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Micropropagation of blueberry 'Bluejay' and 'Pink Lemonade' through in vitro shoot culture

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ABSTRACT

This study systematically evaluated effects of culture media, cytokinins, and auxins on axillary shoot induction as well as auxins and media or substrates on in vitro and ex vitro rooting of a highbush cultivar Bluejay (*Vaccinium corymbosum* L.) and a *V. ashei* rabbiteye hybrid derivative 'Pink Lemonade'. Optimal protocols for in vitro shoot culture of the two cultivars were developed. Two-node stem explants derived from axillary shoots were cultured on Anderson medium supplemented with 13.68 μM 6-(4-hydroxy-3-methylbut-2-enylamino) purine (ZT) and 0.27 μM naphthaleneacetic acid (NAA) for inducing axillary shoots of 'Bluejay' and 9.12 μM ZT and 0.05 μM NAA for 'Pink Lemonade' shoot proliferation. Microcuttings derived from the axillary shoots were cultured on half-strength Anderson medium supplemented with 0.49 μM indole-3-butyric acid (IBA) for in vitro rooting. The rooting percentages for 'Bluejay' and 'Pink Lemonade' were 75% and 65% in two months, respectively. However, by dipping microcuttings into 4.92 μM IBA for 10 s and rooting ex vitro in a substrate composed of 90% peat and 10% perlite, the rooting percentages could be improved to 89% for 'Bluejay' and 97% for 'Pink Lemonade'. After being acclimatized in a shaded greenhouse for three months, rooted plants were ready for planting in the field. The established methods could be used for rapid propagation of highbush and rabbiteye hybrid derivative cultivars for commercial blueberry production.

1. Introduction

Blueberries (*Vaccinium* spp.) are members of the family Ericaceae and belong to small-fruit crops. There are five main groups of blueberries: (1) highbush (*Vaccinium corymbosum* L.), (2) lowbush (*V. angustifolium* Ait., *V. myrtilloides Michx.*, and *V. boreale* Hall and Aald.), (3) half-high derived from crosses between highbush and lowbush, (4) southern highbush developed from crosses between *V. corymbosum, V. darrowii* Camp and other blueberry species, and (5) rabbiteye (*V. ashei* Reade) (Caspersen et al., 2016; Debnath, 2007a). Due to their high nutraceutical and pharmaceutical value (Basu et al., 2010; Miller and Shukitt-Hale, 2012; Whyte and Williams, 2015; Vendrame et al., 2016), blueberry production has been rapidly expanding. For example, worldwide highbush blueberry production increased from 58,400 ha in 2007–110,800 ha in 2014 (Brazelton, 2015). North America represented more than 50% of the production area and accounted for almost 60% of the global highbush blueberry production in 2014 (Brazelton, 2015).

Blueberries are traditionally propagated by stem cuttings (Marino et al., 2014). Cutting propagation may not be suitable for all cultivars due to low rooting percentages (Lyrene, 1981) and also may not be an effective method for quickly increasing numbers of starting materials or propagules for commercial introduction of new cultivars (Miller et al., 2004). Micropropagation has been considered the most effective method for rapid increase of propagules on a year-round basis (Murashige, 1974; Chen and Henny, 2008).

Micropropagation through axillary shoot production was reported in lowbush (Frett and Smagula, 1983), highbush (Tetsumura et al., 2008; Ruzic et al., 2012; Litwinczuk, 2013), and rabbiteye blueberries (Lyrene, 1980). Micropropagated blueberry plants have been reported to produce more fruit (El Shiekh et al., 1996) as well as generate cuttings with higher rooting ability in comparison with traditional cutting

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propagated plants (Lyrene, 1981).

Media used for in vitro shoot culture include MS (Murashige and Skoog, 1962), Anderson (Anderson, 1980), Z-2 (Zimmerman and Broome, 1980), and WPM (Lloyd and McCown, 1981). Shoot proliferation on MS medium was reported to be slow (Ruzic et al., 2012), and shoots tended toward hyperhydricity (Tetsumura et al. (2008). WPM was considered to be more suitable for micropropagation of highbush cultivars than Anderson (Wolfe et al., 1983; Sedlak and Paprstein, 2009), while Tetsumura et al. (2008) observed that the growth of shoots produced on WPM was poorer than those grown on MS or MW (a mixture of equal part of MS and WPM). Growth regulators commonly used for shoot proliferation are 6-γ-γ-(dimethylallylamino)-purine (2iP) or 6-(4-hydroxy-3-methylbut-2-enylamino) purine or zeatin (ZT) with indole-3-acetic acid (IAA) or 2iP alone (Debnath, 2007a; Litwinczuk, 2013). 2iP at high concentrations was reported to cause phytotoxicity (Debnath, 2006). Thus far, effects of different auxins on shoot proliferation of blueberry have been given little attention (Ruzic et al., 2012). Microcuttings derived from axillary shoots may be rooted ex vitro with or without IBA (indole-3-butyric acid), but limited information is available regarding in vitro rooting (Debnath, 2007a; Litwinczuk, 2013). These conflicting results in shoot culture of blueberry suggest that a systematic evaluation of culture media, media pH, growth regulators, and combinations of growth regulators on shoot proliferation and in vitro or ex vitro rooting is needed.

