for each RM per light rack. The fluorescent light regime consisted of four wide spectrum white fluorescent lights producing ~100 µmol m^{-2} s^{-1} PAR on a 16/8 light/dark hours (i.e., long day) photoperiod. The LED light regime consisted of two Tesler 120 watt rectangular indoor grow lights (44 three watt red (630nm) diodes and 11 three watt blue (460nm) diodes) producing ~250 µmol m^{-2} s^{-1} PAR on a long day photoperiod. Light racks were designed so that two of each light regime was tested at the same time. Light conditions varied between light racks, but light conditions were homogenous within racks (table 1). Light rack intensities measurements were taken at the top of each microcutting vessel with a quantum flux meter (Apogee Instruments, Inc.) and the long day photoperiod was achieved by producing 16 hours of continuous light (12:30am-4:30pm) followed by 8 hours of continuous dark (4:20pm-12:30am), as regulated by 24 hour timers. In order to ensure uninterrupted light exposure the RM vessels were placed upon the light

Table 1—Light Rack Specifications. Three light racks were utilized within the experiment 1) Shoot Initiation (SIM) 2) Light Rack 1 (LR1), and 3) Light Rack 2 (LR2). Each SIM contained 24 randomly distributed SIM plates and each LR1 and LR2 contained 12 randomly distributed culture vessels. P-values of a Welch's Two Sample t-test (n=48) and *Welch's Two Sample t-test (n=48) with unequal variances are presented

Light Rack	Light Source	Light Photoperiod (light hours / dark hours)	Trial	Light Intensity (μmol m^{-2} s^{-1} PAR ± SD)	p-value
SIM	Four Sylvania T12 40 W wide spectrum fluorescent tubes	16/8	1	105.88 ± 5.02	0.26*
			2	108.08 ± 8.04	
1	Four Sylvania T12 40 W wide spectrum fluorescent tubes	16/8	1	93.25 ± 6.62	0.35
			2	95.75 ± 6.09	
2	Two Tesler 120 W 4:1 red (630nm) to blue (430nm) diode ratio rectangular indoor LED grow lights	16/8	1	256.75 ± 12.14	0.99
			2	256.75 ± 12.75	

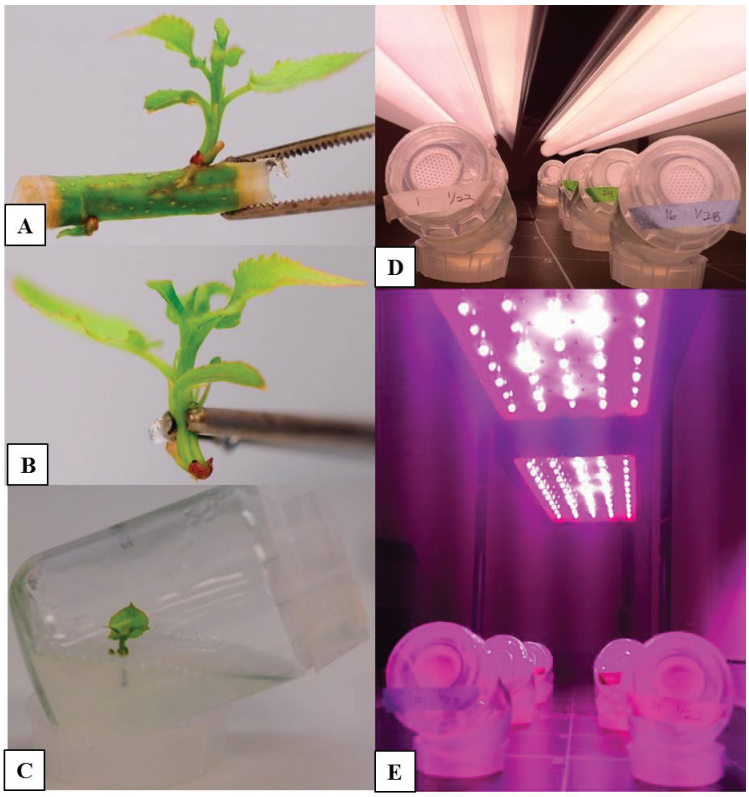

Figure 2—Shoot Excision and Light Regime Assignment. After a shoot reach approximately 2 cm in height it was removed from its SIM plate (A). The shoot was then excised at its base from the stem segment and was thereon termed a microcutting (B). Each microcutting was then randomly assigned a rooting media (RM) and placed into a RM vessel containing that RM (C). The RM vessel was then randomly assigned to, and within, either a wide spectrum fluorescent light rack (D) or a LED light rack (E) where they were exposed to the light regimes for a month.

racks at a 45° angle. Upon any sign of contamination, microcuttings were immediately transferred to a fresh RM vessel containing the same RM, and placed back into its respective position (fig. 2).

