Open Access Articles

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

The Faculty of Oregon State University has made this article openly available. Please share how this access benefits you. Your story matters.



| | |
|---|---|
| Citation | Poothong, S., & Reed, B. M. (2014). Modeling the effects of mineral nutrition for improving growth and development of micropropagated red raspberries. Scientia Horticulturae, 165, 132-141. doi:10.1016/j.scienta.2013.10.040 |
| DOI | 10.1016/j.scienta.2013.10.040 |
| Publisher | Elsevier |
| Version | Version of Record |
| Citable Link | http://hdl.handle.net/1957/47167 |
| Terms of Use | http://cdss.library.oregonstate.edu/sa-termsofuse |

Scientia Horticulturae 165 (2014) 132–141

Contents lists available at ScienceDirect

Scientia Horticulturae

journal homepage: www.elsevier.com/locate/scihorti

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

Sukalya Poothong[a,*], Barbara M. Reed[b]

[a] 4017 Agriculture & Life Sciences Building, Department of Horticulture, Oregon State University, Corvallis, OR 97331-7304, USA
[b] USDA ARS, National Clonal Germplasm Repository, 33447 Peoria Road, Corvallis, OR 97333-2521, USA

ARTICLE INFO

*Article history:*
Received 20 July 2013
Received in revised form 6 September 2013
Accepted 28 October 2013

*Keywords:*
*In vitro* culture
Mineral nutrition
Response surface methodology
*Rubus*
Red raspberry

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.
© 2013 Elsevier B.V. All rights reserved.

## 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

* Corresponding author. Tel.: +1 541 602 9255.
*E-mail addresses:* sukalya_p@hotmail.com, poothons@onid.orst.edu (S. Poothong).

0304-4238/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.scienta.2013.10.040

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) | $CaCl_2{\cdot}2H_2O$<br>$KH_2PO_4$<br>$MgSO_4$ | 0.5–1.5× |
| Group 4 (minors) | $MnSO_4{\cdot}H_2O$<br>$ZnSO_4{\cdot}7H_2O$<br>$CuSO_4{\cdot}5H_2O$<br>KI<br>$CoCl_2{\cdot}6H_2O$<br>$H_3BO_3$<br>$Na_2MoO_4{\cdot}2H_2O$ | 0.5–4.0× |
| Group 5 (iron) | $FeSO_4{\cdot}7H_2O$<br>$Na_2EDTA$ | 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,

**Table 2**
MS-stock based five-factor design including 23 model points, 10 lack-of-fit points, and 11 replicated points, and MS medium controls (points 44–46) for pure error estimation.

| Treatment design points[a] | Factor 1 $NH_4NO_3$ | Factor 2 $KNO_3$ | Factor 3 Mesos | Factor 4 Micros | Factor 5 Fe |
|---|---|---|---|---|---|
| 1 | 0.50 | 1.50 | 1.50 | 0.50 | 2.36 |
| 2 | 1.36 | 0.50 | 0.67 | 0.50 | 4.00 |
| 3 | 0.50 | 1.50 | 0.88 | 2.11 | 0.50 |
| 4 | 1.44 | 1.50 | 0.52 | 4.00 | 3.79 |
| 5 | 1.50 | 1.50 | 1.50 | 4.00 | 4.00 |
| 6 | 0.50 | 0.50 | 0.62 | 3.57 | 3.57 |
| 7 | 0.62 | 0.60 | 1.50 | 0.50 | 3.94 |
| 8 | 0.97 | 1.03 | 0.50 | 4.00 | 0.50 |
| 9 | 1.50 | 0.50 | 1.50 | 4.00 | 0.50 |
| 10 | 1.50 | 0.50 | 0.50 | 0.50 | 0.50 |
| 11 | 0.50 | 1.50 | 0.88 | 2.11 | 0.50 |
| 12 | 1.50 | 0.50 | 0.50 | 0.50 | 0.50 |
| 13 | 0.62 | 0.62 | 1.38 | 3.57 | 0.50 |
| 14 | 1.50 | 0.50 | 1.50 | 4.00 | 0.50 |
| 15 | 1.50 | 1.50 | 1.50 | 4.00 | 4.00 |
| 16 | 0.50 | 1.43 | 1.50 | 1.80 | 4.00 |
| 17 | 0.50 | 1.50 | 0.50 | 4.00 | 4.00 |
| 18 | 1.50 | 1.50 | 0.50 | 0.50 | 2.34 |
| 19 | 0.95 | 0.80 | 0.84 | 1.24 | 2.07 |
| 20 | 0.50 | 0.50 | 1.50 | 0.50 | 0.50 |
| 21 | 1.50 | 1.04 | 1.05 | 4.00 | 0.50 |
| 22 | 1.50 | 0.50 | 0.50 | 4.00 | 4.00 |
| 23 | 1.50 | 1.04 | 1.05 | 4.00 | 0.50 |
| 24 | 0.50 | 0.50 | 0.50 | 4.00 | 0.50 |
| 25 | 1.06 | 1.50 | 1.50 | 2.45 | 0.50 |
| 26 | 1.38 | 0.62 | 1.50 | 4.00 | 3.57 |
| 27 | 1.50 | 0.50 | 1.50 | 0.50 | 4.00 |
| 28 | 1.50 | 1.50 | 0.50 | 0.50 | 2.34 |
| 29 | 0.50 | 1.50 | 0.50 | 4.00 | 4.00 |
| 30 | 1.01 | 1.50 | 0.95 | 0.50 | 4.00 |
| 31 | 0.50 | 1.50 | 1.50 | 4.00 | 0.50 |
| 32 | 1.50 | 1.50 | 1.50 | 0.50 | 0.50 |
| 33 | 0.50 | 0.50 | 1.50 | 4.00 | 4.00 |
| 34 | 1.01 | 1.50 | 0.95 | 0.50 | 4.00 |
| 35 | 1.50 | 1.19 | 0.50 | 1.67 | 4.00 |
| 36 | 0.50 | 1.50 | 1.50 | 4.00 | 0.50 |
| 37 | 0.90 | 1.11 | 1.05 | 3.79 | 2.84 |
| 38 | 0.50 | 0.50 | 0.50 | 0.50 | 4.00 |
| 39 | 1.50 | 0.50 | 0.52 | 3.25 | 1.24 |
| 40 | 1.38 | 0.62 | 1.50 | 0.93 | 0.93 |
| 41 | 0.50 | 1.24 | 0.50 | 0.50 | 0.50 |
| 42 | 1.50 | 1.50 | 0.50 | 4.00 | 0.50 |
| 43 | 1.50 | 1.19 | 0.50 | 1.67 | 4.00 |
| 44 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 |
| 45 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 |
| 46 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 |

