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ORIGINAL PAPER

Modeling optimal mineral nutrition for hazelnut micropropagation

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Abstract Micropropagation of hazelnut (*Corylus avellana* L.) is typically difficult because of the wide variation in response among cultivars. This study was designed to determine the required mineral nutrient concentrations for micropropagation of *C. avellana* cultivars using a response surface design analysis. Driver and Kuniyuki Walnut (DKW) medium mineral nutrients were separated into five factors: NH_4NO_3, $Ca(NO_3)_2$, mesos ($MgSO_4$ and KH_2PO_4), K_2SO_4, and minor nutrients (boron, copper, manganese, molybdenum, and zinc) ranging from 0.5× to 2× the standard DKW medium concentrations with 33 treatments for use in modeling. Overall quality and shoot length for all cultivars were influenced by ammonium and nitrate nitrogen, mesos and minors. Reduced $Ca(NO_3)_2$ improved multiplication while higher amounts increased shoot length for most cultivars. Uptake of nutrients varied among the cultivars. Calcium and magnesium concentrations were greater in the shoots that grew well compared to poorly-growing and control treatments. All five cultivars showed improved growth on some treatments and the models indicated that shoots grown on an optimized medium would be even better. This model indicates that NH_4NO_3, $Ca(NO_3)_2$, mesos, and minors all had significant effects on hazelnut growth and multiplication and should be optimized in future experiments.

Keywords *Corylus* · Growth medium · Micropropagation · Mineral nutrition · Minor nutrients · Nitrogen nutrition

Abbreviations

BA	N^6 benzyladenine
DE	Design Expert Software
DKW	Driver and Kuniyuki Walnut medium
Fe EDTA	Ferric ethylenediaminetetraacetic acid
Fe EDDHA	Ferric ethylenediamine-*N*, *N*'-bis(2-hydroxyphenylacetic acid)
IBA	Indole-3-butyric acid
Mesos	$MgSO_4$ and KH_2PO_4
MS	Murashige and Skoog medium
NCGR-COR	Yu and Reed hazelnut medium
PI	Plant introduction number (US National Plant Germplasm System)
WPM	Woody plant medium

Introduction

Micropropagation provides an option for producing large quantities of clonal cultivars of hazelnuts (*Corylus avellana* L.) and is currently used by commercial nurseries to produce planting stock. Hazelnuts are variable in their response to micropropagation; some grow well while others do not multiply or elongate. Some problems with

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hazelnut culture include lack of multiplication, short shoots, chlorosis, the production of callus, and a milky white exudation (Al Kai et al. 1984; Anderson 1984; Diaz Sala et al. 1990; Yu and Reed 1995; Nas and Read 2001).

The chemical composition of the growth medium is one of the underlying factors that ensures successful micropropagation. General hazelnut micropropagation protocols are often slight modifications of common tissue culture media (Anderson 1984; Diaz Sala et al. 1990; Yu and Reed 1993; Damiano et al. 2005; Jyoti 2013). Anderson (1984) developed a modified Murashige and Skoog Medium (MS) (Murashige and Skoog 1962) basal salt medium for hazelnuts, by reducing both KNO_3 and NH_4NO_3 levels, and replacing KH_2PO_4 with NaH_2PO_4, with iron doubled, and iodine reduced to one third of MS levels. Al Kai et al. (1984) modified MS medium by substituting ferric ethylenediaminetetraacetic acid (Fe-EDTA) with ferric ethylenediamine-*N*, *N*'-bis(2-hydroxyphenylacetic acid) (sequestrene Fe 138, Fe-EDDHA) to produce a greener, healthier hazelnut shoot. The value of sequestrene Fe for hazelnut culture was confirmed by several labs for a range of cultivars and hybrids (Bassil et al. 1992; Yu and Reed 1995; Garrison et al. 2013; Jyoti 2013). Diaz Sala et al. (1990) successfully cultured apical buds and nodal segments of the hazelnut cultivar Tonda Gentille Delle Langhe on modified MS medium composed of half-strength nitrates, double-strength $CaCl_2$ and $MgSO_4$, with 2 mg l^{-1} ascorbic acid. Jyoti (2013) found that adding antioxidants such as ascorbic acid, melatonin, acetylsalicylic, or salicylic acid to DKW medium improved bioreactor-grown hazelnut shoot cultures.

Yu and Reed (1993) compared Driver and Kuniyuki (1984) (DKW) medium, woody plant medium (WPM) (Lloyd and McCown 1980) and Anderson medium (Anderson 1984), and found DKW medium superior to the others. They also determined that substituting 3 % (w/v) glucose for the standard sucrose produced the most shoots on DKW medium. Damiano et al. (2005) tested hazelnut shoots on various basal media: DKW, WPM, Perez-Tornero medium (PT) (Perez-Tornero et al. 2000) and half-strength MS medium. They determined that DKW medium or a combination of DKW medium and WPM were most efficient for multiplication and quality of shoots.

Mineral nutrients play a large role in plant proliferation and development (Leifert et al. 1995; Ramage and Williams 2002). There are 17 essential mineral nutrients required for plant growth that must be available at suitable concentrations (Marschner 1995). Some differences in uptake are likely in vitro as in vitro mineral nutrition involves the direct movement of ions from the medium into plant tissues without the limiting effects imposed by root structure (Williams 1993). Understanding how shoots respond to mineral nutrients in a medium can be complex and developing an optimal medium can be time consuming, as shown by the classic study of Murashige and Skoog (1962). Because of the agar-based growth medium, rootless shoots, and the enclosed environment, in vitro plants have different nutrient requirements compared to in vivo plants. In addition carbohydrate concentrations in the medium may alter nutrient uptake (Adelberg et al. 2010).

Several approaches were used to improve hazelnut medium. Nas and Read (2004) formulated a medium (NRM) based on hazelnut kernel composition and components of DKW medium, MS medium, and WPM. Bacchetta et al. (2008) followed the same procedure using nut and leaf samples from hazelnut and almond in combination with MS medium to formulate a new medium (HM). Another approach is evaluation of spent medium to determine what nutrients the shoots are accumulating (Adelberg et al. 2010).

A computer assisted technique, response surface design statistical software, can model multiple factors and their influence on the overall outcome based on the plant response. This type of analysis was used to examine the effects of nutrients on in vitro plant responses of both callus and shoot cultures (Niedz and Evens 2006, 2007, 2008; Reed et al. 2013a; Wada et al. 2013). A response surface model can test multiple nutrients simultaneously, and optimal nutrient levels can be predicted and further tested to obtain optimized media based on the plant response.

This study was designed to determine mineral factors that have the greatest effects on the growth and development of five diverse hazelnut cultivars using a response surface design analysis. The DKW medium mineral salts and NCGR-COR base medium (Yu and Reed 1995) were used as a starting point for optimization of five mineral-stock solutions for improved growth and multiplication of hazelnut cultivars.