Measurements

Microcuttings were exposed to the light regimes for one month. During the trial, weekly performance ratings were conducted using a modified Kang and others (2009) scale that provided a gradient of poor to excellent performance (table 2). Microcuttings were examined daily for any signs of adventitious rooting. If rooting was observed it was documented so that the number of days to visible adventitious root for each microcutting was known. At the conclusion of the month-long exposure period, each microcutting was checked for any visible signs of rooting to determine rooting success, the number of adventitious roots per microcutting (rooting density), and the average length of each adventitious root. A Nikon D7100 with an attached 105 mm Nikon macro lens was used to document any noteworthy morphological changes such as leaf color variation or leaf necrosis that may have affected the rooting attributes measured.

Statistical Analysis

The study used a completely randomized split-plot design with light regime as the main factor and RM as the sub factor within each light regime. Only one shoot produced any signs of rooting which limited the ability to analyze any rooting attribute. Therefore, the only variable analyzed was the weekly performance ranking. Due to the ordinal scale of the rankings and preliminary analysis that found the data were non-normally distributed with heteroscedastic residuals, the nonparametric aligned rank transformation test was performed using the "art" function within the ARTOOL library (Wobbrock and others 2011) in R (version 3.1.2.) to examine the effects of light regime, RM (i.e., IBA concentration), and their interaction on microcutting performance rating (n=48).

RESULTS AND DISCUSSION

Although there have been several significant *in-vitro Populus* breakthroughs documented within the past 20 years, developing new micropropagation protocols are still susceptible to an array of difficulties due to the numerous microenvironmental factors that can be manipulated and their interactions. Rooting was observed in a sole microcutting that was subjected to the LED light regime and 2mg/L IBA. While the microcutting seemed to be consistent in terms of visual vigor (average performance rating of 4 throughout entire exposure period) the microcutting was produced from a stem segment after only 15 days. In comparison, the mean SIM phase duration across all microcuttings was 21.7 ± 5.45 days. The accelerated SIM phase could represent a superior (e.g., physiologically and genetically) stem segment whose superiority continued throughout subsequent processes such as adventitious root induction and formation.

While confident conclusions cannot be drawn from the minimal rooting, the weekly performance ratings revealed several interesting results. The aligned rank test showed that the average weekly performance rating through two weeks of exposure did not differ by light regime, RM, or their interaction ($p \geq 0.13$). However, after three weeks a significant difference in light regime was found ($p < 0.05$), which continued through the fourth week ($p < 0.01$) (table 3). The statistically significant results suggests that after three weeks of exposure, microcuttings in a wide spectrum fluorescent light regime had a higher ranking (3.96 ± 0.91) than microcuttings in a LED regime (3.26 ± 0.81) by an average of 0.70 (table 4). After an additional week, the average difference between light regimes grew to 0.88. This study's result of a wide spectrum white fluorescent

Table 2—Microcutting Performance Rating Scale. A modified Kang and others (2009) performance scale constructed to visually quantify microcutting performance on a weekly basis through the month long experiment. The scale represents a gradient from poor performance (1) to excellent performance (5)

Performance Rating	Description
1	Microcutting completely died
2	Microcutting nearly dead, leaves exhibiting severe necrosis and/or abnormal morphological changes
3	Microcutting has exhibited no growth since last rating and/or exhibiting moderate sign of necrosis and abnormal morphological changes
4	Microcutting has exhibited limited growth, only lower leaves (i.e., leaves touching media) are exhibiting necrosis and/or abnormal morphological changes
5	Microcutting actively growing, showing limited signs of leaf necrosis and/or abnormal morphological changes

light regime producing higher performing microcuttings supports both Cavusoglu and others (2011) who used cool white fluorescent light (50 µmol m^{-2} s^{-1}) to achieve 80 percent-100 percent rooting of eastern cottonwood clones 89 M 011, 89 M 044, 89 M 048, and 89 M 066 in two weeks and Yadav and others (2009) who used florescent tubes (45 µmol m^{-2} s^{-1}) to induce rooting of clones WIMCO199 and L34 after 4-5 weeks. While these studies did document adequate rooting, our lack of rooting may be due to the higher light intensity (100 µmol m^{-2} s^{-1}) produced by the fluorescent lighting that could have increased the microcutting transpiration rates (Jarvis and Ali 1984, Jarvis and Saheed 1987), resulting in greater exogenous auxin uptake—a factor that can be inhibitory to rhizogenic activity (Brock and Kaufman 1991, Garrido and others 2002). The same reasoning may also explain the lack of rooting and weaker performance of cuttings exposed to the LED light regime.

While there was not a statistical significant effect of IBA concentration over the exposure period, the lowest performance ratings resulted from microcuttings exposed to either 1 mg/L IBA within the LED light regime or the 2 mg/L IBA within the LED light regime each week (table 5). The LED's high light intensity (250 µmol m^{-2} s^{-1}) could have exacerbated the increased transpiration-induced exogenous auxin uptake over the exposure period. Another interesting result was observed of a continual (~0.6 rating points per week) decline in microcutting performance rating exposed to the LED light regime across the IBA concentrations. The continual decline in microcutting performance supports the documentation of high auxin concentrations being stimulatory within the induction phase (~ first 96 hours) but becoming progressively inhibitory after the initial induction phase (De Klerk 2002). However, there is also another light regime factor that was manipulated within the LED light regime, light quality. While the role of red and blue light qualities has been well documented in their regulation of phytochromes and cryptochromes, their role (and potential physiological associations) within *in-vitro* adventitous rooting is still unclear. Li and others (2010) documented a significant decrease