[a] Design points 1–43 were assigned to groups as follows: Group 1 (points 1–15); Group 2 (points 16–29); and Group 3 (points 30–43). One MS point was run with each group (points 44–46).

constant variance assumption, Box Cox plot, outlier $t$ values, predicted *versus* actual value plot, Cook's distance and adequate precision. Detailed descriptions of the statistical methods used to analyze the data can be found in several publications (Niedz and Evens, 2007, 2008).

## 3. Results

Summaries of the ANOVA and results are presented in Tables 3 and 4. Color contour plots of the regions in the 5-factor design space that had the greatest effect on each measured response are presented in Figs. 1–4. The response models were significant in most cases ($p < 0.005$) and many factors had statistically significant effects.

### *3.1. Quality rating (Fig. 1)*

In all five genotypes shoot quality was highly influenced by mesos ($p < 0.001$) and iron ($p < 0.0001$). Minors also influenced shoot quality in 'Indian Summer' ($p = 0.0007$) and 'Willamette' ($p = 0.0132$) (Table 3). There were genotype specific interactions with other factors. For 'Canby', the model showed that improved quality required mesos, $NH_4NO_3$ and $KNO_3$ at high levels with low minors and iron (Fig. 1). In this genotype, the actual data of some treatments are shown on the projection (red dots), and the quality scores were similar to the extrapolated scores from the model. The red dot on the $Y$-axis represents treatment 18 with a mean of quality score 1.0, and the red dot on $X$-axis represents treatment 32 with a mean quality score 2.33. For 'Indian Summer', cultures grown on MS and a design point with high mesos differed greatly in appearance and quality (Fig. 1). The improved quality of 'Indian summer' also required high mesos with low minors and iron. For 'Nootka' and 'Trailblazer', high mesos and low iron were required for best growth. The points indicated on the graphs in both cases showed that cultures grown on high mesos were better than the one on MS (Fig. 1). Finally, for 'Willamette', the best quality model required moderate minors with high mesos over a range of iron concentrations (Fig. 1). 'Willamette' was less sensitive to iron than

