In vitro shoot growth of *Brugmansia* × *candida* Pers

Randall P. Niedz · Terence J. Evens ·
Scott E. Hyndman · Scott Adkins · Daniel O. Chellemi

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Abstract The objective of this study was to improve the growth of *in vitro* shoot cultures of *Brugmansia* × *candida* ‘Creamsickle’. Several mineral nutrient experiments were conducted to determine the effect of NH_4^+ , NO_3^- , K^+ , $\text{FeSO}_4/\text{EDTA}$, ZnSO_4 , MnSO_4 , and CuSO_4 on quality, leaf width and length, size and weight of shoot mass, and shoot number. The experiment to determine the levels of NH_4^+ , NO_3^- , and K^+ , was conducted as a 2-component $\text{NH}_4^+:\text{K}^+$ mixture crossed by $[\text{NO}_3^-]$ and resulted in an experimental design free of ion confounding and capable of separating the effects of proportion and concentration. The results of the $\text{NH}_4^+:\text{K}^+:\text{NO}_3^-$ experiment revealed a region in the design space where growth was significantly improved; the region generally had lower total nitrogen and lower $\text{NH}_4^+:\text{K}^+$ ratios than MS medium. The experiments to determine the appropriate levels of Fe, Zn, Mn, and Cu were conducted at six log levels ranging from 0 to 1 mM. Of the four metal salts tested, MnSO_4 had the least effect on *in vitro* shoot growth and its concentration was reduced from 0.1 mM (MS level) to 0.001 mM. CuSO_4 had large effects on *in vitro* shoot growth and was increased from 0.0001 mM to 0.001 mM. A 2-level factorial of $\text{NH}_4^+:\text{K}^+:\text{NO}_3^-$, $\text{FeSO}_4/\text{EDTA}$, and ZnSO_4 was conducted and several formulations identified for their improvements of quality and growth. In addition to the changes to MnSO_4 and CuSO_4 , these formulations were characterized by lower levels of NH_4^+ , K^+ , NO_3^- and Zn, and higher levels of $\text{FeSO}_4/\text{EDTA}$. Overall, several nutrient

formulations were identified as superior to MS medium for growth of *in vitro* shoot cultures of *B.* ‘Creamsickle’.

Keywords Micropropagation · Mineral nutrition · Ion confounding · Nitrogen · Trace metals · Response surface

Introduction

The genus *Brugmansia* (i.e., Angel’s Trumpet) includes seven species in two sections, *Brugmansia* and *Sphaerocarpium*; species are cross-compatible within a section but not between sections (Hay et al. 2011; International *Brugmansia* and *Datura* Society 2011). Section *Brugmansia* includes four species—*B. aurea*, *B. insignis*, *B. suaveolens*, and *B. versicolor*; it includes various unclassified interspecific hybrids, as well as the botanically named *Brugmansia* × *candida* (*B. aurea* × *B. versicolor*). Section *Sphaerocarpium* includes three species—*B. arborea*, *B. sanguinea*, and *B. vulcanicola*; it includes various unclassified hybrids as well as the botanically named *Brugmansia* × *flava* (*B. arborea* × *B. sanguinea*; syn. *B.* × *flava*). *Brugmansia* is native to South America and can tolerate an average minimal temperature of −3.9 to −6.6 °C equivalent to USDA Hardiness Zone 9 (Preissel and Preissel 2002). *Brugmansia* are popular ornamental plants because of their spectacular trumpet-shaped flowers that vary in size, color, and scent. In addition, they and the closely related *Datura* genus produce tropane alkaloids important for chemotaxonomy (Griffin and Lin 2000) and medical pharmacology (Capasso et al 1997). Species that produce lenticels are easily propagated by rooted cuttings; species without lenticels are propagated via air layering.

Most *in vitro* research with *Brugmansia* has involved hairy root cultures for alkaloid production (Pitta-Alvarez et al 2000) with little research related to tissue culture for horticultural applications such as vegetative multiplication

R. P. Niedz (✉) · T. J. Evens · S. E. Hyndman · S. Adkins ·
D. O. Chellemi
USDA-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

of rare, valuable, or difficult-to-propagate types. Of particular importance given that viruses and viroids are widespread in *Brugmansia* worldwide (Chellemi et al 2011; Verhoeven et al. 2007), would be more efficient micropropagation systems for clonal propagation and maintenance of pathogen-free stocks. Bhatt et al (2004) reported the effect of *in vitro* propagation and cold storage of *B. versicolor* on shoot multiplication potential and scopolamine content. Niedz et al. (2007) reported on the effect of BAP concentration in MS medium on shoot multiplication of *B. × candida*. *Brugmansia* shoot cultures can be multiplied on MS basal medium, but in our experience quality was relatively poor and indicative of inadequate mineral nutrition. The objective of this study was to develop a basal mineral nutrient formulation better suited for *in vitro* shoot growth of *Brugmansia*.

