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Modeling the effects of mineral nutrition for improving growth and development of micropropagated red raspberries

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ABSTRACT

In vitro propagation is important for rapid multiplication of a wide range of nursery crops, including red raspberries. The genetic variation of the many red raspberry cultivars makes it difficult to use one growth medium for all. Although some cultivars grow well on Murashige and Skoog (1962) medium (MS), others display stunting, hyperhydricity, discoloration, callus, leaf spots, or necrosis. This study used response surface methodology (RSM) to determine the effects of MS mineral salts on red raspberry growth and which of these mineral salts are critical for improving growth. *In vitro* growth of five red raspberry cultivars was determined by varying five factors that included NH_4NO_3, KNO_3, mesos salts ($CaCl_2$, KH_2PO_4 and $MgSO_4$), minor elements (Zn–Mn–Cu–Co–Mo–B–I), and EDTA-chelated iron. The effects of these five factors on plant quality, multiplication, shoot length, leaf size, leaf area, leaf color, callus and leaf spots were determined. The effects varied by cultivar for some characteristics, but all cultivars had improved growth or appearance on some experimental treatments when compared to MS medium. Increased mesos was the most significant factor associated with plant quality, multiplication and shoot length in all cultivars. Increasing iron above MS levels decreased quality in all cultivars except 'Willamette'. Decreased KNO_3 with increased mesos and low iron were required to improve shoot multiplication. Increased NH_4NO_3 resulted in greater shoot elongation only in 'Willamette'. Determining the driving mineral factors is the first step in improved medium formulations for micropropagated red raspberries.

1. Introduction

Red raspberries (*Rubus idaeus* L.) have been grown commercially in both Europe and the USA since the beginning of the nineteenth century (Jennings, 1988). European red raspberry cultivars were imported to the USA and were also crossed with native species. Europe, Asia and the Pacific Northwest coast of North America (British Columbia, Washington and Oregon) are the major production areas. Recent interest in raspberries for health and wellness is linked to their high levels of vitamins A and C, fiber and antioxidant activity (Barney et al., 2007). Shoot micropropagation, the technique used to grow shoots under sterile conditions for rapid propagation, is important for small-fruit nursery crop production. Due to the genetic variation of the many red raspberry cultivars (Jennings, 1988) that have diverse mineral nutrition requirements, micropropagation of red raspberry is often difficult (Anderson, 1980; Zawadzka and Orlikowska, 2006; Wu et al., 2009). Growth medium is one of the most important factors in micropropagation, and the most commonly used medium is that of Murashige and Skoog (MS) (1962). Mineral nutrients are the major components of plant growth media and are essential in plant growth and development (Murashige and Skoog, 1962; Anderson, 1984; Williams, 1993; George et al., 2008). They are constituents of essential molecules in plant cells or function as critical parts of cell structure (Epstein, 1972). Mineral elements also affect growth and development by activating enzymes or functioning as co-enzymes or cofactors. There are thirteen essential mineral nutrients classified in two groups. Major essential elements are taken up in relatively large amounts (N, P, K, Ca, Mg, S); minor essential elements are required and taken up in relatively small amounts (Fe, Mn, Zn, Cu, B, Cl, Mo) (Epstein, 1972). Although mineral nutrition is one of the crucial factors of plant micropropagation, little information is available on *in vitro* mineral nutrition.

Optimization of growth media based on mineral nutrition for micropropagation is very challenging due to the diverse nutrition requirements of various plant species and the many interactions of the chemical nutrients. There are many approaches for improving the growth medium based on mineral nutrition. Recent studies noted the effect of mineral nutrients on plant growth and development (Nas and Read, 2004; Adelberg et al., 2010; Halloran and Adelberg, 2011; Greenway et al., 2012). Previously, medium

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optimization or modification based on minerals was developed using traditional or factorial approaches (Murashige and Skoog, 1962; Driver and Kuniyuki, 1984). The traditional approach for optimizing the growth media in plant tissue culture was to vary the concentration of an interesting component or mineral as a single factor at a time. MS medium was developed using this approach with multiple experiments to cover all components. MS (1962) medium was developed for tobacco callus culture and is widely used as a growth medium for many plants, however, it is not suitable for many types of differentiated tissues and shoot cultures (Anderson, 1980; Amiri, 2006; Bell et al., 2009). Another approach for optimizing mineral composition is the triangular method, with varying concentrations of three factors at a time (Hildebrandt et al., 1946). Changes in the concentration of nutrient supply can also affect availability and uptake of mineral nutrients resulting in effects on growth and development (Williams, 1993). The most common way to improve growth media is modification based on changing mineral components compared to MS (Driver and Kuniyuki, 1984). In addition, optimum mineral supply for medium modification was investigated or defined by adapting the concentration of minerals to match the biological mineral components of *in vivo* plants (Morard and Henry, 1998; Monteiro et al., 2000) or field plants (Nas and Read, 2004). Individual plant species or cultivars have a range of mineral requirements for each particular nutrient that provide the optimal growth; both deficiencies and excesses can result from non-optimal concentrations (Ramage and Williams, 2002). The complexity of mineral nutrition with multiple interactions makes mineral optimization very difficult (Murashige and Skoog, 1962; Williams, 1993).

