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MICROPROPAGATION

Mineral nutrition and *in vitro* growth of *Gerbera hybrida* (Asteraceae)

Randall P. Niedz · Scott E. Hyndman · Terrence J. Evens · Albert A. Weathersbee III

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Abstract The effects of mineral nutrient were examined on *in vitro* growth of *Gerbera hybrida* (*G. jamesonii*× *G. viridifolia*), specifically *Gerbera hybrida* cv. Pasadena. Four types of experiments were conducted to quantify the effects of mineral nutrients on four *in vitro* growth responses (quality, shoot number, leaf number, and shoot height) of gerbera and included groups of mineral nutrients (macros/mesos, micros, and Fe), individual salts ($CuSO_4 \cdot 5H_2O$, $MnSO_4 \cdot 4H_2O$, $ZnSO_4 \cdot 7H_2O$, and Fe/EDTA), and the specific ions NO_3^-, NH_4^+, and K^+. Experiments included mixture-amount designs that are essential for separating the effects of proportion and concentration. Highly significant effects were observed in all experiments, but the mineral nutrients with the largest effects varied among the four growth responses. For example, leaf number was strongly affected by the macronutrient group in one experiment and by NH_4^+ and K^+, which were in the macronutrient group, in the $NO_3^-/NH_4^+/K^+$ ion-specific experiment, whereas quality was strongly affected by the micronutrients $ZnSO_4$ and Fe/EDTA. Because mineral nutrient effects varied significantly with the response measured, defining an appropriate formulation requires a clear definition of "optimal" growth.

Keywords Nitrogen · Metals · Minor nutrients · Nitrate · Ammonium · Potassium · Ion-specific · Experimental design · Response surface methodology

R. P. Niedz (✉) · S. E. Hyndman · T. J. Evens · A. A. Weathersbee III
United States Department of Agriculture, Agricultural Research Service, U.S. Horticultural Research Laboratory, 2001 South Rock Road, Ft. Pierce, FL 34945-3030, USA
e-mail: randall.niedz@ars.usda.gov

Introduction

Gerbera hybrida (*G. jamesonii*×*G. viridifolia*) (Bremer 1994; Hansen 1999), commonly called gerbera daisies, are grown and sold as bedding, potted plants, and cut flowers. Because of their worldwide popularity, clonally propagated gerbera daisies are propagated by *in vitro* culture because conventional propagation methods, by division of clumps or cuttings, are not sufficient to economically produce the large numbers of plants required to meet consumer demand. Gerbera daisy micropropagation has been reviewed by Kanwar and Kumar (2008) and Minerva and Kumar (2013).

The majority of studies on gerbera daisy micropropagation have examined the effects of plant growth regulators (PGRs) on shoot culture initiation, proliferation, and rooting (Pierik *et al*. 1973; Meyer and Van Staden 1988; Tyagi and Kothari 2004; Ray *et al*. 2005; Nhut *et al*. 2007; Bhatia *et al*. 2012; Minerva and Kumar 2013), with considerably less information available on the effects of mineral nutrients on *in vitro* growth. Mineral nutrient experiments typically compare various basal media formulations on *in vitro* growth (Pierik *et al*. 1975; Bouman *et al*. 2001; Mohammed and Özzambak 2007; Shabanpour *et al*. 2011; Greenway *et al*. 2012) rather than the effects of specific salts or ions. In one study, the elemental composition of the adult gerbera leaf was used to construct a basal medium (Bouman *et al*. 2001).

Mineral nutrition is of fundamental importance in the growth and development of any organism and is arguably, apart from water relations, the most basic component of a plant tissue culture medium. Understanding the effects of mineral nutrients and how they interact with other medium components such as PGRs, vitamins, carbon sources, and substrate can only improve our ability to successfully culture plant cells, tissues, and organs *in vitro*. We examined the effects of groups of nutrients, salts, and individual ions on the *in vitro* growth of gerbera daisy cv. 'Pasadena'.

Materials and Methodology

Plant material and culture. Shoot cultures of *Gerbera hybrida* cv. 'Pasadena' (hereafter referred to as gerbera) were generously provided by Agri-Starts (Apopka, FL) and subcultured on MS basal medium (Murashige and Skoog 1962) supplemented with the following (mg/L): $NaH_2PO_4 \cdot H_2O$ (85), indole-3-acetic acid (IAA) (2), kinetin (1), thiamine·HCl (30), nicotinic acid (10), *i*-inositol (100), adenine sulfate dehydrate (80), L-tyrosine (100), sucrose (45,000), and agar (10,000) (Murashige *et al.* 1974). Culture media were adjusted to pH 5.8 with NaOH and autoclaved (15 min at 121°C), and 15 ml medium was poured into previously autoclaved flat-bottomed glass culture tubes (25× 100 mm) with polypropylene caps (Magenta Corporation, Chicago, IL). Cultures were grown in a temperature-controlled growth room at 27°C with a 16-h photoperiod under 45–60 μmol photons m^{-2} s^{-1} light provided by cool-white fluorescent lamps.

Experimental approach. Four types of experiments were conducted to quantify the effects of mineral nutrients on *in vitro* growth of gerbera.

1 Macro-micro-Fe—an experiment to determine the effects of the macro/meso, micro, and Fe nutrients of MS medium. The experiment was designed as a 3-factor response surface sufficient for quadratic modeling (Table 1) and was comprised of 26 points—10 model points, 5 replicates for pure error estimation, and 11 lack-of-fit points. The three factors were constructed from MS salts as follows:

   - Factor 1 (macro/meso salts): NH_4NO_3, $CaCl_2 \cdot 2H_2O$, $MgSO_4 \cdot 7H_2O$, KH_2PO_4, KNO_3.
   - Factor 2 (micronutrient salts): H_3BO_3, $CoCl_2 \cdot 6H_2O$, $CuSO_4 \cdot 5H_2O$, $MnSO_4 \cdot 4H_2O$, KI, $Na_2MoO_4 \cdot 2H_2O$, $ZnSO_4 \cdot 7H_2O$.
   - Factor 3 (Fe): $FeSO_4 \cdot 7H_2O$, $Na_2EDTA \cdot 2H_2O$. A Fe/EDTA complex was prepared by dissolving 1.39 g $FeSO_4 \cdot 7H_2O$ and 1.862 g $Na_2EDTA \cdot 2H_2O$ separately into ~250 ml water. The $Na_2EDTA \cdot 2H_2O$ solution was brought to boiling on a hot plate and then added slowly to the $FeSO_4 \cdot 7H_2O$ solution. The combined solutions were then boiled for 1 h, cooled to room temperature, brought to 500 ml volume, and stored in aluminum-foil-wrapped glass bottles at 4°C.

2 Metals (Cu-Mn-Zn-Fe/EDTA)—a set of experiments to determine the effects of $CuSO_4 \cdot 5H_2O$, $MnSO_4 \cdot 4H_2O$, $ZnSO_4 \cdot 7H_2O$, and Fe/EDTA complex (prepared as described above). Each salt was varied using a single-factor design in which the concentration was evaluated at 0, 0.0001, 0.001, 0.01, 0.1, and 1 mM, spanning five log intervals.