Materials and methods

Plant material

Plants of *Brugmansia × candida* ‘Creamsickle’ (Hay et al 2011; herein referred to as *B. ‘Creamsickle’*) were obtained from Native Habitat (Vero Beach, Florida, USA). Shoot cultures were initiated from greenhouse grown mother stock plants grown with standard horticultural practices. Cultures were initiated from lateral buds and shoot tips dissected to about 5 mm to 10 mm, followed by a disinfection protocol that began with 95 % ethanol for 1 min with agitation. After decanting the excess ethanol, the explants were placed into a solution of 0.525 % sodium hypochlorite plus two drops of Tween-20 per 100 ml with agitation. After 15 min, the sodium hypochlorite solution was decanted and the explants were rinsed with sterile water. The lateral buds and shoot tips were further dissected to about 3 mm to 6 mm tall before transferring onto the culture initiation medium. The initiation medium was MS basal medium (Murashige and Skoog 1962), sucrose 30 g/L, myo-inositol 100 mg/L, thiamine HCl 1 mg/L, pyridoxine HCl 1 mg/L, nicotinic acid 1 mg/L, glycine 2 mg/L, BAP 1.1 μ M, pH 5.7, and bacteriological agar (USB Corporation, Cleveland, Ohio, USA) 9.0 g/L. The medium was autoclaved in 500 ml volumes for 20 min at 121 °C and then poured into sterile 25×100 mm flat-bottomed glass culture tubes with polypropylene caps (Magenta Corporation, Chicago, Illinois, USA) with 15 ml of medium per tube. The culture tubes containing the liquid medium were cooled in a vertical position in polypropylene racks (Magenta Corporation). Four weeks after initiation the cultures were transferred to the same medium in polypropylene capped tubes except that the BAP concentration was reduced to 0.5 μ M. Thereafter, the multiplying clumps of precociously enhanced lateral buds were cultured onto

the same medium in 77×77×77 mm polycarbonate vessels with polypropylene closures (Magenta Corporation, Chicago, IL, USA) containing 35 ml medium per vessel. The cultures were maintained in a growth room under low light (26 μ mol m⁻² s⁻¹) provided by cool-white fluorescent lamps, constant 27 °C, and a 16h photoperiod.

Experimental approach

In vitro growth was assessed for nitrogen (NH₄⁺ and NO₃⁻) and K⁺, and metal salts (FeSO₄.7H₂O+Na₂EDTA.2H₂O, ZnSO₄.7H₂O, MnSO₄.4H₂O, and CuSO₄.5H₂O). Fe was chelated with EDTA by heating combined equimolar solutions of FeSO₄.7H₂O and Na₂EDTA.2H₂O. The objective was to determine the optimal types and concentrations of these compounds for *in vitro* shoot growth as assessed by the response variables quality (Niedz et al. 2007), size and weight of shoot mass, and shoot number. Quality is a gestalt ranking ranging from 1 to 3 where 1 is a culture that is readily distinguished as unacceptable and of poor quality and 3 being a culture that is readily distinguished as acceptable and high quality relative to the other treatments; a score of 2 is given if neither 1 nor 3 apply. The base formulation was comprised of MS medium (Murashige and Skoog 1962) supplemented with 0.69 μ M 6-benzylaminopurine (BAP) (Niedz et al. 2007), 30 gL⁻¹ sucrose, and 8 gL⁻¹ agar. The cultures were grown in a temperature-controlled growth room at 27 °C on a 16h photoperiod under 45–60 μ mol photons m⁻² s⁻¹ light provided by cool-white fluorescent lamps.

Optimal levels and proportions of nitrogen (NH₄⁺ and NO₃⁻) and K⁺ were determined by an experimental approach to plant tissue culture mineral nutrition that removed ion confounding and treated pH as a dependent variable (Niedz and Evens 2006, 2008; Evens and Niedz 2008). The experimental design is presented in Table 1. Metal salts FeSO₄.7H₂O+Na₂EDTA.2H₂O, ZnSO₄.7H₂O, MnSO₄.4H₂O, and CuSO₄.5H₂O, were varied individually at 0, 0.0001, 0.001, 0.01, 0.1, and 1 μ M. Lastly, several factors were selected and tested in a 2-level factorial arrangement based on combinations of the optimal levels identified for these factors.