Materials and methods

Plant material and culture conditions

Hazelnut cultivars Dorris (PI 657898), Felix (PI 657901), Jefferson (PI 657902), OSU 880.054 (PI 657899) and Sacajawea (PI 654984) from established cultures were multiplied on NCGR-COR medium consisting of DKW medium salts with 30 g l^{-1} glucose, 200 mg l^{-1} sequestrene 138 (Fe-EDDHA), 2 mg l^{-1} thiamine, 2 mg l^{-1} nicotinic acid, 2 mg l^{-1} glycine, 1 g l^{-1} myo-inositol, 22.2 μM N6-benzyladenine (BA), 0.049 μM indole-3-butyric acid (IBA), and 0.5 % (w/v) agar (PhytoTechnology Laboratories; A1111). Medium was placed into culture vessels (Magenta GA7, Magenta, Chicago, IL), 40 ml per

Table 1 The five nutrient factors used to construct the experimental design space

Factors	DKW medium mineral salts	Range DKW medium
Group 1	NH_4NO_3	0.5–1.5×
Group 2	$Ca(NO_3)_4$	0.5–1.5×
Group 3	K_2SO_4	0.5–1.5×
Group 4 (mesos)	$MgSO_4$	0.5–1.5×
	KH_2PO_4	
Group 5 (minor nutrients)	H_3BO_3	0.5–2.0×
	$CuSO_4$	
	$MnSO_4$	
	Na_2MoO_4	
	$Zn(NO_3)_2$	

The Driver and Kuniyuki Walnut medium (DKW) (Driver and Kuniyuki 1984) salts that constituted the factors and the concentration ranges based on DKW medium × levels. Calcium chloride was not varied from the DKW medium concentration

box, and autoclaved at 121 °C for 20 min. The average growth room illumination measured at the top of the vessels was 80 µmol m^2 s^{-1} with a 16-h photoperiod of half warm-white and half cool-white fluorescent bulbs. Cultures were transferred to new medium at 3-week intervals.

For the experiment, shoots were cut to 2.5 cm with apical meristems removed. For each subsequent transfer, shoots were cut above the basal zone, the lower leaves removed and each piece cut to 2.5 cm. Each treatment consisted of two culture vessels with five shoots for each cultivar (n = 10), that were randomized on the growth room shelf. Study medium was as listed previously, but with 8 µM BA and no IBA (Hand 2013). Shoots were grown on each treatment medium for 10 weeks, with transfers at 3 week intervals with the last growth period for 4 weeks.

Mineral nutrition modeling

The in vitro hazelnut growth response experiment with DKW medium nutrients was developed with the software program Design-Expert® 8 (Design-Expert 2010). Five cultivars were tested on DKW medium nutrients using a multi-factor response surface design to model the nutrient effects. The main DKW medium salt components were separated into five independent factors, creating a five-dimensional experimental design space: NH_4NO_3, $Ca(NO_3)_2$, K_2SO_4, mesos ($MgSO_4$ and KH_2PO_4) and minors [H_3BO_3, $CuSO_4$, $MnSO_4$, Na_2MoO_4, and $Zn(NO_3)_2$] (Table 1). Design points were selected to sample the design space (Niedz and Evens 2007). Treatments were developed from design points and included duplication of some points as a second set of two boxes with five shoots. There were 33 treatments run in two sets with DKW medium controls (#34 and 35) for a total of 35 points (Table 2).

Table 2 Five factor design including 33 treatment points based on ×DKW concentrations of mineral nutrients

Treatment[z]	Factor 1 NH_4NO_3	Factor 2 $Ca(NO_3)_2$	Factor 3 K_2SO_4	Factor 4 Mesos	Factor 5 Minor nutrients
1	0.50	1.15	1.35	1.45	0.50
2	0.77	0.50	0.68	1.10	0.50
3	0.50	1.35	1.50	0.50	2.00
4	0.50	1.50	0.92	1.50	2.00
5	1.02	0.94	1.05	1.03	1.29
6	0.77	0.50	0.68	1.10	0.50
7	1.50	0.50	0.56	1.50	1.97
8	1.34	0.50	1.50	0.50	2.00
9	0.50	0.50	0.69	0.50	2.00
10	0.50	0.50	1.50	0.50	0.80
11	1.50	1.50	1.50	0.87	0.50
12	1.50	0.69	0.50	0.50	0.62
13	1.50	1.50	0.50	1.50	0.50
14	1.50	1.50	0.85	0.50	1.66
15	0.50	1.50	0.50	0.77	0.88
16	0.98	1.26	0.99	0.50	0.50
17	1.01	0.94	1.05	1.02	1.29
18	0.98	1.26	0.99	0.50	0.50
19	1.39	0.50	1.50	1.50	0.50
20	0.50	1.50	1.05	1.12	1.14
21	1.50	0.83	1.04	1.06	1.20
22	1.09	1.21	0.50	0.95	2.00
23	1.14	0.50	0.50	0.87	1.44
24	1.01	0.94	1.05	1.02	1.29
25	0.50	1.32	0.55	1.44	0.50
26	1.09	1.21	0.50	0.95	2.00
27	0.50	0.50	1.50	1.34	2.00
28	1.45	1.50	0.89	1.01	0.95
29	1.50	1.16	1.50	1.50	2.00
30	0.58	0.85	0.50	1.50	1.34
31	1.50	0.83	1.04	1.06	1.20
32	0.96	1.50	1.50	1.50	1.25
33	1.50	0.80	0.84	0.51	1.99
34 control	1.00	1.00	1.00	1.00	1.00
35 control	1.00	1.00	1.00	1.00	1.00

[z] Design points 1–33 were run sequentially in two groups; Group 1 (points 1–16) and Group 2 (points 17–33), one Driver and Kuniyuki Walnut medium (DKW) control (points 34–35) was run with each group

Data collection

Three shoots taken from predetermined points in each culture vessel (diagonal from one corner) were evaluated for eight responses (n = 6). The remaining four shoots were photographed. Shoot quality was a visual assessment of shoot vigor and form: 1 = poor, 2 = moderate, and 3 = good. Shoots longer than 5 mm were counted. The longest shoot of each original shoot was measured. Leaf color was rated 1 = yellow, 2 = light green, and 3 = dark green. A portable Soil–Plant Analysis Development (SPAD) 502 chlorophyll meter (Minolta Camera Co. Ltd., Tokyo, Japan) was used to quantify the chlorophyll content of the second leaf from the top of each shoot. Basal exudation was rated: 1 = high, 2 = moderate, and 3 = none. Callus size was rated: 1 = callus > 2 mm, 2 = callus < 2 mm, and 3 = absent. Leaf size was rated: 1 = small, 2 = medium, 3 = large.

Statistical analysis

The mean plant responses from the six shoots of each cultivar grown in the same treatment were used for modeling by response surface methodology (Design-Expert 2010). Some points were internally replicated as noted above. For the response data, the highest order significant polynomial predictor models were used. Backward elimination regression was used to remove factors from the full model that were not significant (NS). Models and factors with p values ≤ 0.01 were considered significant.

Design Expert optimization

Based on optimization results from DE software the five cultivars were grown on four DE optimized treatments and a DKW medium control (Supplement 1). Growth conditions were the same as mentioned earlier except each treatment consisted of three culture vessels with 5 shoots (2.5 cm) for each genotype. Three shoots taken from standard points in each box were examined for four responses (n = 9). Quality rating, shoot length, shoot number and callus rating were recorded.

Quantitative ionic analysis

Three shoots of each of the five cultivars were used to analyze nutrient uptake [Table 2: good growth (Treatment 4), poor growth (Treatment 10) and control (Treatment 34)]. Fresh and dry weight (oven dried at 70 °C for 3 days) were measured and recorded. Dried samples (0.05 mg) of three shoots combined from each treatment were placed into a muffle oven for 1 h at 500 °C to produce ash (total 24 samples). After cooling, HCl (1 M, 10 ml) was added and the sample dissolved completely. The supernatants were filtered through Whatman filter paper (No. 3) for purification. To measure ion concentrations in the samples, standard solutions of Ca, Mg, Fe, Na, and K were diluted as ion concentrations of 1, 0.5, and 0.01 ppm. An atomic absorption spectrometer (Shimazu, Kyoto, Japan) was employed for the quantitative ion analysis in the samples. Analyses were performed in the Department of Applied Chemistry and Biotechnology, Niihama National College of Technology in Japan.