Table 1. Macro-micro-Fe experiment treatment points and growth response data for gerbera *in vitro* shoot growth

Treatment design points[a]	Factors[b]			Responses			
	Macro/meso	Micro	Fe	Quality	Shoot number	Leaf number	Shoot hgt. (mm)
1	0.1	3	3	2.0	3.0	3.1	25.9
2	0.55	1.65	0.3	1.0	4.7	4.0	19.3
3	0.1	0.3	0.3	1.0	3.0	2.9	16.4
4	0.55	0.3	0.3	2.0	7.2	3.9	23.3
5	0.1	0.3	3	1.0	3.3	3.6	11.6
6	0.1	1.65	3	1.0	4.2	2.9	15.7
7	1	3	3	2.3	5.3	3.8	29.7
8	0.1	0.3	1.65	1.0	4.5	3.5	14.6
9	1	1.65	0.3	1.0	4.2	4.2	16.5
10	1	0.3	1.65	1.3	4.2	3.7	20.0
11	0.55	1.65	1.65	3.0	5.7	3.1	21.0
12	0.1	3	0.3	1.0	1.5	1.9	13.4
13	1	3	0.3	1.0	1.3	4.0	19.8
14	1	3	0.3	1.0	0.8	4.3	20.1
15	0.1	1.65	0.3	1.0	4.0	2.9	15.7
16	0.55	3	1.65	2.8	4.7	3.8	30.6
17	1	1.65	1.65	2.3	6.3	3.5	24.3
18	0.1	3	3	2.0	2.7	3.3	19.8
19	0.55	1.65	1.65	2.3	4.2	4.1	28.7
20	1	0.3	0.3	2.3	5.2	4.1	23.0
21	1	0.3	3	1.1	0.8	3.9	11.8
22	0.55	0.3	3	1.0	4.2	3.4	13.7
23	1	3	3	2.2	4.0	3.6	34.0
24	0.55	0.975	1.65	2.8	9.7	3.1	23.5
25[c]	1	1	1	3.0	8.0	3.6	31.8
26[c]	1	1	1	2.5	3.7	4.2	34.3

Experiment is a three-factor response surface design. The data represent the mean of six duplicate tubes per treatment point

[a] Replicate points are included (e.g., #11 and #19)

[b] Factor levels are expressed as a multiple of MS levels. For example, the factor "Fe" ranged from 0.3× to 3× MS Fe levels

[c] Points #25 and #26 are MS medium

3 Nitrogen and potassium (NH_4^+, NO_3^-, and K^+)—an experiment designed to determine the effects of the three primary ions in MS medium. The experiment was designed as a 2-component mixture-amount experiment that included two mixture components, K^+ and NH_4^+, and one numeric factor, NO_3^- concentration; all other MS mineral nutrient ions were held constant. Sufficient points were selected for quadratic modeling (Table 2, Fig. 1). The proportion of NH_4^+ ranged from 0 to 0.5 and for K^+ from 1 to 0.5. The amount of NO_3^- ranged from 10 to 50 mM. The amount of NH_4^+ plus K^+ equaled the amount of NO_3^- for the treatment; thus, the charge balance, or pH, was equivalent across the design space. The experiment was

Table 2. Mixture-amount treatment points for $NH_4^+/NO_3^-/K^+$ experiment and growth response data for gerbera *in vitro* shoot growth

Treatment design points	Block	Mixture components[a]		Factor	Responses				
		NH_4^+	K^+	NO_3^+ mM	Quality	Shoot number	Leaf number	Shoot hgt. (mm)	pH[b]
1	1	0.250	0.750	10.00	1.67	3.8	14	44.0	5.7
2	1	0.500	0.500	50.00	2.17	5.0	22	45.0	5.8
3	1	0.500	0.500	30.00	1.50	4.7	22	40.0	5.8
4	1	0.000	1.000	20.00	1.50	3.2	13	37.7	5.7
5	1	0.500	0.500	10.00	2.20	3.8	17	57.4	5.7
6	1	0.250	0.750	30.00	2.50	5.7	22	41.2	5.8
7	1	0.250	0.750	30.00	2.17	4.5	18	48.3	5.8
8	1	0.250	0.750	50.00	1.50	4.2	20	41.2	5.8
9	1	0.500	0.500	30.00	2.17	6.3	27	43.3	5.8
10	1	0.000	1.000	50.00	1.33	3.7	14	41.5	5.8
11	1	0.250	0.750	10.00	2.17	4.7	18	37.3	5.7
12	1	0.500	0.500	50.00	2.67	5.8	25	43.5	5.8
13	1	0.000	1.000	50.00	1.33	2.3	10	51.0	5.8
14	2	0.500	0.500	10.00	1.33	5.0	23	52.5	5.7
15	2	0.000	1.000	10.00	1.00	2.8	9	43.0	5.7
16	2	0.125	0.875	30.00	2.00	5.2	21	47.2	5.8
17[c]	2	0.500	0.500	40.00	1.50	3.8	21	41.7	5.8
18	2	0.250	0.750	50.00	1.50	5.0	20	45.5	5.8
19	2	0.250	0.750	50.00	1.83	4.5	20	46.7	5.8
20[c]	2	0.500	0.500	40.00	1.67	5.0	25	44.8	5.8
21	2	0.500	0.500	10.00	1.17	4.7	22	43.2	5.7
22	2	0.000	1.000	40.00	1.00	2.3	12	42.5	5.8
23	2	0.000	1.000	10.00	1.50	3.5	14	42.7	5.7
24	2	0.000	1.000	10.00	1.33	2.7	12	50.3	5.7
25	2	0.250	0.750	20.00	1.83	4.7	18	58.2	5.8
26	2	0.000	1.000	40.00	1.33	3.0	15	41.3	5.8

Experiment is a two-component mixture of NH_4^+ and K^+ with NO_3^- concentration as a quantitative factor. The mixture components are listed as proportions with the actual amounts matched to the amount of NO_3^-. For example, treatment point #1 included 2.5 mM NH_4^+, 7.5 mM K^+, and 10 mM NO_3^-. The data represent the mean of six duplicate tubes per treatment point

[a] Expressed as a proportion that sums to 1. The concentration of NH_4^+:K^+ is equal to the mM of NO_3^+ to achieve charge neutrality

[b] Calculated from the chemical equilibrium modeling software MINEQL+ver. 4.5 (Schecher and McAvoy, 2003), temperature corrected and assumed open to the atmosphere with a P_{CO2} at sea level of $10^{-3.50}$ atm

[c] Points #17 and #20 are MS medium

designed with two blocks and 26 treatment design points: 9 model points, 12 replicates for pure error estimation, and 5 lack-of-fit points. Ion confounding was removed using the method of Niedz and Evens (2006) and the software application ARS-Media (freely available for download at http://www.ars.usda.gov/services/software/download.htm?softwareid=148 or by request).

4 Chelated Fe—an experiment designed to determine the proportional and concentration effects of Fe and EDTA chelation. The experiment was designed as a 2-component mixture-amount that included two mixture components, NaFeEDTA and $FeSO_4 \cdot 7H_2O$, and one quantitative factor, Fe concentration (μM); all other MS mineral nutrient ions were held constant. Sufficient points were selected for modeling a quadratic response surface (Table 3). The design geometry and treatment point coordinates Fig. 2.

Responses measured. To reduce potential carryover effects, shoot clumps were cultured for three growth cycles (4 wk per growth cycle) on the treatment formulations; data were then collected at the end of the third culture cycle. Six culture tubes (pseudo-replicates) were used per treatment. Five true replicates were used to provide an estimate of pure error. For each experiment, five responses were measured as follows:

1. Quality—an overall assessment using a gestalt ranking of 1–3 where 1 was a culture readily distinguished as

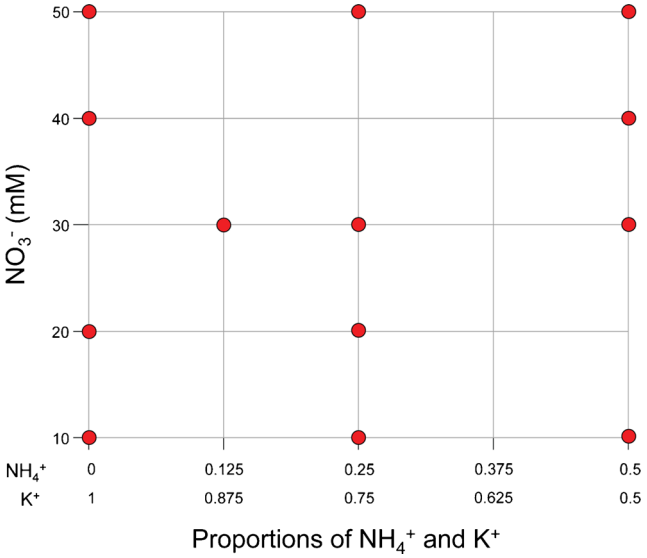

Figure 1. Design geometry and point locations for the 2-component mixture-amount $NH_4^+/NO_3^-/K^+$ experiment.