The experimental vessel was a 25×100 mm flat-bottomed glass tube with a polypropylene cap and 15 ml of medium. Five mm long shoot tips from multiplying clump cultures described above were cultured onto the experimental formulations with a 4 week subculture. Following the first subculture, the basal clump was recultured onto the same experimental medium in separate culture tubes for an additional 4 weeks to reduce carryover effects. Six pseudo-replicates were used per treatment. Three true replicates (i.e., each composed of six pseudo-replicates) were used to provide an estimate of pure error.

Table 1 Mixture-amount $\text{NH}_4^+ \text{-NO}_3^- \text{-K}^+$ treatments and data for *Brugmansia* shoot multiplication. Experiment is a two-component mixture-amount design of $\text{NH}_4^+ \text{-K}^+$ and $[\text{NO}_3^-]$. The two 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. Replicated points are included (e.g., Points #3 and #9). Points #17 and #20 are MS medium

Treatment design points	Block	Mixture components		Factor NO_3^- mM	Measured responses					
		NH_4^+	K^+		Quality	Shoot #	Shoot mass width (mm)	Shoot clump weight (g)	Final media pH	pH ^a
1	1	0.250	0.750	10.00	2.83	3.67	21.33	0.62	5.4	5.7
2	1	0.500	0.500	50.00	2.67	3.83	27.17	0.61	5.2	5.8
3	1	0.500	0.500	30.00	2.67	2.83	27.33	0.59	5.8	5.8
4	1	0.000	1.000	20.00	1.67	1.83	18.17	0.30	5.8	5.7
5	1	0.500	0.500	10.00	2.17	3.00	22.33	0.54	5.3	5.7
6	1	0.250	0.750	30.00	1.67	1.83	17.50	0.44	5.4	5.8
7	1	0.250	0.750	30.00	2.17	2.67	21.50	0.50	5.3	5.8
8	1	0.250	0.750	50.00	2.00	2.33	24.33	0.58	5.3	5.8
9	1	0.500	0.500	30.00	2.50	3.67	24.00	0.68	5.1	5.8
10	1	0.000	1.000	50.00	1.00	0.67	18.17	0.25	5.8	5.8
11	1	0.250	0.750	10.00	2.67	3.33	20.83	0.59	5.4	5.7
12	1	0.500	0.500	50.00	2.33	2.67	27.67	0.58	5.1	5.8
13	1	0.000	1.000	50.00	1.00	0.33	15.00	0.16	5.8	5.8
14	2	0.500	0.500	10.00	2.00	2.00	21.83	0.41	5.3	5.7
15	2	0.000	1.000	10.00	1.50	1.17	17.50	0.28	6.0	5.7
16	2	0.125	0.875	30.00	2.17	2.50	25.67	0.47	5.4	5.8
17	2	0.500	0.500	40.00	2.50	2.83	27.17	0.62	5.2	5.8
18	2	0.250	0.750	50.00	1.00	0.67	18.50	0.25	5.5	5.8
19	2	0.250	0.750	50.00	2.00	1.83	21.33	0.48	5.4	5.8
20	2	0.500	0.500	40.00	2.17	3.00	25.67	0.60	5.3	5.8
21	2	0.500	0.500	10.00	2.33	2.67	24.00	0.48	5.3	5.7
22	2	0.000	1.000	40.00	1.33	1.50	19.17	0.23	5.9	5.8
23	2	0.000	1.000	10.00	1.00	0.33	17.83	0.26	6.0	5.7
24	2	0.000	1.000	10.00	1.00	0.50	12.33	0.21	6.0	5.7
25	2	0.250	0.750	20.00	2.33	3.00	25.50	0.47	5.4	5.8
26	2	0.000	1.000	40.00	1.00	0.50	18.33	0.19	6.0	5.8

^a Calculated from the chemical equilibrium modelling software MINEQL+ (Ver. 4.5; Schecher and McAvoy 1998), temperature corrected and assumed open to the atmosphere with a P_{CO_2} at sea level of $10^{-3.50}$ atm

Data was collected at the end of the second 4 week subculture period.

