In Vitro Shoot Proliferation of Apple Rootstocks ‘B.9’, ‘G.30’, and ‘G.41’ Grown under Red and Blue Light

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Additional index words. light quality, gibberellin, *Malus × domestica*, shoot growth

Abstract. The influence of red and blue light wavelengths was tested to improve the initial in vitro multiplication of apple (*Malus × domestica*) rootstock cultivars Budagovsky 9 (B.9), Geneva 30 (G.30), and Geneva 41 (G.41). Single-node segments were established in semisolid Murashige and Skoog media and then transferred to proliferation media and cultured 40 days under white, red, or blue light irradiance. In a second experiment, G.30 was cultured under red, blue, or white light with and without gibberellic acid (GA₃). The three rootstocks responded similarly under white light in terms of shoot number, length of the longest shoot, and the number of elongated shoots. Red light increased the number of shoots, length of the longest shoot, and the number of elongated shoots of B.9 and G.30 when compared with white or blue light. Red light increased the number of elongated B.9 and G.30 shoots to five per explant compared with one per explant under white light. In contrast, shoot growth of G.41 showed no difference under the three light quality treatments, and the number of elongated shoots per explant was less than one. When compared with an absence of GA₃, a concentration of GA₃ at 0.5 mg·L⁻¹ promoted in vitro shoot growth of G.30 under red and blue light.

Micropropagation, a technique used to rapidly increase available stock of new cultivars, can be used for propagation of apple rootstocks (Webster and Jones, 1989). Micropropagation is also useful for production of stock plants with improved rooting ability (Quamme and Hogue, 1994; Webster and Jones, 1991). For apple, micropropagation is inefficient in the proliferation stage (Aklan et al., 1997; Dobránszki and Teixeira da Silva, 2010; Isutsa et al., 1998; Pua et al., 1983; Webster and Jones, 1989). Efficiency of proliferation requires a rapid increase in

the number of new shoots that elongate sufficiently to transfer to the rooting stage. For some woody plant taxa or genotypes, in vitro shoot growth is stunted with poor axillary branching and shoot tips that fail to elongate (Geng et al., 2012; Moreira da Silva and Debergh, 1997; Naik et al., 1999; Tao and Sugiura, 1992; Webster and Jones, 1989).

Some otherwise promising rootstocks have limited commercial use because propagation is difficult (Nelson, 1976; Quamme and Hogue, 1994). For example, poor shoot proliferation and insufficient shoot elongation occur with ‘Ottawa 3’ (Ott.3), ‘Malling 9’ (M.9), ‘Malling 26’ (M.26), ‘Budagovsky 9’ (B.9), and ‘P 2’ (Aklan et al., 1997; Lane and McDougald, 1982; Pua et al., 1983; Webster and Jones, 1991). New rootstocks in the Geneva series such as G.41 have been selected for reduced suckering and lack of burr knots, but with the undesirable consequence of being difficult to propagate (Robinson et al., 2011). Previous research on micropropagation of the Geneva series focused on rooting and establishment of viable explants (Isutsa et al., 1998), but methods to enhance shoot proliferation are necessary to decrease the time for rapid propagation of newly released cultivars.

Axillary shoot proliferation and elongation can be enhanced by altering the spectral light quality of in vitro plantlets (Chee and Pool, 1989; Economou and Read, 1987). Compared with white light, blue light increases in vitro shoot number in *Amelanchier* (Behrouz and Lineberger, 1981) and plum (Baraldi et al., 1988), but decreases shoot number and shoot length in *Spiraea* and apple (Norton et al., 1988a, 1988b). Red light increases the length of axillary shoots in apple (Muleo and Morini, 2006, 2008), plum (Muleo and Thomas, 1997), *Azorina vidalii* (Moreira da Silva and Debergh, 1997), and *Vaccinium corymbosum* (Noë et al., 1998), but not in grape (Chee and Pool, 1989). The inconsistent responses may be the result of variation in physiology among taxa or in conditions among experiments (Baraldi et al., 1988; Economou and Read, 1987; Norton et al., 1988a). At low intensity, blue light has little effect on shoot growth of tobacco, but at higher intensity, it enhances shoot growth compared with red or yellow light (Seibert et al., 1975). At low intensity, red light produces the greatest proliferation rate, but under higher intensity, red light does not differ from white or blue light in its effect on shoot proliferation (Baraldi et al., 1988). Altering light quality can be an efficient method for improving shoot proliferation, because it does not increase the duration of the propagation cycle, unlike prolonged subculturing (Webster and Jones, 1991).