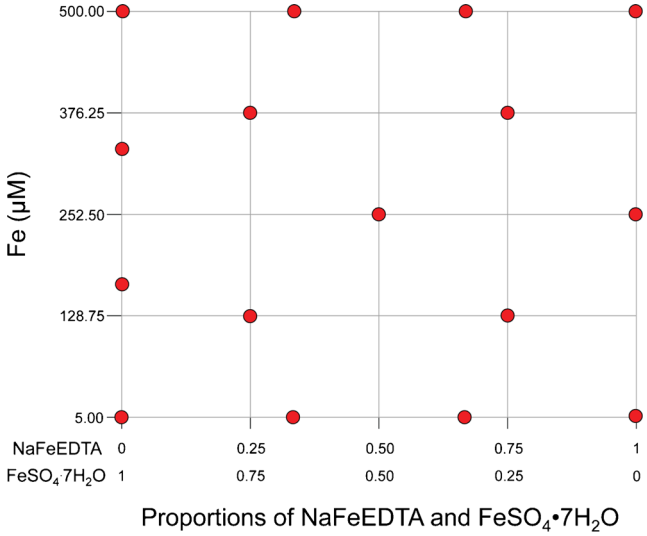

Figure 2. Design geometry and point locations for the 2-component mixture-amount NaFeEDTA+$FeSO_4 \cdot 7H_2O$ (chelated Fe) experiment.

unacceptable and of poor quality, and 3 a culture readily distinguished as acceptable and high quality relative to the other treatments; a score of 2 was given if neither 1 nor 3 applied (Niedz *et al*. 2007). A high-quality gerbera culture was scored a 3 if the clump had a "healthy and vigorous" green appearance, included up to five shots in the clump, 2–3 leaves per shoot, and a height of 2–3 cm; optimally, the culture would be suitable for continued micropropagation or rooting and transfer to greenhouse. Cultures that were scored as 1 would be too large, too small, and/or chlorotic or necrotic.

Table 3. Mixture-amount Fe/EDTA treatment points for chelated Fe experiment and growth response data for gerbera *in vitro* shoot multiplication

Treatment design points[a]	Mixture components		Factor	Responses			
	NaFeEDTA	$FeSO_4 \cdot 7H_2O$	Fe (µM)	Quality	Shoot number	Leaf number	Shoot hgt. (mm)
1	0	1	500	1	2.5	13.7	23.8
2	1	0	500	1	1	9.0	22.0
3	0.75	0.25	376.25	1.8	4.3	23.0	31.2
4	0.75	0.25	128.75	2.3	5.2	26.0	41.2
5	1	0	252.50	2	5.8	28.8	33.5
6	0.25	0.75	376.25	2.5	7.3	31.3	54.7
7	0.33	0.67	500	2.5	6.5	28.0	52.2
8	1	0	252.50	2.3	5	25.5	33.3
9	0.67	0.33	5	1	1	4.2	24.7
10	0	1	5	1	1	4.5	27.3
11	0	1	335	1	1	5.5	29.3
12	1	0	500	1	0	0.0	0.0
13	0	1	170	1	1	6.3	24.7
14	1	0	5	1	1	4.2	25.0
15	0	1	500	1	1.5	9.3	26.5
16	0.67	0.33	500	1.7	3.3	20.3	30.5
17	0	1	5	1	1	3.8	24.0
18	0.33	0.67	5	1	1	4.0	25.2
19	1	0	5	1	1	3.5	26.0
20	0.25	0.75	128.75	1	5	26.3	35.2
21	0.50	0.50	252.50	2.7	5.5	27.3	44.3

Experiment is a two-component mixture of NaFeEDTA and $FeSO_4 \cdot 7H_2O$ with Fe concentration as a quantitative factor. The mixture components are listed as proportions. A specific treatment formulation would include Fe in the proportions and amount specified. For example, treatment design point #4 includes 128.75 µM Fe as 96.56 µM NaFeEDTA (75%)+32.19 µM $FeSO_4 \cdot 7H_2O$. The data represent the mean of six duplicate tubes per treatment point

2. Leaf number—all visible leaves including immature unfolded leaves were counted.
3. Shoot number—number of shoots per clump. The callused base was removed, the shoots separated, and the shoots counted; the total shoot number included the original shoot.
4. Shoot clump height—measured from the base of the callus to the tip of the tallest leaf.

Data analysis. The ANOVA data for each treatment design point were the means of the six culture vessels (pseudo-replicates) at the end of the third growth cycle. The three Cu-Mn-Zn-Fe/EDTA salt experiments were analyzed by one-way ANOVA, which was then followed by Dunnett's multiple comparison test to compare each concentration level to the MS level. The software application Prism 5 (GraphPad Software, La Jolla, CA) was used for the ANOVA and multiple comparison analyses. For the response surface designs, all possible models from the mean to cubic polynomial were calculated, and the highest-order polynomial model in which additional model terms were significant at the 0.05 level was analyzed by ANOVA. Detailed descriptions of these statistical methods are discussed by Niedz and Evens (2007, 2011). The software application Design-Expert® 8 (Stat-Ease, Inc, Minneapolis, MN) was used for response surface experimental design construction, model evaluation, analysis, and graphics.

Table 4. ANOVA model terms, p values (Prob. > F), lack-of-fit, and R^2 statistics for the effect of macro, micro, and Fe salts on quality, shoot number, leaf number, and shoot height (mm) of *Gerbera in vitro* shoot cultures

Source	Quality[a]	Shoot number[b]	Leaf number[c]	Shoot hgt.[d] (mm)
Model	<0.0001	0.0062	0.0002	<0.0001
A–macro	0.0160	0.4916	<0.0001	<0.0001
B–micro	0.0844	0.1361	–	<0.0001
C–Fe	0.1224	0.9630	0.9248	0.1012
AC–macro×Fe	–	–	0.0234	0.0706
BC–micro×Fe	<0.0001	0.0086	–	<0.0001
A^2–macro^2	0.0750	0.0454	–	0.0199
C^2–Fe^2	0.0013	0.0550	–	0.0002
Lack of fit	0.0047	0.5313	0.6257	0.3546
R^2	0.77	0.58	0.58	0.91
R^2 adj	0.69	0.45	0.52	0.88
R^2 pred	0.56	0.28	0.42	0.83
Model type[e]	Quadratic	Quadratic	2FI	Quadratic

Model equations are presented in terms of coded factors in the table footnotes

[a] 1/Sqrt(quality) = 0.64 – 0.063*A – 0.045*B – 0.039*C – 0.14*B*C + 0.090*A^2 + 0.18*C^2

[b] Shoot # = 6.24 + 0.26*A – 0.60*B – 0.018*C + 1.26*B*C – 1.56*A^2 – 1.48*C^2

[c] Leaf # = 3.47 + 0.42*A – 8.643E–003*C – 0.24*A*C

[d] (Shoot hgt.)$^{-1.46}$ = 8.949E–003 – 2.847E–003*A – 3.331E–003*B + 9.544E–004*C + 1.163E–003*A*C – 5.602E–003*B*C + 2.745E–003*A^2 + 4.837E–003*C^2

[e] Model reduction by backward elimination. *2FI* 2-factor interaction.