Data analysis

The ANOVA data were the means of the six culture vessels (i.e., pseudo-replicates) that comprised each treatment. The treatment points for the $\text{NH}_4^+ \text{-NO}_3^- \text{-K}^+$ experiment were selected by D-optimality criteria to sample the experimental design space. For each response, all possible models from the mean to quadratic polynomial were calculated and the highest order polynomial model where additional model terms were significant at the 0.05 level was analyzed by ANOVA. Initial model selection was based on a battery of adequacy

tests (Anderson and Whitcomb 2005). Normality and constant variance were determined graphically; a Box-Cox plot was used to choose the correct transformations (Box and Cox 1964). Overly influential data points were identified with DFFITS and DFBETAS plots (Belsley et al 1980). Adequate precision of the model was determined by comparing the range of the predicted values at the design points ($\hat{y}$) to the average variance ($V\text{-bar}$) of the prediction (Anderson and Whitcomb 2005). Potential outlier points were checked with externally studentized “outlier-t” (Weisberg 1986; Myers 1990) and Cook’s Distance (Cook and Weisberg 1982) graphical plots. R^2 , adjusted- R^2 (R_{adj}^2), and predicted- R^2 (R_{pred}^2), were estimated for each selected model (Myers and Montgomery 2002). Detailed descriptions of the statistical

Table 2 ANOVA p-values (Prob. > F) and summary statistics for the effect of NH_4^+ , NO_3^- , and K^+ on quality, shoot number, shoot mass width, and shoot clump weight of *Brugmansia* in vitro shoot cultures

	Source	Quality	Shoot #	Shoot mass width (mm)	Shoot clump weight (g)	Final medium pH
	Model	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
	Linear mixture	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
	$\text{NH}_4^+ * \text{K}^+$	0.0140	0.0244	–	0.0002	<0.0001
	$\text{NH}_4^+ * [\text{NO}_3^-]$	0.3419	0.2942	0.0708	0.0427	–
	$\text{K}^+ * [\text{NO}_3^-]$	0.3356	0.5023	0.9416	0.0419	–
	$\text{NH}_4^+ * \text{K}^+ * [\text{NO}_3^-]$	0.0114	0.0106	–	–	–
^a QxL quadratic mixture x linear $[\text{NO}_3^-]$; $rLxQ$ reduced linear x quadratic; $rQxL$ reduced quadratic x linear; $cQxM$ combined quadratic x mean; $rQxQ$ reduced quadratic x quadratic	$\text{NH}_4^+ * [\text{NO}_3^-]^2$	–	–	0.9349	–	–
	Lack of fit	0.4853	0.3965	0.1143	0.0411	0.0004
	R^2	0.79	0.78	0.72	0.89	0.93
	R^2 adj	0.74	0.72	0.65	0.87	0.93
	R^2 pred	0.62	0.59	0.53	0.82	0.91
^b Model reduction by backward elimination	Model type ^a	QxL	QxL	rLxQ	rQxL	cQxM

methods have been provided in the literature (Niedz and Evens 2008; Niedz et al 2007; Niedz and Evens 2007). The software application Design-Expert® 7 (Stat-Ease, Inc, Minneapolis, MN) was used for experimental design construction, model evaluation, and analysis.

The eight treatments from the two-level factorial plus MS medium were analyzed by ANOVA and, then the means separated by Duncan's Multiple Range Test at $\alpha=0.01$.

Results

$\text{NH}_4^+ - \text{NO}_3^- - \text{K}^+$ experiment

Five responses were measured—quality, shoot number, 3rd leaf width, shoot mass width, clump weight, and final medium pH. The results for each response are as follows:

1. **Quality.** Quality ranged from 1 to 2.83 (Table 1) and indicated that NH_4^+ , K^+ , and NO_3^- affected quality. A summary of the ANOVA, lack-of-fit test and three R^2 statistics for quality is presented in Table 2 and a contour plot of the quality response over the design space in Fig. 1. A quadratic $\text{NH}_4^+ : \text{K}^+$ mixture $\times$ linear NO_3^- amount polynomial model was selected ($p < 0.0001$). Residual and model diagnostics were within acceptable limits. The lack-of-fit test was not significant ($p = 0.4853$) and indicated that additional variation in the residuals could not be removed with a better model. R^2 , R_{adj}^2 and R_{pred}^2 statistics ranged from 0.79 to 0.62. The single largest effect on quality was the linear mixture term ($p < 0.0001$). A significant linear mixture term shows that quality at the two extremes ends (vertices) of the mixture design space was significantly different; it is not a measure of the proportional effects of the three ions NH_4^+ , NO_3^- , and K^+ . The region of the

design space that resulted in the highest quality had lower concentration and lower $\text{NH}_4^+ : \text{K}^+$.