Few studies have measured variation in response to light quality among apple rootstock cultivars. In MM.106 apple, culturing under red or blue light reduces in vitro shoot number compared with white or green light, but red light increases shoot length (Muleo and Morini, 2006). In contrast, shoot number and shoot length of the M.9 apple are increased by red light relative to blue, yellow, green, white, or far-red light (Muleo and Morini, 2008).

GA₃, added to proliferation media in combination with cytokinin and auxin, improves shoot proliferation in apple (Pua et al., 1983), but responses may vary with explant condition (Elliot, 1972). Light quality can affect phytohormone balance in developing shoots, with red and blue light altering endogenous auxin and gibberellins (Baraldi et al., 1995). We hypothesized that light quality may alter responses of apple rootstocks to exogenous GA₃.

The purpose of this study was to test the effect of blue and red light on in vitro shoot growth of three apple rootstocks, B.9, G.30, and G.41, which vary in their ease of propagation. Additionally, we tested the effect of blue and red light in combination with GA₃ on in vitro shoot growth of G.30.

Materials and Methods

Plant material. Three apple rootstock cultivars, B.9, G.30, and G.41, were used in this study. Propagation research on protected Geneva® rootstock material was conducted with consent from the lead breeder. One-year-old trees were stored at 1 °C for ≈1 month and then transferred to a heated,

Received for publication 21 Nov. 2014. Accepted for publication 7 Jan. 2015.

Funded in part by a grant from the Maine Agricultural Center and by the National Institute of Food and Agriculture.

Mention of a trademark, proprietary product, or vendor does not constitute a guarantee or warranty of the product, and does not imply its approval to the exclusion of other products that also may be suitable. Maine Ag. and Forest Exp. Stn. publication no. 3395. ¹Current address: College of Horticulture, Life Science Building, Nanjing Agricultural University, Nanjing 210095, China.

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glass-glazed greenhouse at the University of Maine (Orono, ME) at the end of February in both 2012 and 2013. The rootstocks were planted into 3.8-L plastic containers filled with MetroMix 560 (Scotts-Sierra Horticultural Products Company, Marysville, OH) in 2012 and Fafard 3B Mix (Sunagro, Agawam, MA) in 2013 and watered as needed. Testing with Agdia kits revealed no virus infections of the sprouts (Agdia, Inc., Elkhart, IN). After 2 months in the greenhouse, young softwood shoots were cut off in the early morning to provide the raw explants for aseptic establishment of shoots *in vitro*.

Aseptic culture establishment. Collected shoots were immediately placed in a beaker containing distilled water and transferred to a tissue culture laboratory. To reduce transpiration, collected shoots were defoliated and trimmed to 1 to 1.5 cm with one or two nodes. The nodal cuttings were surface-disinfested for 30 s in 70% ethanol and soaked for 10 min in a solution containing 0.6% sodium hypochlorite and several drops of Tween 20 (Agdia® Inc.). All nodal cuttings were rinsed three to five times under a laminar hood using sterilized, distilled water.

MS basic medium with 30 g·L⁻¹ sucrose and 8 g·L⁻¹ agar (Sigma Chemical Co., St. Louis, MO) was used in all experiments. The pH of all media was adjusted to 5.8 ± 0.2 with 1 mol·L⁻¹ NaOH or 1 mol·L⁻¹ HCl solution after adding agar. An aliquot of 10 mL of the medium was pipetted into glass tubes, capped, and autoclaved at 121 °C for 30 min.

Each nodal cutting was vertically inserted into 60-mL disposable culture tubes (borosilicate glass, 25 × 150 mm) containing 10 mL MS culture media. Tubes with explants were set in tube racks and sealed in a plastic bag. Cultures were arranged randomly on shelves under cool-white fluorescent lamps (Philips 60-Watt 4 FT T8 type; Somerset, NJ) and incubated at a temperature of 27.2 ± 1.9 °C with a 16-h photoperiod. Photosynthetic photon flux of normal white light was 25 μmol·m⁻²·s⁻¹ as measured using a LQM 70-10 quantum light meter (Apogee Instruments Inc., Logan, UT). The relative humidity in the culture room was 70% ± 5%. After 40 d of culture, newly sprouted shoots were used to test the effect of light quality on shoot elongation and shoot multiplication.