Results

Macro-micro-Fe experiment. Four responses were measured for the macro-micro-Fe experiment: quality, shoot number, leaf number, and shoot height. Quality ranged from 1 to 3, shoot number from 0.8 to 9.7, leaf number from 1.9 to 4.3, and shoot height from 11.6 mm to 34.3 mm (Table 1). Data transformations, used to meet the statistical assumptions of ANOVA, were required for the quality and shoot height data. Significant models were obtained for each of the responses (Table 4) $p<0.0001$ (quality and shoot height), $p=0.0002$ (leaf number), and $p=0.0062$ (shoot number). The lack-of-fit test was significant for quality and could not be improved with other models. To use this model, it would be necessary to utilize confirmation runs to identify useful formulations. The ranges between R^2, R^2_{adj}, and R^2_{pred} statistics for each of the four models was less than 0.2 (Anderson and Whitcomb 2005), indicating models potentially useful for prediction; however, overall values varied and ranged from a R^2_{pred} low of 0.28 for shoot number to an R^2_{pred} high of 0.83 for shoot height, the "best" modeled response.

The factors that influenced each response varied. The interaction effect of the micronutrients and Fe (Micro×Fe) had a strong effect on quality, shoot number, and shoot height, but had no effect on leaf number and was not included in the model for leaf number. The main effect of the macronutrients on leaf number and shoot height was highly significant ($p<0.0001$), and on quality, slightly significant ($p=0.0160$). The main effect of the micronutrients on shoot height was highly significant ($p<0.0001$). From these results, the remaining three experiments were designed to determine examine specific mineral nutrient effects in greater detail.

Metals (Cu-Mn-Zn-Fe/EDTA) experiment. Summary statistics of the effect of each metal salt on the four measured responses were calculated (Table 5) and the data analyzed (Table 6). Representative images of gerbera plants on all of the metal salt treatments are presented (Fig. 3).

$CuSO_4 \cdot 5H_2O$. The effect of $CuSO_4$ on the four growth responses was primarily detrimental. The effects were significant ($p<0.001$) for shoot number, leaf number, and shoot height, but had little effect ($p=0.0627$) on quality. $CuSO_4$ at 1 mM was the only treatment that tested significantly different from the 0.0001 mM MS level, and was toxic. Plants grown at 0 mM

Table 5. Summary data of the effect of $CuSO_4$, $ZnSO_4$, $MnSO_4$, and Fe/EDTA on shoot quality, shoot and leaf number, and shoot height of gerbera *in vitro* shoot cultures

Concentration (mM)	Salt	Quality	Shoot number	Leaf number	Shoot hgt. (mm)
0	$CuSO_4$	1.50±0.34	7.83±1.25	34.83±6.32	37.67±3.06
0.0001		1.50±0.22	5.17±0.65	26.00±2.28	43.17±1.74
0.001		1.83±0.31	7.00±1.57	34.33±7.80	44.17±1.08
0.01		1.83±0.31	7.33±1.52	36.33±6.16	48.00±1.88
0.1		1.00±0	3.00±0.52	18.17±3.63	41.67±3.09
1		1.00±0	1.00±0	4.00±0.52	23.83±2.41
0	$ZnSO_4$	1.00±0	0.00±0	5.33±0.17	23.00±1.71
0.0001		1.00±0	0.00±0	2.67±0.49	16.00±1.39
0.001		1.00±0	0.00±0	13.50±1.20	20.00±2.71
0.01		1.17±0.17	4.50±0.92	24.83±4.76	37.67±3.08
0.1		2.50±0.22	6.50±0.34	44.33±3.44	51.33±3.75
1		1.00±0	1.00±0	4.50±0.43	18.00±2.93
0	$MnSO_4$	1.33±0.21	5.67±0.92	32.50±4.74	34.33±2.22
0.0001		1.50±0.22	7.67±0.56	41.17±5.41	41.83±3.47
0.001		1.67±0.33	7.50±0.50	39.83±4.82	39.50±2.50
0.01		1.67±0.33	6.17±0.65	32.17±3.52	38.83±2.95
0.1		1.67±0.21	6.17±0.87	32.00±3.89	33.50±2.57
1		2.00±0.37	7.17±0.98	35.67±5.04	32.67±1.74
0	Fe/EDTA	1.00±0	0.00±0	0.00±0	0.00±0
0.0001		1.00±0	0.00±0	0.00±0	0.00±0
0.001		1.00±0	0.00±0	5.00±0.52	27.17±1.05
0.01		1.83±0.41	6.67±0.49	33.50±2.51	38.50±2.79
0.1		2.33±0.52	5.17±0.95	33.50±4.84	48.00±1.90
1		1.50±0.55	3.83±0.40	21.50±2.17	42.83±3.53

Data presented as mean ± S.D.

were not distinguishable from the 0.0001 mM MS level. Thus, optimal growth is in the range of 0 to 0.1 mM (Fig. 4).

$MnSO_4$ $4H_2O$. The effect of $MnSO_4$ on each of the four growth responses was not significant. The effect on shoot height had a $p=0.1003$ and was probably detecting a reduction in growth at 1 mM. Thus, optimal growth is in the range of 0 to 1 mM; MS has 0.1 mM Mn^{2+}.

$ZnSO_4$ $7H_2O$. The effect of $ZnSO_4$ on each of the four growth responses was significant ($p<0.0001$). The optimal level was 0.1 mM; growth declined substantially below and above this level. Thus, optimal growth is in the range of 0.01 to 1 mM; MS has 0.037 mM Zn^{2+}.

Fe/EDTA. The effect of Fe/EDTA on each of the four growth responses was significant ($p<0.0001$). Overall, the best treatment was 0.1 mM (MS level). However, there was some variation for each of the four responses around the 0.1 mM level. For example, for shoot number, there was no significant difference between 0.1, 0.01, and 1 mM, whereas for shoot height, 0.1 vs. 0.01 mM was significant (p value 0.01 to 0.05) and 0.1 vs. 1 mM was not significant ($p>0.05$). Optimal growth is in the range of 0.01 to 1 mM.

$NH_4^+/NO_3^-/K^+$ experiment. Four growth responses were measured for the $NH_4^+/NO_3^-/K^+$ experiment: quality, shoot number, leaf number, and shoot height. In addition, the pH of the initial medium was measured. Quality ranged from 1 to 2.67, shoot number from 2.3 to 6.3, leaf number from 9 to 27, shoot height from 37.3 to 58.2 mm, and pH was 5.7–5.8 (Table 2). Data transformation was not required. Significant models were obtained for three of the four responses (Table 7): quality ($p=0.0068$), shoot number ($p<0.0001$), and leaf number ($p<0.0001$). The model for shoot height was not significant, indicating that NH_4^+, NO_3^-, and K^+ had no detectable effect. The lack-of-fit test was not significant for any of the four responses; indicating that the information contained within the data was captured and could not be improved with better models. The range between R^2, R^2_{adj}, and R^2_{pred} statistics for quality, shoot number, and leaf number was less than 0.2, indicating models potentially useful for prediction; however, overall values varied and ranged from a R^2_{pred} low of 0.18 for quality to an R^2_{pred} high of 0.68 for leaf number, the "best" modeled response. The greatest effect on quality, shoot number, and leaf number was in the linear mixture term. The linear mixture term compares the response at the extreme ends of the NH_4^+:K^+ mixture design space, 0% NH_4^+:100% K^+ vs. 50% NH_4^+:50% K^+; it is not a measure of the effects of NH_4^+ and