2. **Shoot number.** Shoot number ranged from 0.3 to 3.83 (Table 1) and indicated that NH_4^+ , K^+ , and NO_3^- affected shoot number. A summary of the ANOVA, lack-of-fit test and three R^2 statistics for shoot number are presented in Table 2. A quadratic $\text{NH}_4^+ : \text{K}^+$ mixture $\times$ linear NO_3^- amount polynomial model was selected ($p < 0.0001$). Residual and model diagnostics were within acceptable limits. The lack-of-fit test was not significant ($p = 0.3965$) and the R^2 , R_{adj}^2 and R_{pred}^2 statistics ranged from 0.78 to 0.59. The single largest effect on shoot number was the linear mixture term ($p < 0.0001$).
3. **Shoot mass width.** Shoot mass width ranged from 12 to 28 mm (Table 1) and indicated that NH_4^+ , K^+ , and NO_3^- affected shoot mass width. A summary of the ANOVA,

Fig. 1 Contour plot of quality of *Brugmansia* shoot cultures from the $\text{NH}_4^+ - \text{NO}_3^- - \text{K}^+$ experiment

Fig. 2 Contour plot of final medium pH of *Brugmansia* shoot cultures from the NH_4^+ – NO_3^- – K^+ experiment

lack-of-fit test and three R^2 statistics for shoot mass are presented in Table 2. A reduced linear $\text{NH}_4^+:\text{K}^+$ mixture x quadratic NO_3^- amount polynomial model was selected ($p < 0.0001$). Residual and model diagnostics were within acceptable limits. The lack-of-fit test was not significant ($p = 0.1143$) and the R^2 , R_{adj}^2 and R_{pred}^2 statistics ranged from 0.72 to 0.53. The single largest effect on shoot mass width was the linear mixture term ($p < 0.0001$).

4. **Shoot clump weight.** Shoot clump weight ranged from 0.16 to 0.68 g (Table 1) and indicated that NH_4^+ , K^+ , and NO_3^- affected clump weight. Shoot clump weight included the weight of the shoots and the large callus mass typically associated with each culture. A summary of the ANOVA, lack-of-fit test and three R^2 statistics for shoot clump weight are presented in Table 2. A quadratic $\text{NH}_4^+:\text{K}^+$ mixture x linear NO_3^- amount polynomial model was selected ($p < 0.0001$). Residual and model diagnostics

were within acceptable limits with the exception of run #18 for the difference in fits diagnostic DFFITS. DFFITS is calculated by measuring the change in the predicted value when a point is deleted; the larger the change the larger the effect of that point on the fitted model. The point was removed from the analysis. The lack-of-fit test was significant ($p = 0.0411$) and indicated that 1) there remained additional signal in the response not captured by the model or, 2) low experimental error. The R^2 , R_{adj}^2 and R_{pred}^2 statistics ranged from 0.89 to 0.82. The single largest effect on shoot clump weight was the linear mixture term ($p < 0.0001$). A smaller secondary effect was contributed by the $\text{NH}_4^+:\text{K}^+$ ($p = 0.0002$).

5. **Final medium pH.** Final medium pH was measured with a surface pH probe at the end of the culture period and ranged from 5.08 to 6.02 (Table 1) and indicated that NH_4^+ , K^+ , and NO_3^- affected final medium pH. A summary of the ANOVA, lack-of-fit test and three R^2 statistics for final medium pH are presented in Table 2 and a contour plot of the final medium pH response over the design space in Fig. 2. A quadratic $\text{NH}_4^+:\text{K}^+$ mixture x linear NO_3^- amount polynomial model was selected ($p < 0.0001$). Residual and model diagnostics revealed several anomalies with run #16 including a slight departure from normality identified from the normal probability plot and as an outlier from the Externally Studentized Residuals test (i.e., Outlier t test). Accordingly, run #16 was removed from the analysis. The lack-of-fit test was significant ($p = 0.0004$). R^2 , R_{adj}^2 and R_{pred}^2 statistics ranged from 0.93 to 0.91. The ANOVA contained two significant terms with $p < 0.0001$, the linear mixture and $\text{NH}_4^+ * \text{K}^+$ terms; however, the linear mixture was the single largest effect with an F value of 949 vs. 53 for $\text{NH}_4^+ * \text{K}^+$. The result was an inverse relationship between the proportion of NH_4^+ or K^+ and final medium pH. As the proportion of NH_4^+ increased, or conversely the proportion of K^+ decreased, final medium pH decreased.