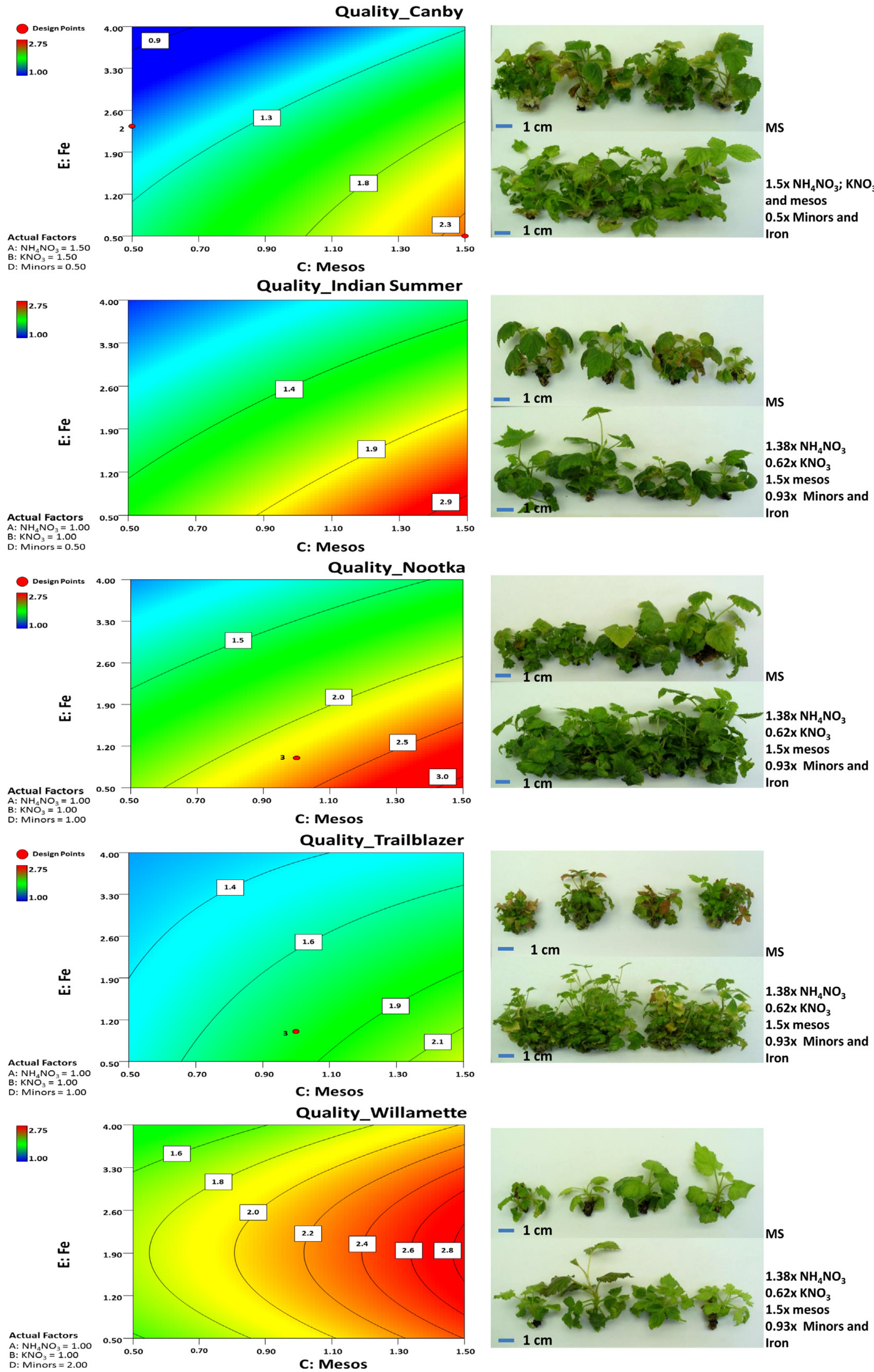


**Fig. 1.** The projected graphs of mineral nutrition effects on overall quality [Rated 1 poor (dark blue)–3 good (red)] of five red raspberry cultivars (left) and the growth appearance on standard MS compared to high mesos medium (right). Greatest response is in red, median in yellow-green and least in blue. Red dots indicate design points. Mean rating responses displayed in boxes.

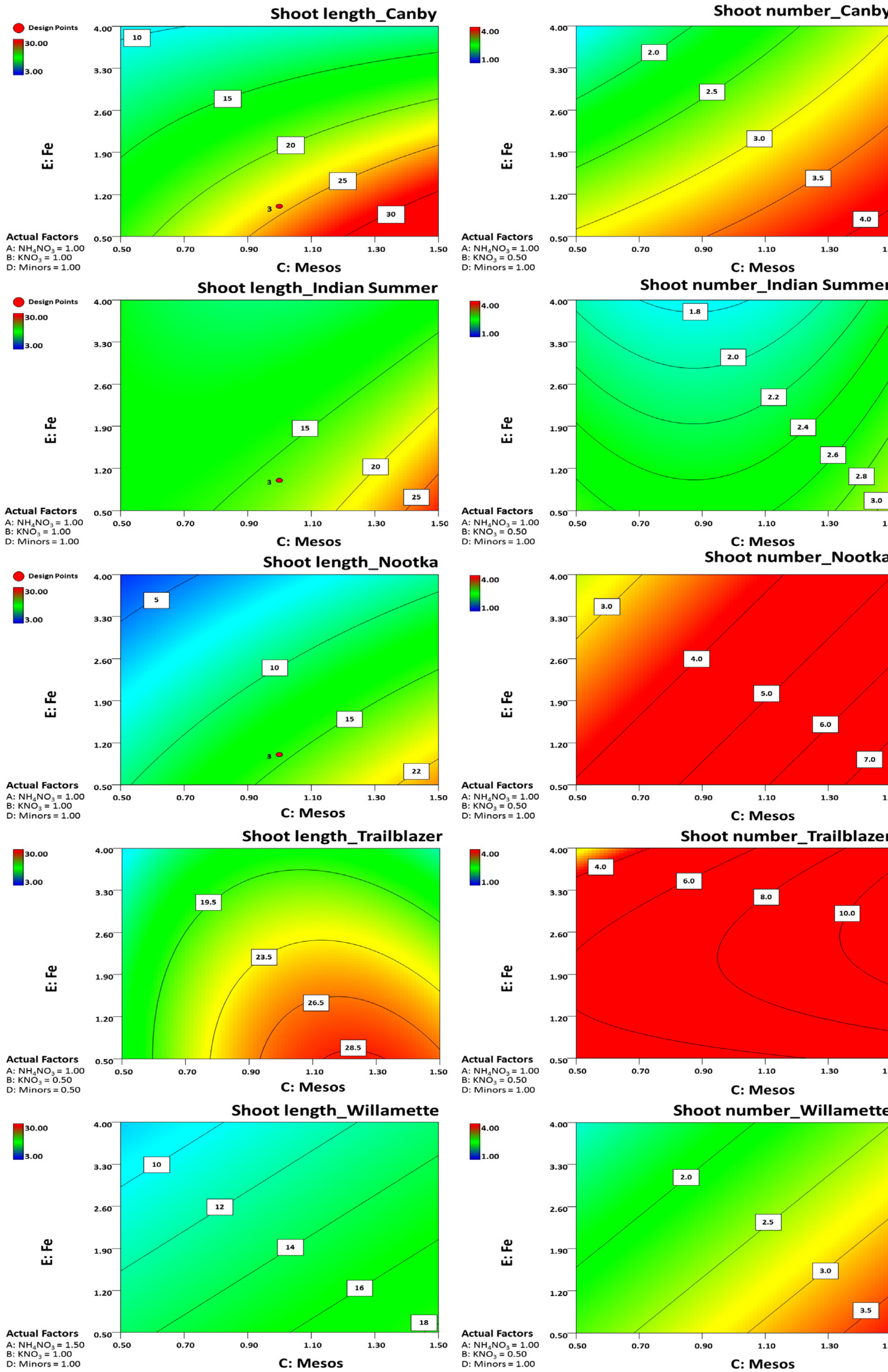


**Fig. 2.** The projected graphs of mineral nutrition effects on shoot length (left in mm) and shoot number (right in number of shoots) of five red raspberry cultivars [(Rated 1 low (dark blue)–3 high (red)]. Greatest response is in red, median in yellow-green and least in blue. Red dots indicate design points. Mean rating responses displayed in boxes.

