

# Improving *in vitro* mineral nutrition for diverse pear germplasm

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**Abstract** Mineral nutrition in the media used for growth of *in vitro* plants is often difficult to optimize due to complex chemical interactions of required nutrients. The response of plant tissue to standard growth media varies widely due to the genetic diversity of the plant species studied. This study was designed as the initial step in determining the optimal mineral nutrient requirements for micropropagation of shoot tips from a collection of genetically diverse pear germplasm. Five mineral nutrient factors were defined from Murashige and Skoog salts:  $\text{NH}_4\text{NO}_3$ ,  $\text{KNO}_3$ , mesos ( $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ – $\text{KH}_2\text{PO}_4$ – $\text{MgSO}_4$ ), micronutrients (B, Cu, Co, I, Mn, Mo, and Zn), and Fe-EDTA. Each factor was varied over a range of concentrations. Treatment combinations were selected using response surface methods. Five pears in three species (*Pyrus communis* ‘Horner 51,’ ‘Old Home×Farmingdale 87,’ ‘Winter Nelis,’ *Pyrus dimorphophylla*, and *Pyrus ussuriensis* ‘Hang Pa Li’) were grown on each treatment combination, responses were measured, and each response was analyzed by analysis of variance. The analyses resulted in the identification of the following factors with the single largest effects on plant response: shoot quality (mesos), leaf spotting/necrosis (mesos), leaf size (mesos), leaf color (mesos,  $\text{NH}_4\text{NO}_3$ , and  $\text{KNO}_3$ ), shoot number ( $\text{NH}_4\text{NO}_3$  and Fe), nodes ( $\text{NH}_4\text{NO}_3$  and  $\text{KNO}_3$ ), and shoot length (mesos and Fe). Factors with the largest effects (mesos and

Fe) were similar among the genotypes. This approach was very successful for defining the appropriate types and concentrations of mineral nutrients for micropropagation of diverse pear genotypes.

**Keywords** *Pyrus* · Micropropagation · Mineral nutrition · Growth medium · Surface response design

## Introduction

Micropropagation is important for both multiplication and preservation of a wide range of nursery crops, including many fruit crops. A number of studies exist on optimization of growth for one or two cultivars, but often these results cannot be transferred to additional genotypes because individual cultivars may differ greatly in their cultural requirements. Therefore, genotype-specific growth medium formulations are usually empirically developed for many plants including pear (Singha 1986; Zhao and Gu 1990; Bell and Reed 2002). Pear cultivars and species are often recalcitrant to tissue culture manipulations (Bell and Reed 2002) and most media formulations use Murashige and Skoog (1962) (MS) basal nutrient medium at full or half strength or with slight modifications. The media formulations of Lepoivre (Quoirin and Lepoivre 1977), Driver-Kuniyuki Walnut (Driver and Kuniyuki 1984), and Woody Plant Medium (Lloyd and McCown 1980) are also used and differ from MS mostly in types or amounts of calcium and nitrogen. The major differences in macronutrients for these four basal media are the concentrations of ammonium, nitrate, and total ion concentration (Bell *et al.* 2009). Despite the availability of many media formulations, no information on the most suitable medium for diverse pear genotypes is available.

Developing growth media for specific and unique cultivars is complex and time-consuming. The development of

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MS medium (Murashige and Skoog 1962) provided some insight into the many interactions of minerals in the medium and their uptake by plant tissues. New media are typically developed by minor modifications to “standard” media formulations, usually MS. Improved experimental design and statistical software currently allow much more efficient approaches to be utilized for the improvement of micropropagation media and conditions (Niedz and Evens 2007). Response surface methods interpret each medium component as a geometric dimension, resulting in a geometric volume with  $n$  dimensions. This volume is the experimental design space that is sampled according to the objectives of the experiment. The media formulations are constructed to correspond to the points used to sample the design space. The resulting responses are measured at each of the design points, and practical information can be determined for the types of formulations that provide the “best” response. The nutritional factors that cause a cultivar to respond well or poorly in the various formulations can then be determined.

The field-grown pear germplasm collection at the US Department of Agriculture-Agricultural Research Service National Clonal Germplasm Repository (NCGR) in Corvallis, Oregon includes nearly 1,000 accessions of 26 *Pyrus* species and over 1,400 *Pyrus communis* cultivars. The *in vitro* pear collection is used for distribution and serves as a security backup for approximately 200 pear species and cultivars; expansion of the collection to other diverse genotypes is limited by poor *in vitro* growth. Given the known difficulties of pear *in vitro* culture, a systematic and non-haphazard approach seems essential for further progress in this diverse genus. In this study, we tested the surface response, geometric mineral-medium development approach using MS as the basal medium for five pears in three species: three moderately growing and two slow-growing pears.

## Materials and Methods

**Plant material.** Genotypes tested were two very slow-growing genotypes (*Pyrus dimorphophylla* and *Pyrus ussuriensis* ‘Hang Pa Li’) and three moderately growing genotypes (*P. communis* ‘Winter Nelis,’ ‘Old Home × Farmingdale 87’ (OH × F87), and ‘Horner 51’).

**Multiplication.** Stock shoot cultures were grown in Magenta GA7 boxes (Magenta Corp., Chicago, IL) with 40-ml medium per box with transfer to new medium every 3 wk. The base medium contained MS salts with 0.5× KNO<sub>3</sub>, MS vitamins, 2.5 mg/l thiamine, and (per liter): 4.4 μMN<sup>6</sup>-benzyladenine, 3 g agar (Phytotech Agar, PhytoTechnology Labs, Shawnee Mission, KS),

and 1.75 g Gelrite at pH 5.7 (Reed 1995). Cultures were grown at 25°C under a 16-h photoperiod with an average 80 μMm<sup>-2</sup>s<sup>-1</sup> irradiance provided by a combination of cool and warm white florescent bulbs.

**Experimental design.** The software application Design-Expert<sup>®</sup> 8 (Design-Expert 2010) was used for experimental design construction, model evaluation, and analyses. The experimental design was a 5-factor response surface design where the design points (*i.e.*, combinations of the five factors) were selected using modified D-optimal criteria suitable for fitting a quadratic polynomial. Each factor (Table 1) was varied over a concentration range expressed in relation to MS medium (1×=MS concentration). A factorial design would require 3,125 treatments (5<sup>5</sup>), but the response surface design with quadratic resolution used in this study only required 43 treatment points (Table 2) to sample the same experimental design space. The basic strategy follows the methods of Niedz and Evens (2007).