Table 3 ANOVA p-values (Prob. > F) and summary statistics for the effect of FeSO_4 /EDTA on quality, shoot number, shoot mass width, and shoot clump weight of *Brugmansia* in vitro shoot cultures

Source	Quality	Shoot #	Shoot mass width (mm)	Shoot clump weight (g)
Model	<0.0001	<0.0001	<0.0001	<0.0001
FeSO_4 /EDTA	<0.0001	<0.0001	<0.0001	<0.0001
FeSO_4 /EDTA ²	<0.0001	0.3669	–	–
FeSO_4 /EDTA ³	–	<0.0001	–	–
Lack of fit	<0.0001	<0.0001	0.0051	<0.0001
R^2	0.83	0.92	0.65	0.76
R^2 adj	0.83	0.91	0.64	0.75
R^2 pred	0.81	0.90	0.61	0.74
Model type	quadratic ^a	cubic ^b	linear	linear ^b

^a Data power transformed (Quality^{-2.25}) per Box Cox analysis

^b Data log10 transformed per Box Cox analysis. Log₁₀(Shoot #+0.06), Log₁₀(Clump weight)

Metal experiments

Four metal experiments were conducted that included Fe, Zn, Mn, or Cu salts in six concentrations ranging from 0 to 1 mM in five log concentration intervals. The objective was to identify the order of magnitude concentration for each metal. Four responses were measured—quality, shoot number, shoot mass width, and clump weight. The results were as follows:

Fig. 3 Effects of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O} + \text{Na}_2\text{EDTA} \cdot 2\text{H}_2\text{O}$, $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$, or $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ on growth of *Brugmansia* shoot cultures. 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 each image and are as follows: $\text{FeSO}_4 \cdot 7\text{H}_2\text{O} + \text{Na}_2\text{EDTA} \cdot 2\text{H}_2\text{O}$ (0.10 mM), $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ (0.36 mM), $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$ (0.10 mM), and $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (0.0001 mM)

1. **$\text{FeSO}_4 \cdot 7\text{H}_2\text{O} + \text{Na}_2\text{EDTA} \cdot 2\text{H}_2\text{O}$** —The effect of EDTA-chelated Fe on the four growth responses is summarized in Table 3 and Fig. 3. The model for each of the four growth responses was significant at $p < 0.0001$. The lack-of-fit test was significant for all four responses. $\text{FeSO}_4/\text{EDTA}$ from 0 to 0.001 mM was too low to support growth, at 0.01 mM growth was supported but chlorosis was severe, and good growth occurred at 0.1 mM (MS level) and 1 mM, with some

signs of toxicity at 1 mM. Optimal growth lies between 0.1 mM and 1 mM.

2. **ZnSO₄·7H₂O**—The effect of ZnSO₄ on the four growth responses is summarized in Table 4 and Fig. 3. Three of the four models were significant at $p < 0.0001$ with the model for shoot mass width only significant at $p = 0.0003$. Residual and model diagnostics were within acceptable limits for shoot number, shoot mass width, and clump weight. However, run #10 was identified as an outlier for quality and was removed from the analysis of quality. The lack-of-fit test was not significant for quality but significant for the remaining three responses. ZnSO₄ at 1 mM resulted in little to no growth and lateral shoots were stunted and chlorotic. Optimal growth lies between 0.001 and 0.1 mM (MS level).
3. **MnSO₄·4H₂O**—The effect of MnSO₄ on four growth responses is summarized in Table 5 and Fig. 3. The models for quality and shoot clump weight had low significance at $p = 0.0108$ and $p = 0.0269$, but the R^2 values were low, indicating that the models are of little value for prediction. MnSO₄ at 0 mM resulted in growth but plants had some interveinal chlorosis, at 1 mM the plants were chlorotic. Optimal growth lies between 0.001 mM and 0.1 mM (MS level).
4. **CuSO₄·5H₂O**—The effect of CuSO₄ on the four growth responses is summarized in Table 6 and Fig. 3. The model for each of the four growth responses was significant at $p < 0.0001$. Residual and model diagnostics were within acceptable limits. The lack-of-fit test was significant for the quality response indicating additional curvature not accounted for in the quadratic model. CuSO₄ at 0 mM resulted in some growth but plants were

Table 4 ANOVA p-values (Prob. > F) and summary statistics for the effect of ZnSO₄ on quality, shoot number, shoot mass width, and shoot clump weight of *Brugmansia* in vitro shoot cultures

Source	Quality	Shoot #	Shoot mass width (mm)	Shoot clump weight (g)
Model	<0.0001	<0.0001	0.0003	<0.0001
ZnSO ₄	<0.0001	<0.0001	0.0006	0.0255
ZnSO ₄ ²	<0.0001	<0.0001	0.0166	<0.0001
ZnSO ₄ ³	<0.0001	<0.0001	—	0.0016
Lack of fit	0.4701	0.0028	0.0400	0.0145
R ²	0.83	0.77	0.39	0.62
R ² adj	0.81	0.75	0.35	0.59
R ² pred	0.80	0.71	0.25	0.53
Model type	cubic ^a	cubic ^b	quadratic	cubic ^c

^a Data power (Quality^{-1.73}) transformed per Box Cox analysis

^b Data square root transformed per Box Cox analysis. Sqrt(Shoot # + 0.50)