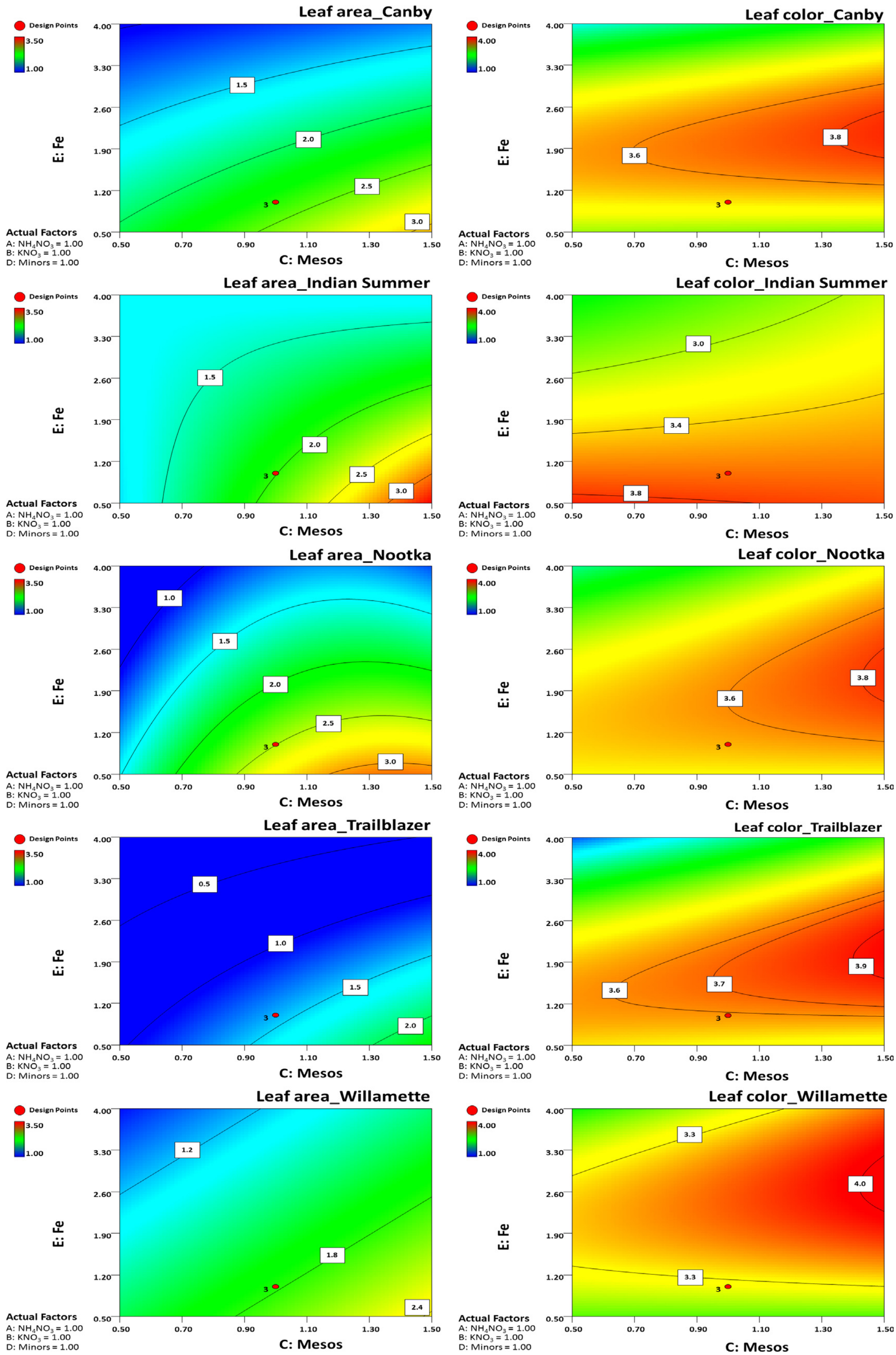


**Fig. 3.** The projected graphs of mineral nutrition effects on leaf area (left in $cm^2$) and leaf color (right with scores 1 yellow–4 dark green) of five red raspberry cultivars. Greatest response is in red, median in yellow-green and least in blue. Red dots indicate design points. Mean rating responses displayed in boxes.