Results

Mineral nutrients

The response surface design of the experiments were visualized in graphs that were projections of the best treatments based on the data collected from the design points (treatments). For each cultivar the two most significant factors were used as the axes in the design graphs. The optimum concentration of Fe-EDDHA was determined in a preliminary experiment so iron was not included in this study (Hand 2013).

Quality

The quality rating was a subjective evaluation of plant health that included many of the other metrics evaluated separately (Niedz et al. 2007). There were significant models ($p < 0.01$) for quality for 'Felix' and 'Jefferson' (Table 3) and they required a complex combination of nutrients often including nitrogen compounds, mesos, and minors to improve the growth response compared to the control shoots (Fig. 1). Shoots of 'Felix' required high NH_4NO_3, $Ca(NO_3)_2$, mesos, and minors; 'Jefferson' produced high quality ratings with higher $Ca(NO_3)_2$ and minors. Mesos was the only significant factor for 'Sacajawea'. Plant quality rating was projected to be significantly greater than the DKW medium control (1×) for all cultivars: 'Dorris' projected quality rating was 0.7 points greater than the control; 'Felix' was 0.6 greater; 'Jefferson,' OSU 880.054, and 'Sacajawea' were each 0.4 greater (Fig. 1). Shoots grown on some of the treatments showed overall improved growth and vigor compared to the controls (Fig. 1) although these are not necessarily located in the maximum quality areas. Improved general appearance was seen for all five genotypes, but none would be considered optimum at this point. Overall, quality ratings provided a good subjective evaluation of the micropropagated shoots.

Shoot length

Shoot length models were significant ($p < 0.01$) for all of the cultivars except 'Felix' (Table 3). Most of the cultivars

Table 3 Significant responses for quality, shoot length, and shoot number of hazelnut cultivars to mineral nutrients

Cultivar	Quality	Shoot length	Shoot number
Dorris	Model[z] (0.0065)(3.71)	Model (0.0002)(6.24)	Model NS
	NS	$Ca(NO_3)_2$ (0.008)(8.41)	NS
		mesos (0.009)(8.13)	
		$Ca(NO_3)_2$ × minors (0.009)(7.97)	
Felix	Model (0.0008)(4.87)	Model NS	Model (0.0014)(4.48)
	$Ca(NO_3)_2$ (0.0006)(16.45)	$Ca(NO_3)_2$ × Minors (0.01)(7.46)	K_2SO_4 × minors (0.008)(8.75)
	minors (0.01)(8.21)		
	$Ca(NO_3)_2$ × minors (0.01)(7.70)		
Jefferson	Model (0.0004)(5.52)	Model (0.0002)(6.26)	Model (0.0060)(4.15)
	$Ca(NO_3)_2$ (0.0002)(20.42)	NH_4NO_3 (0.008)(8.37)	$Ca(NO_3)_2$ (0.002)(11.81)
	NH_4NO_3 × $Ca(NO_3)_2$ (0.004)(10.54)	$Ca(NO_3)_2$ (0.008)(8.37)	
	NH_4NO_3 × mesos (0.001)(15.25)	NH_4NO_3 × $Ca(NO_3)_2$ (0.003)(10.41)	
	$Ca(NO_3)_2$ × K_2SO_4 (0.005)(10.23)	NH_4NO_3 × mesos (0.009)(7.95)	
OSU 880.054	Model (0.0033)(3.92)	Model (<0.0001)(8.96)	Model (0.0004)(6.09)
	NS	NH_4NO_3 (<0.0001)(36.44)	$Ca(NO_3)_2$ (0.003)(10.69)
		minors (0.005)(9.58)	minors (0.009)(8.06)
Sacajawea	Model[y] NS	Model (0.006)(5.12)[y]	Model (0.007)(4.96)[y]
	mesos (0.006)(8.91)	mesos (0.01)(7.46)	$Ca(NO_3)_2$ (0.009)(7.95)

[z] ANOVA models based on quadratic models unless otherwise noted. (p values) (F values)

[y] ANOVA model linear with natural log transformation

NS not significant at $p \leq 0.01$

showed multiple nutrient effects, but mesos was the only significant factor for 'Sacajawea'. Both 'Dorris' and 'Felix' had a $Ca(NO_3)_2$×minors interaction. Shoot length of 'Dorris' 'Felix', and OSU 880.054 was projected to be the greatest with high mesos and minors, but with low NH_4NO_3 (Fig. 2). Shoots of 'Jefferson' were long on all treatments, but were mostly influenced by high $Ca(NO_3)_2$ and low NH_4NO_3. Shoot length was best for 'OSU 880.054' with low NH_4NO_3, higher minors and mesos, while 'Sacajawea' required only high mesos to increase shoot length (Fig. 2). All cultivars showed increased shoot length, but the most important were increases in the shortest cultures, 'Felix' and 'Dorris'.

Shoot number

Analysis of shoot number resulted in significant models ($p < 0.01$) for four of five cultivars (Table 3). The model for 'Dorris' was not significant. Fewer factors influenced shoot number than some of the other responses. The best multiplication projections ranged from 2.4 to 2.8 shoots per original shoot (Fig. 2). An interaction of K_2SO_4 × minors for 'Felix' produced the most shoots on low NH_4NO_3, $Ca(NO_3)_2$, and minors with high mesos and K_2SO_4; The most significant factor was $Ca(NO_3)_2$ for 'Jefferson' and increased shoots required low NH_4NO_3 and $Ca(NO_3)_2$. OSU 880.054 was significant for both $Ca(NO_3)_2$ and minors at low levels, and also required low mesos. 'Sacajawea,' multiplication was best with low $Ca(NO_3)_2$ as the only significant factor (Table 3; Fig. 2). As expected, factors that positively influenced shoot multiplication decreased shoot length.

Leaf responses

Analysis of leaf responses (leaf size, leaf color rating, and SPAD) resulted in highly significant models ($p < 0.0001$) in all but one case (Table 4). The highest $Ca(NO_3)_2$ produced large leaves for all cultivars while the amount of NH_4NO_3 required varied by cultivar (Fig. 3). Both 'Felix' and OSU 880.054 required high levels of mesos and minors for the largest leaf size. A moderate leaf size (a rating of two) usually was produced with low NH_4NO_3 and $Ca(NO_3)_2$ concentrations. SPAD ratings of 26–31 were considered to be good color for in vitro shoots. Nitrogen type and concentration significantly affected chlorophyll production (SPAD meter) for all cultivars, and mesos and interactions of several factors were significant for some cultivars (Table 4; Fig. 3). Leaf color ratings (data not shown) reflected the SPAD values.