Five mineral nutrient factors used were based on MS salts: (1) NH<sub>4</sub>NO<sub>3</sub>, (2) KNO<sub>3</sub>, (3) mesos (CaCl<sub>2</sub>·2H<sub>2</sub>O, KH<sub>2</sub>PO<sub>4</sub>, and MgSO<sub>4</sub>), (4) micronutrients (MS salts of B, Cu, Co, I, Mn, Mo, and Zn), and (5) Fe-EDTA (Table 1). Response data (described below) at each design point were calculated from the mean of three of the five shoots in each of two Magenta boxes ( $n=6$ ). Boxes were assigned random numbers for arrangement on the growth room shelf. Shoots were transferred to the same medium at 3-wk intervals and harvested after 9 wk. Three plants were evaluated from predetermined locations (at a diagonal from the label) in each box ( $n=6$ ), and the two remaining plants were photographed ( $n=4$ ). The experiment was performed in three blocks with two control boxes containing MS medium

**Table 1.** The five factors (stock solutions) used to construct the five-dimensional design space, their component MS salts, and concentration range expressed as ×MS levels

Factors	MS salts	Range
Group 1	NH <sub>4</sub> NO <sub>3</sub>	0.5–1.5×
Group 2	KNO <sub>3</sub>	0.5–1.5×
Group 3 (mesos)	CaCl <sub>2</sub> ·2H <sub>2</sub> O KH <sub>2</sub> PO <sub>4</sub> MgSO <sub>4</sub> MnSO <sub>4</sub> ·H <sub>2</sub> O	0.5–1.5×
Group 4 (micros)	ZnSO <sub>4</sub> ·7H <sub>2</sub> O CuSO <sub>4</sub> ·5H <sub>2</sub> O KI CoCl <sub>2</sub> ·6H <sub>2</sub> O H <sub>3</sub> BO <sub>3</sub> Na <sub>2</sub> MoO <sub>4</sub> ·2H <sub>2</sub> O	0.5–4×
Group 5 (Fe)	FeSO <sub>4</sub> ·7H <sub>2</sub> O Na <sub>2</sub> EDTA	0.5–4×

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

Treatment design points <sup>z</sup>	Factor 1 NH <sub>4</sub> NO <sub>3</sub>	Factor 2 KNO <sub>3</sub>	Factor 3 Mesos	Factor 4 Micros	Factor 5 Fe
1	0.50	1.50	1.50	0.50	2.36
2	1.36	0.50	0.67	0.50	4.00
3	0.50	1.50	0.88	2.11	0.50
4	1.44	1.50	0.52	4.00	3.79
5	1.50	1.50	1.50	4.00	4.00
6	0.50	0.50	0.62	3.57	3.57
7	0.62	0.60	1.50	0.50	3.94
8	0.97	1.03	0.50	4.00	0.50
9	1.50	0.50	1.50	4.00	0.50
10	1.50	0.50	0.50	0.50	0.50
11	0.50	1.50	0.88	2.11	0.50
12	1.50	0.50	0.50	0.50	0.50
13	0.62	0.62	1.38	3.57	0.50
14	1.50	0.50	1.50	4.00	0.50
15	1.50	1.50	1.50	4.00	4.00
16	0.50	1.43	1.50	1.80	4.00
17	0.50	1.50	0.50	4.00	4.00
18	1.50	1.50	0.50	0.50	2.34
19	0.95	0.80	0.84	1.24	2.07
20	0.50	0.50	1.50	0.50	0.50
21	1.50	1.04	1.05	4.00	0.50
22	1.50	0.50	0.50	4.00	4.00
23	1.50	1.04	1.05	4.00	0.50
24	0.50	0.50	0.50	4.00	0.50
25	1.06	1.50	1.50	2.45	0.50
26	1.38	0.62	1.50	4.00	3.57
27	1.50	0.50	1.50	0.50	4.00
28	1.50	1.50	0.50	0.50	2.34
29	0.50	1.50	0.50	4.00	4.00
30	1.01	1.50	0.95	0.50	4.00
31	0.50	1.50	1.50	4.00	0.50
32	1.50	1.50	1.50	0.50	0.50
33	0.50	0.50	1.50	4.00	4.00
34	1.01	1.50	0.95	0.50	4.00
35	1.50	1.19	0.50	1.67	4.00
36	0.50	1.50	1.50	4.00	0.50
37	0.90	1.11	1.05	3.79	2.84
38	0.50	0.50	0.50	0.50	4.00
39	1.50	0.50	0.52	3.25	1.24
40	1.38	0.62	1.50	0.93	0.93
41	0.50	1.24	0.50	0.50	0.50
42	1.50	1.50	0.50	4.00	0.50
43	1.50	1.19	0.50	1.67	4.00
44	1.00	1.00	1.00	1.00	1.00
45	1.00	1.00	1.00	1.00	1.00
46	1.00	1.00	1.00	1.00	1.00

<sup>z</sup>Design points 1–43 were randomly assigned to blocks as follows: Block 1 (points 1–15); Block 2 (points 16–29); and Block 3 (points 30–43). Additionally, one MS point was run with each block (points 44–46)

included with each block, for a total of 43 treatments plus three MS controls.

**Data.** The following plant responses were measured: quality, a subjective rating of plant appearance (1=poor quality, 2=acceptable quality, and 3=good quality); shoot length (longest shoot measured); shoot multiplication (shoots counted); leaf spotting/necrosis (1=no spots, 2=some spots, and 3=heavily spotted); leaf width (mean in mm) of top three leaves on each plant; leaf size (1=small, 2=medium, and 3=large); and leaf color (1=dark green, 2=light green, 3=yellow, and 4=red/actual soil plant analysis development (SPAD) meter) readings.

**Statistical analysis.** The plant response at each design point was estimated from the mean of three shoots from each of two Magenta boxes. Replicated points used the mean of another set of two duplicate Magenta boxes. For each measured response, the highest-order polynomial model where additional model terms were significant at the 0.05 level was analyzed by analysis of variance (ANOVA; (Design-Expert 2010). The responses of shoots at tested points and projected responses at untested points are presented graphically. Detailed descriptions of the statistical methods used to analyze the data are available (Niedz and Evens 2006, 2007; Evens and Niedz 2008).