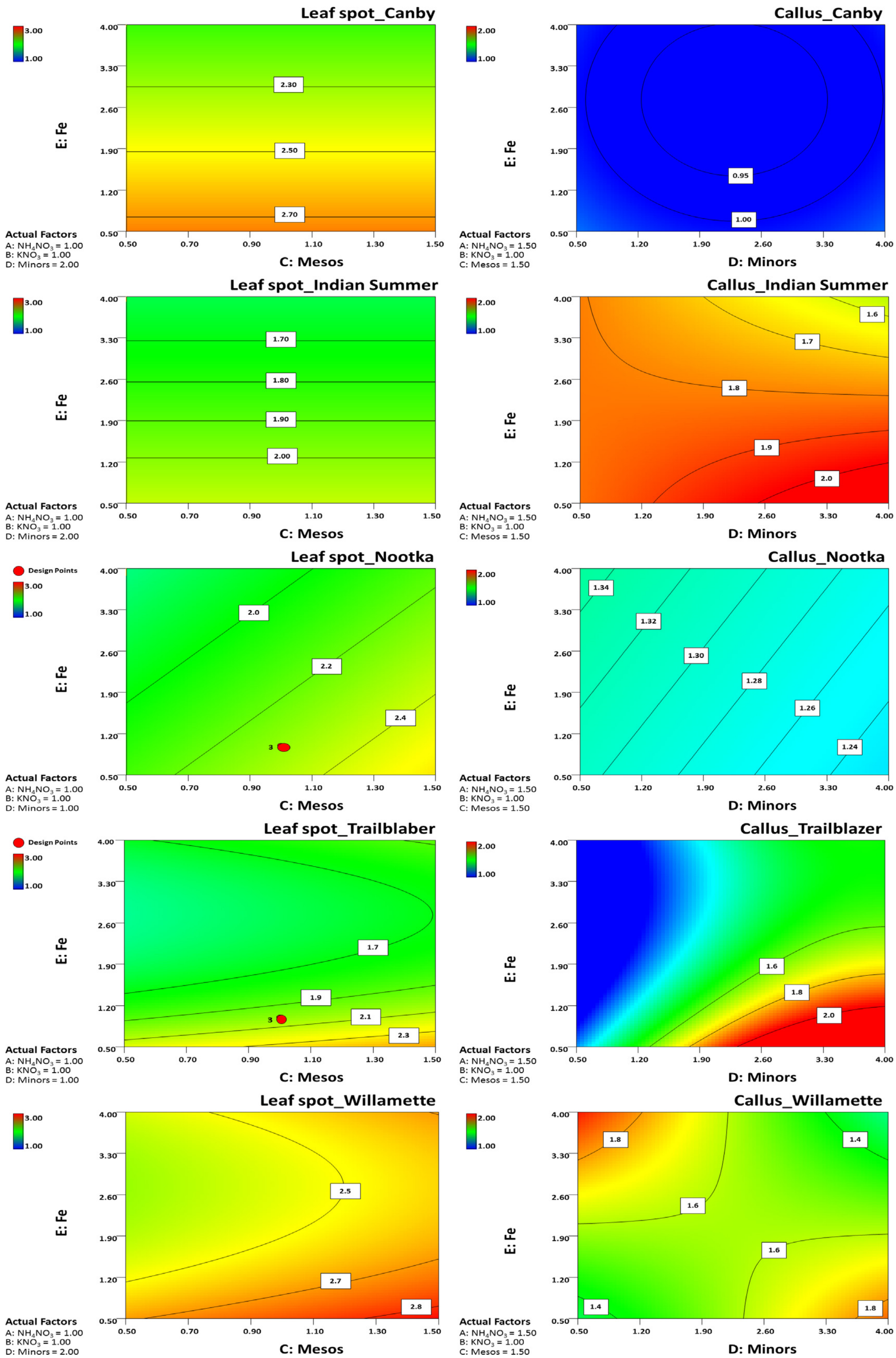


**Fig. 4.** The modeled graphs of mineral nutrition effects on leaf spot (left) and callus (right) of five red raspberry cultivars [(Rated 1 low (dark blue)–3 high (red)]. Greatest response is in red, median in yellow-green and least in blue. Red dots indicate design points. Mean rating responses displayed in boxes.

**Table 3**
Mineral nutrient factors that had the greatest effects on plant quality for each genotype as indicated by the *p*-value of the overall model and the factors including interactions.

| Sources | Genotypes | | | | |
|---|---|---|---|---|---|
| | Canby | Indian Summer | Nootka | Trailblazer | Willamette |
| Model | <0.0001** | <0.0001** | <0.0001** | <0.0001** | <0.0001** |
| A-$NH_4NO_3$ | 0.7132 | 0.9912 | 0.2595 | NS | 0.5214 |
| B-$KNO_3$ | 0.7548 | 0.9932 | 0.5951 | 0.3562 | 0.0047* |
| C-Mesos | 0.0008* | <0.0001** | <0.0001** | <0.0001** | <0.0001** |
| D-Minors | 0.3473 | 0.0007* | 0.3094 | 0.0540 | 0.0132* |
| E-Fe | <0.0001** | <0.0001** | <0.0001** | <0.0001** | 0.0202* |
| AB | NS | NS | NS | NS | NS |
| AC | NS | 0.0938 | NS | NS | NS |
| AD | 0.0069* | NS | NS | NS | 0.0445* |
| AE | NS | NS | 0.0056* | NS | 0.0026* |
| BC | <0.0001** | NS | 0.0596 | 0.0029* | NS |
| BD | NS | NS | NS | NS | NS |
| BE | NS | NS | NS | NS | NS |
| CD | 0.0005* | 0.0054* | 0.1298 | NS | NS |
| CE | NS | 0.0405* | 0.0449* | 0.0038* | NS |
| DE | 0.0438* | 0.0169* | 0.0361* | NS | 0.0088 |

* Significant with *p*-value < 0.05.
** Significant with *p*-value < 0.0001.

the other cultivars; at high mesos 'Willamette' quality remained high with moderate MS iron. In addition a moderate concentration of minors was required for high quality. High mesos alone was not sufficient to improve the quality of 'Willamette' shoots.

### 3.2. Shoot length (Fig. 2)

For micropropagated red raspberries, the optimal shoot length was considered to be 25–40 cm. Increased mesos ($p < 0.0001$) and low iron ($p \leq 0.0001$) were significant for all cultivars (Table 4). For 'Canby', 'Indian Summer' and 'Nootka' production of taller shoots required high mesos and low iron. 'Trailblazer' elongated best with moderate to high mesos, low iron and $KNO_3$. For 'Willamette' greater shoot length required high mesos and $NH_4NO_3$ with low iron.

### 3.3. Shoot number (Fig. 2)

Multiplication was highly influenced by increased mesos ($p < 0.001$) and low $KNO_3$ ($p < 0.0001$) for all cultivars and low iron ($p < 0.0010$) for all except 'Trailblazer' (Table 4). The optimal shoot number was considered to be four shoots per initial shoot. For all cultivars the model showed that the greatest number of shoots required high mesos with low iron and $KNO_3$. 'Trailblazer' had high shoot numbers compared to the other cultivars and produced the optimal shoot numbers with moderate minors.