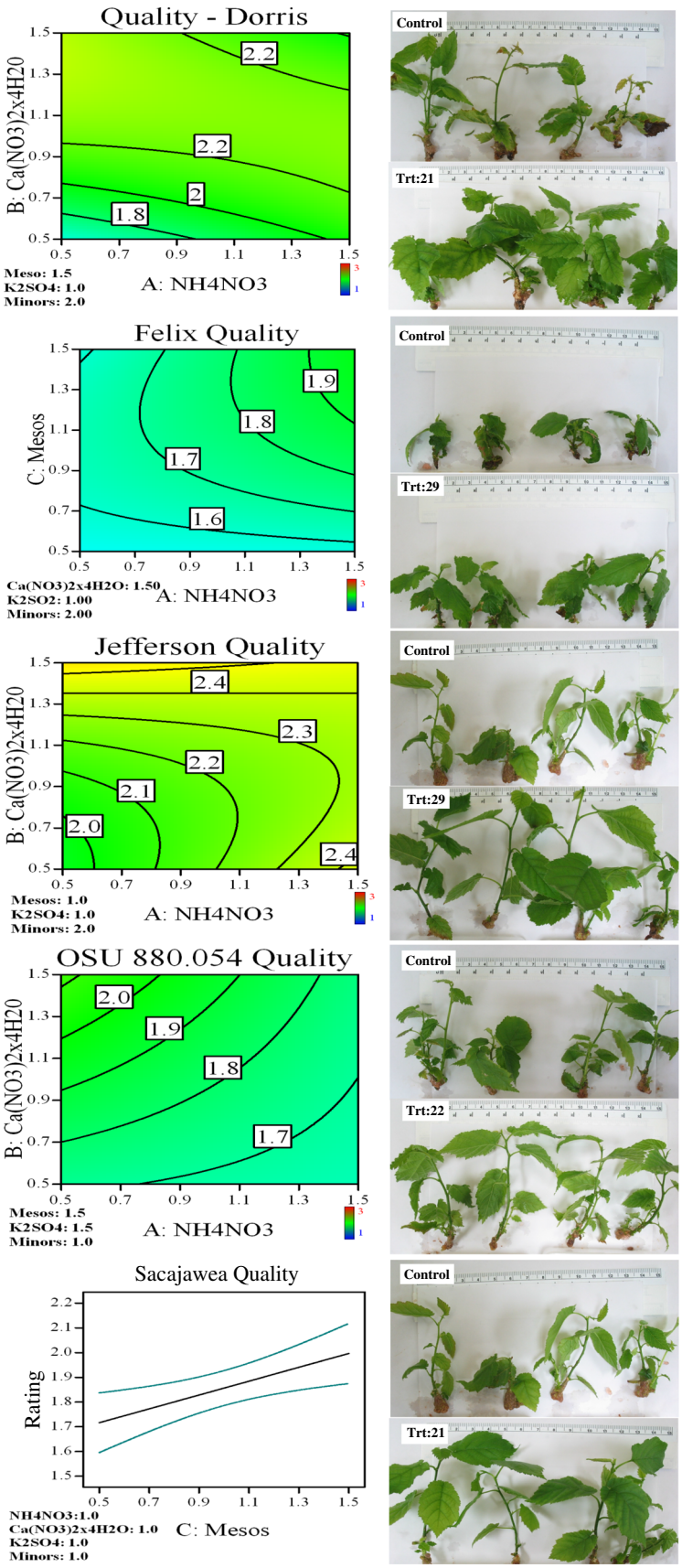

Fig. 1 Quality rating responses of hazelnut shoot cultures. *Left* Response surface graphs of mineral nutrient effects on the quality of five hazelnut cultivars. The two most significant factors were displayed as *x*- and *y-axis*, the other factors were set to concentrations that maximize the response. Responses were indicated from highest rating (3) to lowest rating (1) (*red*, *yellow*, *green*, *blue*). *Right* Shoot cultures of each cultivar grown on control medium or a treatment (Trt) with good growth. (Color figure online)

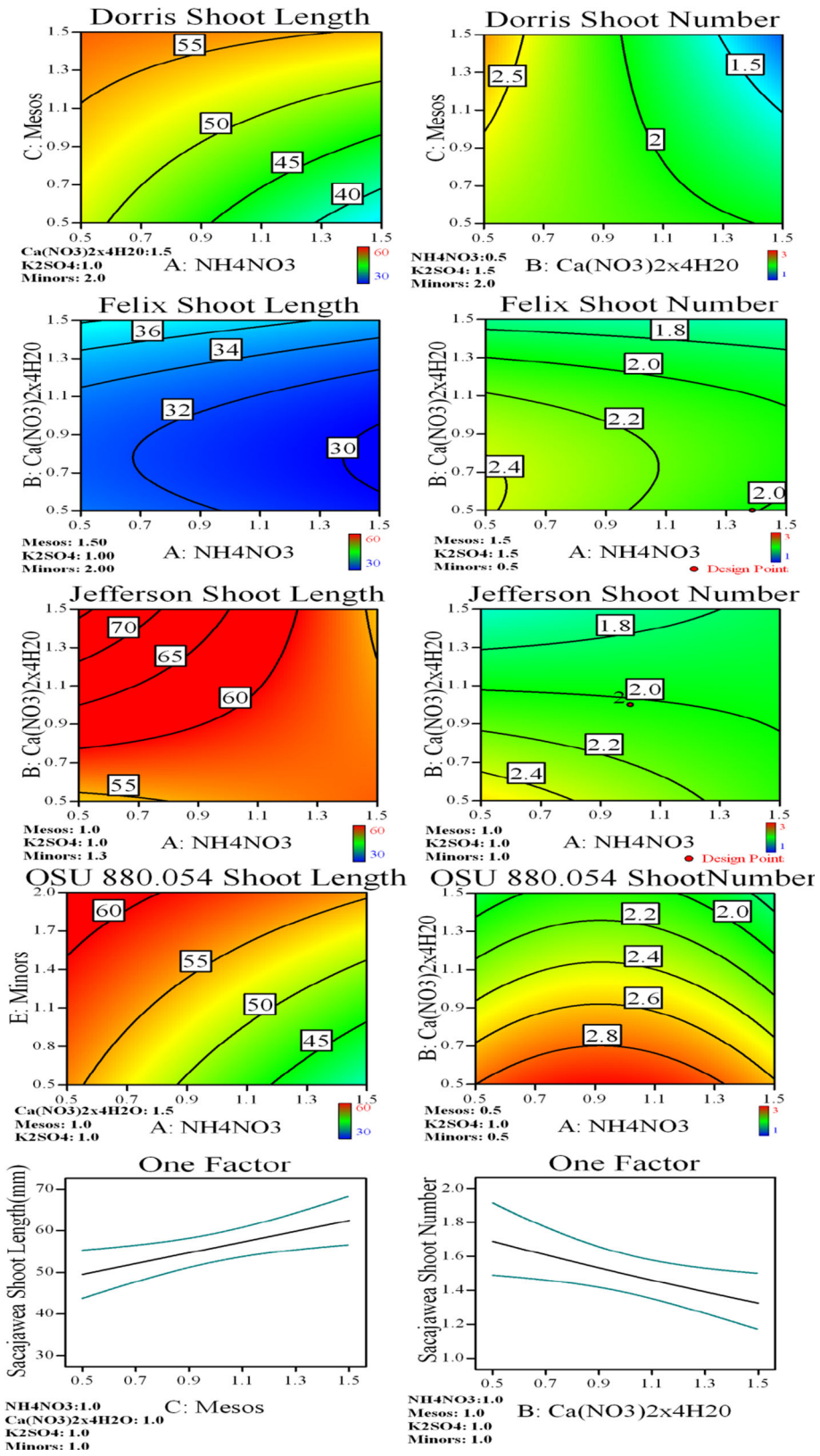

Fig. 2 Response surface graphs of mineral effects modeled for hazelnut: *Left* the shoot length (mm) and *Right* shoot number of five hazelnut cultivars. Responses were indicated from highest rating (3) to lowest rating (1) (*red*, *yellow*, *green*, *blue*). Design points represented in a particular graph were indicated by a *dot*, and if replicated, by the number of replications (i.e. 2 or 4). (Color figure online)