## Results

The surface-response design models plant responses based on the design treatment points, so the graphical data are projections of the modeled response rather than a presentation of data for the 43 points tested in the 3,125-point design space. The graphical information indicates the trends interpolated from actual data points via least-squares fitting (Figs. 1, 2, 3, and 4). Summary data indicating the driving factors for each response and *p* values are presented in Table 3. The majority of models were significant at a *p* value of less than 0.0001 with clear identification of the factor(s) driving each response. Mesos, ammonium nitrate, and iron were significant in many of the responses, while micros were significant in only a few responses.

**Quality rating.** The quality rating is a subjective determination made by personnel experienced in pear tissue culture, based on identifying the ideal plant and capturing aspects of quality not represented by any single feature (Niedz and Evens 2007). Overall, ANOVA showed the largest effect from mesos, a smaller effect by iron, and a small interaction of mesos and micros as shown by the sample ANOVA for 'OH×F87' (Table 4). The factors with the largest effects included mesos ('OH×F87', 'Winter Nelis', 'Horner 51,' and

*P. dimorphophylla*), iron (all genotypes), and micros ('Hang Pa Li'). For each genotype, the two factors with the largest effects on quality are shown as contour plots (Fig. 1). Increased meso concentrations (1.3× to >1.5×) produced the best quality plants for all but 'Hang Pa Li', where less than or equal to 1× micros and mesos produced the best quality. Iron at the 0.5× to 1× MS concentrations provided the best quality for all genotypes. The best quality for 'Horner 51' and 'OH×F87' was a relatively large area of moderate to high mesos and moderate to low iron; the best quality of 'Winter Nelis' and *P. dimorphophylla* required high mesos and low iron (Fig. 1). Quality of 'Hang Pa Li' did not improve as much as the other genotypes and was best with low micros and low to moderate mesos. The poorest quality plants for the three *P. communis* cultivars and *P. dimorphophylla* were with high iron and low mesos (Fig. 1A), while the best were low iron and high mesos (Fig. 1B); *P. ussuriensis* cultivar Hang Pa Li had a lesser response to increasing mesos.

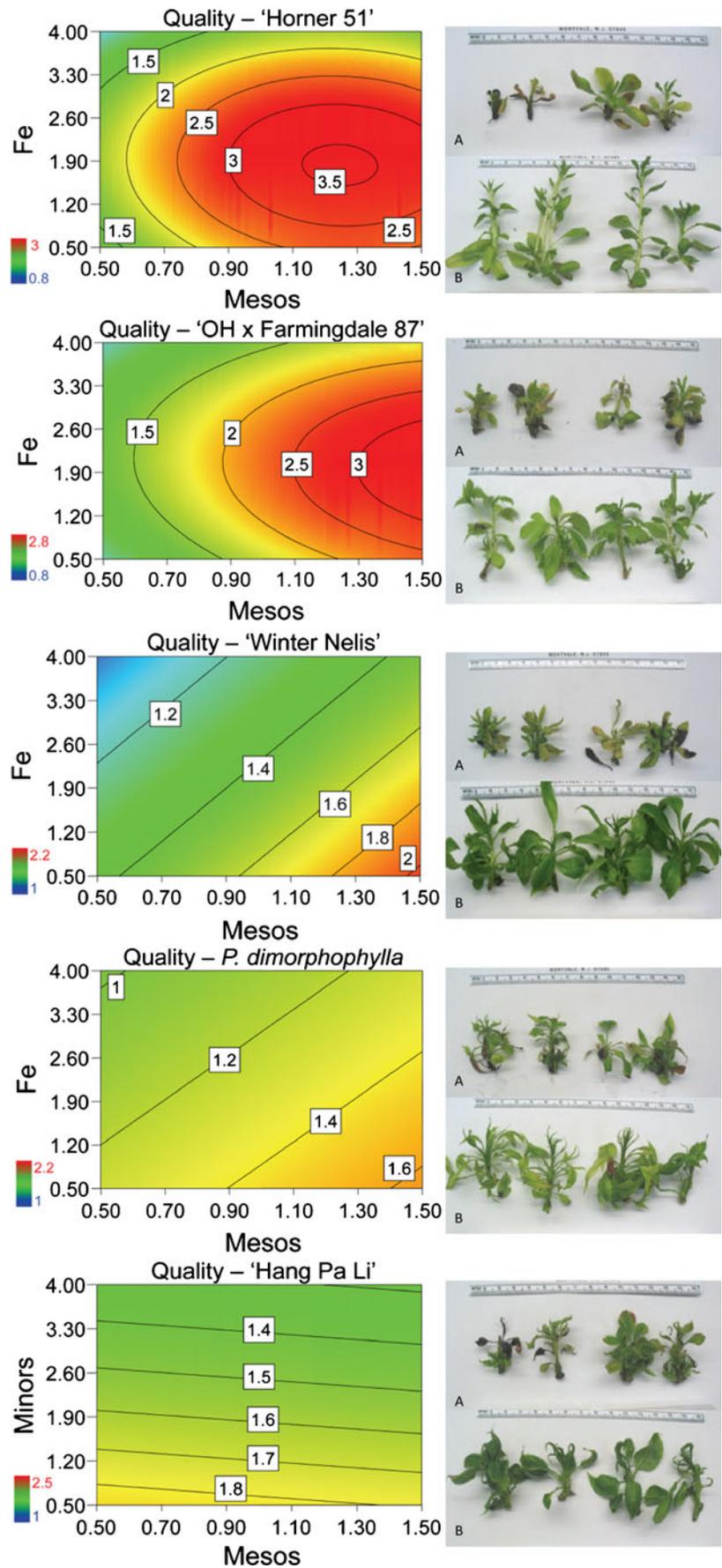
**Shoot number.** The factors with the largest effects on shoot multiplication included NH<sub>4</sub>NO<sub>3</sub> (all genotypes), iron (all genotypes except 'Hang Pa Li'), and mesos ('Hang Pa Li'). Low concentrations (≤1×) of ammonium nitrate and iron resulted in the most shoots for all the genotypes (Fig. 2). In some cases, increased iron (OH×F87) or lower mesos ('Hang Pa Li') also contributed to more shoots.