Expt. I: Effects of light quality on *in vitro* shoot growth of B.9, G.30, and G.41. The established *in vitro* shoots of B.9, G.30, and G.41 were dissected and transferred into Magenta™ vessels (GA-7, 77 × 77 × 97 mm; Sigma Chemical Co., St. Louis, MO) containing 50 mL MS base medium supplemented with 6-benzyladenine (6-BA) at a dose of 1.0 mg·L⁻¹, indole-3-butyric acid (IBA) at 0.1 mg·L⁻¹, and GA₃ at 0.5 mg·L⁻¹. The GA₃, which is not heat-stable, was dissolved in 95% alcohol in a laminar hood and pipetted into sterilized media cooled to ≈60 to 70 °C after autoclaving. Shoots were cultured under the same conditions as those used during the establishment phase, but with white, red, or blue light quality. Blue and red light qualities were provided by plastic filters

Table 1. Analysis of variance *P* values for the effect of light quality on microshoot growth of B.9, G.30, and G.41 apple rootstock cultivars.

Source	No. of shoots/explant	No. of elongated shoots/explant	Length of the longest shoot
Light	0.0002	0.0005	<0.0001
Cultivar	0.0002	<0.0001	<0.0001
Light × cultivar	0.0016	NS ^y	<0.0001

^aElongation is defined as a minimum shoot length of 1.5 cm.

^yNS = nonsignificant at alpha = 0.05.

(Rosco® Roscolux #74 for blue light and #27 medium red for red light; Rosco Laboratories Inc., Stamford, CT) that allowed primarily blue (420 to 520 nm) or red (600 to 740 nm) wavelength to pass. Both light filters were made of coextruded polycarbonate plastic and dyed polyester with a transmission rate of 4%. Irradiance in the blue range (400 to 500 nm) was estimated at 5.7 μmol·m⁻²·s⁻¹ and that in the red range (600 to 700 nm) was estimated at 6.6 μmol·m⁻²·s⁻¹ based on a blue output of 23% and a red output of 26% from cool-white fluorescent lighting (Chee and Pool, 1989).

Expt. II: Effects of gibberellic acid and light quality on *in vitro* shoot growth of G.30. The established G.30 *in vitro* shoots were dissected and transferred into Magenta™ square vessels containing 50 mL MS medium supplemented with 1.0 mg·L⁻¹ 6-BA and 0.1 mg·L⁻¹ IBA. Media in half the cultures contained no GA₃, whereas media the other half contained 0.5 mg·L⁻¹ GA₃ added after autoclaving. Shoots were cultured under white, red, or blue light in the same culture room as in Expt. I.

Data collection. After culturing for 40 d, the number of microshoots per explant, the length of the longest microshoot per explant, and the number of elongated microshoots (defined as microshoots 1.5 cm or greater) were measured. Shoots were characterized as elongated (score of 1) or non-elongated (score of 0). For each treatment combination, multiplication rate was calculated as the percentage of explants with two or more shoots, and elongation rate was calculated as the percentage of microshoots with a shoot length of 1.5 cm or longer.

Experimental design and statistical analysis. In both experiments, we used a completely randomized design with at least four replicates per treatment per rootstock and eight explants per replicate. All data were analyzed using Statistical Analysis Systems (Version 9.3; SAS Institute Inc., Cary, NC). Analysis of variance was used to test for variation in the maximum *in vitro* shoot length. The multiplication rate and elongation rate were analyzed as binomial data, and the number of microshoots and elongated microshoots was analyzed as negative binomial data. Means separation was carried out using Fischer's least significant difference method with an alpha of 0.05.

Results and Discussion

Expt. I. Light quality affected the number of shoots per explant, although the effects

Fig. 1. Microshoot growth of B.9, G.30, and G.41 apple rootstocks after 40 d of proliferation culture under blue, red, or white light. Means followed by the same letter are not significantly different by Fisher's least significant difference (LSD) test at $\alpha = 0.05$.

varied by cultivar (Table 1). For G.41, light quality had no effect on the number of shoots per explant, number of elongated shoots per explant, or length of the longest shoot (Fig. 1). Blue light decreased shoot number of B.9 compared with white light and slightly, but non-significantly, decreased shoot number for G.30. In contrast, the red light treatment enhanced shoot number for both B.9 and G.30 and increased both the number of elongated shoots and length of the longest shoot (Figs. 1 and 2). Similar results occurred for the multiplication rate (data not shown). In general, B.9 and G.30 demonstrated the potential to respond to light quality during *in vitro* growth, whereas G.41 did not.