^c Data square root transformed per Box Cox analysis. Sqrt(Clump weight)

Table 5 ANOVA p-values (Prob. > F) and summary statistics for the effect of MnSO₄ on quality, shoot number, shoot mass width, and shoot clump weight of *Brugmansia* in vitro shoot cultures

Source	Quality	Shoot #	Shoot mass width (mm)	Shoot clump weight (g)
Model	0.0108	ns	ns	0.0269
MnSO ₄	0.0785			0.0269
MnSO ₄ ²	0.0117			—
Lack of fit	0.7398	0.1861	0.6487	0.7880
R ²	0.24			0.14
R ² adj	0.19			0.11
R ² pred	0.12			0.045
Model type	quadratic ^a	mean	mean	linear ^a

^a Data log₁₀ transformed per Box Cox analysis. Log₁₀(Quality), Log₁₀(Clump weight)

chlorotic, at 0.1 and 1 mM plants were chlorotic and stunted. Optimal growth lies between 0.001 and 0.01 mM, 10 and 100 times MS levels (0.0001), respectively.

Optimization

Based on the results from the NH₄⁺-NO₃⁻-K⁺ and metals experiments, eight treatments were selected from combinations of the factors (Table 7 and Fig. 4) with the greatest effect on growth including ZnSO₄ (0.01 and 0.1 mM), FeSO₄/EDTA (0.25 and 0.50 mM), and NH₄⁺-K⁺-NO₃⁻ (2.5-7.5-10 and 15-15-30 mM), and run in a 2-level factorial arrangement. The objective was to determine if a significant improvement in growth could be achieved. In all treatment

Table 6 ANOVA p-values (Prob. > F) and summary statistics for the effect of CuSO₄ on quality, shoot number, shoot mass width, and shoot clump weight of *Brugmansia* shoot cultures

Source	Quality	Shoot #	Shoot mass width (mm)	Shoot clump weight (g)
Model	<0.0001	<0.0001	<0.0001	<0.0001
CuSO ₄	0.0301	0.0021	<0.0001	<0.0001
CuSO ₄ ²	<0.0001	<0.0001	<0.0001	<0.0001
Lack of Fit	0.0001	0.6394	0.3881	0.1607
R ²	0.60	0.75	0.73	0.82
R ² adj	0.57	0.73	0.71	0.81
R ² pred	0.54	0.70	0.69	0.79
Model type	quadratic ^a	quadratic ^b	quadratic	quadratic ^c

^a Data power transformed (Quality^{-1.39}) per Box Cox analysis

^b Data square root transformed per Box Cox analysis. Sqrt(Shoot #)

^c Data log₁₀ transformed per Box Cox analysis. Log₁₀(Clump weight)

Table 7 Treatment combinations derived from optimal levels of NH_4^+ - K^+ - NO_3^- and metals experiments to improve *Brugmansia* *in vitro* shoot cultures. CuSO_4 was increased from 0.0001 to 0.001 mM and MnSO_4 was reduced to 0.001 mM in treatments A-H

Treatment	ZnSO_4 (mM)	$\text{FeSO}_4/\text{EDTA}$ (mM)	NH_4^+ - K^+ - NO_3^- (mM)
MS	0.37	0.10	20.6-20.0-39.4
A	0.01	0.25	2.5-7.5-10
B	0.10	0.25	2.5-7.5-10
C	0.01	0.50	15-15-30
D	0.10	0.50	15-15-30
E	0.01	0.25	15-15-30
F	0.10	0.25	15-15-30
G	0.01	0.50	2.5-7.5-10
H	0.10	0.50	2.5-7.5-10

combinations, CuSO_4 was increased from 0.0001 mM (i.e., MS level) to 0.001 mM and MnSO_4 was reduced from 0.1 mM (i.e., MS level) to 0.001 mM. Because the ANOVA for each of the four measured responses was significant, the means of the treatments were analyzed by Duncan's Multiple Range Test at $\alpha=0.01$ to provide information on the specific differences. For quality, all treatments were better than MS. For shoot number, treatment F was the best (vs. MS). For shoot mass width, treatment E had the greatest width (vs. MS). For shoot clump weight, treatments

C, F, and E had the greatest weight. B. 'Creamsickle' shoot cultures developed a large mass of callus at the base of the shoot clump that was little affected in the NH_4^+ - K^+ - NO_3^- or metal experiments suggesting that mineral nutrition is not a significant factor in its development and growth.