**Shoot length.** The factors with the largest effects on shoot length included iron and mesos ('Horner 51', 'OH×F87', 'Winter Nelis', and *P. dimorphophylla*), and micros ('Hang Pa Li'). Shoot length increased with lower levels of iron and higher levels of mesos for four pears (Fig. 2). Optimal shoot length for tissue culture typically required the longest shoots to be at least 30 mm. The response of OH×F87 and 'Winter Nelis' for shoot length was better over a wider range of iron and mesos than was seen for other genotypes. For 'Hang Pa Li', the micronutrients were the only significant factor with the longest shoots produced with moderate micros (1.5 to 2×). For 'Horner 51' and *P. dimorphophylla*, the longest shoots were produced with low levels of iron and high mesos.

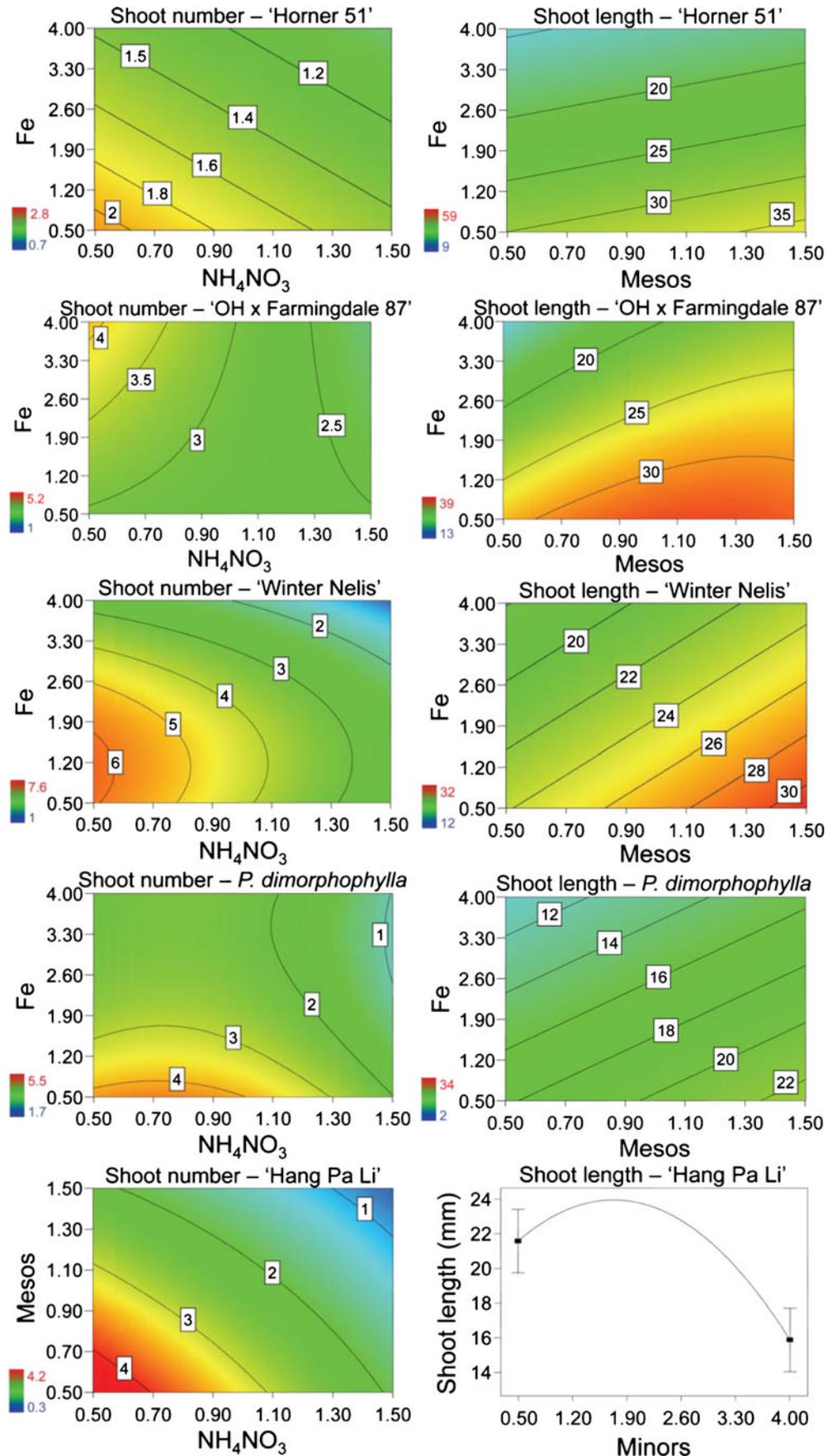
**Number of nodes.** The number of nodes on the longest shoot was influenced by several factors, but mesos played a part for all genotypes (Table 3). High mesos were best for OH×F87. 'Winter Nelis' had increased nodes when KNO<sub>3</sub> and mesos were high. 'Horner 51' and *P. dimorphophylla* nodes were maximized on low iron with increased mesos. Low mesos and micros produced more nodes on 'Hang Pa Li'.

**Leaf size/width.** Leaf size ratings and leaf width measurements were both included in the analysis. The factors with the largest effects included mesos ('OH×F87', 'Winter Nelis', 'Horner 51'), micros ('Winter Nelis' and *P. dimorphophylla*),

