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In vitro proliferation and *ex vitro* rooting of microshoots of commercially important rabbiteye blueberry (*Vaccinium ashei* Reade) using spectral lights

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ABSTRACT

Efficient protocols for *in vitro* shoot growth and *ex vitro* root formation from *in vitro*-derived shoots under light-emitting diodes (LEDs) with simple ventilation were developed for rabbiteye blueberry cv. 'Titan'. Red LEDs promoted shoot elongation while blue LEDs induced short shoots with enhanced accumulation of leaf chlorophyll contents. A combined treatment of 80% red with 20% blue LEDs was found the most suitable for plant growth, and more effective than 50% red mixed with 50% blue LEDs or fluorescent lamps as the control treatment. The use of ventilated vessels with ambient CO_2 enrichment from a growth chamber resulted in healthy plants having greatly improved shoot length, leaf expansion, leaf chlorophyll contents and dry weight of both shoots and roots. *In vitro*-cultured shoots could be directly rooted at 100% under non-sterile conditions using a perlite-peat mixture with careful controls of high humidity. These results suggest that LEDs, particularly when coupled with ventilated vessels, should be used as a primary light source for replacing conventional fluorescent tubes in large-scale production of rabbiteye blueberries.

1. Introduction

The rabbiteye blueberry (*Vaccinium ashei* syns. *V. virgatum, V. amoenum*), a member of the family Ericaceae, is native to the North American subtropical regions and mostly found in the southeastern United States (Prodorutti et al., 2007; Yadong et al., 2003). Similar to other *Vaccinium* species including highbush blueberry (*V. corymbosom*), lowbush blueberry (*V. angustifolium*) and half-highbush blueberry (*V. corymbosum* × *V. angustifolium*), rabbiteye blueberry shrubs are of commercial importance, owing to their small fruits containing high contents of antioxidant phenolic compounds for human health benefits, such as anthocyanins, flavonols and phenolic acids (Debnath, 2007, 2009; Howell, 2009; Tetsumura et al., 2012). Despite this similarity, rabbiteye blueberry cultivars can produce tall plants of up to 20 feet with much longer fruitful lives (approx. 20 years) and lesser vulnerability to diseases than those of other blueberry species (Prodorutti et al., 2007; Yadong et al., 2003).

Propagation of blueberry crops using true seeds exhibits high rates of heterozygosity (Prodorutti et al., 2007; Sedlak and Paprstein, 2009). Therefore, blueberry bushes are conventionally propagated vegetatively by stem nodal cuttings for preserving their genetic structure and uniformity, however, this conventional method is costly, labour-intensive, time-consuming, and commonly faces challenges in rooting capacity (Litwińczuk, 2013; Litwińczuk et al., 2005; Meiners et al., 2007; Zhao et al., 2011). These shortcomings can be overcome through the use of *in vitro* propagation techniques to allow rapid multiplication of elite geno-

Abbreviations: B, 100% blue LEDs; FL, Fluorescent lamps; LEDs, Light-emitting diodes; R, 100% red LEDs; R5B5, 50% red plus 50% blue LEDs; R8B2, 80% red plus 20% blue LEDs; MS, Murashige and Skoog; MW, Medium containing 50% MS plus 50% WPM; WPM, Woody Plant Medium.

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types with high capacity of root formation, greater uniformity of plantlet progenies and complete independence from environmental or seasonal impacts (Litwińczuk et al., 2005; Meiners et al., 2007). However, there are very few reports on micropropagation of rabbiteye (Tetsumura et al., 2012; Yadong et al., 2003) in comparison with highbush (Finn et al., 1991; Meiners et al., 2007; Reed and Abdelnour-Esquivel, 1991; Sedlak and Paprstein, 2009) and lowbush (Debnath, 2007, 2009; Finn et al., 1991; Zhang et al., 2006) blueberries. This is probably due to the 'difficult-to-micropropagate' nature of many rabbiteye blueberry genotypes, particularly the cultivar 'Titan'. So far, blueberry micropropagation has been frequently successful with culture media and plant hormones (Debnath, 2007, 2009; Meiners et al., 2007; Sedlak and Paprstein, 2009; Tetsumura et al., 2012; Yadong et al., 2003). Whereas, other environmental conditions are little studied in spite of their remarkable effects on the *in vitro* plant growth and morphogenesis (Finn et al., 1991; Reed and Abdelnour-Esquivel, 1991; Zhang et al., 2006).

Ventilation as a type of gas exchange is one of the important environmental factors that can exert strong influences on plant growth and development (Kozai et al., 2005; Pérez et al., 2015). Gas exchange is reported to have relationships with physiological responses including leaf chlorophyll content change, stomatal opening, transpiration and water potential in soil-cultivated blueberry species (Erbl et al., 1991; Wright et al., 1993). However, there is little investigation on how or whether gas exchange can also have beneficial effects on blueberry growth under *in vitro* or closed environments using airtight culture vessels with small air volume as previously extensively described in numerous plant species (Kozai et al., 2005).