**Table 4**
Mineral nutrient factors that had the largest effects on six responses for each red raspberry genotype as indicated by the *p*-values for the overall model and factors.

| Measured responses[a] | Genotypes | | | | |
|---|---|---|---|---|---|
| | Canby | Indian summer | Nootka | Trailblazer | Willamette |
| Shoot length | *Model (<0.0001)*<br>Mesos (<0.0001)<br>Iron (<0.0001) | *Model (<0.0001)*<br>Mesos (<0.0001)<br>Iron (<0.0001) | *Model (<0.0001)*<br>Mesos (<0.0001)<br>Iron (<0.0001) | *Model (0.0007)*<br>$KNO_3$ (0.0137)<br>Mesos (0.0068)<br>Minors (0.0075)<br>Iron (0.0002) | *Model (<0.0001)*<br>$NH_4NO_3$ (0.0041)<br>Mesos (<0.0001)<br>Iron (0.0001) |
| Shoot number | *Model (<0.0001)*<br>$KNO_3$ (<0.0001)<br>Mesos (<0.0001)<br>Iron (0.0001) | *Model (<0.0001)*<br>$NH_4NO_3$ (0.0438)<br>$KNO_3$ (<0.0001)<br>Mesos (0.0009)<br>Iron (<0.0001) | *Model (<0.0001)*<br>$NH_4NO_3$ (0.0017)<br>$KNO_3$ (<0.0001)<br>Mesos (<0.0001)<br>Iron (0.0056) | *Model (<0.0001)*<br>$KNO_3$ (0.0001)<br>Mesos (<0.0001) | *Model (<0.0001)*<br>$KNO_3$ (0.0028)<br>Mesos (<0.0001)<br>Iron (0.0002) |
| Leaf area | *Model (<0.0001)*<br>Mesos (<0.0001)<br>Iron (<0.0001) | *Model (<0.0001)*<br>Mesos (<0.0001)<br>Iron (<0.0001) | *Model (<0.0001)*<br>Mesos (<0.0001)<br>Minors (0.0004)<br>Iron (<0.0001) | *Model (<0.0001)*<br>Mesos (<0.0001)<br>Iron (<0.0001) | *Model (<0.0001)*<br>Mesos (<0.0001)<br>Iron (<0.0001) |
| Leaf color | *Model (<0.0001)*<br>$KNO_3$ (0.0049)<br>Mesos (0.0230)<br>Iron (0.0468) | *Model (0.0014)*<br>Iron (0.0109) | *Model (<0.0001)*<br>$KNO_3$ (0.0124)<br>Mesos (0.0425)<br>Iron (0.0488) | *Model (<0.0001)*<br>$KNO_3$ (0.0122)<br>Mesos (<0.0001)<br>Iron (<0.0001) | *Model (<0.0001)*<br>$KNO_3$ (0.0002)<br>Mesos (0.0012)<br>Iron (0.0465) |
| Leaf spot | *Model (<0.0001)*<br>Iron (<0.0001) | *Model (0.0003)*<br>Iron (<0.0001) | *Model (<0.0001)*<br>$NH_4NO_3$ (0.0184)<br>Mesos (0.0025)<br>Iron (0.0030) | *Model (0.0002)*<br>Iron (0.0004) | *Model (<0.0001)*<br>$NH_4NO_3$ (0.0109)<br>Mesos (0.0066)<br>Iron (0.0091) |
| Callus | *Model (<0.0001)*<br>$NH_4NO_3$ (0.0207)<br>Minors (0.0387) | *Model (0.0001)*<br>$NH_4NO_3$ (0.0204)<br>Mesos (0.0002) | *Model*<br>(not significant) | *Model (<0.0001)*<br>$NH_4NO_3$ (0.0073)<br>Minors (0.0157)<br>Iron (0.0273) | *Model (0.0026)*<br>Minors × Iron (0.0002) |

[a] The *p*-value is provided in parentheses.

### 3.4. Leaf area (Fig. 3)

All cultivars were highly influenced by increased mesos ($p < 0.0001$) and low iron ($p < 0.0001$) for leaf area (Table 4). For micropropagated red raspberries, the optimal leaf area was assigned as 2.5–3.0 $cm^2$. For all cultivars, leaf area increased with high mesos and standard to low iron. 'Trailblazer' responded to mesos and iron as well, but the leaf area was smaller than the optimum range. Leaf size ratings gave similar results (data not shown).

### 3.5. Leaf color (Fig. 3)

Leaf color of all cultivars was highly influenced by iron (Table 4). Standard or moderate iron provided the greenest leaves combined with standard or high mesos except in 'Indian Summer' where the model showed that increasing iron resulted in pale green leaves.

### 3.6. Leaf spot (Fig. 4)

Appearance of spots and leaf edge necrosis is a problem of micropropagated red raspberry and was observed in 'Indian Summer' and 'Canby'. Leaf spot was influenced by iron, $NH_4NO_3$ and mesos (Table 4) with genotype-specific interactions for other factors. 'Canby' and 'Indian Summer' required low iron with intermediate minors for leaf spot reduction. 'Willamette' also required moderate minors. 'Nootka' and 'Trailblazer' had less leaf spot with low iron and high mesos (Fig. 4).

### 3.7. Callus (Fig. 4)

Callus was seen as cell clusters at the base of shoots. Callus production was influenced by $NH_4NO_3$ ($p < 0.05$) in three cultivars, minors ($p < 0.05$) in two, mesos ($p = 0.0002$) in one and iron ($p = 0.0273$) in one cultivar (Table 4). For all cultivars, based on the models, the interaction between low iron and moderate to high minors decreased callus production when combined high $NH_4NO_3$ and mesos (Fig. 4).

## 4. Discussion

In red raspberry, mineral nutrient effects on growth and development are mostly studied as the effects of fertilizer on field plants. The influence of minerals on raspberry growth and development is based on the amount in the soil, as well as mineral accumulation in plant tissues. Fertilization is very important for improving raspberry crop production, and N, P and K are commonly applied for crop production along with B and Ca, based on soil analysis (Kowalenko, 1994; Hart et al., 2006).

Red raspberries have high genetic variation in nutritional requirements which makes studying or optimizing nutrients very complicated (Anderson, 1980; Reed, 1990; Zawadzka and Orlikowska, 2006; Wu et al., 2009). Mineral availability and uptake in the *in vitro* culture system is very different compared to soil culture which is a complex buffer system. When MS medium is used for the *in vitro* culture of raspberries, abnormal growth, stunted plants, hyperhydricity, necrosis and discoloration may occur at various levels, depending on the genotype. This study investigated the effects of mineral nutrition in medium on growth and development of micropropagated red raspberries using RSM.