Response surface methodology (RSM) is now employed for modeling or optimizing the most important mineral component factors for *in vitro* plant growth (Niedz and Evens, 2007; Reed et al., 2013; Wada et al., 2013). Computer aided experimental design allows the study of several factors with fewer treatments, compared to the traditional studies or factorial designs (Niedz and Evens, 2007, 2008).

Plants cultured on MS medium (1962) often exhibit suboptimal growth and symptoms such as stunting, hyperhydricity, discoloration, callus, leaf spots, or necrosis (Singha et al., 1987; Dantas et al., 2001; Greenway et al., 2012; Reed et al., 2013). Shoot cultures of many of the red raspberry germplasm accessions at the USDA-ARS National Clonal Germplasm Repository had poor growth and showed these symptoms, indicating that MS medium was not providing optimum nutrition (Reed, 1990). In this study, we modeled the effects of MS mineral nutrients using response surface methodology and determined the most critical mineral salts for improved shoot quality and plant growth responses in five genetically-diverse micropropagated red raspberries.

2. Materials and methods

2.1. Plant materials and establishment of shoot cultures

Five red raspberry cultivars, Canby, Indian Summer, Nootka, Trailblazer and Willamette were grown on MS (Murashige and Skoog, 1962) medium with LS vitamins (Linsmaier and Skoog, 1965), 4.44 μM N^6-benzyladenine (BA), 0.49 μM indole-3-butyric acid (IBA), 0.29 μM gibberellic acid (GA), 30 g l^{-1} sucrose, 3.5 g l^{-1} agar (PhytoTechnology A111) and 1.45 g l^{-1} gellan gum (PhytoTechnology G434) at pH 5.7 and autoclaved. Shoot cultures were grown in Magenta GA7 boxes (Magenta Corp., Chicago, IL) with 40 ml of medium per box and transferred to fresh medium every 3 weeks. All plants were grown at 24 ± 1 °C and a 16 h photoperiod with 70–90 μM m^2 s^{-1} irradiance provided by a combination of cool- and warm-white fluorescent bulbs.

Table 1
The five factors used to construct the 5-dimensional design space, their component MS salts, and concentration range expressed as *X* MS levels.

Factors	MS salts	Range
Group 1	NH_4NO_3	0.5–1.5×
Group 2	KNO_3	0.5–1.5×
Group 3 (mesos)	$CaCl_2{\cdot}2H_2O$ KH_2PO_4 $MgSO_4$	0.5–1.5×
Group 4 (minors)	$MnSO_4{\cdot}H_2O$ $ZnSO_4{\cdot}7H_2O$ $CuSO_4{\cdot}5H_2O$ KI $CoCl_2{\cdot}6H_2O$ H_3BO_3 $Na_2MoO_4{\cdot}2H_2O$	0.5–4.0×
Group 5 (iron)	$FeSO_4{\cdot}7H_2O$ Na_2EDTA	0.5–4.0×

2.2. Growth medium experimental design

The first step, the experimental design, was a five-factor RSM design where the design points (combinations of the five factors) were selected using a modified D-optimal design using the software application Design-Expert®8 (Design-Expert, 2010). These points were suitable for fitting a quadratic polynomial equation (Niedz and Evens, 2007, 2008). Five mineral nutrient factors were based on MS salts: (1) NH_4NO_3, (2) KNO_3, (3) mesos ($CaCl_2{\cdot}2H_20$, KH_2PO_4, $MgSO_4{\cdot}7H_2O$), (4) micronutrients (B, Cu, Co, I, Mn, Mo, Zn), and (5) Fe-EDTA. Each factor was varied over a range of concentrations expressed in relation to MS medium (1× is the MS concentration) (Table 1). This experiment was setup as three sequential groups of treatments with MS points run with each group. There were 23 model points, 10 lack-of-fit points and 11 replicated points either within or on the surface of the five-dimensional design space (Table 2). For the next step, shoot-tips, about 1.5 cm, were cultured on a set of treatment combinations. Each treatment included five plantlets in each of two boxes. Shoots were transferred to the same medium at three week intervals and harvested after 9 weeks.

2.3. Data collection and statistical analysis

Plant response models using RSM (Design-Expert, 2010) were generated based on data taken from plants grown for 9 weeks: response data (described below) at each design point were estimated from the mean of six shoots from the two boxes or more if the points were internally replicated. Three plants were evaluated from predetermined locations in each box ($n = 6$) and the two remaining plants were photographed ($n = 4$). Graphical models for each response were extrapolated by modeling a map of the response as a function of two factors while holding the others constant. Quality ratings were assigned to each plant on a scale of 1 (poor quality), 2 (moderate quality) and 3 (good quality), leaf size rating of 1 (small), 2 (moderate) and 3 (large), leaf area of the typical leaf was measured in cm^2. Leaf color was scored from 1 (red or brown), 2 (yellow), 3 (pale green) and 4 (green), leaf spots/necrosis ranged on a scale of 1 (major symptoms on 2 or more leaves), 2 (minor) and 3 (absent), callus 1 (major > 2 mm), 2 (minor) and 3 (absent), number of shoots counted and shoot length of the longest shoot measured in mm (from base to shoot tip). The best fitting polynomial regression or extrapolated model was obtained for each measured response. The *F* value and *P* value of overall models analyzed by ANOVA significant at the 0.05 levels and lack of fit tests including R^2 were constructed. Model adequacy was tested by Design-Expert®8 (Design-Expert, 2010) for normality assumption,