Table 6. The effect of individual salts $CuSO_4$, $ZnSO_4$, $MnSO_4$, and Fe/EDTA on shoot quality, shoot and leaf number, and shoot height of gerbera *in vitro* shoot cultures

Metal salts (MS level)	Quality		Shoot number		Leaf number		Shoot hgt. (mm)	
	ANOVA *p* value	MS level vs. other levels ($p<0.05$)	ANOVA *p* value	MS level vs. other levels ($p<0.05$)	ANOVA *p* value	MS level vs. other levels ($p<0.05$)	ANOVA *p* value	MS level vs. other levels ($p<0.05$)
$CuSO_4$ (0.0001 mM)	0.0627	ns - all	0.0004	vs. 0 ns	0.0005	vs. 0 ns	<0.0001	vs. 0 ns
				vs. 0.001 ns		vs. 0.001 ns		vs. 0.001 ns
				vs. 0.01 ns		vs. 0.01 ns		vs. 0.01 ns
				vs. 0.1 ns		vs. 0.1 ns		vs. 0.1 ns
				vs. 1*		vs. 1*		vs. 1***
$MnSO_4$ (0.10 mM)	0.7035	ns - all	0.3533	ns - all	0.5707	ns - all	0.1003	ns - all
$ZnSO_4$ (0.037 mM)[a]	<0.0001	vs. 0***	<0.0001	vs. 0***	<0.0001	vs. 0***	<0.0001	vs. 0***
		vs. 0.0001***		vs. 0.0001***		vs. 0.0001***		vs. 0.0001***
		vs. 0.001***		vs. 0.001***		vs. 0.001***		vs. 0.001***
		vs. 0.01 ***		vs. 0.01 **		vs. 0.01 ***		vs. 0.01 **
		vs. 1***		vs. 1***		vs. 1***		vs. 1***
Fe/EDTA (0.10 mM)	<0.0001	vs. 0***	<0.0001	vs. 0***	<0.0001	vs. 0***	<0.0001	vs. 0***
		vs. 0.0001***		vs. 0.0001***		vs. 0.0001***		vs. 0.0001***
		vs. 0.001***		vs. 0.001***		vs. 0.001***		vs. 0.001***
		vs. 0.01 ns		vs. 0.01 ns		vs. 0.01 ns		vs. 0.01*
		vs. 1**		vs. 1 ns		vs. 1 **		vs. 1 ns

The effect of each salt was analyzed by one-way ANOVA followed by Dunnett's multiple comparison test to compare each concentration against the MS level, except where noted. *ns p* value>0.05
***p value <0.001; **p value 0.001 to 0.01; *p value 0.01 to 0.05
[a] For $ZnSO_4$, 0.10 mM (rather than the MS level of 0.037 mM) was compared to the other levels

K^+. Plants grew best at the extreme range of the mixture (0.5 NH_4^+ and 0.5 K^+), the MS proportion, suggesting that extending the design space to higher proportions of NH_4^+ may be beneficial.

Chelated Fe experiment. Four responses were measured for the chelated Fe experiment: quality, shoot number, leaf number, and shoot height. Quality ranged from 1 to 2.7, shoot number from 0 to 7.3, leaf number from 0 to 31.3, and shoot height from 0 to 54.7 mm (Table 3). Data transformation was required for each of the four responses to meet the normality and constant variance assumptions of ANOVA and included power (quality and shoot height), inverse square root (shoot number), and base 10 log (leaf number) transforms. Models significant at $p<0.0001$ were obtained for all four responses (Table 8). The same seven terms were adequate for describing each of the four responses. The regions of the design space where each response was greatest were similar. This region is colored using a red (greatest response) to blue (least response) range and is pictured in Fig. 5 for quality. The lack-of-fit statistic was significant for quality, shoot number, and leaf number. Higher-order models were tested that improved the lack of fit, but resulted in more complex contour plots. The final models selected represented what we considered as the "best" representation of the biological effects, namely, that (1) the proportion of Fe that is chelated and the amount of Fe needed for good growth are inversely related and (2) good growth was not observed without some chelated Fe. Additionally, the amount of Fe for "best" growth was dependent on the proportion of chelated Fe but ranged from 2–5× the MS level. Figure 5 shows pictures of plants grown on the unique 16 treatment points overlaid on a contour plot of the treatments to visually represent the effects on growth over the design space. The range between each of the three R^2 statistics was less than 0.2 for each of the responses and indicated that the models are potentially useful for prediction. The quality response had the greatest R^2.

Because the experiment was designed as a mixture-amount experiment, the proportional effects of NaFeEDTA and $FeSO_4 \cdot 7H_2O$ could be determined. Only quality had a significant linear mixture term ($p=0.0009$). The AB term quantifies blending synergies and antagonisms and was highly significant for all four responses. This is of particular interest because it means that NaFeEDTA and $FeSO_4 \cdot 7H_2O$ exhibit nonlinear blending effects, specifically synergistic blending; in other words, the responses are greater, which is evident from Fig. 5, than what would be predicted from a linear relationship. The terms ABC and AC^2 are the interactions between the