Table 4 Significant responses for leaf characteristics of hazelnut cultivars to mineral nutrients

Cultivar	Factors		
	Leaf size	Leaf color	SPAD
Dorris	Model[z] (<0.0001)(10.30)	Model (<0.0001)(10.61)	Model (<0.0001)(24.22)
	NH_4NO_3 (0.001)(12.43)	NH_4NO_3 (<0.0001)(33.83)	NH_4NO_3 (< 0.0001)(62.11)
	$Ca(NO_3)_2$ (0.0004)(15.79)	$Ca(NO_3)_2$ (< 0.0001)(30.54)	$Ca(NO_3)_2$ (<0.0001)(29.42)
Felix	Model (<0.0001)(10.57)	Model (<0.0001)(12.64)	Model NS
	NH_4NO_3 (0.0006)(16.34)	NH_4NO_3 (<0.0001)(67.32)	$NH_4NO_3 \times K_2SO_4$ (0.05)(4.40)
	$Ca(NO_3)_2$ (0.001)(34.22)	$Ca(NO_3)_2$ (0.002)(11.37)	
	Mesos (<0.0001)(32.93)	minors (0.01)(7.35)	
	Mesos × minors (0.005)(9.72)		
Jefferson	Model (< 0.0001)(7.91)	Model (<0.0001)(16.59)	Model (<0.0001)(19.03)
	$Ca(NO_3)_2$ (0.002)(12.89)	NH_4NO_3 (<0.0001)(41.57)	NH_4NO_3 (<0.0001)(84.58)
	minors (0.001)(14.09)	$Ca(NO_3)_2$ (0.01)(7.27)	$Ca(NO_3)_2$ (0.0006)(14.96)
	NH_4NO_3 × mesos (0.01)(7.18)		
	$Ca(NO_3)_2$ × mesos (<0.0001)(30.50)		
	K_2SO_4 × minors (0.0003)(18.86)		
OSU 880.054	Model (<0.0001)(7.82)	Model (<0.0001)(8.47)	Model (<0.0001)(21.80)
	mesos (0.0003)(17.76)	NH_4NO_3 (0.0002)(17.64)	NH_4NO_3 (<0.0001)(137.50)
	$Ca(NO_3)_2$ × minors (0.0002)(18.72	NH_4NO_3 × Ca(NO3)2 (0.004)(9.79)	$Ca(NO_3)_2$ (< 0.0001)(45.35)
			K_2SO_4 (0.002)(13.30)
			mesos × minors (0.001)(14.0)
			NH_4NO_3 × minors (0.002)(12.44)
			$Ca(NO_3)_2 \times K_2SO_4$ (0.004)(10.28)
Sacajawea	Model[y] (<0.0001)(9.45)	Model[y] (0.0001)(9.46)	Model[y] (<0.0001)(19.01)
	$Ca(NO_3)_2$ (0.01)(7.04)	NH_4NO_3 (0.0002)(18.05)	NH_4NO_3 (<0.0001)(52.14)
	mesos (0.002)(12.43)		$Ca(NO_3)_2$ (<0.0001)(23.89)

[z] ANOVA models based on quadratic models unless otherwise noted. (p values), (F values)

[y] ANOVA model linear with natural log transformation

NS not significant at $p \leq 0.01$

Callus

A range of callus production was observed in hazelnut shoot cultures. Models of callus response were significant ($p < 0.01$) for three of the four cultivars that produced callus (Table 5). 'Felix' rarely produced callus. For 'Dorris,' the model was significant and high levels of NH_4NO_3 and $Ca(NO_3)_2$ reduced callus production (Fig. 4). For 'Jefferson' increased NH_4NO_3 was significant, and for OSU 880.054, high NH_4NO_3 and $Ca(NO_3)_2$ significantly reduced callus. For 'Sacajawea', multiple interactions affected callus production. Higher levels of NH_4NO_3 produced the least callus for the four cultivars although all still had some callus (Fig. 4).

Exudation

A milky white exudate was often associated with callus production in hazelnut shoot cultures. Analysis of exudation response resulted in significant models ($p < 0.009$) for three of the four cultivars where it occurred and there were significant interactions (Table 5). For three cultivars, higher concentrations of NH_4NO_3 reduced exudation (Fig. 4). 'Dorris' showed a significant interaction