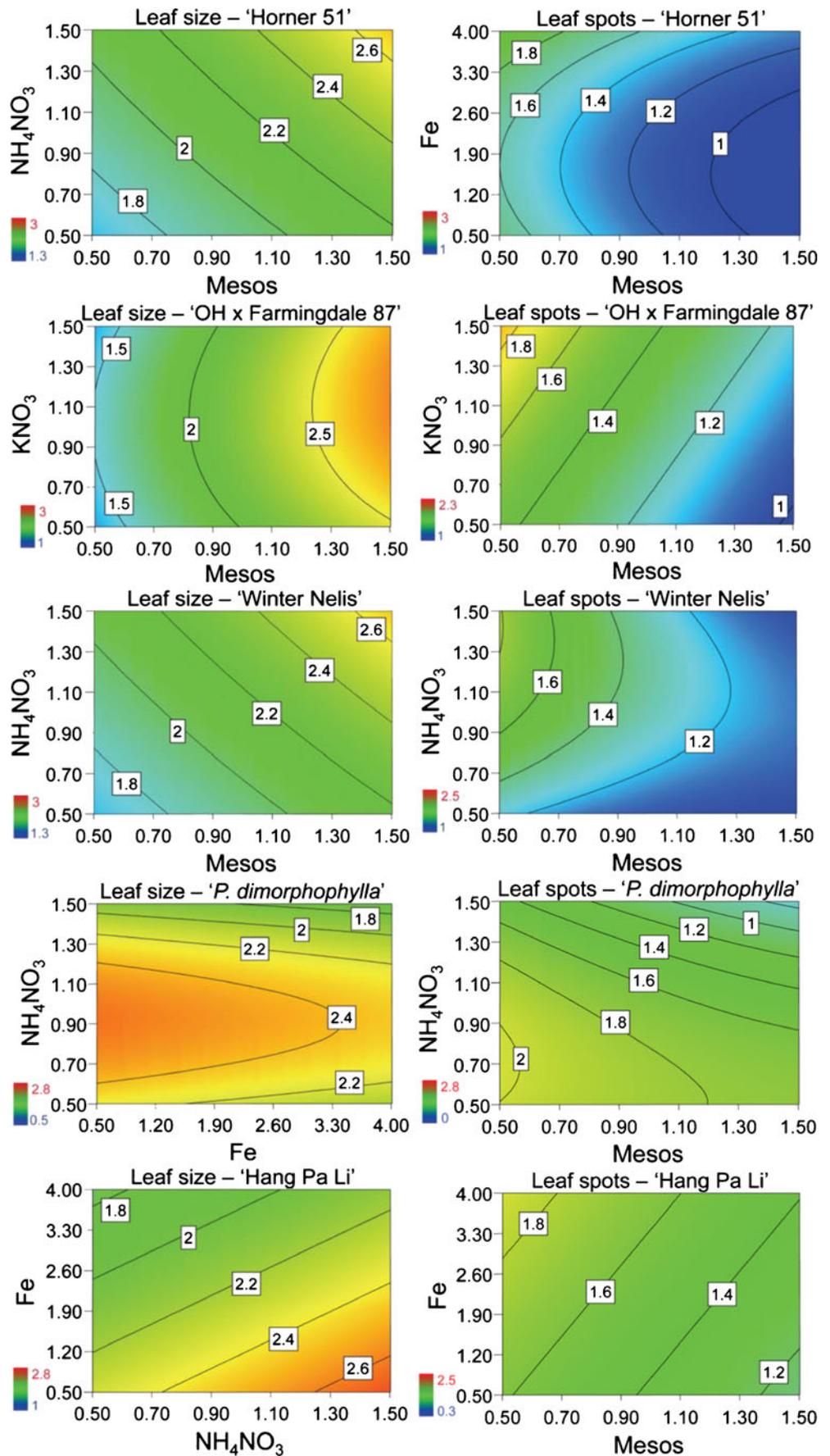
**Figure 1.** *Left*, surface response graphs of mineral nutrient effects on the quality of five pear genotypes. Greatest response (best quality) is in *red*, median in *yellow* and *green*, and least in *blue*. *Right*, shoot cultures of each genotype grown on high iron, low mesos treatment (A); grown on low iron, high mesos treatment (B).



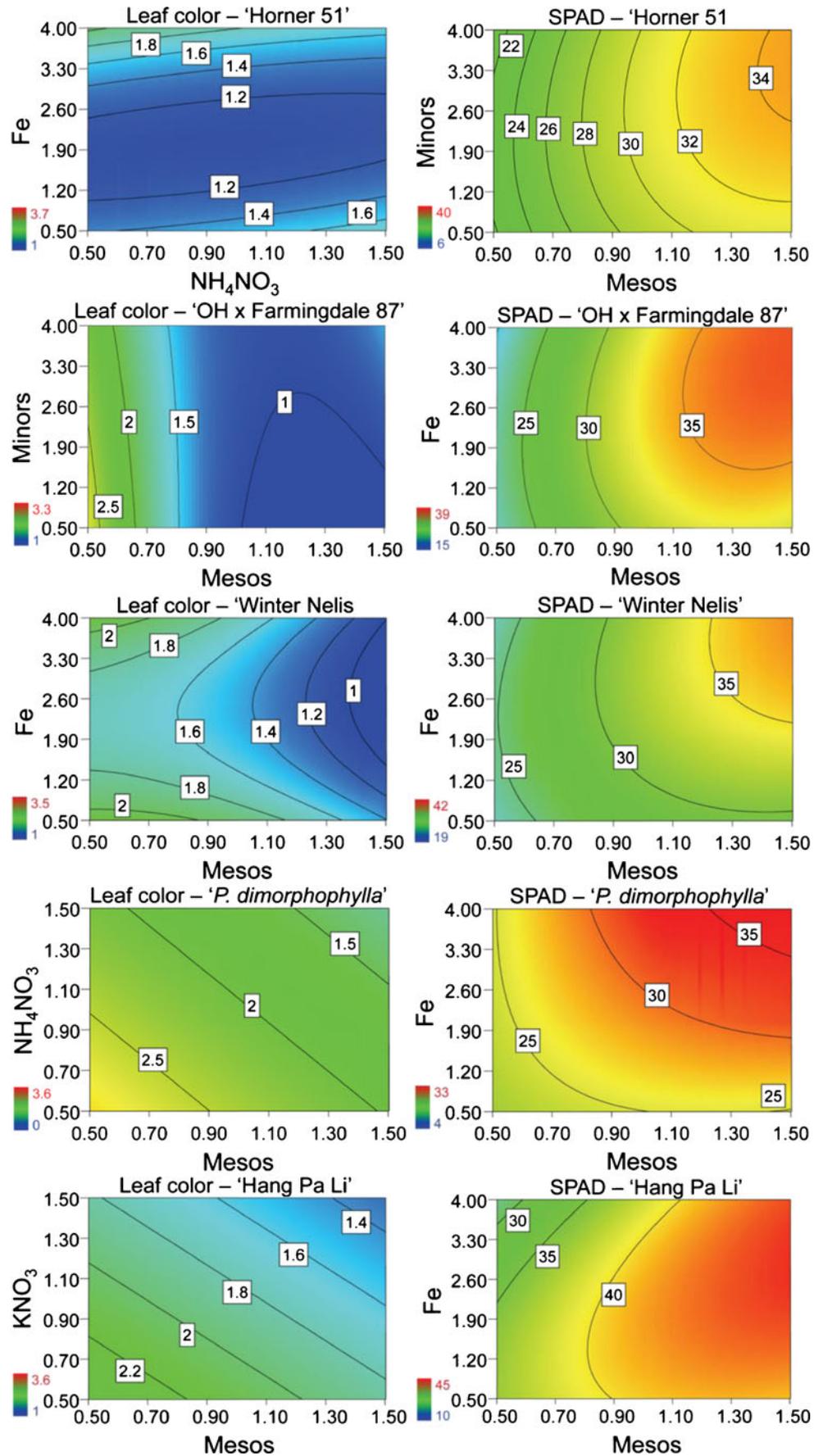
**Figure 2.** Surface response graphs of mineral nutrition effects on the shoot number (left) and shoot length (right) of five pear genotypes. Greatest response is in red, median in yellow and green, and least in blue. Minors was the only factor that affected shoot length of ‘Hang Pa Li’ therefore, a single-factor effect plot is presented. The range symbols on the effect plots are the least significant difference (LSD) calculations performed at the 95% confidence level. Because the LSD bars do not overlap, it is assumed that the points are significantly different.