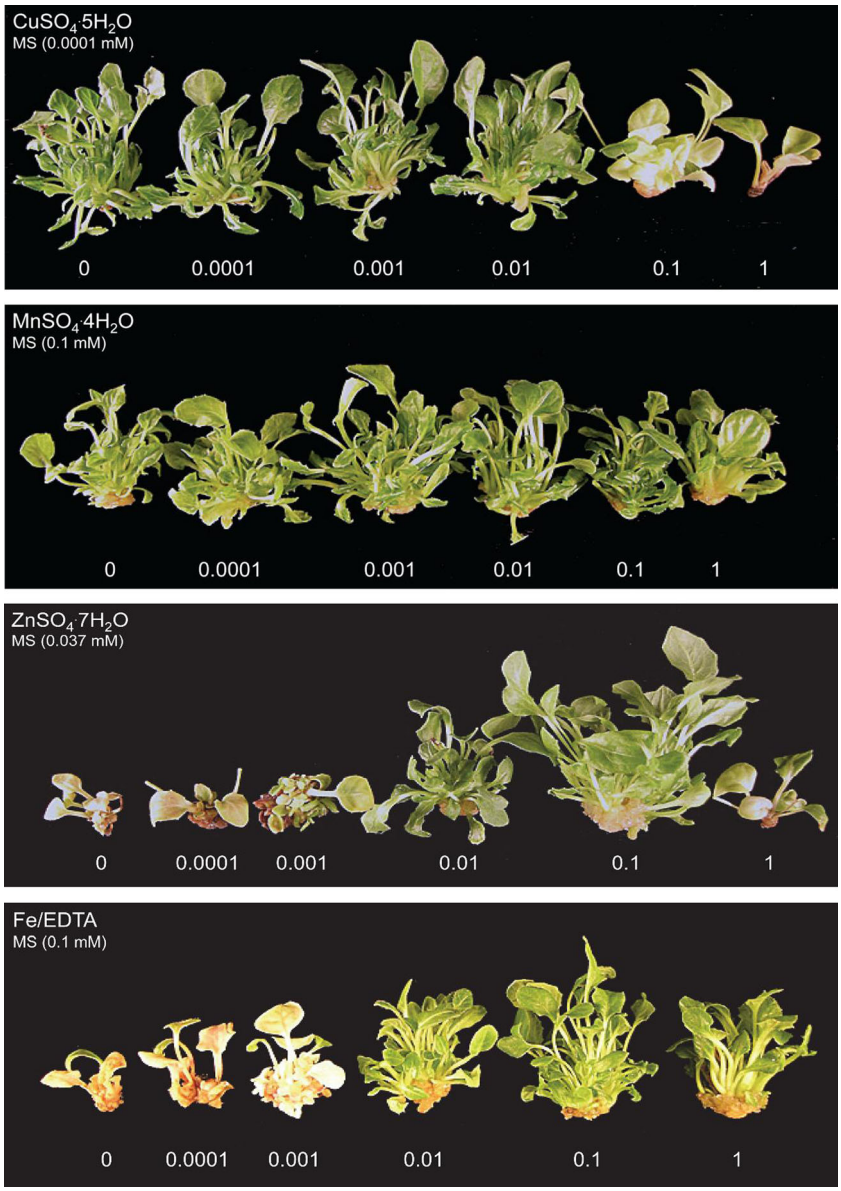

Figure 3. Effects of $CuSO_4 \cdot 5H_2O$, $MnSO_4 \cdot 4H_2O$, $ZnSO_4 \cdot 7H_2O$, or $FeSO_4 \cdot 7H_2O$+ $Na_2EDTA \cdot 2H_2O$ (Fe/EDTA) on growth of gerbera shoot cultures (Metals experiment). Each metal salt was varied over five log intervals—0, 0.0001, 0.001, 0.01, 0.1, and 1 mM. The objective was to identify, for each metal, the order of magnitude concentration for shoot growth. MS levels are listed in the upper left of each image and are as follows: $CuSO_4 \cdot 5H_2O$ (0.0001 mM), $MnSO_4 \cdot 4H_2O$ (0.10 mM), $ZnSO_4 \cdot 7H_2O$ (0.037 mM), and $FeSO_4 \cdot 7H_2O$+$Na_2EDTA \cdot 2H_2O$ (Fe/EDTA, 0.10 mM).

mixture components (NaFeEDTA and $FeSO_4 \cdot 7H_2O$) and Fe concentration, and the mixture component NaFeEDTA and Fe concentration, respectively. The interaction between NaFeEDTA and Fe concentration (AC^2) was highly significant ($p<0.0001$) and had the single greatest effect on all responses.

Discussion

This research emphasized *in vitro* responses rather than the recovery of large numbers of plants. The effects of various mineral nutrients on *in vitro* growth of shoot cultures of the gerbera daisy cv. 'Pasadena' were studied using both salt- and ion-based experiments. Salt-based experiments can be useful when there is little information on the effects of mineral nutrients. Treating with groups of salts, as was done in the macro-micro-Fe experiment, is a type of crude screening experiment to detect effects that can then be explored in further detail. Because the factors are groups of salts, with the exception of Fe, the effects of individual salts or specific ions cannot be determined. This approach can also identify formulations that result in better growth, as was done in the case of citrus embryogenic and nonembryogenic cell lines (Niedz and Evens 2007) and pear micropropagation (Reed *et al*. 2013; Wada *et al*. 2013). The majority of gerbera micropropagation studies used a single mineral nutrient formulation to determine the effects of various PGRs. Where mineral nutrition has been studied, comparisons are typically

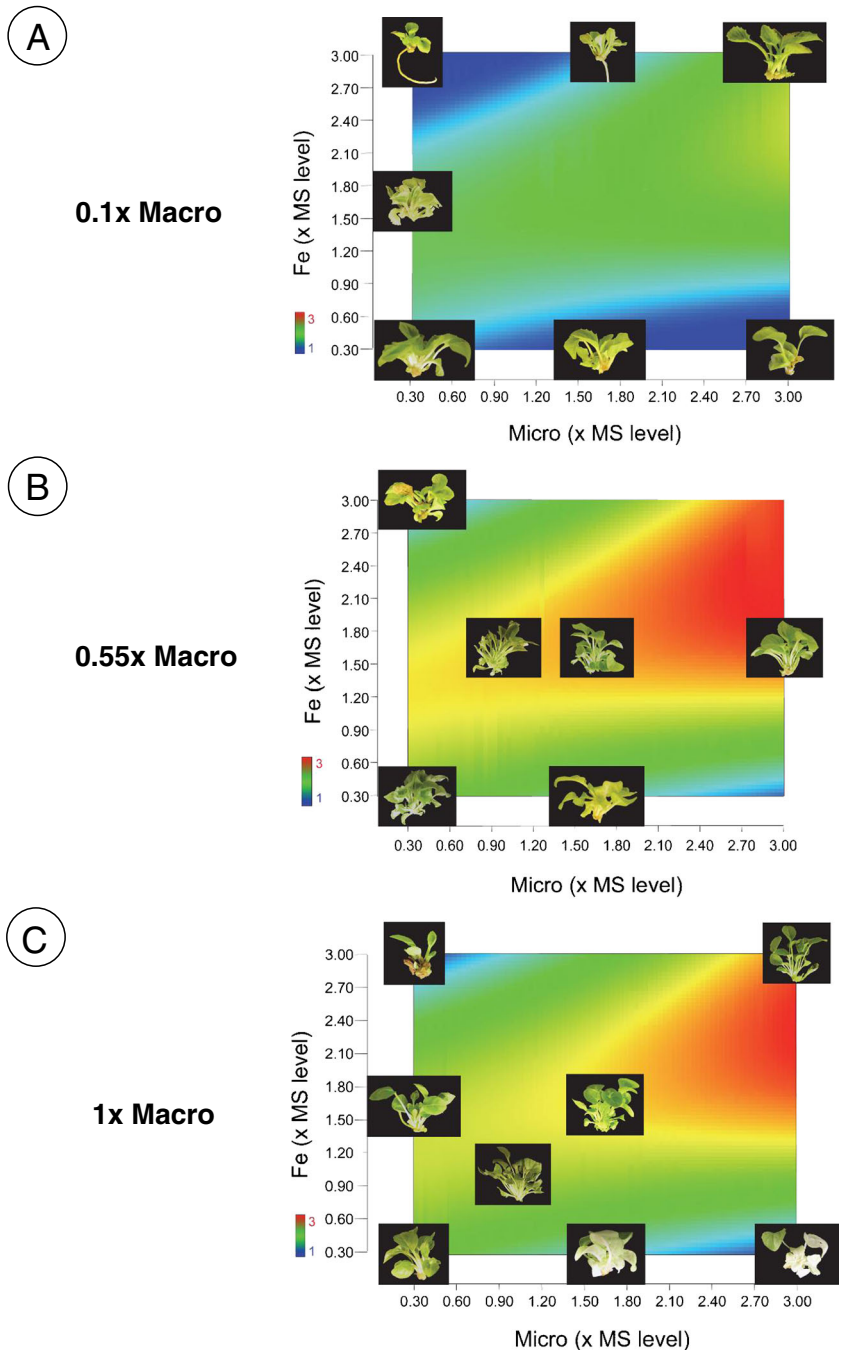

Figure 4. Contour plot of quality for the 3-factor response surface macro-micro-Fe experiment. The effect of each treatment is shown by a picture of gerbera shoot cultures grown on each treatment coordinate (listed in Table 1). Because the design included three factors, the design space is 3D and is represented by Fe×micro contour plots at each of the three macro levels. *a* 0.1× MS level, *b* 0.55× MS level, *c* 1× MS level.

between full- and half-strength concentrations of popular formulations, most commonly MS. A recent approach used a set of diverse formulations to identify the formulation that resulted in the "best" response (Greenway *et al*. 2012). Using this approach, the effects of four formulations (MS, B5, BDS, and BABI) on gerbera micropropagation were tested; no significant differences were observed. The results from the macro-micro-Fe experiment showed that the greatest effects varied with response and suggested additional experimentation to explore these relationships in greater detail.