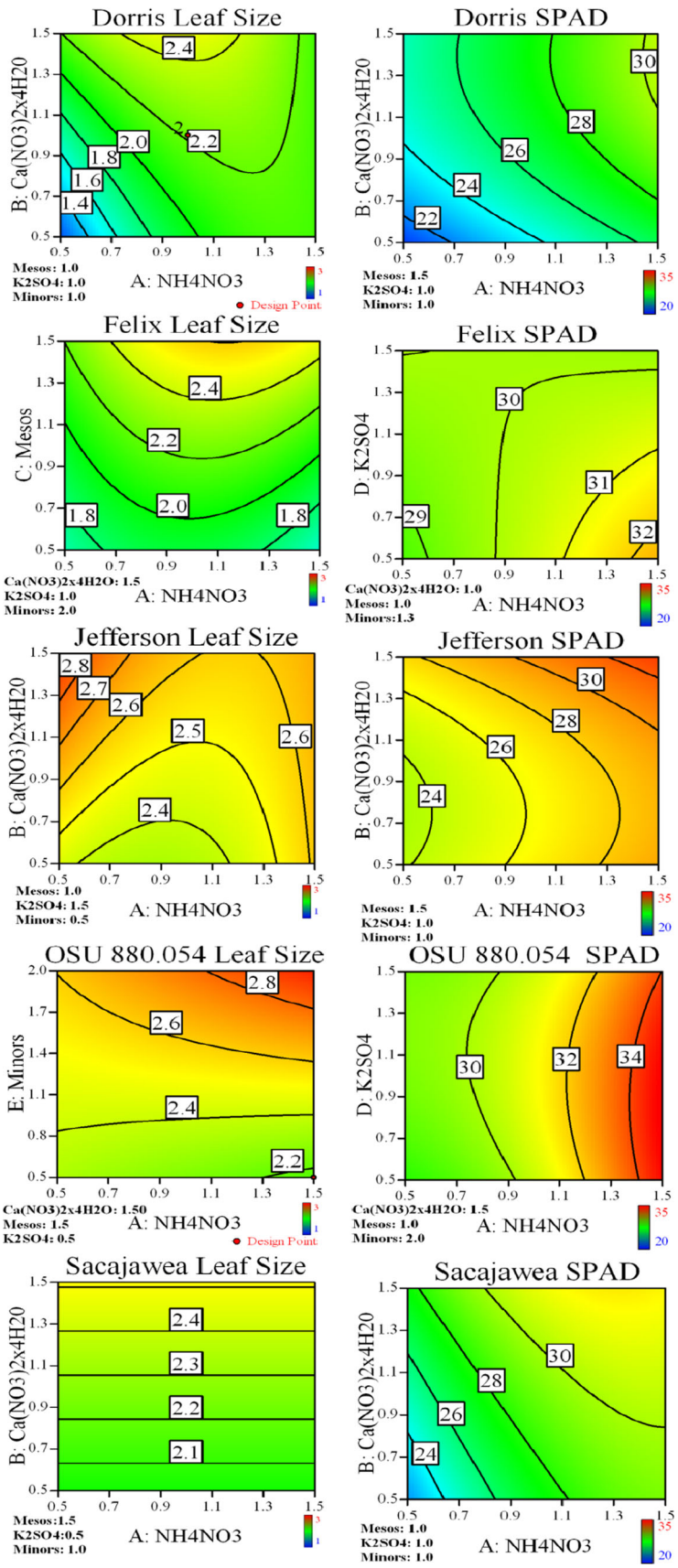

Fig. 3 Response surface graphs of mineral effects on: *Left* leaf size rating and *Right* chlorophyll content (SPAD) of five cultivars. Larger leaf size and increased chlorophyll content (SPAD) were indicated from highest rating (3) to lowest rating (1) (*red*, *yellow*, *green*, *blue*). Design points represented in a particular graph were indicated by a *dot*, and if replicated, by the number of replications (i.e. 2 or 4). (Color figure online)

Table 5 Significant responses of callus and exudation of hazelnut cultivars to mineral nutrients

Cultivar	Callus	Exudation
Dorris	Model[z] (0.002)(4.92)	Model NS
		$Ca(NO_3)_2$ × minors (0.005)(4.42)
Felix	NS	NS
Jefferson	Model NS	Model (<0.0001)(10.32)
	NH_4NO_3 (0.005)(9.61)	NH_4NO_3 (0.0002)(18.40)
		NH_4NO_3 × mesos (0.002)(11.51)
OSU 880.054	Model (<0.0001)(9.13)	Model (0.0002)(6.82)
	NH_4NO_3 (<0.0001)(36.02)	NH_4NO_3 (0.005)(9.23)
	$Ca(NO_3)_2$ × minors (0.01)(6.90)	NH_4NO_3 × minors (0.005)(9.39)
Sacajawea	Model[y] (<0.0001)(14.18)	Model[y] (0.008)(3.90)
	NH_4NO_3 (<0.0001)(54.68)	NH_4NO_3 (0.002)(11.01)
	Mesos (0.0003)(18.92)	
	Minors (<0.0001)(33.90)	
	NH_4NO_3 × mesos (0.001)(13.97)	
	NH_4NO_3 × minors (0.0002)(20.67)	
	$Ca(NO_3)_2$ × mesos (0.001)(13.98)	
	Mesos × minors (0.001)(14.37)	

[z] ANOVA models based on quadratic models unless otherwise noted. (*p* value) (F value)

[y] ANOVA model linear with natural log transformation

NS Not significant at $p \leq 0.01$

($p < 0.005$) such that either low $Ca(NO_3)_2$ and high minors, or high $Ca(NO_3)_2$ and low minors reduced exudation. 'Jefferson' had a significant interaction ($p < 0.002$) on high NH_4NO_3 and low mesos with low exudation; OSU 880.054 had the least exudate with high NH_4NO_3 and there was a significant interaction ($p < 0.005$) with the minor nutrients; 'Sacajawea' exudation was slightly reduced on medium with high NH_4NO_3.

Design Expert optimization

The DE optimization function was used to predict the best combination of mineral nutrients for each genotype. Data from the responses were compared to set criteria of an ideal shoot, and predicted combinations of the nutrient factors were produced. The results were generally inconclusive, probably due to the number of significant factors involved in shoot response (Supplement 1). For individual genotypes there were significant differences in quality compared to the control (Treatment C) for 'Dorris' (Treatment B) and 'Felix' (Treatment A). Shoot length was significantly greater for 'Felix' on Treatment A. Callus was significantly decreased for 'Dorris' with all treatments compared to the control and for OSU 880.054 on treatment E (Supplement 1).

Quantitative ionic analysis

Shoots grown on mineral nutrient combinations (Table 2) with the good-growth treatment (Trt) 4, control medium nutrients (Trt 34), and poor growth (Trt 10) were compared (Fig. 5). Nutrient content varied with the treatment and the cultivar. The concentrations of Ca in the shoots on Trt 4 (1.5× mesos) were generally greater than those with poor growth (Trt 10; 0.5× mesos) and the control (1× mesos). The high Ca content in 'Sacajawea' may be responsible for the impact of mesos on shoot quality (Fig. 1). The Mg concentrations increased with increasing mesos concentrations except for 'Sacajawea'. All cultivars had higher K content on the good growth treatment (Trt 4; 1.5× mesos and 0.9× K_2SO_4) compared to the poor growth treatment and many on the control (Fig. 5). 'Felix' accumulated more calcium than the other cultivars on the poor and control treatments and much less on the good treatment. Much less Na was present in shoots on the good treatment compared to poor or control shoots except for 'Dorris' where the amount was relatively constant. Sodium content of 'Felix' controls was much higher than the other cultivars and may be the cause of the stunted growth seen in control shoots (Fig. 1).

Discussion

The response surface design used in this study was a systematic approach for determining the mineral nutrient factors that influence the growth of specific hazelnut cultures as well as hazelnuts as a group. Plant quality rating, a response that encompasses a subjective evaluation of leaf factors, multiplication, shoot length, and physiological abnormalities, was an important indicator of plant health that varied with changes in the mineral nutrients. Individual responses could be studied and modeled to improve selected growth factors of interest. The value of this quality rating system was also evident in studies of in vitro mineral nutrition of pear and raspberry, (Reed et al. 2013a, b; Wada et al. 2013; Poothong and Reed 2014).

Developing a universal hazelnut mineral nutrient medium can be difficult because of cultivar variation, and the results of various studies often seem contradictory. Medium development has typically involved testing existing formulations to find one that provides adequate growth and