Our modeled responses, based on polynomial regression analysis, display the effects of the MS mineral nutrients or nutrient combinations. Mesos ($CaCl_2$, $MgSO_4$ and $KH_2PO_4$) had significant effects on the shoot quality, multiplication and shoot length of micropropagated red raspberries (Tables 3 and 4 and Figs. 1 and 2). This was the most critical factor for greatly improving plant growth development and quality compared to the MS controls (Table 3 and Fig. 1). These are macronutrients that are provided in relatively moderate amounts as compared to the nitrogen compounds. The effects of increased mesos in this study are similar to studies of several other plants. Increasing calcium to 4× MS, doubling Cu and reducing iron to 0.5× MS provided the best growth for three micropropagated *Vriesea* bromeliads (Aranda-Peres et al., 2009). Increased calcium not only improved growth, but also facilitated the uptake of nitrogen, potassium, zinc, manganese and boron (Aranda-Peres et al., 2009). Similarly in micropropagated yellow passion fruit, increased calcium salts, magnesium and phosphorus increased growth (Monteiro et al., 2000). Increased mesos components were the main factor required for improving the growth and development of pear shoot cultures (Reed et al., 2013; Wada et al., 2013). Improved quality of *Curcuma longa* L. plantlets was due to higher uptake of calcium, magnesium, and phosphate (Halloran and Adelberg, 2011). In *Hemerocallis* culture, phosphorous, calcium, and magnesium were indirectly limited due to low solubility associated with inorganic precipitation (Adelberg et al., 2010). These examples indicate that adjusting mineral components would increase availability of most critical nutrients and optimize growth and development. Our results showed that increasing mesos components greatly improved growth and plant quality for all the genotypes tested (Fig. 1).

The form of nutrient used by plants is dependent on the morphogenic growth stage. Nitrate, phosphorus, calcium, potassium and sulfur were associated with plant morphogenesis and growth (Ramage and Williams, 2002). Doubling the concentration of MS macronutrients provided the best growth and development of shoots of the sweet cherry rootstock 'Gisela 5', and this study showed the effects of nitrogen and phosphorus on growth and multiplication with highest uptake compared to the other salts (Ruzic et al., 2000). Our results similarly showed the effect of mesos (phosphorus) and nitrogen on shoot multiplication and shoot length (Table 4 and Fig. 2). Nitrogen and its forms have significant effects on plant growth based on the total amount of nitrogen or the proportions of ammonium and nitrate (Niedz and Evens, 2008). Nitrogen salts were reduced in modified MS medium for increasing plant growth of yellow passion fruit (Monteiro et al., 2000), but in our study the proportions of nitrogen salts likely affected multiplication, shoot length and callus formation in some cultivars (Table 4 and Figs. 2 and 4).

The effects of iron in our study were statistically significant, and the lower concentrations produced the best overall quality, shoot length and multiplication. Minor nutrients affected some responses of some of the cultivars. This result is similar to the bromeliad study where reduced iron concentrations with double copper and high calcium increased growth (Aranda-Peres et al., 2009). However, in micropropagation of yellow passion fruit, increasing iron and copper concentrations combined with low zinc concentrations reduced chlorosis. This result is somewhat similar to our study, as we found that increased iron with high mesos concentrations improved leaf color (Table 4 and Fig. 3).

Our goal in this study was to establish a systematic approach for determining the most significant nutrients driving important facets of *in vitro* growth and thereby improving the growth of these diverse red raspberry cultures. The response surface methodology allowed modeling of the effects of mineral nutrients using fewer treatments compared to factorial experimental designs. Although the assessment of mineral nutrition effects on the growth of micropropagated red raspberries is very complicated, this approach was a significant step for determining the most significant components. The model analyses showed that the most critical nutrients affecting overall plant quality and appearance were the mesos components and the nitrogen factors (Tables 2 and 3 and Figs. 1–4). Additional testing of the effects of individual mesos and nitrogen components will be required to finalize the optimization of growth medium to produce the best quality and growth of red raspberries.

## Acknowledgements

This project was supported by funds from the U.S. Department of Agriculture, Agricultural Research Service CRIS project 5358-21000-033D. Sukalya Poothong acknowledges the financial support of a Royal Thai Government Scholarship.

## References


Adelberg, J.W., Delgado, M.P., Tomkins, J.T., 2010. Spent medium analysis for liquid culture micropropagation of *Hemerocallis* on Murashige and Skoog medium. In Vitro Cell. Dev. Biol. – Plant 46, 95–107.

Amiri, M.E., 2006. Effect of mineral concentration on *in vitro* explant growth of almond (*Prunus amygdalus* var. Binazir). J. Appl. Hortic. 8, 62–64.

Anderson, W.C., 1980. Tissue culture propagation of red and black raspberries, *Rubus idaeus* and *R. occidentalis*. Acta Hortic. 112, 13–20.

Anderson, W.C., 1984. Revised tissue culture medium for shoot multiplication of *Rhododendron*. J. Am. Soc. Hortic. Sci. 109, 343–347.

Aranda-Peres, A.N., Martinelli, A.P., Pereira-Peres, L.E., Higashi, E.N., 2009. Adjustment of mineral elements in the culture medium for the micropropagation of three *Vriesea* bromeliads from the Brazilian Atlantic Forest: the importance of calcium. HortScience 44 (1), 106–112.

Barney, D.L., Bristow, P., Cogger, C., Fitzpatrick, S.M., Hart, J., Kaufman, D., Miles, C., Miller, T., Moore, P.P., Murray, T., Rempel, H., Strik, B., Tanigoshi, L., 2007. Commercial Red Raspberry Production in the Pacific Northwest. Pacific Northwest Extension Publications, Oregon State University, Corvallis, OR, pp. 108.