The simplest approach for development of media formulations would be to define the type of *in vitro* growth desired, select formulations that match or approximate the growth objective(s), and test those formulations. Additional formulations could be identified by utilizing the polynomial models that describe *in vitro* growth over the design regions examined. This is numerical optimization and requires statistical software. Numerical optimization selects locations (formulations) in the design space predicted by the models to result in the desired type of growth. For example, from the

Table 7. ANOVA model terms, p values (Prob. > F), lack-of-fit, and R^2 statistics for the effect of NH_4^+, K^+, and NO_3^+ on quality, shoot number, leaf number, and shoot mass height of gerbera *in vitro* shoot cultures

Source	Quality[a]	Shoot number[b]	Leaf number[c]	Shoot hgt.[d] (mm)
Model	0.0068	<0.0001	<0.0001	0.4960
Linear mixture	0.0131	<0.0001	<0.0001	0.4960
A–NH_4^+				
B–K^+				
AB–NH_4 * K	0.0313	0.0112	–	–
AC–NH_4 * [NO_3]	–	–	0.0523	–
BC–K * [NO_3]	–	–	0.5190	–
BC^2–K * [NO_3]^2	–	–	0.0831	–
Lack of fit	0.1906	0.5121	0.7137	0.1418
R^2	0.35	0.63	0.80	0.02
R^2 adj	0.30	0.60	0.76	−0.02
R^2 pred	0.18	0.54	0.68	−0.14
Model type[e]	$Q\times M$	$Q\times M$	$rL\times Q$	$L\times M$

Model equations are presented in terms of coded factors in the table footnotes
[a] Quality=1.81*A+1.31*B+1.45*A*B
[b] Shoot #=4.89*A+2.99*B+3.16*A*B
[c] Leaf #=22.93*A+15.42*B+1.92*A*C+0.61*B*C–3.34*B*C^2
[d] Shoot hgt=45.90*A+44.13*B
[e] Model reduction by backward elimination. $Q\times M$ quadratic×mean, $rL\times Q$ reduced linear×quadratic, $L\times M$ linear×mean

Table 8. ANOVA model terms, p values (Prob. > F), lack-of-fit, and R^2 statistics for the effect of % EDTA-chelated Fe and the amount of $FeSO_4$ on shoot quality, shoot number, leaf number, and shoot height of gerbera *in vitro* shoot cultures

Model and model terms	Quality[a]	Shoot number[b]	Leaf number[c]	Shoot hgt.[d] (mm)
Model	<0.0001	<0.0001	<0.0001	<0.0001
Linear mixture	0.0009	0.2521	0.1030	0.3048
A–NaFeEDTA				
B–$FeSO_4{\cdot}7H_2O$				
AB–NaFeEDTA× $FeSO_4{\cdot}7H_2O$	<0.0001	0.0002	0.0014	0.0001
AC–NaFeEDTA×[Fe]	0.6251	0.8887	0.0301	0.0024
BC–$FeSO_4{\cdot}7H_2O$×[Fe]	0.6141	0.0261	0.0157	0.7886
ABC–NaFeEDTA× $FeSO_4{\cdot}7H_2O$×[Fe]	<0.0001	0.0228	0.1835	0.0013
AC^2–NaFeEDTA×[Fe]^2	<0.0001	<0.0001	<0.0001	<0.0001
Lack of fit	<0.0001	0.0479	0.0165	0.4837
R^2	0.93	0.89	0.87	0.89
R^2 adj	0.90	0.84	0.81	0.81
R^2 pred	0.89	0.81	0.77	0.71
Model type[e]	$rQ\times Q$	$rQ\times Q$	$rQ\times Q$	$rQ\times Q$

To allow direct comparison of coefficients, model equations are presented in terms of coded factors in the table footnotes
[a] (Quality)$^{-3}$ =0.097*A+1.00 *B–1.90*A*B+0.034*A*C–0.034*B*C–1.99*A*B*C+0.90*A*C^2
[b] 1/Sqrt(shoot #)=0.43*A+0.88*B–1.18*A*B+7.711E–003*A*C–0.13*B*C–0.74*A*B*C+0.59*A*C^2
[c] Log10(leaf #)=1.46*A+0.85*B+1.30*A*B+0.18*A*C+0.20*B*C+0.57*A*B*C–0.72*A*C^2
[d] (Shoot hgt.)$^{-2.48}$ =1.690E–004*A+3.081E–004*B–6.777E–004*A*B+1.108E–004*A*C–8.000E–006*B*C
[e] Model reduction by backward elimination. $rQ\times Q$ reduced quadratic×quadratic

macro-micro-Fe experiment assume the objective is to predict formulations that will result in a quality score ≥2.5, shoot number of 3–5, and shoot height of 20–30 mm. The polynomial models for each of these three responses would be queried to identify the region in the design space predicted to equal each response objective—the polynomial model for quality would predict where quality was ≥2.5, and so on for shoot number and shoot height. Where the three regions intersect, the region that meets the criteria for all three objectives, would be where new formulations would be selected for testing. For this example, a typical formulation from the region predicted to result in gerbera growth per the three stated objectives would be 1× macro, 2.3× micro, 2× Fe Fig. 4. Formulations could be similarly generated from the other experiments. This approach would generate a set of formulations for testing against current in-house formulations.

Comparisons of these results and approaches to other mineral nutrient studies summarized as follows:

1. Bouman *et al*. (2001)—The experimental design was a single qualitative factor (basal medium composition) with three levels (basal medium formulations DKW, MS, and GAM+MS micronutrients). Unfortunately, no statistical analyses were conducted and there is little information on the materials and methods used, making it difficult to interpret the results. The authors did mention that hyperhydricity was observed only in the DKW and MS treatments, but not in the GAM+MS micronutrient treatment. Hyperhydricity was not observed, or measured, in our experiments. Also, the "best" growth in our experiments occurred at the greatest proportion of NH_4^+ (0.5 NH_4^+ and 0.5 K^+), which is greater than the GAM+MS micronutrient treatment proportion of 0.16 NH_4^+: 0.84 NO_3^-.
2. Greenway *et al*. (2012)—The experimental design was a single qualitative factor (basal medium composition) with five levels (basal medium formulations MS, B5, BDS, BABI, and FN-Lite) that measured one response variable ("number of divisions per culture cycle"); there were no significant differences between formulations. This type of experiment can determine what formulation(s) work "best" and can be useful as an initial screen, as the authors proposed.
3. Mohammed and Özzambak (2007)—The experimental design was a single qualitative/quantitative factor (MS vs. ½ MS) that measured one response variable