The red light treatment produced greater shoot multiplication and elongation for some rootstocks despite a light intensity that was 25% lower than that of the white light treatments, suggesting that light quality, instead of intensity or photon flux, was the primary driver of the observed differences in shoot number, elongation, and length of the longest shoot. Red light promotes axillary shoot elongation at the low light intensity that occurs in culture rooms, whereas blue light does not effectively promote axillary bud development at low light intensity (Baraldi et al., 1988; Muleo and Morini, 2003). Because Roscolux red light filters transmit a substantial component of far-red wavelengths, the developmental differences among treatments may have resulted from red to far-red light ratios detected by the phytochrome system in existing foliage. The red light filter allowed transmission of both red and far-red light, but cool-white fluorescent lights produce only $\approx 1\%$ far-red output (Chee and Pool, 1989). Although far-red light promotes internode elongation of apple shoots, Muleo and Morini (2008) found that total shoot length is not increased because fewer nodes are formed. We could not determine conclusively whether the increased shoot elongation observed in our study was the result of red or far-red light, because they co-occurred in the red treatment and the number of nodes per shoot was not counted. The use of light-emitting diode lighting could change the results as a result of differences in far-red output, higher light intensity, and lower radiant temperatures compared with cool-white fluorescent lights (Kim et al., 2004; Morrow, 2008).

The overall decrease in growth that resulted from the blue light treatment compared with white light likely resulted from the lower light intensity in the blue treatment in our study. However, the poor performance of cultivar G.41 in the blue light treatment (Fig. 1) despite the four times greater irradiance under the white lights suggests either that the low light level under the blue treatment was sufficient to saturate light requirements of explants of that cultivar or that factors other than quantum flux are regulating the developmental process.

Expt. II. Both GA₃ and light quality affected in vitro microshoot proliferation of G.30 rootstock after 40 d of culturing in the proliferation stage with no interaction between GA₃ and light quality (Table 2). GA₃ significantly increased the number of shoots per explant and the length of the longest shoot, but not the number of elongated shoots. Light quality had a significant effect on microshoot growth, including the number of shoots per explant, the length of the longest shoot, and the number of elongated shoots. Blue and red light increased the number of shoots per explant compared with white light (Fig. 3). Red light increased the number of elongated shoots compared with white and blue light. Red light also increased the length of the

Fig. 2. Microshoot growth of G.30 apple rootstock under (A) blue, (B) red, or (C) white light.

Table 2. Analysis of variance *P* values for the effect of light quality and gibberellic acid (GA₃) on microshoot growth of G.30 apple rootstock.

Source	No. of shoots/explant	No. of elongated ^z shoots/explant	Length of the longest shoot
GA ₃	0.0344	NS ^y	0.0059
Light	0.0326	<0.0001	0.0004
GA ₃ × light	NS	NS	NS

^zElongation is defined as a minimum shoot length of 1.5 cm.

^yNS = nonsignificant at alpha = 0.05.

longest shoot compared with blue and red light.

Previous studies showed an average in vitro multiplication rate for apple of six to seven shoots per explant (Druart, 2003). However, the length of time in culture in our study was much shorter and involved the initial subculture after the establishment phase. Although the multiplication rate of rootstocks under blue and white light was typical for the first subculture, red light produced rates of multiplication exceeding those achieved in previous studies (Sun et al., 2014; Webster and Jones, 1991). However, this was not true of the G.41 rootstocks, which failed to proliferate and did not respond to light quality. Additional rounds of subculturing may increase the multiplication rate of apple rootstock cultivars (Grant and Hammatt, 1999; Webster and Jones, 1991), a practice that could be investigated for its effect on proliferation of G.41.

The effect of red light on G.30 was more pronounced in Expt. I than in Expt. II, despite the similar media and culture room conditions. Different stock plants were used in each experiment, and conditions in the greenhouse varied as well. It is not clear how growing conditions for stock plants affect responses of explants in vitro, but differences in growth persisting with subculturing have been demonstrated among rootstocks of M.9 (Grant and Hammatt, 1999; Webster and Jones, 1989).

Conclusions

The influence of light environment and GA₃ on growth of apple rootstocks in culture is highly variable by cultivar. Culturing under red light increased the number of shoots, maximum shoot length, and number of elongated shoots for B.9 and G.30 but not G.41. For these same cultivars, addition of GA₃ enhanced the increase in shoot number under blue light and the increase in elongated shoots under red light. Under white light, the addition of GA₃ did not influence shoot growth meaningfully. The effects of GA₃

Fig. 3. Microshoot growth of G.30 apple rootstock after 40 d of proliferation culture on media with and without gibberellic acid (GA₃) and under blue, red, or white light. Means followed by the same letter are not significantly different by Fisher's least significant difference (LSD) test at $\alpha = 0.05$.

concentration and phenological age of the explants (Elliot, 1972) on shoot proliferation of apple rootstocks should be investigated to further enhance multiplication rates during micropropagation.

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