Bell, R.L., Srinivasan, C., Lomberk, D., 2009. Effect of nutrient media on axillary shoot proliferation and preconditioning for adventitious shoot regeneration of pears. In Vitro Cell. Dev. Biol. – Plant 45, 708–714.

Dantas, A.K., Majada, J.P., Fernández, B., Cañal, M.J., 2001. Mineral nutrition in carnation tissue cultures under different ventilation conditions. Plant Growth Regul. 33, 237–243.

Design-Expert, 2010. Design-Expert 8. Stat-Ease, Inc., Minneapolis, MN.

Driver, J.A., Kuniyuki, A.H., 1984. *In vitro* propagation of paradox walnut rootstock. HortScience 19, 507–509.

Epstein, E., 1972. Mineral Nutrition of Plants: Principles and Perspective. John Wiley and Sons Inc., London, pp. 412.

George, E.F., Hall, M.A., De Klerk, G.J., 2008. The components of plant tissue culture media I: macro- and micro-nutrients. In: George, E.F., Hall, M.A., De Klerk, G.J. (Eds.), Plant Propagation by Tissue Culture. , 3rd ed. Springer, New York, pp. 65–113.

Greenway, M.B., Phillips, I.C., Lloyd, M.N., Hubstenberger, J.F., Phillips, G.C., 2012. A nutrient medium for diverse applications and tissue growth of plant species *in vitro*. In Vitro Cell. Dev. Biol. – Plant 48, 403–410.

Halloran, S.M., Adelberg, J., 2011. A macronutrient optimization platform for micropropagation and acclimatization: using turmeric (*Curcuma longa* L.) as a model plant. In Vitro Cell. Dev. Biol. – Plant 47, 257–273.

Hart, J., Strik, B., Rempel, H., 2006. Nutrient Management Guide: Caneberry. Ore. State Univ. Ext. Serv. EM 8903-E., pp. 1–8.

Hildebrandt, A.C., Riker, A.J., Duggar, B.M., 1946. The influence of the composition of the medium on growth *in vitro* of excised tobacco and sunflower tissue. Am. J. Bot. 33, 591–597.

Jennings, D.L., 1988. Raspberries and Blackberries – Their Breeding, Disease, and Growth. Academic Press, London, pp. 230.

Kowalenko, C.G., 1994. Growing season dry matter and macroelement accumulations in Willamette red raspberry and related soil-extractable macroelement measurements. Can. J. Soil Sci. 74, 565–571.

Linsmaier, E.M., Skoog, F., 1965. Organic growth factor requirements of tobacco tissue cultures. Physiol. Plant. 18, 100–127.

Monteiro, A.C.B.d.A., Higashi, E.N., Goncalves, A.N., Rodriguez, A.P.M., 2000. A noval approach for the definition of the inorganic medium components for micropropagation of yellow passionfruit (*Passiflora edulis* Sims, F-flavicarpa Deg.). In Vitro Cell. Dev. Biol. – Plant 36, 527–531.

Morard, P., Henry, M., 1998. Optimization of the mineral composition of *in vitro* culture media. J. Plant Nutr. 21, 1565–1576.

Murashige, T., Skoog, F., 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15, 473–497.

Nas, M.N., Read, P.E., 2004. A hypothesis for the development of a defined tissure culture medium of higher plants and micropropagation of hazelnuts. Sci Hortic. 101, 189–200.

Niedz, R.P., Evens, T.J., 2007. Regulating plant tissue growth by mineral nutrition. In Vitro Cell. Dev. Biol. – Plant 43, 370–381.

Niedz, R.P., Evens, T.J., 2008. The effects of nitrogen and potassium nutrition on the growth of nonembryogenic and embryogenic tissue of sweet orange (*Citrus sinensis* (L.) Osbeck). BMC Plant Biol. 8, 126.

Ramage, C.M., Williams, R.R., 2002. Mineral nutrition and plant morphogenesis. In Vitro Cell. Dev. Biol. – Plant 38, 116–124.

Reed, B.M., 1990. Multiplication of *Rubus* germplasm *in vitro*: a screen of 256 accessions. Fruit Var. J. 44, 141–148.

Reed, B.M., Wada, S., DeNoma, J., Niedz, R.P., 2013. Improving *in vitro* mineral nutrition for diverse pear germplasm. In Vitro Cell. Dev. Biol. – Plant 49, 343–355.

Ruzic, D., Saric, M., Cerović, R., Culafic, L., 2000. Relationship between the concentration of macroelements, their uptake and multiplication of cherry rootstock Gisela 5 *in vitro*. Plant Cell Tissue Organ Cult. 63, 9–14.

Singha, S., Oberly, G.H., Townsend, E.C., 1987. Changes in nutrient composition and pH of the culture medium during *in vitro* shoot proliferation of crabapple and pear. Plant Cell Tissue Organ Cult. 11, 209–220.

Wada, S., Niedz, R.P., DeNoma, J., Reed, B.M., 2013. Mesos components ($CaCl_2$, $MgSO_4$, $KH_2PO_4$) are critical for improving pear micropropagation. In Vitro Cell. Dev. Biol. – Plant 49, 356–365.

Williams, R.R., 1993. Mineral nutrition *in vitro* – a mechanistic approach. Aust. J. Bot. 41, 237–251.

Wu, J.-H., Miller, S.A., Hall, H.K., Mooney, P.A., 2009. Factors affecting the efficiency of micropropagation from lateral buds and shoot tips of *Rubus*. Plant Cell Tissue Organ Cult. 99, 17–25.

Zawadzka, M., Orlikowska, T., 2006. Factors modifying regeneration *in vitro* of adventitious shoots in five red raspberry cultivars. J. Fruit Ornam. Plant Res. 14, 105.