**Figure 3.** Surface response graphs of mineral nutrition effects on the leaf size (left) and leaf spots (right) and necrosis of five pear genotypes. Larger leaf size and increased amount of necrosis are indicated by red color, and smaller leaves and no necrosis are indicated by dark blue.



**Figure 4.** Surface response graphs of mineral nutrition effects on leaf color ratings (left) and SPAD analysis (right) of five pear genotypes. A dark green leaf color is indicated by blue, and the greatest SPAD meter reading (chlorophyll content) is in red.



**Table 3.** Mineral nutrient factors (stock solutions) that had the largest effects on eight responses for each genotype

Genotype	Quality	Shoot number	Shoot length	Number of nodes	Leaf size	Leaf spots	Leaf color	SPAD mean
Homer 51	Model (<0.0001) <sup>a</sup>	Model (<0.0001)	Model (<0.0001)	Model (<0.0001)	Model (<0.0001)	Model (<0.0001)	Model (<0.0001)	Model (<0.0001)
	Mesos (0.0003)	NH <sub>4</sub> NO <sub>3</sub> (<0.0002)	Iron (<0.0001)	Mesos (<0.0003)	Mesos (<0.0001)	Mesos (<0.0001)	Mesos (<0.0001)	Mesos (<0.0001)
	Iron (0.0002)	Iron (<0.0001)		Iron (<0.0001)	Iron (0.0009)	Iron (<0.0001)		Minors×NH <sub>4</sub> NO <sub>3</sub>
OH×F87	Model (<0.0001)	Model (<0.0001)	Model (<0.0001)	Model (<0.0003)	Model (<0.0001)	Model (<0.0001)	Model (<0.0001)	Model (<0.0001)
	Mesos (<0.0001)	NH <sub>4</sub> NO <sub>3</sub> (<0.0001)	Mesos (<0.0001)	Mesos (<0.0001)	Mesos (<0.0001)	KNO <sub>3</sub> (0.0002)	Mesos (<0.0001)	Mesos (<0.0001)
	Iron (0.01)	Iron (<0.0001)	Iron (<0.0001)			Mesos (<0.0001)		
Winter Nelis	Model (<0.0001)	Model (<0.0001)	Model (<0.0001)	Model (<0.0001)	Model (<0.0001)	Model (<0.0001)	Model (<0.0001)	Model (<0.0001)
	Mesos (<0.0001)	NH <sub>4</sub> NO <sub>3</sub> (<0.0001)	Mesos (<0.0001)	KNO <sub>3</sub> (0.0023)	NH <sub>4</sub> NO <sub>3</sub> (<0.0001)	NH <sub>4</sub> NO <sub>3</sub> (0.0047)	Mesos (<0.0001)	Mesos (<0.0001)
	Iron (0.0002)	Iron (<0.0001)	Iron (<0.0001)	Minors (<0.0001)	Mesos (<0.0001)	Mesos (<0.0001)		NH <sub>4</sub> NO <sub>3</sub> (<0.0001)
Hang Pa Li	Model (<0.0001)	Model (<0.0001)	Model (0.0009)	Model (<0.0001)	Model (<0.0001)	Model (<0.0001)	Model (<0.0001)	Model (<0.0001)
	Minors (0.0011)	NH <sub>4</sub> NO <sub>3</sub> (<0.0003)	Minors (0.0026)	Mesos (<0.0077)	NH <sub>4</sub> NO <sub>3</sub> (0.0008)	Mesos (<0.0001)	NH <sub>4</sub> NO <sub>3</sub> (<0.0001)	Mesos (<0.0001)
	Iron (0.0315)	Mesos(0.0001)		Minors (0.0001)	Iron (<0.0001)	Iron (0.0271)	Mesos (<0.0001)	Mesos (<0.0001)
<i>Pyrus dimorphophylla</i>	Model (0.0009)	Model (<0.0001)	Model (0.0008)	Model (<0.0004)	Model (<0.0001)	Model (<0.0001)	Model (<0.0001)	Model (<0.0001)
	Mesos (0.035)	NH <sub>4</sub> NO <sub>3</sub> (<0.0001)	Mesos (<0.0174)	Mesos (<0.042)	Iron (0.0002)	NH <sub>4</sub> NO <sub>3</sub> (0.0017)	Mesos (<0.0001)	Mesos (<0.0001)
	Iron (0.0273)	Iron (<0.0002)	Iron (<0.0001)	Iron (<0.0044)	Minors (0.0135)	Mesos (0.0076)		Minors×iron