Table 3—Aligned Rank ANOVA. Results from the nonparametric aligned rank ANOVA test used to examine the effects of light regime, rooting media (RM) and their interaction over a four week period. Significance was assessed at the α = 0.05 level

Week	Factor	Sum of Squares	F Value	P Value
1	Light Regime	470.14	2.45	0.13
	RM	184.41	0.46	0.64
	Light Regime x RM	494.50	1.27	0.29
2	Light Regime	345.41	1.73	0.20
	RM	188.36	0.46	0.63
	Light Regime x RM	519.85	1.33	0.28
3	Light Regime	1441.35	8.24	<0.05
	RM	533.50	1.36	0.27
	Light Regime x RM	384.93	0.96	0.39
4	Light Regime	1735.02	10.66	<0.005
	RM	70.09	0.18	0.84
	Light Regime x RM	96.14	0.24	0.79

Table 4—Mean Performance Ratings by Light Regime. Mean (± 1 standard deviation) weekly performance ratings by light regime using a modified Kang and others (2009) scale

Light Regime	Week 1 Rating (±SD)	Week 2 Rating (±SD)	Week 3 Rating (±SD)	Week 4 Rating (±SD)
Wide Spectrum Fluorescent	4 ± 0.98	4.17 ± 0.87	3.96 ± 0.91*	3.58 ± 1.02**
Tesler 4:1 LEDs	4.43 ± 0.59	3.91 ± 0.79	3.26 ± 0.81*	2.7 ± 0.86**

* statistical significance at the 0.05 level
** statistical significance at the 0.005 level

Table 5—Average Weekly Performance Rating by Light Regime and IBA Concentration. Microcuttings were randomly assigned one of three IBA concentrations, light regime, and position within light regime to investigate their effects on adventitious rooting stimulation over a month long exposure period. Weekly performance ratings were taken using a modified Kang and other (2009) scale

Light	IBA (mg/L)	Week 1 Rating (±SD)	Week 2 Rating (±SD)	Week 3 Rating (±SD)	Week 4 Rating (±SD)
Wide Spectrum Fluorescent	0.5	3.88 ± 0.83	4.25 ± 0.71	4.00 ± 0.76	3.75 ± 0.89
	1	3.88 ± 0.99	3.75 ± 1.04	3.63 ± 1.10	3.38 ± 1.06
	2	4.31 ± 1.16	4.50 ± 0.76	4.25 ± 0.89	3.63 ± 1.19
Tesler 4:1 LEDs	0.5	4.63 ± 0.52	4.13 ± 0.83	3.63 ± 0.92	2.63 ± 0.92
	1	4.29 ± 0.76	4.00 ± 0.82	3.00 ± 0.58	2.57 ± 0.53
	2	4.38 ± 0.52	3.63 ± 0.74	3.13 ± 0.83	2.88 ± 1.13

in root activity of upland cotton (*Gossypium hirsutum* L.) microcuttings cultured under a 3:1 red (660 nm) to blue (460 nm) LED, standard white flourescent, and blue LED light regimes in comparison to microcuttings cultivated under a red light regime. Our study's 4:1 red (630 nm) to blue (430 nm) LED seems to be an unfavorable light quality and may be made more effective by decreasing the blue diode component, a light quality that has also been documented to be inhibitory to birch (*Betula pendula*) microcutting rooting (Pinker and others 1989) and loblolly pine (*Pinus taeda*) hypocotyl explant adventitous root elongation (Amerson and others 1988). Merkle and others (2005) also discussed the implications of red and blue LEDs when they documented a positive effect on germination, conversion, and early growth of *Pinus taeda, Pinus elliottii* Engelm.,and *Pinus palustris* Mill. somatic embryos with red wavelengths. However, the researchers were hesitate to designate the cause of the documented effects to a stimulatory effect of red wavelengths because they also documented a consistent poorer result from blue wavelengths in comparison to both red wavelengths and standard white flourescents. Those results could imply an inhibtory effect of blue wavelengths instead of a stimulatory effect of red wavelengths. In consideration of potential light quality and auxin interaction during *in-vitro* adventitious rooting, Rossi and others (1993) documented no significant differences in root induction or formation of damson plum (*Prunus insititia*) clone GF655-2 microcuttings exposed to blue, red, and white light qualties supplemented with either an absence or application of exogenus auxin.

Our study's results suggest that a wide spectrum flourescent light regime will produce better performing eastern cottonwood 110412 microcuttings regardless of IBA concentration. However, in developing future micropropagation protocols for the 110412 clone, other potential microenvironmental manipulations may include a lower light intensity (e.g., 50 µmol m^{-2} s^{-1}), relatively unstable auxin (e.g., indole-3-acetic acid) that can be relatively quickly photo-oxidized (De Klerk 2002, Fett-Neto and others 2001), and use of either white or red light qualities.

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