Light, a primary source of energy important for signalizing tissue-cultured plants, is another major environmental factor known to remarkably affect the plant physiological processes and metabolism (Folta et al., 2014). Blueberries are sensitive to light environment with respect to light intensity, light quality and photoperiod (Kim et al., 2011; Lobos et al., 2012). Until now, the artificial lighting sources used for the cultivation of blueberries and other plants have primarily consisted of fluorescent lamps, high pressure sodium lamps, incandescent lamps or metal halide lamps. Recently, light-emitting diodes (LEDs) have been reported as a novel lighting system for improving plant growth in closed research rooms (Gupta and Jatothu, 2013).

The objective of this research was to use LEDs coupled with enhanced ventilation for improving the micropropagation of rabbiteye blueberry cv. 'Titan'. Specifically, red, blue and combined red and blue LEDs were used together with fluorescent tubes (as control) during (1) *in vitro* growth of shoots and (2) subsequent *ex vitro* rooting of *in vitro* shoots cultured in plastic vessels with lids having gas permeable filters.

2. Materials and methods

2.1. Source of explants and medium preparation

Six-week-old multiple-shoots of rabbiteye blueberry cv. 'Titan' obtained from the plant tissue culture laboratory of the LED Agri-bio Fusion Technology Research Center, Chonbuk National University, Iksan, South Korea were used as the initial source of explants. The *in vitro* multiple-shoots were excised into micro-cuttings at 1.5 cm in length before culturing onto growth medium consisting of 50% MS (Murashige and Skoog, 1962; Duchefa Biochemie, Haarlem, The Netherlands) and 50% WPM (Lloyd and McCown, 1980; Duchefa Biochemie, Haarlem, The Netherlands) micro-salts, macro-salts and vitamins (referred to as MW medium hereafter). The MW medium was supplemented with 3% (w/v) sucrose and 1 mg L^{-1} zeatin riboside (Duchefa Biochemie, Haarlem, The Netherlands) with pH adjusted to 5.0, and solidified with 2.15% (w/v) gelrite-agar prior to autoclaving at 121 °C, 105 kPa for 20 min (Meiners et al., 2007).

2.2. Vessel preparation for ventilation and nodal cutting culture

The MW medium was dispensed into 600 mL clear polypropylene vessels (100 mL per vessel) that were equipped with membrane-vented polypropylene lids of 80 mm in diameter (40 mm microporous polypropylene membrane and 0.053 mm pore size; SPL Life Sciences Co., Pocheon-si, South Korea; referred to as 'Ventilation' hereafter). Control vessels were capped without membrane filters (referred to as 'Non-ventilation' hereafter). Five micro-cuttings were transferred into each vessel and ten vessels were used for each treatment.

2.3. Light treatments

All micro-cutting cultures were incubated in a growth chamber for shoot proliferation during 6 weeks at 22 °C under five following light sources (Fig. 1A) with a 16-h photoperiod and photosynthetic photon flux density (PPFD) uniformly controlled at 50 μmol m^{-2} s^{-1}:

(1) Cool-white fluorescent lamps (OSRAM, Korea) as control (FL);
(2) 100% red LEDs (R);
(3) 80% red plus 20% blue LEDs (R8B2);
(4) 50% red plus 50% blue LEDs (R5B5); or
(5) 100% blue LEDs (B).

The above bar-type LED apparatus was designed and provided by the OD-Tech Co. (Jeollabuk-do, Korea) as previously described by Hung et al. (2015). Briefly, each LED lighting system consisted of 8 LED bars (Fig. 1A) mounted in parallel on a rectangular aluminium board and linked with a central controller (Version 2.0) for adjusting the photon flux, spectral distribution and lighting cycle. Each LED bar was equipped with an electric current and a pipe of running cold water to reduce heat generated during irradiation. Spectral distributions in relative energy of the red (peak wavelength: 660 nm, semiconductor material: AlGaInP, forward voltage: 2.0–2.2), blue (peak wavelength: 460 nm, semiconductor material: InGaN, forward voltage: 2.9–3.3), and red plus blue (1:1 photon flux density) LEDs were calculated by a spectroradio meter (LI-250A, LI-COR, USA) ranging from 300 to 800 nm with half bandwidths of peak wavelengths at ±20 nm.

2.4. Ex vitro rooting of microshoots

After 6-week light treatments, microshoots obtained from ventilated and unventilated vessels were carefully rinsed off gelrite-agar, divided into single segments of 3 cm in length (referred to as macro-cuttings hereafter), and then transferred into 600 mL clear polypropylene vessels with and without ventilation, respectively, following the method as described above. All vessels contained 200 mL of non-sterile perlite and peatmoss mixture (Top Blueberry Co., Cheonan, South Korea; v/v = 1:6) that was well-wetted by tap water with pH adjusted to 5.0. All the macro-cutting cultures were maintained for 6 weeks at 22 °C in a growth chamber under five corresponding light sources with the same photoperiod and PPFD as used for shoot proliferation phase (Fig. 1A).

2.5. Data collection

The variables recorded consisted of the lengths and weights of shoots and roots, the numbers of shoots, nodes, leaves and roots per