Data include the overall model and factors with the largest effects

<sup>a</sup> The *p* value is provided in parentheses

**Table 4.** Sample ANOVA and summary statistics for shoot quality of 'OH×F87'

Source	Sum of squares	df	Mean square	F value	p value (Prob>F)
Model	1.8400	9	0.2000	9.6400	<0.0001
NH <sub>4</sub> NO <sub>3</sub>	0.0077	1	0.0077	0.3600	0.5516
KNO <sub>3</sub>	0.0021	1	0.0021	0.1000	0.7525
Mesos	1.1000	1	1.1000	51.7100	<0.0001
Micros	0.0061	1	0.0061	0.2900	0.5949
Fe	0.1600	1	0.1600	7.4000	0.0100
NH <sub>4</sub> NO <sub>3</sub> ×KNO <sub>3</sub>	0.0690	1	0.0690	3.2400	0.0801
Mesos×micros	0.1100	1	0.1100	5.2100	0.0284
Mesos <sup>2</sup>	0.1400	1	0.1400	6.6300	0.0143
Fe <sup>2</sup>	0.1600	1	0.1600	7.6400	0.0089
Residual	0.7600	36	0.0210		
Lack of fit	0.4500	24	0.0190	0.7300	0.7495
Pure error	0.3100	12	0.0260		
Cor total	2.6000	45			
SD	0.1500		R squared	0.7068	
Mean	0.7700		Adj R squared	0.6335	
CV%	19.0200		Pred R squared	0.5202	
Press	1.2500		Adeq Precision	12.7960	

<sup>a</sup>ANOVA for Response Surface Reduced Quadratic Model

<sup>b</sup>Analysis of variance table (classical sum of squares—type II)

NH<sub>4</sub>NO<sub>3</sub> ('Winter Nelis' and 'Hang Pa Li'), and iron ('Horner 51', 'Hang Pa Li', *P. dimorphophylla*) (Table 3; Fig. 3). Increased mesos resulted in larger leaves for the *P. communis* cultivars in conjunction with increased nitrogen. Low iron and increased NH<sub>4</sub>NO<sub>3</sub> increased leaf size for *P. dimorphophylla* and 'Hang Pa Li'. Most plants had moderate leaf size with standard MS medium. Leaf width measurement results were similar to leaf size ratings (data not shown).

**Necrosis and leaf spots.** Dark or discolored spots and leaf edge necrosis were common for all genotypes, and the mineral nutrients significantly affected the number of leaf spots and amount of necrosis. The factors with the largest effects included mesos (all genotypes), KNO<sub>3</sub> (OH×F87), NH<sub>4</sub>NO<sub>3</sub> ('Winter Nelis' and *P. dimorphophylla*), and iron ('Hang Pa Li': Fig. 3). In general, increased meso levels reduced the number of leaf spots (lower ratings). *P. dimorphophylla* had a different response to nitrogen, as higher NH<sub>4</sub>NO<sub>3</sub> levels combined with high mesos reduced leaf spots; lower nitrogen led to reduced leaf spots in other genotypes.

**Leaf color ratings.** Leaf color ratings of the genotypes were all significantly affected by mesos; 'Hang Pa Li' was also affected by NH<sub>4</sub>NO<sub>3</sub> (Table 3). Projections of the best leaf color for 'Hang Pa Li' and *P. dimorphophylla* indicated that high mesos combined with high nitrogen would produce the greenest leaves (Fig. 4); increased mesos combined with moderate to high iron or micros produced the darkest leaves for all *P. communis* cultivars.

**SPAD meter readings.** SPAD readings (correlated to actual chlorophyll content) of all genotypes were significantly influenced by mesos (Table 3). In addition, 'Horner 51' was significantly affected by an interaction of micros and NH<sub>4</sub>NO<sub>3</sub>, and 'Winter Nelis' was influenced by NH<sub>4</sub>NO<sub>3</sub> and iron. 'Horner 51' had an interaction of NH<sub>4</sub>NO<sub>3</sub>×micros; and *P. dimorphophylla* an interaction of micros×iron (Table 3). High mesos and increased iron were projected to produce better chlorophyll readings for all genotypes (Fig. 4).

**Genotype effects.** Differences among genotypes were high, even among the three *P. communis* cultivars. Design Expert (2010) can be used to predict the "best" ratings for plants that simultaneously meet designated criteria. Based on the following criteria: quality greater than 2.5, shoot number at least 4, shoot length between 6 and 8 cm, leaf spots are minimized, and leaf size between 2 and 2.5 cm, the predicted optimized medium for each genotypes is described as follows: OH×F87 was most influenced by the mesos group, with shoot number influenced negatively by ammonium nitrate and iron. The best medium was MS with at least 1.5× mesos. Slight improvements could be made with slightly decreased micros. Lower ammonium increased shoot numbers but decreased general quality. 'Winter Nelis' was most influenced by mesos and iron for quality. Ammonium nitrate and iron influenced leaf size and shoot number. Shoot number was also influenced by an interaction of mesos and micros. The best medium would be MS with 1.5× mesos and 1.5× KNO<sub>3</sub>. 'Horner 51' was most influenced

by mesos. Shoot number increased with ammonium nitrate and iron, while shoot length decreased with iron. The best medium was MS with 1.34× mesos and 0.5× NH<sub>4</sub>NO<sub>3</sub>. ‘Hang Pa Li’ quality, shoot length, and nodes were influenced by the micros. Ammonium nitrate and mesos decreased shoot numbers. This may indicate that micros should be carefully balanced for this species. The best medium was low iron and micros (0.5×) and high KNO<sub>3</sub> (1.5×) with medium ammonium nitrate and mesos (0.7×). *P. dimorphophylla* had decreased shoot number with increased ammonium nitrate. It appears that decreased ammonium nitrate would greatly improve growth of this species. The best medium was MS with low iron (0.5×) and high mesos and KNO<sub>3</sub> (1.5×).

## Discussion

Many possible approaches exist for optimizing the growth medium for plant tissue culture. The initial methods of approach by Murashige and colleagues (Murashige and Skoog 1962; Murashige and Nakano 1965; Murashige 1974) for tobacco callus cultures were performed as single-factor changes on a single type of callus tissue. The complexity of medium constituent interactions was noted by Murashige and Skoog (1962) in the discussion of their ground-breaking study. They also noted wide genotype variation when their medium was used for growth of other tobacco genotypes; some grew poorly and others did not grow at all. Growth medium composition is not easy to develop or optimize (Williams 1993; Ramage and Williams 2002; Adelberg *et al.* 2010). Our own experience with shoot culture of the diverse pear germplasm indicated that several variations of MS medium were not suitable for a large percentage of pear germplasm and many genotypes had poor initiation success or poor growth *in vitro* (Reed, unpublished data). Due to the difficulties involved in developing new formulations, many recent studies rely on comparisons of established media (Thakur and Kanwar 2008; Bell *et al.* 2009).