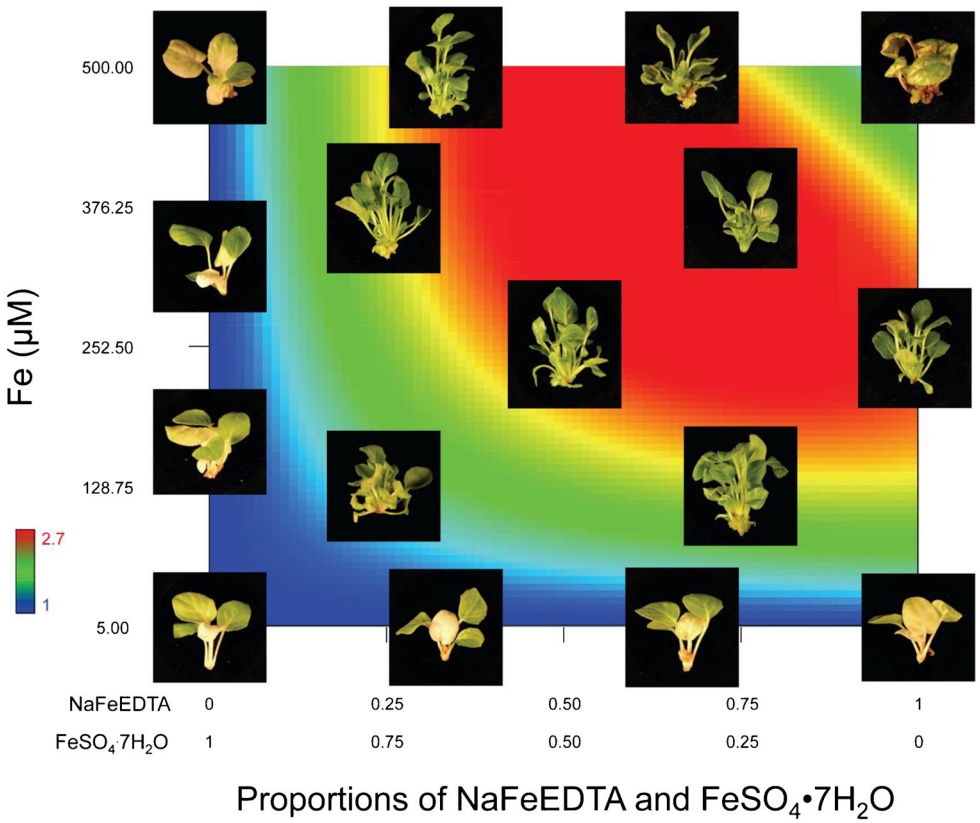

Figure 5. Contour plot of quality for the 2-component mixture-amount NaFeEDTA/$FeSO_4 \cdot 7H_2O$ (chelated Fe) experiment. The effect of each treatment is shown by a picture of gerbera shoot cultures grown on each treatment coordinate (listed in Table 3). Because the design included two mixture components and one factor for concentration, the design space is 2D.

(rooting); no significant difference was observed between MS and ½ MS.

4. Pierik *et al.* (1975)—The experimental design is not well-described but appeared to be a single qualitative factor (basal medium composition) with five levels (basal medium formulations) that measured "shoot development." The results were reported as an anecdotal data analysis; no summary statistics or statistical analyses were reported.
5. (Shabanpour *et al.* 2011)—The experimental design was a single qualitative factor (basal medium composition) with three levels (basal medium composition) that measured one response variable ("shoot proliferation"); MS medium resulted in the greatest number of proliferating shoots, compared to MS–½N and ½ B5.

From the results of the macro-micro-Fe experiment, three experiments were designed that were either salt- or ion-based (*i.e.*, the factors were individual salts or ions). One limitation of salt-based experiments is the potential for ion confounding. Ion confounding occurs when the objective is to determine the effect of a specific mineral nutrient using a salt-based experiment. Because the concentration of the ion of interest is not varied independently of its co-ion counterpart in the salt, the main effect of that ion is indistinguishable from, or confounded with, its co-ion. However, where there is a large concentration difference between the two ions from the salt, ion confounding can probably be ignored, though technically present, and the ion-specific effects reasonably estimated from the salt. This is the situation with the metals and is why we designed the metals (Cu-Mn-Zn-Fe/EDTA) experiments using salts. For example, MS medium contains 0.0001 mM Cu that is delivered as $CuSO_4 \cdot 5H_2O$, but SO_4^{2-} is present at 1.74 mM because of the other sulfate salts; thus, $CuSO_4 \cdot 5H_2O$ contributes only about 0.006% of the SO_4^{2-}. At the 1 mM $CuSO_4 \cdot 5H_2O$ treatment level, the ion confounding is significant: SO_4^{2-} was increased by 1 mM to a total of 2.74 mM, or a 57% increase over MS SO_4^{2-} levels. Thus, the effect on growth at the 1 mM level for each of the metals cannot be clearly attributed to the metal alone and should be attributed to the factor varied, which is the salt used. The objective of these experiments was to identify a working range for further exploration for each of these metals. It was clear that gerbera responded quite differently to these metals. For example, gerbera exhibited a much narrower range of growth for Zn and Fe/EDTA than for Cu and Mn. One interpretation is that because gerbera has a narrow optimization level for these two metals, further optimization to identify the optimum may be useful, particularly for commercial producers. This reasoning resulted in designing the chelated Fe experiment.

Fe is unique among the mineral nutrients because it is typically delivered in plant tissue culture formulations in a chelated form. We studied two effects: the degree of chelation and the concentration of Fe in the medium. To determine these effects, we used a mixture-amount experimental design. To

the best of our knowledge, this is the first use of a mixture approach to study Fe chelation; the vast majority of studies compare different chelating agents and Fe salts. A mixture design determines the effect of the *proportion* of Fe that is chelated while holding Fe concentration constant. Adding Fe concentration as a second factor to the mixture design results in a mixture-amount design; this approach separates and quantifies the effects of 'proportion of chelation', Fe amount, and the interaction between 'proportion of chelation' and Fe amount. The results showed that as the 'proportion of chelation' increased, less Fe was required, and demonstrated a significant synergy between NaFeEDTA and $FeSO_4 \cdot 7H_2O$. In addition to finding that the optimal Fe concentration was 2–5× the MS level, we found that the optimal delivery of Fe may not necessarily require that 100% of the Fe be in a chelated form.

We designed the $NH_4^+/NO_3^-/K^+$ experiment because the 'macro' factor had a large effect on shoot number and leaf number in the macro-micro-Fe experiment. We selected NH_4^+, K^+, and NO_3^- to test because these are the dominant ions in most tissue culture media formulations. To determine the specific effects of these ions on growth, we designed the experiment to (1) treat NH_4^+, K^+, and NO_3^-, rather than their salts, as the factors to be varied (Niedz and Evens 2008); (2) fix the concentration of all other inorganic ions in MS medium; and (3) calculate the salt/acid/base formulations required to achieve the NH_4^+–K^+–NO_3^- levels specified for each treatment combination using the ion/salt linear programming algorithm (Niedz and Evens 2006) and the software ARS-Media. The resulting design was a mixture-amount design and was free of ion confounding. As a mixture-amount design, it separated the effects of NH_4^+ and K^+ proportion and the amount of NO_3^-. Because the three ions are all monovalent, treating NH_4^+ and K^+ as a 2-component mixture matched to the amount of NO_3^- resulted in a design space of near-uniform pH. Thus, pH was not directly controlled but was treated as a response. The experimental design space in this gerbera study is actually a subset of points that describe a plane through a triangular prism defined by the 3-component mixture of NH_4^+, K^+, and NO_3^- projected through a third dimension of amount (total mM). All of the points falling on this plane have a pH near 5.8. This is an inherent property of the solutions on this plane, not the result of adjusting the pH of the solutions. Because the results of the $NH_4^+/NO_3^-/K^+$ experiment revealed, not surprisingly, significant growth differences over the design space, this raises a question: how important is the starting pH of the culture medium? A follow-up experiment using sets of mineral nutrient ions as statistical factors would provide information on the effect of pH. Because the ions are the independent causal agents of pH, varying the ions would cause the pH to vary widely throughout the design space, and the resulting correlation between growth and pH would provide information on the importance of pH. This type of experiment has been reported for protein precipitation (Evens and Niedz 2008) and algal growth (Evens and Niedz 2011). Are there regions in the larger "ion space" where gerbera would grow as well as or better than on the plane sampled in these experiments? Given that growth was not uniform across the plane but confined to a specific region, we cannot assume that there may not be even better regions for growth that lie above or below this plane. Sampling the larger design space would have the advantage of permitting the plants to "select" the ion nutrient combinations most suited to growth without imposing a pH bias constraint.

In this study, we quantified the effects of various mineral nutrients on the growth of *in vitro* gerbera shoot cultures. The initial experiment screened groups of nutrients and the results led to experiments to examine the effects of the metal salts of Cu, Mn, Zn, and Fe/EDTA; the effects of the dominant major ions NH_4^+, NO_3^-, and K^+; and the effects of the proportion of Fe chelation and Fe concentration. Because highly significant effects were observed in all experiments, information about growth from these experiments should be useful in providing a better understanding of plant nutrition, but also in devising formulations for improved *in vitro* growth of gerbera daisies.

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