The objective of this study was to develop reliable protocols for in vitro shoot culture of blueberries through a systematic evaluation of different culture media, media pH, cytokinins, auxins, and combinations of cytokinin with auxin for in vitro shoot multiplication of a highbush and a *V. ashei* rabbiteye hybrid derivative cultivars. This study also evaluated in vitro and ex vitro rooting of microcuttings. Optimal medium, auxin treatment, and substrate were identified for rooting of the two cultivars.

2. Materials and methods

2.1. Plant materials

Young stems (about 12 cm) of 'Bluejay' (*Vaccinium corymbosum* L.) and 'Pink Lemonade' (a *V. ashei* Reade rabbiteye hybrid derivative) were collected from greenhouse-grown stock plants. After defoliation, stems were washed with running tap water for 1.5 h and cut to about 6 cm. The cut stems were immersed in 20% Clorox (1.2% NaOCl) solution with agitation for 20 min and rinsed with sterile distilled water three times under aseptic conditions. The stems were soaked in 75% ethanol for 10 s and rinsed with sterile distilled water three times. The stems were further cut as single nodes (about 1 cm) and placed in sterile petri dishes for initiation of in vitro culture.

2.2. Initial culture

Anderson medium (Anderson, 1980) (Product ID: A267, PhytoTechnology, Shawnee Mission, KS) was supplemented with 3% sucrose and 0.8% agar. The medium was autoclaved at 121 °C for 20 min after pH was adjusted to 5.0. When the temperature dropped to about 50 °C, filter-sterilized stock solutions of ZT and IAA were added, resulting in a final concentration of ZT at 2.28 μM and IAA at 0.57 μM. The medium was aliquoted to glass baby food jars at about 20 mL each. Sterilized single-node stem explants of 'Bluejay' and 'Pink Lemonade' were cultured on the medium and maintained in a culture room described below for initial induction of axillary shoots. Axillary shoots produced after eight weeks of culture were cut to two-node stem explants (about 1 cm) and cultured on Anderson medium supplemented with 4.56 μM ZT and 0.57 μM IAA for producing more axillary shoots.

2.3. Selection of basal media

Three culture media: Anderson, MS (Product ID: M524, PhytoTechnology, Shawnee Mission, KS), and WPM (Product ID: L154, PhytoTechnology, Shawnee Mission, KS) were tested. Each medium contained 3% sucrose and 0.8% agar with pH being adjusted to 5.0 before autoclaving at 121 °C for 20 min. When the temperature dropped to about 50 °C, filter-sterilized stock solutions of ZT and IAA were added to each medium, ending up 4.56 μM ZT and 0.57 μM IAA. Media were respectively aliquoted to baby food jars at 20 mL each. Two-node stem explants of 'Bluejay' and 'Pink Lemonade' were cultured on the media, four explants per jar and eight jars per treatment. After eight weeks of culture, shoot numbers per explant and shoot lengths were recorded.

2.4. Evaluation of medium pH effects

The pH of Anderson medium supplemented with 3% sucrose and 0.8% agar was adjusted to 4.5, 5.0, 5.5, and 6.0 respectively before autoclaving at 121 °C for 20 min. After autoclaving, ZT and IAA were added to the medium resulting in ZT at 4.56 μM and IAA at 0.57 μM. The medium was aliquoted to baby food jars at 20 mL each. Four two-node stem explants of the two cultivars were cultured on the medium with different levels of pH, and each treatment had eight jars. Shoot numbers per explant and shoot lengths were recorded after eight weeks of culture.

2.5. Selection of cytokinins

Autoclaved Anderson medium with pH at 5.0 was supplemented with BA (6-benzylaminopurine) at 2.22, 4.44, and 6.66 μM or ZT at 2.28, 4.56, and 6.84 μM, each with 0.57 μM IAA. Four two-node stem explants of the two cultivars were cultured on the medium, and there were four jars per treatment. Shoot numbers and shoot lengths were recorded eight weeks later.

2.6. Selection of auxin

IAA or NAA (naphthaleneacetic acid) were added to autoclaved Anderson medium with pH 5.0, which resulted in IAA at 0.29, 0.57, and 1.14 μM and NAA at 0.27, 0.54, and 1.07 μM, each with 4.56 μM ZT. Four two-node stem explants of the two cultivars were cultured on the medium, four replications per treatment. Shoot numbers and shoot lengths were recorded after eight weeks of culture.

2.7. Optimization of multiplication medium

Anderson medium with a pH of 5.0 was supplemented with 4.56, 9.12, and 13.67 μM ZT in a factorial combination with 0.05, 0.27, and 1.34 μM NAA. Four two-node stem explants of each cultivar were cultured on the medium with four jars per treatment. Shoot numbers per explant were recorded after eight weeks of culture. Based on collected data, a ZT and NAA combination that induced the highest numbers of axillary shoots per explant were identified for each cultivar, and they were considered to be the optimal medium for shoot proliferation. The media were then used to produce more axillary shoots for the following rooting experiments.

2.8. Rooting medium selection

Axillary shoots with a height about 2.5 cm were excised as microcuttings and rooted in Anderson, MS, and WPM media, each with a pH of 5.0, supplemented with 2.46 μM indole-3-butyric acid (IBA). There were four microcuttings per jar and four jars per treatment for each cultivar. Rooting percentage, root numbers, and mean root length were recorded after two months of culture.