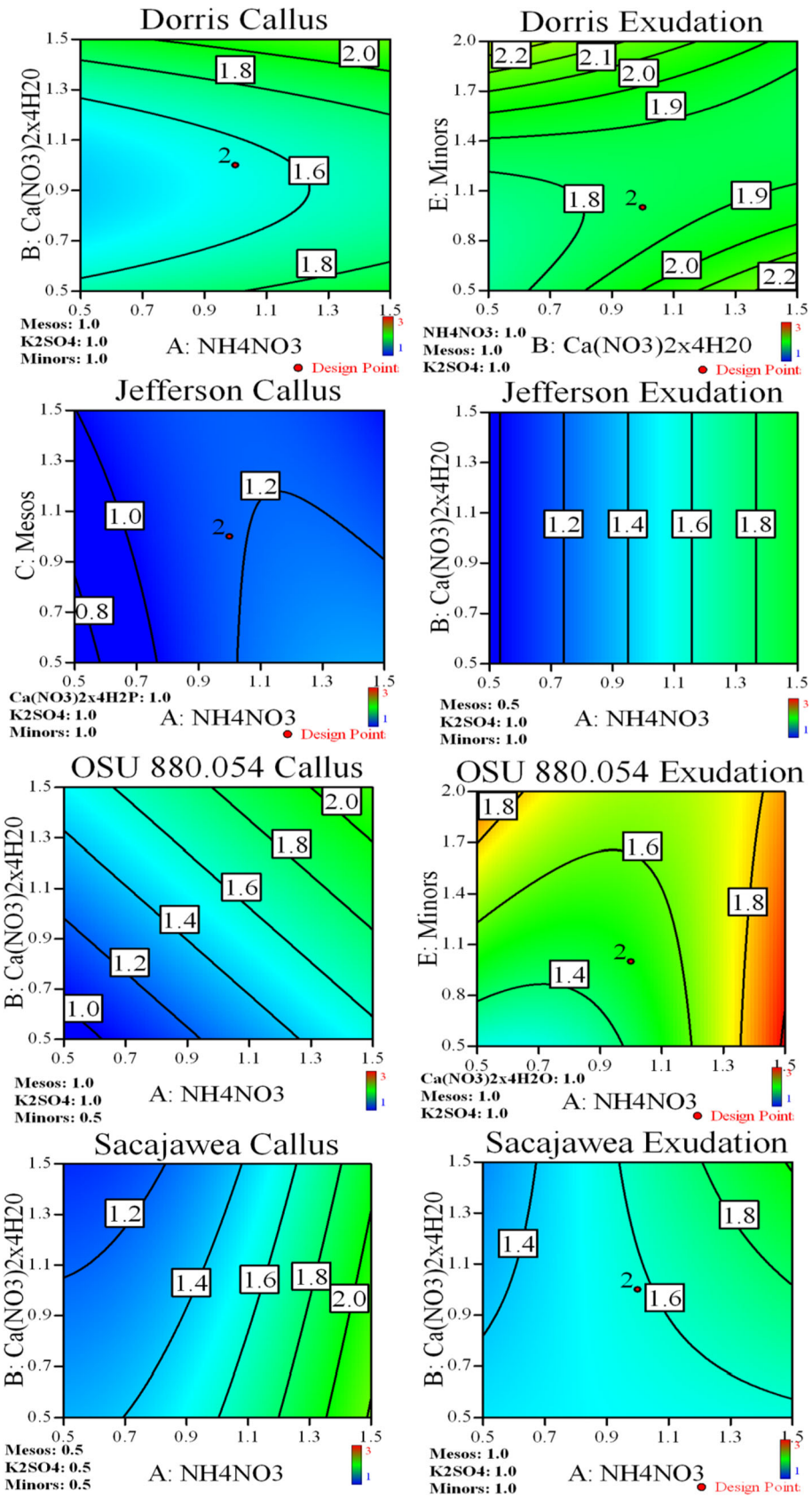

Fig. 4 Response surface graphs of mineral effects on: *Left* callus, and *Right* exudation. Both were rated on a one to three scale where one was a high amount and three was a low amount of response. Responses were indicated from highest rating (3) to lowest rating (1) (*red*, *yellow*, *green*, *blue*). Design points represented in a particular graph were indicated by a *dot*, and if replicated, by the number of replications (i.e. 2 or 4). (Color figure online)

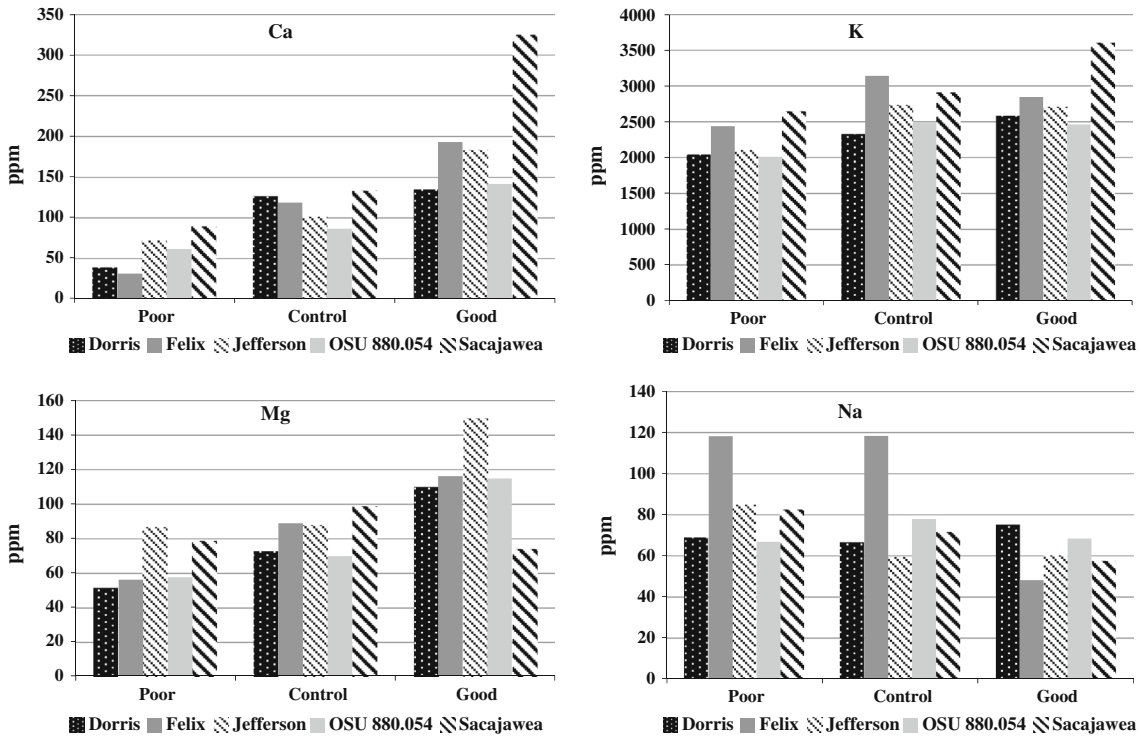

Fig. 5 Concentrations of mineral elements (PPM) found in three pooled shoots grown on the poor growth (Treatment 10), control (Treatment 34), and good growth (Treatment 4) treatments for all five cultivars

development, but this type of study does not produce an optimum medium for any diverse plant species (Reed et al. 2013a). It is likely that more than one medium will be needed for optimum growth of hazelnut species and cultivars.

Nitrogen is always an important nutrient, but the type and amount required is often cultivar dependent. In our study increased $Ca(NO_3)_2$ (1.5× DKW medium) significantly improved overall quality in two of the five hazelnut cultivars and was a significant factor for shoot multiplication and length for the others (Table 3). These $Ca(NO_3)_2$ concentrations were much higher than those of WPM, NRM, MS medium and HM medium (Table 6). The quality response of these five *C. avellana* cultivars to nitrogen type and concentration was variable. One cultivar responded best to low NH_4NO_3 and high $Ca(NO_3)_2$, two required high $Ca(NO_3)_2$ and high NH_4NO_3, while one required high amounts of $Ca(NO_3)_2$ at medium to low NH_4NO_3 concentrations (Fig. 1). Nas and Read (2004) used an average of MS medium and DKW medium for the NH_4NO_3 and $Ca(NO_3)_2$ concentrations in NRM, and Bacchetta et al. (2008) used MS medium concentrations of NH_4NO_3 and $Ca(NO_3)_2$ in their HM medium. Pear cultures of several species optimized for mineral components also had variable requirements for nitrogen compounds (Reed et al. 2013a). These cultivar and species differences in nitrogen requirements may account for the disparities among research studies. WPM and NRM have lower total nitrogen compared to the other media (Table 6). Our results indicated that screening hazelnut cultivars for nitrogen requirement was important for determining a final medium for cultivars that do not respond well to standard formulations. Final optimal concentrations of NH_4^+ and NO_3^- will be determined in a future experiment.