Discussion

We observed that B. 'Creamsickle' *in vitro* shoot cultures grew relatively poorly on MS basal medium and sought to improve growth by defining a more appropriate nutrient medium formulation. Because NH_4^+ , K^+ , and NO_3^- are the dominant ions in most tissue culture media formulations, an experiment to determine the levels of each of these ions for optimal growth in MS medium was conducted. The experiment was designed to 1) treat the ions NH_4^+ , K^+ , and NO_3^- , as opposed to their salts, as the factors to be varied; 2) fix the concentration of all other inorganic ions to their MS levels; 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 and the software ARS-Media (Niedz and Evens 2006). The resulting design was thus free of ion confounding (Niedz and Evens 2006). Proportionality and amount were incorporated into the design to separate the proportional and amount effects on growth. A detailed

Fig. 4 *Brugmansia* shoot cultures grown on MS medium or eight media formulations from a 2-level 3-factor factorial of $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, $\text{FeSO}_4 \cdot 7\text{H}_2\text{O} + \text{Na}_2\text{EDTA} \cdot 2\text{H}_2\text{O}$, and NH_4^+ - K^+ - NO_3^- . The eight test formulations also included 0.001 mM CuSO_4 and 0.001 mM MnSO_4 . Listed as mM for $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, $\text{FeSO}_4 \cdot 7\text{H}_2\text{O} + \text{Na}_2\text{EDTA} \cdot 2\text{H}_2\text{O}$, and NH_4^+ - K^+ - NO_3^- the treatments are as follows: MS) 0.37-0.1-20.6-20-39.4; A) 0.01-0.25-2.5-7.5-10; B) 0.1-0.25-2.5-7.5-10; C) 0.01-0.5-15-15-30; D) 0.1-0.5-15-15-30; E) 0.01-0.25-15-15-30; F) 0.1-0.25-15-15-30; G) 0.01-0.5-2.5-7.5-10; H) 0.1-0.5-2.5-7.5-10. Treatments were ranked from low to high for each of the four measured growth responses and the means analyzed per Duncan's multiple range test. Treatments connected by the same line are not significantly different at the 1 % level

Quality	MS	G	A	B	H	C	D	E	F
Shoot #	MS	G	H	B	A	D	E	C	F
Shoot mass width (mm)	MS	G	H	A	F	D	C	B	E
Shoot clump weight (g)	G	A	H	MS	D	B	C	F	E

description of this design approach was recently reported by Niedz and Evens (2008). 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—i.e., pH was not directly controlled but treated as a dependent variable. The results of the $\text{NH}_4^+ \text{-K}^+ \text{-NO}_3^-$ experiment revealed a region in the design space where growth was significantly improved; the region generally had lower total nitrogen and lower $\text{NH}_4^+ \text{:K}^+$ ratios than MS medium. This raises the question of the importance of the starting pH of the culture media. Shoot growth was not uniform across the experimental design space even though the pH was the same. Also, there was little relationship between growth and the final pH of the medium. The experimental design space in this study is actually a subset of points that describe a plane through an implied 3-dimensional experimental design space defined by the 3-component mixture of NH_4^+ , K^+ , and NO_3^- projected through an amount dimension. The resulting design geometry can be visualized as a triangular prism. Are there regions in the full experimental design space where shoots would grow as well or better than on the pH 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 shoot growth without imposing a pH bias constraint.

The effects of the four metals Fe, Zn, Mn, and Cu were tested from 0 to 1 mM at six log levels. Not knowing the requirements of *B. 'Creamsickle'* for these metal nutrients, the experiments were designed to sample a wide range in order to determine the appropriate magnitude. The assumption was made that the use of salts was reasonable for these ions because of the small amounts involved (e.g., SO_4^{2-} makes up 1.85 % of MS medium). It is noted that ion confounding did exist in each of these experiments per the SO_4^{2-} counter ion. MnSO_4 had the least effect on *in vitro* shoot growth of the four metals tested. MnSO_4 and CuSO_4 levels were adjusted and fixed in the optimization experiment. Because CuSO_4 was required in substantially higher levels than MS, its concentration was increased 10x. Compared to plant tissue, copper is generally in lower concentrations in plant tissue culture media and increasing Cu levels has been observed to promote better growth (Dahleen 1995; Nirwan and Kothari 2003; Kintzios et al. 2001; Nas and Read 2004; George and de Klerk 2008). ZnSO_4 and $\text{FeSO}_4/\text{EDTA}$ both strongly influenced *in vitro* shoot growth but their effective ranges were broad enough to include in the final optimization experiment.

The final optimization experiment was composed of eight treatments in a 2-level factorial arrangement. Formulations were identified that provided significantly better *in vitro* shoot growth than MS. These formulations had increased Cu, reduced Mn, increased Fe, and altered NH_4^+ , K^+ , and NO_3^- levels.

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