Advanced experimental design and surface response methods were used by Niedz and coworkers to improve growth medium for several callus and shoot culture types (Niedz and Evens 2006, 2007; Niedz *et al.* 2007). Statistical software can be used to design experiments to determine the best combinations of nutrients (and other media components) from experiments that are resource efficient for the amount of knowledge that they generate.

A complimentary approach is spent medium analysis that determines which nutrients are in the greatest demand by a specific plant (Adelberg *et al.* 2010). This information is used to design experiments to optimize the levels of the identified nutrients. Potassium, phosphorous, sodium, iron, and copper concentrations all decreased in medium with

rapidly growing *Hemerocallis* shoots; however, the phosphorous concentration found in the harvested shoots remained below optimal concentrations for greenhouse-grown plants. Analysis of plants after growth on nutrient media or analysis of field plants can also indicate plant nutrient requirements in various stages of growth and differentiation (Kintzios *et al.* 2004; Nas and Read 2004) and the effects of minerals in agar on plant growth (Singha *et al.* 1985).

Our results indicate distinct effects of mineral nutrients on morphogenesis. Shoots grown on high iron concentrations were always stunted (Fig. 1(A)), while those on high mesos grew vigorously and produced large leaves (Figs. 1(B) and 3). Phosphorous was rapidly used by shoot-forming tobacco leaf cultures but not by non-differentiating cultures, and for those cultures, MS concentrations were optimal (Ramage and Williams 2002). Higher phosphorous levels decreased shoot production in tobacco cultures, possibly due to decreased uptake of iron or calcium ions. It is likely that the improved growth of pears and increased shoot and leaf size on high mesos treatments is linked to phosphorous in this study. Williams (1993) found that uptake is not always the same as use; plants can take up more than is needed at the time and sequester the minerals for later use. Little attention has been paid to the effect of mineral nutrition on plant morphogenesis (Ramage and Williams 2002). Preece (1995) found interactions between plant growth regulators (PGRs) and mineral nutrients in plant tissue culture media, as well as effects of nutrient formulations on morphogenesis. Plant growth on suboptimal nutrient media was compensated by higher PGR concentrations, and optimal nutrient media required less PGR for good plant growth.

Development of tobacco meristems and their resulting shoots was significantly related to the uptake of nitrate, phosphorous, and potassium and somewhat with calcium; magnesium and iron were not involved in differentiation. Ramage and Williams (2002) note that nitrogen, phosphorus, and calcium appear essential for growth and differentiation while potassium, magnesium, and sulfur are required in lesser amounts. Nutrient uptake of key minerals varies with the stages of differentiation and growth of melon cultures (Kintzios *et al.* 2004); potassium uptake results in a decrease in differentiation of the callus cultures. We did not observe a decrease in growth with increased potassium; however, the highest treatment levels were probably below that which would inhibit growth. Additional study will be needed to determine the optimal concentration of potassium in pears. Organogenesis of melon was better with a phosphorous-enriched medium, but somatic embryogenesis was improved with more magnesium. Nitrogen promotes differentiation in some genotypes of melon but not others (Kintzios *et al.* 2004). Increased boron and sodium chloride greatly affected the uptake of many other important mineral

nutrients in pear (Sotiropoulos *et al.* 2006). The amount of calcium in medium also affects the uptake of many other minerals; N, K, Zn, Mn, and B increased in bromeliad tissues with higher calcium concentrations of up to 4× MS (Aranda-Peres and Martinelli 2009). All of these studies illustrate the complexity of determining optimal *in vitro* mineral nutrition.

The objective of our experiment was to determine the effects of mineral nutrition on the growth of *in vitro* pear shoot cultures. The surface response design and related software allowed for projections of optimal mineral combinations and are of great value in designing further experiments to optimize growth medium. Our data analysis showed that the mineral nutrients had significant effects on growth and quality of all five genotypes. The mineral nutrient factors (Table 1) having the greatest impacts on each of the five measured responses for each cultivar (Table 3) show that shoot quality was significantly affected by the mesos stock (CaCl<sub>2</sub>·2H<sub>2</sub>O, KH<sub>2</sub>PO<sub>4</sub>, and MgSO<sub>4</sub>) with increased quality as the concentration increased. As shown with bromeliads, increased calcium promotes the uptake of additional minerals and may be the greatest influence in improving growth (Aranda-Peres and Martinelli 2009). Many of the factors we analyzed were involved in overall quality: shoot length, shoot number, leaf color, leaf size, and leaf spots. This overall rating was a quick way to visually determine the best treatments based on what plants were “desirable” or “undesirable” (Niedz *et al.* 2007). Closer analysis of each of these traits provided insight into the effects of the five stock solutions. As expected, there were differences in response for each of the three species studied (Fig. 1).

Shoot length and leaf size were significantly increased with increasing mesos for most of the pear genotypes, and leaf spots and necrosis were significantly decreased in all pears (Table 3). The increase in shoot growth was likely the result of increased phosphorous and calcium, while improved leaf quality may be due to improved calcium and magnesium nutrition (Aranda-Peres and Martinelli 2009). Shoot numbers were significantly higher with standard or minimal concentrations of iron (0.5–1×) and NH<sub>4</sub>NO<sub>3</sub> (0.8–0.5×) for all five pears (Fig. 2). As expected, factors that increased shoot length tended to decrease shoot number. The ideal medium appears to be one with standard 1× or 0.5× iron and high mesos, while the nitrogen concentrations are quite variable among the genotypes.

The main stock solutions used in the preparation of MS medium have both positive and negative impacts on the growth of diverse pear genotypes. Our results indicate that the mesos, nitrogen, and minor factors need further evaluation. Because of the significant influence of the mesos group on most of the parameters studied, we increased the mesos stock concentration of MS medium to 1.5× for use as a multiplication medium for the all genotypes. Because the

mesos factor affected the most responses and the most genotypes, it should be the first to be evaluated in more detail, followed by nitrogen. Mesos were important in some cases and should be tested separately for the final optimization step.

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