The mesos component ($MgSO_4$ and KH_2PO_4) was significant for better quality for two of the five cultivars (Table 3); the 1.5× DKW medium concentrations were higher than those found in MS medium, WPM and HM, while NRM used even higher concentrations of both (Table 6). Analysis of hazelnut cultures grown on treatments that produced poor, intermediate (control), or good growth, and with correspondingly low, medium, and high Ca and Mg, revealed that when more of these nutrients were available, more were taken up by the shoots for most of the cultivars (Fig. 5). Treatment 29 (Table 2) which produced high quality shoots included high (1.5× DKW medium) P and Mg compared to MS medium and WPM, but not as high as NRM (Table 6). Uptake of Na from the agar was generally higher in treatment with poor growth (Trt 10) compared to the good growth treatment (Fig. 5). The high uptake of Na by 'Felix' might be responsible for the slow growth of this cultivar and perhaps for other

Table 6 The mineral nutrients of five growth media used for micropropagation of hazelnut

Mineral nutrients (mM or μM)	DKW[a]	DKW 1.5×	HM	MS	NRM	WPM
Macro nutrients (mM)						
Nitrogen						
NH_4NO_3	17.69	26.53	20.61	20.61	6.62	5.00
$Ca(NO_3)_2{\cdot}4H_2O$	8.30	12.45	–	–	2.96	2.35
KNO_3	–	–	18.20	18.79	5.44	–
Mesos						
$MgSO_4{\cdot}7H_2O$	3.00	4.50	0.92	1.50	6.49	1.50
KH_2PO_4	1.90	2.85	0.59	1.25	9.55	1.25
$CaCl_2{\cdot}2H_2O$	1.00	1.00	1.30	2.99	0.61	0.65
K_2SO_4	8.95	13.43	0.42	–	–	5.68
Minor nutrients (μM)						
H_3BO_3	77.62	116.43	109.96	100.26	100.26	100.26
$CuSO_4{\cdot}5H_2O$	1.00	1.50	0.09	0.10	10.01	1.00
$MnSO_4{\cdot}H_2O$	198.21	297.32	388.08	99.99	118.34	131.94
$Na_2MoO_4{\cdot}2H_2O$	1.61	2.42	0.00	1.03	10.33	1.03
$ZnSO_4{\cdot}7H_2O$	–	–	8.94	29.91	30.60	29.91
$Zn(NO_3)_2{\cdot}6H_2O$	57.14	85.72	–	–	–	–
Sequestrene 138 Fe	459.98	459.98	–	–	229.99	–
$FeSO_4{\cdot}7H_2O$	–	–	103.30	99.99	–	99.99
EDTA		–	100.20	100.20	–	100.20
KI	–	–	5.12	5.00	–	–
$CoCl_2{\cdot}6H_2O$	–	–	0.13	0.11	–	–

[a] Modified DKW medium (Driver and Kuniyuki, 1984) as adapted for NCGR-COR medium with sequestrene 138 Fe and glucose as the carbon source (Yu and Reed 1995), HM (Bacchetta et al. 2008), MS (Murashige and Skoog 1962), NRM (Nas and Read 2004), and WPM (Lloyd and McCown 1980)

cultivars on suboptimal media. Toxic uptake of Na can be inhibited by higher Ca concentrations through a protein mediated pathway (Tester and Davenport 2003), and it was likely that this effect was reflected in the increased Ca in the control and good treatments in this study.

The minor nutrients of various growth media differ considerably for some components (Table 6). Our results indicated that increasing the minor minerals to 2× DKW medium produced better quality shoots for three of the five cultivars (Fig. 1). There were interactions of the minor nutrients with other medium components as well (Table 3). At 2× DKW medium, most of these minor nutrient concentrations were much higher than MS medium and WPM (Table 6). Increasing certain minor elements also agreed with Nas and Read (2004) who recommended $CuSO_4$ at 10× and Na_2Mo_4 at 6.4× DKW medium for hybrid hazelnuts. Bacchetta et al. (2008) did not find any Mo in their leaf samples and therefore did not include it in the medium, but they did increase Mn to 1.95× DKW medium. Our study did not examine each individual minor nutrient, however because of the importance of this group in improved shoot growth, further examination of the individual minor mineral components is important and will be the next step for optimization.

Shoot length and shoot proliferation are important components of quality for micropropagation. Proper nutrition provides healthy shoots with more nodal sections for multiplication. Nas and Read (2004) and Bacchetta et al. (2008) both noted that longer shoots allow for more potential multiplication, since shoots were usually cut into nodal sections for propagation. Our study found that increased $Ca(NO_3)_2$, mesos and minors increased shoot length for four of the five cultivars; 'Sacajawea' required high mesos and moderate NH_4NO_3 (Fig. 2). The cultivars studied here produced shoots that ranged in length from 20 to 75 mm. Nas and Read (2004) had 7 to 15 mm shoots before adding extra Cu and myo-inositol and Bacchetta et al. (2008) cultivars were 18-24 mm. Nas and Read (2004) concluded that increasing Cu and myo-inositol resulted in increased shoot length up to three-fold and almost doubled shoot multiplication. Hazelnuts in their study cultured with 2.55 mg l^{-1} $CuSO_4$ plus 400 mg l^{-1} myo-inositol, produced about 35-50 mm length in four cultivars and about five to seven axillary buds per shoot. Having additional basal shoots is also important for propagation. Nas and Read (2004) and Bacchetta et al. (2008) found low shoot proliferation. Shoot proliferation was influenced by both $Ca(NO_3)_2$ and minor nutrients in this study, so as those are optimized in future experiments the multiplication should increase. We held the BA concentration low (8 μM) for this study so the shoot numbers were 1.5–2 per shoot, but increased basal proliferation was

controlled by cytokinin concentrations and could be further improved by further optimizing BA.

We have often observed callus and basal exudation in hazelnut cultures. However, there was little or no mention of these issues in the literature. Decreased callus and exudation were observed on treatments with increased NH_4NO_3 (Fig. 4). It was our observation that shoots that grew vigorously often produced more callus and more exudation. 'Felix' did not grow vigorously and it produced little or no callus or exudation, while the more vigorous cultures of 'Jefferson' and 'Sacajawea' produced the most. Reduction in callus production would be useful as it might channel growth into new shoots or increased shoot growth instead of unorganized cells.

Conclusions

There is a need in the micropropagation industry for a practical procedure for the development of specific medium formulations for new crops. Medium development has typically involved testing existing formulations to find one that provides adequate growth and development. The response surface design used in this study was a systematic approach for determining the mineral nutrient factors that influence the growth of hazelnut shoots. Plant quality rating was an important indicator of plant health, and could be improved through changes in the mineral nutrients of the growth medium. The overall response of these cultivars to changes in mineral nutrients indicated that DKW medium mineral nutrient concentrations were not optimal for any of the five cultivars tested. The number of nutrient interactions observed in this study indicated the complexity involved in determining an optimal nutrient medium for hazelnut. *Corylus avellana* cultivar response to mineral nutrients varied somewhat, however all showed improved growth on some treatments, and models indicated that even greater improvements were possible. Tests of DE-suggested "optimal" media were inconclusive, likely because of the number of factors that had significant effects on these cultures (Supplement 1). New medium formulations will require optimization for higher $Ca(NO_3)_2$ and NH_4NO_3, mesos and minors.

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